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CHAPTER 1

Cell Culture

INTRODUCTION

Cell biology traces its roots to the introduction of the concept of “cells” by Robert Hooke in the second half of the 17th century. However, not until nearly halfway through the 20th century were techniques for the culture of cells developed. In fact, 1998 marked the golden anniversary of the first continuous mammalian cell line. Cell culture has become such an integral part of cell biology that it is somewhat difficult to imagine the field in the B.C. (“Before Culture”) era. Cell culture also represents the primary way in which cell biology reaches into related disciplines, since the maintenance and propagation of cells has become an important component of biochemistry, biophysics, genetics, immunology, physiology, molecular biology, and neuroscience. Accordingly, it is altogether fitting that the first chapter of *Current Protocols in Cell Biology* should present methods related to the culture of cells.

The immediate aim of cell culture is to maintain or expand a population of cells, and the single most important consideration is cell viability. Determining the number of cells and their viability is important in standardizing culture and experimental conditions. As viable cells replicate in culture, passaging of the cells allows their number to be expanded to meet experimental needs. The ability to freeze, store, and recover cells provides an essential safeguard against losing a cell line to contamination, incubator malfunction, or an error on the part of the investigator. In addition to preserving the cells, maintenance of a frozen stock is desirable to avoid cellular senescence and genetic drift. Chapter 1 therefore begins with protocols for passaging cells, freezing and thawing cells, and determining cell number and viability (*UNIT 1.1*).

Success in cell culture is highly dependent on the choice of a medium. At minimum, a medium must provide the nutritional requirements of the cells as well as any required growth factors, and maintain pH and osmolarity compatible with survival. The historical development of a wide variety of culture media has influenced significantly the types of cells that can be studied experimentally, since cell lines that proliferate in a particular environment are always selected at the expense of those that do not. The second unit of Chapter 1 therefore focuses on media used in culturing cells and provides descriptions of standard, serum-free, and selective media, as well as the use of soft agar for anchorage-independent growth (*UNIT 1.2*).

The next three units of this chapter deal with microbial contamination of cell cultures. *UNIT 1.3* describes basic aseptic techniques and the laminar flow hoods that are the main weapons in the constant battle against contamination. *UNIT 1.4* provides protocols related to sterilization, namely filtration and heat sterilization (e.g., autoclaving), as well as the use of disinfectants. *UNIT 1.5* describes methods for detecting microbial contaminants (bacteria, fungi, and mycoplasmas). While the best way to deal with such contamination may well be to review the previous unit on autoclaving and faithfully apply its precepts, situations do arise where an attempt to salvage a contaminated culture is warranted. *UNIT 1.5* details the use of antibiotics for this purpose.

Of course there are cell biologists who do not see the growth of fungi as an annoying contamination of their mammalian cell cultures but as a desirable goal. For scientists who

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wish to propagate yeast, *UNIT 1.6* provides recipes for media and descriptions of some basic culture methodologies.

UNIT 1.7 represents the first unit of Chapter 1 dealing with culture of plants cells, specifically the culture and transformation of BY-2 cells derived from tobacco. BY-2 cells have been described as the HeLa cell of higher plants.

Future units in Chapter 1 will cover specialized systems for cell culture (e.g., cell cloning, polarized cells, and three-dimensional cultures), as well as additional units on the propagation of plant cells, cells from other so-called simpler organisms, and viruses.

For additional information on mammalian cell culture, readers are directed to Freshney (1993).

LITERATURE CITED

Freshney, R.I. 1993. Culture of Animal Cells. A Manual of Basic Techniques, 3rd ed. Wiley-Liss, New York.

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Basic Techniques for Mammalian Cell Tissue Culture

Tissue culture technology has found wide application in the field of cell biology. Cell cultures are utilized in cytogenetic, biochemical, and molecular laboratories for diagnostic as well as research studies. In most cases, cells or tissues must be grown in culture for days or weeks to obtain sufficient numbers of cells for analysis. Maintenance of cells in long-term culture requires strict adherence to aseptic technique to avoid contamination and potential loss of valuable cell lines (see UNIT 1.3).

An important factor influencing the growth of cells in culture is the choice of tissue culture medium. Many different recipes for tissue culture media are available and each laboratory must determine which medium best suits their needs. Individual laboratories may elect to use commercially prepared medium or prepare their own. Commercially available medium can be obtained as a sterile and ready-to-use liquid, in a concentrated liquid form, or in a powdered form. Besides providing nutrients for growing cells, medium is generally supplemented with antibiotics, fungicides, or both to inhibit contamination. Medium preparation is discussed in UNIT 1.2.

As cells reach confluency, they must be subcultured or passaged. Failure to subculture confluent cells results in reduced mitotic index and eventually cell death. The first step in subculturing monolayers is to detach cells from the surface of the primary culture vessel by trypsinization or mechanical means. The resultant cell suspension is then subdivided, or reseeded, into fresh cultures. Secondary cultures are checked for growth, fed periodically, and may be subsequently subcultured to produce tertiary cultures, etc. The time between passing cells depends on the growth rate and varies with the cell line.

The Basic Protocol describes subculturing of a monolayer culture grown in petri plates or flasks; the Alternate Protocol 1 describes passaging of suspension cultures. Support Protocols describe freezing of monolayer cells, thawing and recovery of cells, counting cells using a hemacytometer, and preparing cells for transport. Alternate Protocol 2 describes freezing of suspension cells.

CAUTION: When working with human blood, cells, or infectious agents, appropriate biosafety practices must be followed.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

TRYPsinIZING AND SUBCULTURING CELLS FROM A MONOLAYER

A primary culture is grown to confluency in a 60-mm petri plate or 25-cm² tissue culture flask containing 5 ml tissue culture medium. Cells are dispersed by trypsin treatment and then reseeded into secondary cultures. The process of removing cells from the primary culture and transferring them to secondary cultures constitutes a passage, or subculture.

Materials

Primary cultures of cells

HBSS (APPENDIX 2A) without Ca²⁺ and Mg²⁺, 37°C

Trypsin/EDTA solution (see recipe), 37°C

Complete medium with serum: e.g., supplemented DMEM (APPENDIX 2A) with 10% to 15% (v/v) FBS (complete DMEM-10 or -15), 37°C

BASIC PROTOCOL

Sterile Pasteur pipets
37°C warming tray *or* incubator
Tissue culture plasticware or glassware including pipets and 25-cm² flasks
or 60-mm petri plates, sterile

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

1. Remove all medium from primary culture with a sterile Pasteur pipet. Wash adhering cell monolayer once or twice with a small volume of 37°C HBSS without Ca²⁺ and Mg²⁺ to remove any residual FBS that may inhibit the action of trypsin.

Use a buffered salt solution that is Ca²⁺ and Mg²⁺ free to wash cells. Ca²⁺ and Mg²⁺ in the salt solution can cause cells to stick together.

If this is the first medium change, rather than discarding medium that is removed from primary culture, put it into a fresh dish or flask. The medium contains unattached cells that may attach and grow, thereby providing a backup culture.

2. Add enough 37°C trypsin/EDTA solution to culture to cover adhering cell layer.
3. Place plate on a 37°C warming tray 1 to 2 min. Tap bottom of plate on the countertop to dislodge cells. Check culture with an inverted microscope to be sure that cells are rounded up and detached from the surface.

If cells are not sufficiently detached, return plate to warming tray for an additional minute or two.

4. Add 2 ml 37°C complete medium. Draw cell suspension into a Pasteur pipet and rinse cell layer two or three times to dissociate cells and to dislodge any remaining adherent cells. As soon as cells are detached, add serum or medium containing serum to inhibit further trypsin activity that might damage cells.

If cultures are to be split 1/3 or 1/4 rather than 1/2, add sufficient medium such that 1 ml of cell suspension can be transferred into each fresh culture vessel.

5. Add an equal volume of cell suspension to fresh plates or flasks that have been appropriately labeled.

Alternatively, cells can be counted using a hemacytometer or Coulter counter and diluted to the desired density so a specific number of cells can be added to each culture vessel. A final concentration of $\sim 5 \times 10^4$ cells/ml is appropriate for most subcultures.

For primary cultures and early subcultures, 60-mm petri plates or 25-cm² flasks are generally used; larger vessels (e.g., 150-mm plates or 75-cm² flasks) may be used for later subcultures.

Cultures should be labeled with date of subculture and passage number.

6. Add 4 ml fresh medium to each new culture. Incubate in a humidified 37°C, 5% CO₂ incubator.

If using 75-cm² culture flasks, add 9 ml medium per flask.

Some labs now use incubators with 5% CO₂ and 4% O₂. The low oxygen concentration is thought to simulate the in vivo environment of cells and to enhance cell growth.

For some media it is necessary to adjust the CO₂ to a higher or lower level to maintain the pH at 7.4.

7. If necessary, feed subconfluent cultures after 3 or 4 days by removing old medium and adding fresh 37°C medium.
8. Passage secondary culture when it becomes confluent by repeating steps 1 to 7, and continue to passage as necessary.

PASSAGING CELLS IN SUSPENSION CULTURE

A suspension culture is grown in culture flasks in a humidified 37°C, 5% CO₂ incubator. Passaging of suspension cultures is somewhat less complicated than passaging of monolayer cultures. Because the cells are suspended in medium rather than attached to a surface, it is not necessary to disperse them enzymatically before passaging. However, before passaging, cells must be maintained in culture by feeding every 2 to 3 days until they reach confluency (i.e., until the cells clump together in the suspension and the medium appears turbid when the flask is swirled).

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

1. Feed cells as follows every 2 to 3 days until the cultures are confluent:
 - a. Remove flask of suspension cells from incubator, taking care not to disturb those that have settled to the flask bottom.
 - b. Aseptically remove and discard about one-third of the medium from flask and replace with an equal volume of prewarmed (37°C) medium. If the cells are growing rapidly, add an additional 10% medium by volume in order to maintain optimum concentration of 1×10^6 cells/ml. Gently swirl flask to resuspend cells.
 - c. Return flask to incubator. If there is <15 ml of medium in the flask, incubate flask in horizontal position to enhance cell/medium contact.

At higher volumes of medium the flask can be incubated in the vertical position.

If using a 25-cm² flask, there should be 20 to 30 ml of medium in the flask at confluency.

2. On the days cultures are not being fed, check them by swirling flask to resuspend cells and observing color changes in the medium that indicate good metabolic growth.
3. When cultures are confluent ($\sim 2.5 \times 10^6$ cells/ml), passage culture as follows:
 - a. Remove flask from incubator and swirl flask so that cells are evenly distributed in the medium.
 - b. Aseptically remove half of the volume of cell suspension and place into a fresh flask.
 - c. Feed each flask with 7 to 10 ml prewarmed medium and return flask to incubator.

Some labs prefer to split the cells 1:3 or 1:4, although increasing the split ratio will result in a longer interval before subcultures reach confluency.

FREEZING HUMAN CELLS GROWN IN MONOLAYER CULTURES

It is sometimes desirable to store cell lines for future study. To preserve cells, avoid senescence, reduce the risk of contamination, and minimize effects of genetic drift, cell lines may be frozen for long-term storage. Without the use of a cryoprotective agent freezing would be lethal to the cells in most cases. Generally, a cryoprotective substance such as dimethylsulfoxide (DMSO) is used in conjunction with complete medium for preserving cells at -70°C or lower. DMSO acts to reduce the freezing point and allows a slower cooling rate. Gradual freezing reduces the risk of ice crystal formation and cell damage.

Materials

Log-phase monolayer culture of cells in petri plate
Complete medium (e.g., supplemented DMEM, APPENDIX 2A)
Freezing medium: complete medium supplemented with 10% to 20% (v/v)
FBS and 5% to 10% (v/v) DMSO, 4°C
Benchtop clinical centrifuge (e.g., Fisher Centrifig or Clay Adams Dynac)
with 45°C fixed-angle or swinging-bucket rotor

1. Trypsinize cells from plate (see Basic Protocol, steps 1 to 4).

It is best to use cells in log-phase growth for cryopreservation.

2. Transfer cell suspension to a sterile centrifuge tube and add 2 ml complete medium with serum. Centrifuge 5 min at 300 to 350 × g (~1500 rpm in Fisher Centrifig rotor), room temperature.

Cells from three or more dishes from the same subculture of the same source can be combined in one tube.

3. Remove supernatant and add 1 ml of 4°C freezing medium. Resuspend pellet.
4. Add 4 ml of 4°C freezing medium, mix cells thoroughly, and place on wet ice.
5. Count cells using a hemacytometer (see Support Protocol 3). Dilute with more freezing medium as necessary to get a final cell concentration of 10⁶ or 10⁷ cells/ml.

To freeze cells from a nearly confluent 25-cm² flask, resuspend in ~3 ml freezing medium.

6. Pipet 1-ml aliquots of cell suspension into labeled 2-ml cryovials. Tighten caps on vials.
7. Place vials 1 hr to overnight in a -70°C freezer, then transfer to liquid nitrogen storage freezer.

Alternatively, freeze cells in a freezing chamber in the neck of a Dewar flask according to manufacturer's instructions. Some laboratories place vials directly into the liquid nitrogen freezer, omitting the gradual temperature drop. Although this is contrary to the general recommendation to gradually reduce the temperature, laboratories that routinely use a direct-freezing technique report no loss of cell viability on recovery.

Keep accurate records of the identity and location of cells stored in liquid nitrogen freezers. Cells may be stored for many years and proper information is imperative for locating a particular line for future use.

ALTERNATE PROTOCOL 2

FREEZING CELLS GROWN IN SUSPENSION CULTURE

Freezing cells from suspension culture is similar in principle to freezing cells from monolayer. The major difference is that suspension cultures need not be trypsinized.

1. Transfer cell suspension to a centrifuge tube and spin 10 min at 300 to 350 × g (~1500 rpm in Fisher Centrifig centrifuge), room temperature.
2. Remove supernatant and resuspend pellet in 4°C freezing medium at a density of 10⁶ to 10⁷ cells/ml.

Some laboratories freeze lymphoblastoid lines at the higher cell density because they plan to recover them in a larger volume of medium and because there may be a greater loss of cell viability upon recovery as compared to other types of cells (e.g., fibroblasts).

3. Transfer 1-ml aliquots of cell suspension into cryovials and freeze as for monolayer cultures.

THAWING AND RECOVERING HUMAN CELLS

When cryopreserved cells are needed for study, they should be thawed rapidly and plated at high density to optimize recovery.

CAUTION: Protective clothing, particularly insulated gloves and goggles, should be worn when removing frozen vials or ampules from the liquid nitrogen freezer. The room containing the liquid nitrogen freezer should be well-ventilated. Care should be taken not to spill liquid nitrogen on the skin.

Additional Materials (also see *Basic Protocol*)

70% (v/v) ethanol

Complete medium/20% FBS (e.g., supplemented DMEM-20, *APPENDIX 2A*), 37°C

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

1. Remove vial from liquid nitrogen freezer and immediately place it into a 37°C water bath. Agitate vial continuously until medium is thawed.

The medium usually thaws in <60 sec.

Cells should be thawed as quickly as possible to prevent formation of ice crystals that can cause cell lysis. Try to avoid getting water around the cap of the vial.

2. Wipe top of vial with 70% ethanol before opening.

Some labs prefer to submerge the vial in 70% ethanol and air dry before opening.

3. Transfer thawed cell suspension into a sterile centrifuge tube containing 2 ml warm complete medium/20% FBS. Centrifuge 10 min at 150 to 200 × g (~1000 rpm in Fisher Centrifuge), room temperature. Discard supernatant.

Cells are washed with fresh medium to remove residual DMSO.

4. Gently resuspend cell pellet in small amount (~1 ml) of complete medium/20% FBS and transfer to properly labeled culture plate containing the appropriate amount of medium.

Cultures are reestablished at a higher cell density than that used for original cultures because there is some cell death associated with freezing. Generally, 1 ml of cell suspension is reseeded in 5 to 20 ml medium.

5. Check cultures after ~24 hr to ensure that cells have attached to the plate.

6. Change medium after 5 to 7 days or when pH indicator (e.g., phenol red) in medium changes color. Keep cultures in medium with 20% FBS until cell line is reestablished.

If recovery rate is extremely low, only a subpopulation of the original culture may be growing; be especially careful of this when working with cell lines known to be mosaic.

DETERMINING CELL NUMBER AND VIABILITY WITH A HEMACYTOMETER AND TRYPAN BLUE STAINING

Determining the number of cells in culture is important in standardization of culture conditions and in performing accurate quantitation experiments. A hemacytometer is a thick glass slide with a central area designed as a counting chamber.

The exact design of the hemacytometer may vary; the one described here is the Improved Neubauer from Baxter Scientific (Fig. 1.1.1). The central portion of the slide is the counting platform which is bordered by a 1-mm groove. The central platform is divided into two counting chambers by a transverse groove. Each counting chamber consists of

a silver footplate on which is etched a 3×3 -mm grid. This grid is divided into nine secondary squares, each 1×1 mm. The four corner squares and the central square are used for determining the cell count. The corner squares are further divided into 16 tertiary squares and the central square into 25 tertiary squares to aid in cell counting.

Accompanying the hemacytometer slide is a thick, even-surfaced coverslip. Ordinary coverslips may have uneven surfaces that can introduce errors in cell counting; therefore, it is imperative that the coverslip provided with the hemacytometer is used in determining cell number.

Cell suspension is applied to a defined area and counted so cell density can be calculated.

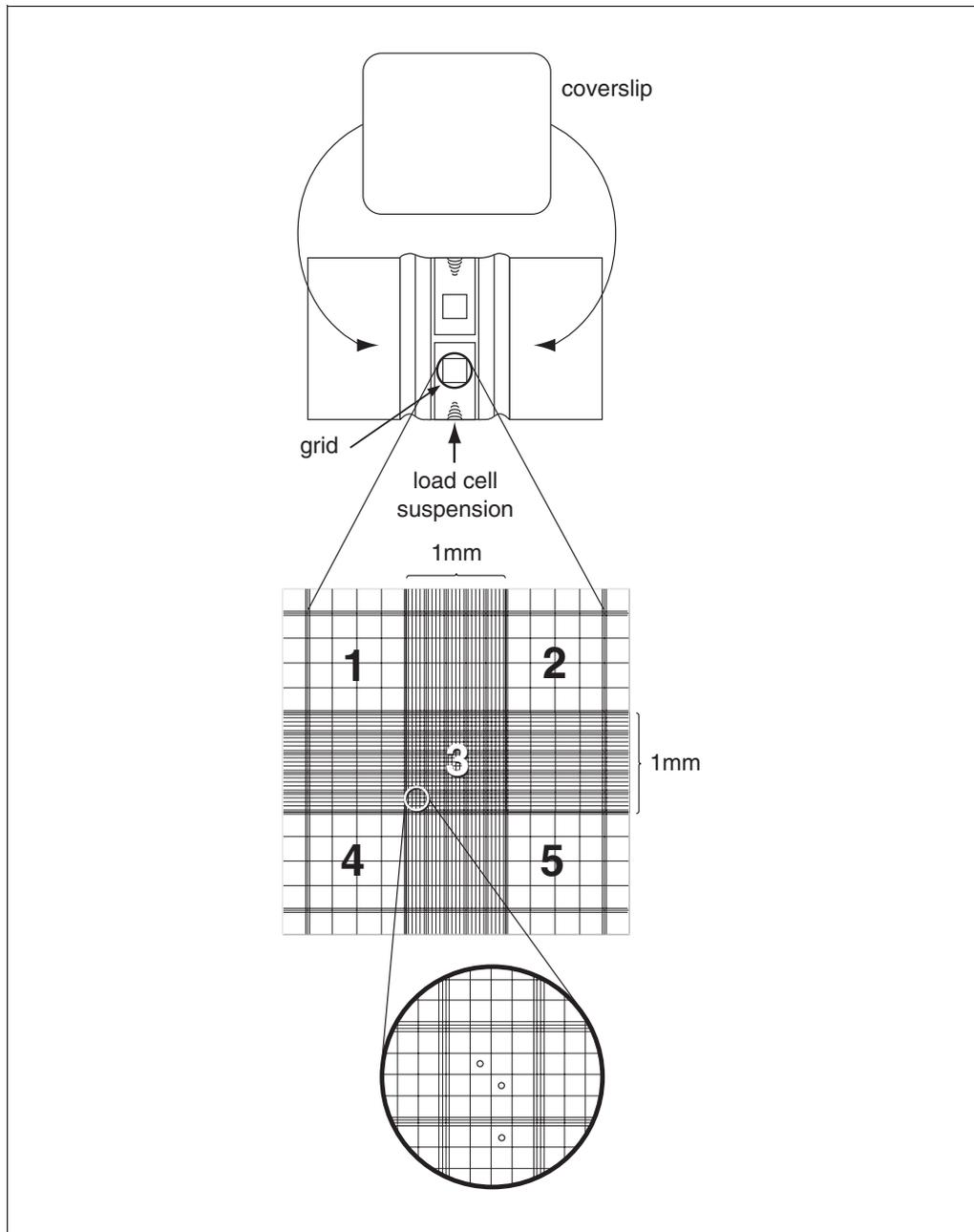


Figure 1.1.1 Hemacytometer slide (Improved Neubauer) and coverslip. Coverslip is applied to slide and cell suspension is added to counting chamber using a Pasteur pipet. Each counting chamber has a 3×3 -mm grid (enlarged). The four corner squares (1, 2, 4, and 5) and the central square (3) are counted on each side of the hemacytometer (numbers added).

Materials

- 70% (v/v) ethanol
- Cell suspension
- 0.4% (w/v) trypan blue *or* 0.4% (w/v) nigrosin prepared in HBSS (APPENDIX 2A)
- Hemocytometer with coverslip (Improved Neubauer, Baxter Scientific)
- Hand-held counter

Prepare hemacytometer

1. Clean surface of hemacytometer slide and coverslip with 70% alcohol.
Coverslip and slide should be clean, dry, and free from lint, fingerprints, and watermarks.
2. Wet edge of coverslip slightly with tap water and press over grooves on hemacytometer. The coverslip should rest evenly over the silver counting area.

Prepare cell suspension

3. For cells grown in monolayer cultures, detach cells from surface of dish using trypsin (see Basic Protocol, steps 1 to 4).
4. Dilute cells as needed to obtain a uniform suspension. Disperse any clumps.

When using the hemacytometer, a maximum cell count of 20 to 50 cells per 1×1 -mm square is recommended.

Load hemacytometer

5. Use a sterile Pasteur pipet to transfer cell suspension to edge of hemacytometer counting chamber. Hold tip of pipet under the coverslip and dispense one drop of suspension.

Suspension will be drawn under the coverslip by capillary action.

The hemacytometer should be considered nonsterile. If cell suspension is to be used for cultures, do not reuse the pipet and do not return any excess cell suspension in the pipet to the original suspension.

6. Fill second counting chamber.

Count cells

7. Allow cells to settle for a few minutes before beginning to count. Blot off excess liquid.
8. View slide on microscope with 100 \times magnification.

A 10 \times ocular with a 10 \times objective = 100 \times magnification.

Position slide to view the large central area of the grid (section 3 in Fig. 1.1.1); this area is bordered by a set of three parallel lines. The central area of the grid should almost fill the microscope field. Subdivisions within the large central area are also bordered by three parallel lines and each subdivision is divided into sixteen smaller squares by single lines. Cells within this area should be evenly distributed without clumping. If cells are not evenly distributed, wash and reload hemacytometer.

9. Use a hand-held counter to count cells in each of the four corner and central squares (Fig. 1.1.1, squares numbered 1 to 5). Repeat counts for other counting chamber.

Five squares (four corner and one center) are counted from each of the two counting chambers for a total of ten squares counted.

Count cells touching the middle line of the triple line on the top and left of the squares. Do not count cells touching the middle line of the triple lines on the bottom or right side of the square.

Calculate cell number

10. Determine cells per milliliter by the following calculations:

$$\text{cells/ml} = \text{average count per square} \times \text{dilution factor} \times 10^4$$

$$\text{total cells} = \text{cells/ml} \times \text{total original volume of cell suspension} \\ \text{from which sample was taken.}$$

The number 10^4 is the volume correction factor for the hemacytometer: each square is 1×1 mm and the depth is 0.1 mm.

Stain cells with trypan blue to determine cell viability

11. Determine number of viable cells by adding 0.5 ml of 0.4% trypan blue, 0.3 ml HBSS, and 0.1 ml cell suspension to a small tube. Mix thoroughly and let stand 5 min before loading hemacytometer.

Either 0.4% trypan blue or 0.4% nigrosin can be used to determine the viable cell number. Nonviable cells will take up the dye, while live cells will be impermeable to dye.

12. Count total number of cells and total number of viable (unstained) cells. Calculate percent viable cells as follows:

$$\% \text{ viable cells} = \frac{\text{number of unstained cells}}{\text{total number of cells}} \times 100$$

13. Decontaminate coverslip and hemacytometer by rinsing with 70% ethanol and then deionized water. Air dry and store for future use.

SUPPORT PROTOCOL 4

PREPARING CELLS FOR TRANSPORT

Both monolayer and suspension cultures can easily be shipped in 25-cm² tissue culture flasks. Cells are grown to near confluency in a monolayer or to desired density in suspension. Medium is removed from monolayer cultures and the flask is filled with fresh medium. Fresh medium is added to suspension cultures to fill the flask. *It is essential that the flasks be completely filled with medium to protect cells from drying if flasks are inverted during transport.* The cap is tightened and taped securely in place. The flask is sealed in a leak-proof plastic bag or other leak-proof container designed to prevent spillage in the event that the flask should become damaged. The primary container is then placed in a secondary insulated container to protect it from extreme temperatures during transport. A biohazard label is affixed to the outside of the package. Generally, cultures are transported by same-day or overnight courier.

Cells can also be shipped frozen. The vial containing frozen cells is removed from the liquid nitrogen freezer and placed immediately on dry ice in an insulated container to prevent thawing during transport.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Trypsin/EDTA solution

Prepare in sterile HBSS or 0.9% (w/v) NaCl:

0.25% (w/v) trypsin

0.2% (w/v) EDTA

Store ≤ 1 year (until needed) at -20°C

Specific applications may require different concentrations of trypsin; the appropriate protocols should be consulted for details.

Trypsin/EDTA solution is available in various concentrations including 10 \times , 1 \times , and 0.25% (w/v). It is received frozen from the manufacturer and can be thawed and aseptically divided into smaller volumes. Preparing trypsin/EDTA from powdered stocks may reduce its cost; however, most laboratories prefer commercially prepared solutions for convenience.

EDTA (disodium ethylenediamine tetraacetic acid) is added as a chelating agent to bind Ca^{2+} and Mg^{2+} ions that can interfere with the action of trypsin.

COMMENTARY

Background Information

At its inception in the early twentieth century, tissue culture was applied to the study of tissue fragments in culture. New growth in culture was limited to cells that migrated out from the initial tissue fragment. Tissue culture techniques evolved rapidly, and since the 1950s culture methods have allowed the growth and study of dispersed cells in culture (Freshney, 1993). Cells dispersed from the original tissue can be grown and passaged repeatedly to give rise to a relatively stable cell line.

Four distinct growth stages have been described for primary cells maintained in culture. First, cells adapt to the *in vitro* environment. Second, cells undergo an exponential growth phase lasting through ~ 30 passages. Third, the growth rate of cells slows, leading to a progressively longer generation time. Finally, after 40 or 50 passages, cells begin to senesce and die (Lee, 1991). It may be desirable to study a particular cell line over several months or years, so cultures can be preserved to retain the integrity of the cell line. Aliquots of early-passage cell suspensions are frozen, then thawed and cultures reestablished as needed. Freezing cultures prevents changes due to genetic drift and avoids loss of cultures due to senescence or accidental contamination (Freshney, 1993).

Cell lines are commercially available from a number of sources, including the American Type Culture Collection (ATCC) and the Human Genetic Mutant Cell Repository at the Coriell Cell Repository (CCR; SUPPLIERS APPENDIX). These cell repositories are a valuable re-

source for researchers who do not have access to suitable patient populations.

Critical Parameters

Use of aseptic technique is essential for successful tissue culture. Cell cultures can be contaminated at any time during handling, so precautions must be taken to minimize the chance of contamination. All supplies and reagents that come into contact with cultures must be sterile and all work surfaces should be kept clean and free from clutter.

Cultures should be 75% to 100% confluent when selected for subculture. Growth in culture will be adversely affected if cells are allowed to become overgrown. Passaging cells too early will result in a longer lag time before subcultures are established. Following dissociation of the monolayer by trypsinization, serum or medium containing serum should be added to the cell suspension to stop further action by trypsin that might be harmful to cells.

When subculturing cells, add a sufficient number of cells to give a final concentration of $\sim 5 \times 10^4$ cells/ml in each new culture. Cells plated at too low a density may be inhibited or delayed in entry into growth stage. Cells plated at too high a density may reach confluence before the next scheduled subculturing; this could lead to cell loss and/or cessation of proliferation. The growth characteristics for different cell lines vary. A lower cell concentration (10^4 cells/ml) may be used to initiate subcultures of rapidly growing cells, and a higher cell concentration (10^5 cells/ml) may be used to

initiate subcultures of more slowly growing cells. Adjusting the initial cell concentration permits establishment of a regular, convenient schedule for subculturing—e.g., once or twice a week (Freshney, 1993).

Cells in culture will undergo changes in growth, morphology, and genetic characteristics over time. Such changes can adversely affect reproducibility of laboratory results. Nontransformed cells will undergo senescence and eventual death if passaged indefinitely. The time of senescence will vary with cell line, but generally at between 40 and 50 population doublings fibroblast cell lines begin to senesce. Cryopreservation of cell lines will protect against these adverse changes and will provide a backup in case of contamination.

Cultures selected for cryopreservation should be in log-phase growth and free from contamination. Cells should be frozen at a concentration of 10^6 to 10^7 cells/ml. Cells should be frozen gradually and thawed rapidly to prevent formation of ice crystals that may cause cells to lyse. Cell lines can be thawed and recovered after long-term storage in liquid nitrogen. The top of the freezing vial should be cleaned with 70% alcohol before opening to prevent introduction of contaminants. To aid in recovery of cultures, thawed cells should be reseeded at a higher concentration than that used for initiating primary cultures. Careful records regarding identity and characteristics of frozen cells as well as their location in the freezer should be maintained to allow for easy retrieval.

For accurate cell counting, the hemacytometer slide should be clean, dry, and free from lint, scratches, fingerprints, and watermarks. The coverslip supplied with the hemacytometer should always be used because it has an even surface and is specially designed for use with the counting chamber. Use of an ordinary coverslip may introduce errors in cell counting. If the cell suspension is too dense or the cells are clumped, inaccurate counts will be obtained. If the cell suspension is not evenly distributed over the counting chamber, the hemacytometer should be washed and reloaded.

Anticipated Results

Confluent cell lines can be successfully subcultured in the vast majority of cases. The yield of cells derived from a monolayer culture is directly dependent on the available surface area

of the culture vessel (Freshney, 1993). Overly confluent cultures or senescent cells may be difficult to trypsinize, but increasing the time of trypsin exposure will help dissociate resistant cells. Cell lines can be propagated to get sufficient cell populations for cytogenetic, biochemical, and molecular analyses.

It is well accepted that anyone can successfully freeze cultured cells; it is thawing and recovering the cultures that presents the problem. Cultures that are healthy and free from contamination can be frozen and stored indefinitely. Cells stored in liquid nitrogen can be successfully thawed and recovered in >95% of cases. Several aliquots of each cell line should be stored to increase the chance of recovery. Cells should be frozen gradually, with a temperature drop of $\sim 1^\circ\text{C}$ per minute, but thawed rapidly. Gradual freezing and rapid thawing prevents formation of ice crystals that might cause cell lysis.

Accurate cell counts can be obtained using the hemacytometer if cells are evenly dispersed in suspension and free from clumps. Determining the proportion of viable cells in a population will aid in standardization of experimental conditions.

Time Considerations

Establishment and maintenance of mammalian cell cultures require a regular routine for preparation of media and feeding and passaging of cells. Cultures should be inspected regularly for signs of contamination and to determine if the culture needs feeding or passaging.

Literature Cited

- Freshney, R.I. 1993. *Culture of Animal Cells. A Manual of Basic Techniques*, 3rd ed. Wiley-Liss, New York.
- Lee, E.C. 1991. Cytogenetic Analysis of Continuous Cell Lines. *In The ACT Cytogenetic Laboratory Manual*, 2nd ed. (M.J. Barch, ed.) pp. 107-148. Raven Press, New York.

Key Reference

Lee, 1991. See above.

Contains pertinent information on cell culture requirements including medium preparation and sterility. Also discusses trypsinization, freezing and thawing, and cell counting.

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In culturing mammalian cells *in vitro*, one attempts to reproduce in a culture vessel the physiological environment and characteristic responses of individual cell types. This simple idea is not easily realized because the physiological environments of most cells are not yet completely characterized, and because the complex regulatory interactions between neighboring cells within tissues are still being elucidated. However, a body of knowledge about the survival and growth requirements of cells has been empirically derived over the past 90 years, making it possible to maintain and to propagate a variety of normal, immortalized, and transformed cell types *in vitro*. This received knowledge has directly influenced the compositions of culture media and culture substrata that are in use today, and it continues to evolve as the requirements of individual cell types become more explicitly defined. Part of this evolution may be founded on the ongoing discovery of novel growth factors, growth mediators, and survival factors that comes about as the range of specialized cells that can be propagated *in vitro* is extended. It may be further shaped by advances in understanding of the signaling mechanisms by which culture substrata and other external stimuli influence cells to proliferate, differentiate, become quiescent, or die.

At a minimum, the fluid medium in which cells are cultured must provide for their nutritional requirements, provide an energy source, maintain pH, and provide a level of osmolarity compatible with cell viability. In addition, the environment in which cultures are kept, typically a water-jacketed CO₂ incubator, must provide a constant temperature, humidity to prevent evaporation of medium, O₂ for respiration, and CO₂ for maintenance of the pH of bicarbonate-buffered medium. Culture media commonly used today consist of two parts: a basal nutrient medium and supplements. The basal nutrient medium, such as Dulbecco's modified Eagle's medium (DMEM; also known as Dulbecco's minimal

Table 1.2.1 Tissue Culture Products and Suggested Suppliers^a

Supplier	Culture-related products
Bayer	Fatty acid-free BSA
Becton Dickinson Labware	Growth factors, hormones, Matrigel
Cell Systems	Specialized serum-free media
Chemicon International	Low-density lipoprotein
Clonetics	Specialized serum-free media
Corning Costar	Filter-sterilization apparatus, tissue culture vessels
Difco Laboratories	Agar
Gelman Sciences	Filter-sterilization apparatus
Life Technologies	Basal nutrient media, antibiotics
Hyclone Laboratories	Sera
J.T. Baker	Chemicals
Millipore	Water-purification system
Nalgene	Filter-sterilization apparatus
Research Organics	HEPES
Sigma Chemical	Inorganic and organic chemicals, BSA fraction V, FBS, sterols, antibiotics, growth factors, hormones, tissue culture vessels
Steraloids	Sterols
Upstate Biotechnology Inc. (UBI)	Insulin, transferrin, FBS, type I collagen, growth factors, hormones

^aSee *SUPPLIERS APPENDIX* for suppliers' addresses.

**BASIC
PROTOCOL 1**

Eagle's medium), RPMI 1640, or Ham's F-12, is a buffered aqueous solution of inorganic salts, vitamins, amino acids and other anabolic precursors, energy sources such as glucose and glutamine, and trace elements. Supplements are either undefined, such as fetal bovine serum (FBS), tissue extracts, and conditioned medium, or defined, such as hormones and growth factors, transport proteins, and attachment factors. The compositions of basal nutrient media and medium supplements may vary considerably; however, both components of the complete medium are necessary for support of cell viability and proliferation.

The basic protocols in this unit describe the preparation of serum-supplemented medium (see Basic Protocol 1); serum-reduced or serum-free medium (see Basic Protocol 2); selective medium, such as HAT (see Basic Protocol 3) or cholesterol- and serum-free medium (see Alternate Protocol); and soft agar for anchorage-independent growth (see Basic Protocol 4). The support protocols discuss the use of pH buffers (see Support Protocol 1) and antibiotics (see Support Protocol 2) in culture media. Suppliers for culture products are listed in Table 1.2.1.

NOTE: For all solutions and media, use water purified using a reverse osmosis system (e.g., Milli-Q system from Millipore) and vessels that are thoroughly rinsed with water after use but never washed with detergent.

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

PREPARATION OF SERUM-CONTAINING MEDIA

Many cell types, especially fibroblasts and transformed cells, survive and proliferate to some extent when cultured in a basal nutrient medium supplemented with 5% to 20% (v/v) serum. The degree of cell proliferation achieved under these nonoptimized conditions may be sufficient for the goals of the experiment as long as the cellular properties of interest are not lost. It is worth remembering that if tissue samples consisting of more than one cell type are cultured, this culture environment may confer a growth advantage to fibroblasts. In addition, the medium may select against the propagation of some types of cells: a number of epithelial cell types cease proliferating and differentiate in the "physiological" Ca^{2+} concentrations of commonly used serum-supplemented basal media. Finally, a serum-supplemented nutrient solution does not approximate a physiological environment for any cells except those in a healing wound.

Materials

Basal nutrient medium, such as DMEM, Ham's F-12, or RPMI 1640 (*APPENDIX 2B*; see Critical Parameters for discussion of medium selection)

HEPES (e.g., Research Organics)

Sodium bicarbonate (e.g., J.T. Baker)

Glutamine and pyruvic acid (e.g., Sigma)

Penicillin G and streptomycin sulfate (e.g., Sigma)

5 N NaOH (e.g., J.T. Baker)

Serum (e.g., Hyclone, Sigma, UBI; see Critical Parameters for discussion of fetal bovine serum)

Filter sterilization units (e.g., Nalge Nunc, Corning, Gelman)

0.2- μm -pore-size filters (e.g., Nalge Nunc, Corning, Gelman)

Additional reagents and equipment for culture of mammalian cells (*UNIT 1.1*)

1. Dissolve powdered medium with constant stirring in a 0.8 \times to 0.9 \times volume of water.

If a commercially prepared liquid medium is being used, add penicillin and streptomycin from commercial stock solutions and proceed to step 8.

2. Add an amount of HEPES that yields a concentration of 15 mM in the final volume of medium.

Omit this step if the powdered medium is formulated with HEPES.

3. Add the amount of sodium bicarbonate recommended by the medium supplier for use in a CO₂-controlled atmosphere (e.g., 14 to 36 mM in 5% CO₂ atmosphere).

Omit this step if the powdered medium contains sodium bicarbonate.

4. Add glutamine to give a final concentration of 2 mM and pyruvic acid to give a final concentration of 0.01% (w/v).

5. Add penicillin G to give a final concentration of 100 IU/ml and streptomycin to give a final concentration of 50 µg/ml.

Other antibacterial agents or antifungal agents should not be routinely included in culture medium. Gentamicin at a final concentration of 50 µg/ml or kanamycin at 100 µg/ml may be useful in eliminating gram-positive and gram-negative bacteria from primary cultures or from irreplaceable cultures, but it is best to discard any cultures that are contaminated with bacteria, yeast, or fungi.

6. Adjust the pH of the medium to 7.4 with 5 N NaOH, and add water to achieve the final (1×) volume. Readjust the pH of the medium to 7.4, if necessary.

7. Sterilize the medium by filtration through a 0.2-µm filter. Store the medium at 4°C in the dark.

Vacuum-operated filtering units or bottle-top filters are useful for small volumes of medium (0.1 to 2 liters), whereas filter capsules (2 to 5 liters) or filter stands (>10 liters) that are used under positive pressure are more suitable for larger volumes.

8. Add serum to the desired final concentration at the time of use.

Basal nutrient medium and the serum supplement should be stored individually at 4°C, and the complete medium should be made up at the time of use and only in the volume necessary. Working volumes of serum should be stored at 4°C and used within several weeks. Serum should not be subjected to repeated freezing and thawing, but it can be stored for at least 2 years at -20°C with little deterioration in growth-promoting activity. In this way, medium components are not wasted, and the chances of detecting, isolating, and eliminating contamination with minimal losses are increased.

PREPARING MEDIA FOR REDUCED-SERUM OR SERUM-FREE GROWTH

The most obvious advantages of serum-free cell culture are that it costs less and it simplifies the purification of cell products. However, it is in the increased knowledge of cell physiology that the real value of serum-free medium lies. Both reduced-serum and serum-free media are intermediates in a continuum between completely undefined mixtures of biological fluids and chemically defined, protein-free medium. Although mixtures of biological fluids and protein-free medium have useful applications, neither extreme provides a suitable environment for studies of cellular physiology. At the current time, the most physiologically relevant culture medium for an isolated cell type is a defined, protein-supplemented medium consisting of required components at optimal concentrations and extracellular matrix constituents. This set of conditions can be approached for an increasing number of cell types, but for some cell types, a reduced-serum medium is the best that can be achieved. This state of affairs reflects incomplete knowledge of cell growth requirements, and it suggests that there are novel mediators of cell proliferation and differentiation that remain to be discovered.

BASIC PROTOCOL 2

Cell Culture

1.2.3

Table 1.2.2 Components of Reduced-Serum and Serum-Free Medium^a

Component	Final concentration	Stock concentration	Suggested supplier(s)
Undefined supplements			
Sera ^b	5% to 20% (v/v)	100% (v/v)	Hyclone, Sigma, or UBI
Pituitary extract	5 to 100 µg/ml	1 to 2 mg/ml	UBI, Clonetics
Conditioned medium	5% to 50% (v/v)	100% (v/v)	Not commercially available ^c
Energy sources			
Glucose	1 to 4.5 g/liter	None	Sigma
Glutamine	1 to 2 mM	None	Sigma
Attachment factors			
Collagen type I	10 to 50 µg/ml	3 to 4 mg/ml	UBI, Sigma
Fibronectin	1 to 10 µg/ml	0.5 to 1 mg/ml	Sigma
Vitronectin	1 to 10 µg/ml	0.5 to 1 mg/ml	Sigma
Hormone			
Insulin	1 to 10 µg/ml	1 mg/ml	Sigma, UBI
Carrier proteins			
Transferrin	5 to 30 µg/ml	1 mg/ml	Sigma, UBI
BSA, fatty acid-free	0.5 to 4 mg/ml	50 mg/ml	Bayer
Trace element			
Selenium, sodium salt	1 to 20 nM	2 µM	Sigma
Lipids and lipid precursors			
Ethanolamine	1 to 20 µM	2 mM	Sigma
Fraction V BSA	0.05 to 5 mg/ml	50 mg/ml	Sigma
Unsaturated fatty acids	1 to 10 µg/ml	20 to 50 mg/ml	Sigma
Sterols	1 to 20 µg/ml	2 to 4 mg/ml	Steraloids, Sigma
Low-density lipoprotein	1 to 20 µg/ml	1 to 2 mg/ml	Chemicon International

^aNonsterile stock solutions should be sterilized by filtration. Add glucose and glutamine as dry powder (or frozen aliquots that have been thawed) to reconstituted powdered medium.

^bSee Critical Parameters for discussion of FBS.

^cConditioned medium must be made in the investigator's laboratory, and the choice of cells used depends on the investigator's purpose.

If all the growth requirements of the cells of interest can be satisfied, the undefined medium supplement can be completely eliminated. The limitation of this approach is that not all of the cell growth regulators operating within tissues have been discovered. As the variety of cell types that can be cultured *in vitro* for extended periods expands, new growth activities may be identified and novel growth factors purified.

Materials

Basal nutrient medium, such as DMEM, Ham's F-12, or RPMI 1640 (*APPENDIX 2B*; see Critical Parameters for discussion of medium selection)

Nutrients: inorganic salts, amino acids, and vitamins (e.g., Sigma)

Trace elements (e.g., Sigma)

Supplements: growth factors and hormones (e.g., Sigma, UBI, Becton Dickinson Labware) and other assorted medium components (Table 1.2.2)

Additional reagents and equipment for culture of mammalian cells (*UNIT 1.1*)

Optimize nutrient medium

1. Starting with a complete medium empirically determined to best support the growth of the cells of interest, reduce the concentration of the undefined medium supplement until cell proliferation is suboptimal but cell viability remains high.

2. Vary the concentrations of individual components of the nutrient medium in a cell proliferation assay. Vary the concentrations of components in the following groupings, which have been found to be limiting for proliferation of at least one cell type:
 - a. energy sources (glucose and glutamine);
 - b. trace elements and electrolytes (Na^+ , Cl^- , K^+ , Ca^{2+} , Mg^{2+} , Zn^{2+} , Cu^{2+} , Fe^{2+} , selenium, H_2PO_4^- , and HCO_3^-);
 - c. amino acids (glutamine, cystine, cysteine, histidine);
 - d. vitamins (biotin, vitamin B_{12});
 - e. lipids and lipid precursors (oleic acid, linoleic acid, or cholesterol conjugated to fatty acid-free BSA; undefined lipids bound to fraction V BSA; low-density lipoprotein; ethanolamine or phosphoethanolamine).

Determine the optimal concentration range for the most limiting factor, and fix its concentration in the center of that range. Repeat the growth assays to sequentially identify and optimize each limiting factor.

Concentrations of 0.1 \times , 1 \times , and 10 \times those listed in the chosen medium formulation are initially useful to determine which components are limiting factors for proliferation.

3. Lower the concentration of the undefined supplement and repeat the optimization procedure. Continue optimizing the nutrient medium until reductions in the concentration of the undefined medium supplement can no longer be compensated for.

Each new supplement concentration is tested on an individual culture for a finite period (3 to 10 days). The best concentration, supporting the most cell growth, is incorporated into the next round of testing on new cultures.

Optimize medium supplements

4. Optimize medium supplements such as growth factors, hormones, transport proteins, and attachment factors either independently of or in conjunction with the nutrient medium components.

The set of defined supplements that are required for an individual cell type may be less complex in the presence of an optimized nutrient medium. If an optimized nutrient medium is not being used, then a nutrient-rich basal medium such as DMEM/F-12 (see recipe) is recommended for supplement optimization (see Critical Parameters). As with nutrient optimization, the strategy is to decrease the concentration of an undefined supplement to give suboptimal growth with high cell viability and then to restore proliferation with optimized concentrations of defined supplements.

PREPARATION OF SELECTIVE MEDIA: HAT MEDIUM

Culture medium can be used to intentionally select mammalian cells with desired properties from a mixed population of cells. This strategy is founded on the proven effectiveness of selective media in selecting strains of mutant microorganisms that, for example, cannot grow in minimal media but thrive in a medium supplemented with one particular nutrient. Although mammalian cells with desired characteristics proliferate in selective media, in this case selective pressure is applied to inhibit or, preferably, to kill cells that do not possess those characteristics. Thus, the efficacy of a selective medium depends on the efficiency with which it eliminates unwanted cells and on the degree to which the selected phenotypic trait is expressed. Because monoclonal antibodies are commonly used reagents in cell and molecular biology, the use of selective media that target differences in metabolic pathways is illustrated with two protocols for selecting hybridomas (see Background Information for further discussion). In both protocols, unfused spleen cells do not survive 7 to 10 days.

**BASIC
PROTOCOL 3**

Cell Culture

1.2.5

Materials

Spleen cell × myeloma fusion products (10:1)
RD medium (Life Technologies) with 10% FBS (Hyclone; see recipe)
 4×10^{-5} M aminopterin (A solution; 100× stock in 0.1 N NaOH)
 1×10^{-5} M hypoxanthine/ 1.6×10^{-3} M thymidine in water (HT solution; 100× stock)
HAT medium: RD/10% FBS/1× A solution/1× HT solution
96-well tissue culture plates

Additional reagents and equipment for culture of mammalian cells (UNIT 1.1)

1. Resuspend the fusion products in RD/10% FBS at a concentration of 1×10^6 myeloma cells per ml. Add 0.01× volumes of A and HT solutions to the cell suspension to make HAT selection medium.
2. Plate 0.1 ml of cell suspension per well into 96-well plates. Incubate the plates in a humidified 37°C, 5% CO₂ incubator.
3. Every 2 or 3 days, remove half the existing medium from the wells by aspiration and replace with fresh HAT medium.
4. After 21 days, screen hybridoma supernatants for the presence of the antibodies of interest.
5. Wean hybridomas stepwise from HAT medium by transferring them to HT-supplemented medium and then to RD/10% FBS over a 2-week period. Replace 50% of the medium with HT medium four times at 3-day intervals. Replace 50% of the medium with RD/10% FBS at 3-day intervals.

Aminopterin is toxic, so it is advisable to wean hybridomas from HAT medium as soon as possible.

ALTERNATE PROTOCOL

PREPARATION OF SELECTIVE MEDIA: CHOLESTEROL-FREE, SERUM-FREE MEDIUM

Most of the mouse myeloma cell lines survive in serum-supplemented medium, but they die in the absence of cholesterol (see Background Information for explanation). This conditional lethal defect has been exploited to create an alternative selection process for hybridomas using cholesterol-free medium. Because they are produced by fusion with spleen cells capable of producing cholesterol, NS-1 hybridomas survive in cholesterol-free medium, but the parent NS-1 myeloma cells are selected against. This selective medium allows for the outgrowth of up to 10 times as many hybridomas as HAT medium. This procedure can be used with any myeloma cell line that is unable to synthesize cholesterol.

Materials

Spleen cell × NS-1 myeloma fusion products (10:1)
RD medium with 5F supplement (see recipes)
100× BSA–oleic acid conjugate solution: fatty acid–free BSA (e.g., Bayer; 50 mg/ml) conjugated with oleic acid (e.g., Sigma; 500 µg/ml) in PBS
96-well tissue culture plates

Additional reagents and equipment for culture of mammalian cells (UNIT 1.1)

1. Make complete RD/5F medium by adding appropriate volumes of the stock solutions of insulin, transferrin, ethanolamine, 2-mercaptoethanol, and sodium selenite (e.g., Sigma) to RD medium. Supplement the medium with 1/100 vol of 100× BSA–oleic acid conjugate.

BSA–oleic acid conjugate is available from Sigma. For a protocol, see Kawamoto et al. (1983).

2. Resuspend fusion products at 2 or 3×10^5 NS-1 cells per milliliter of medium.
3. Plate 0.1 ml of cell suspension per well in 96-well plates. Incubate the plates in a humidified 37°C , 5% CO_2 incubator.
4. Add $0.5\times$ volume of fresh medium to the wells every 2 or 3 days after removing half the existing medium by aspiration.
5. Screen hybridoma supernatants for antibodies of interest after 10 to 14 days. Maintain hybridomas in RD/SF medium supplemented with BSA–oleic acid.

GROWTH OF TRANSFORMED CELLS IN SOFT AGAR

Malignantly transformed cells can differ from their normal counterparts in a number of respects. Chief among these differences are a loss of contact-inhibited growth, the acquisition of an infinite life span, and the ability to form tumors in animal hosts. Freedman and Shin (1974) found that there was a general correlation between the tumorigenic potential of transformed cells *in vivo* and their ability to grow in an anchorage-independent manner *in vitro*. Although this generalization does not hold for every transformed cell, growth in soft agar can be used as a surrogate *in vitro* assay for transformation and tumorigenicity. The advantages of this method are that it is relatively easy, it may take much less time than an *in vivo* tumorigenesis assay, and it does not require the maintenance and care of experimental animals. However, if growth in soft agar is used as a measure of transformation of cells that have been manipulated *in vitro*, an *in vivo* tumorigenesis assay is required to determine whether the cells are malignantly transformed.

Materials

2% (w/v) agar (e.g., Difco; see recipe)

Basal nutrient medium, such as DMEM, Ham's F-12, or RPMI 1640 (*APPENDIX 2B*; see Critical Parameters for discussion of medium selection), with 24.6% and 20% FBS

Single-cell suspension

12-well culture plates (e.g., Corning Costar)

15-ml polycarbonate conical centrifuge tubes (e.g., Sarstadt), sterile

1. For each set of replicate wells, add 2.25 ml of 2% agar solution to 9.75 ml of medium containing 24.6% FBS to give a solution of 0.375% agar in 20% FBS. Dispense 2-ml aliquots into five sterile 15-ml polypropylene conical tubes and incubate at 45°C .
2. To separate tubes, add 50, 100, 200, 500, or 1000 cells to a final volume of 0.5 ml medium with 20% FBS. Prepare an additional set of tubes for each set of replicate cultures.
3. Add each cell suspension to an aliquot of agar solution, mix, and quickly pour into a well of a 12-well plate.
4. Incubate the plate at 37°C in a humid atmosphere of 5% CO_2 until cell colonies appear.
5. Count colonies >32 cells (five doublings) under phase contrast with an inverted microscope, and calculate colony formation efficiency (percentage of plated cells that formed colonies).

To prevent any cell attachment to the plastic substratum, cells in 0.3% agar can be overlaid on a preformed layer of 0.5 ml of 0.5% agar in medium supplemented with 20% FBS.

pH CONTROL IN MEDIA

Most cell lines proliferate in medium with a pH of 7.4, and they exhibit decreased viability and rates of proliferation as the medium becomes progressively more acidic or more basic (Eagle, 1973). Culture media must buffer the CO₂ and lactic acid produced as cells metabolize glucose and glutamine. Historically, bicarbonate, HCO₃⁻, in conjunction with atmospheric CO₂, has been used as a buffering system. Each basal medium formulation has a recommended concentration for sodium bicarbonate, usually 20 to 40 mM, to maintain pH and osmolarity. Media that are to be incubated in an elevated CO₂ atmosphere contain higher concentrations of bicarbonate than those designed to be used at ambient CO₂ levels. Most cell culture media require an atmosphere of 5% CO₂ to maintain pH 7.4. However, certain media contain levels of bicarbonate that require different amounts of CO₂. For example, DMEM containing 3700 mg/liter of sodium bicarbonate equilibrates to ~pH 7.6 in a 5% CO₂ environment and requires 10% CO₂ to maintain pH 7.4. The low pK_a of bicarbonate (pK_a = 6.1) makes it a poor buffer around pH 7.4, and, in the absence of atmospheric CO₂, the breakdown of H₂CO₃ formed from bicarbonate releases CO₂ that comes out of solution, causing a rise in pH. With the development of Good buffers (Good et al., 1966), nontoxic buffering agents effective in the pH range of 6 to 8, such as PIPES (pK_a = 6.8), MOPS (pK_a = 7.2), TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; pK_a = 7.5), and HEPES (pK_a = 7.55), became available to the research community. HEPES in a concentration range of 10 mM to 25 mM has become a standard buffer in serum-free medium, but it is used in addition to and not in place of the bicarbonate and CO₂ system. Phenol red is an indicator dye that is commonly added to medium to provide a visual assessment of pH. Red at pH 7.4, it becomes orange (pH 7.0) and then yellow (pH 6.5) as the pH decreases; it turns violet (pH 7.6) and purple (pH 7.8) as the pH rises. Culture medium should generally be replaced as the phenol red changes from orange to yellow, which reflects the accumulation of lactic acid.

Materials

Powdered medium without NaHCO₃ or HEPES
HEPES (e.g., Research Organics)
NaHCO₃ (e.g., J.T. Baker)

1. Dissolve powdered medium in water with gentle stirring.
2. Add HEPES (mol. wt. 238.3) to give a final concentration of 15 mM, and stir until dissolved.
3. Add NaHCO₃ to the recommended concentration for the basal nutrient medium being used and stir.
4. Add other medium components, and adjust the pH to 7.4 (see Basic Protocol 1).
5. Filter sterilize the medium (see Basic Protocol 1, step 7).

Filtering medium under vacuum may cause the pH to increase slightly. A small change in pH need not be compensated because other factors such as medium supplements, temperature, atmospheric pressure, and atmospheric CO₂ levels can also affect pH.

6. Check the pH of the complete medium after it has equilibrated with incubator temperature and CO₂ atmosphere.

USE OF ANTIBIOTICS IN MEDIA

Antibiotics can be added to culture media to eliminate microbial contaminants (Perlman, 1979). The most common contaminants encountered are bacteria, yeast, other fungi, and mycoplasma, and the most common routes of contamination are operator error and nonsterile medium components. Of the common microbial contaminants, yeast and other fungi are very difficult to eradicate, and it is recommended that all heavily contaminated cultures be discarded unless the cells cannot be replaced. Thus, it is wise to maintain duplicate cultures of important cells and to cryopreserve cell lines as soon as possible. Penicillin and streptomycin are broad-spectrum antibacterial agents that are often added to culture media (see Basic Protocol 1 and Table 1.2.3). However, the routine use of antibiotics in media is not recommended, because when used to compensate for poor aseptic technique, they may select for antibiotic-resistant strains of microorganisms. Antibiotics may be used to best effect in primary cultures of cells for which the sterility of the tissue samples is in doubt. The antifungal agent amphotericin B should be used sparingly, as it is toxic to mammalian cells (Perlman, 1979), and it may select for the property of cholesterol auxotrophy (Sato et al., 1987). Mycoplasma and viruses are too small to be retained by 0.2- μm sterilization filters; mycoplasma can be treated with gentamicin or kanamycin, but viruses cannot be treated with antibiotics. There is no reliable method for eliminating viral contaminants from cell cultures.

To be effective in culture, antibiotics must have the following characteristics: they must completely eliminate the microbial contaminant; they must not affect the viability or metabolism of mammalian cells; and they must be compatible with medium components in an aqueous environment. In addition, because the identity of a contaminating species is usually unknown, antibiotics should act on a broad spectrum of microorganisms. A list of antibiotics commonly used in culture media is provided in Table 1.2.3.

Materials

Antibiotic
Sterile solvent
0.2- μm -pore-size sterilizing filter

1. Dissolve the antibiotic at a 100 \times or greater concentration in an appropriate sterile solvent. If it is highly soluble in an aqueous solvent, use water or PBS.
2. If the antibiotic was not obtained in a sterile form, filter the solution through a 0.2- μm filter.
3. Store the antibiotic solution at 4°C prior to use or at -20°C for long-term storage.
4. Add the antibiotic to medium immediately prior to use.

Table 1.2.3 Some Antibiotics Used in Culture Media and Their Microbial Targets

Antibiotic	Concentration	Microbial targets
Amphotericin B	2.5 $\mu\text{g}/\text{ml}$	Yeast and other fungi
Ampicillin	100 $\mu\text{g}/\text{ml}$	Gram-positive and -negative bacteria
Chloramphenicol	5 $\mu\text{g}/\text{ml}$	Gram-negative bacteria
Gentamicin	50 $\mu\text{g}/\text{ml}$	Gram-positive and -negative bacteria, mycoplasma
Kanamycin	100 $\mu\text{g}/\text{ml}$	Gram-positive and -negative bacteria, mycoplasma
Penicillin G	100 IU/ml	Gram-positive bacteria
Streptomycin	100 $\mu\text{g}/\text{ml}$	Gram-positive and -negative bacteria
Tetracycline	10 $\mu\text{g}/\text{ml}$	Gram-positive and -negative bacteria, mycoplasma

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Agar

Dissolve 2% (w/v) agar (e.g., Difco) in water by boiling it or heating it in a microwave. Maintain soft agar at 45°C.

DMEM/F12 medium, supplemented

Make 1:1 (v/v) mixture of DMEM and F-12. Filter sterilize the medium and store at 4°C. Add the appropriate volume of serum at the time of use.

Five factors (5F) supplement

Make the following 200× stock solutions:

2 mg/ml insulin in 10 mM HCl

2 mg/ml transferrin in PBS

2 mM ethanolamine in water

2 mM 2-mercaptoethanol in water

2 μM sodium selenite in water

Filter sterilize the stock solution and store at 4°C prior to use

Add 0.5 ml of each stock solution per 100 ml of medium

RD medium, supplemented

Make 1:1 (v/v) mixture of RPMI 1640 and DMEM. Add glutamine to 2 mM, penicillin G to 100 IU/ml, and streptomycin to 50 μg/ml. Filter sterilize the medium and store at 4°C. Add the appropriate volume of serum at the time of use.

COMMENTARY

Background Information

It is beyond the scope of this unit to describe all of the medium formulations that have been developed for mammalian cell culture. The following references, which provide surveys of media and conditions that have been used successfully to culture numerous cell lines and a variety of cell types, are recommended as sources of this information: Bottenstein et al. (1979); Jacoby and Pastan (1979); Barnes et al. (1984); Freshney (1987, 1992); Baserga (1989); Davis (1994); and Sato et al. (1994).

Because the immediate aim of the cell culturist is to maintain or expand a population of cells, culture media have been developed with an emphasis on maintaining cell viability and stimulating cell proliferation. These goals have necessarily led to culture media and culture conditions that select for cells that can proliferate at the expense of those that cannot. Thus, the choice of culture medium and the culture conditions used affect the properties and types of cells that can be studied in vitro. As an example of selection, normal rodent cells cultured on a plastic substratum in the presence of fetal bovine serum often go through a period of "crisis" in which a minority population of ane-

uploid cells survive and proliferate as immortalized but not completely transformed cells. Another example of unintentional selection through in vitro cell culture is the outgrowth of basal keratinocytes from cultured epidermal cells; this occurs because keratinocytes in the suprabasal layers of the epidermis differentiate and lose the ability to proliferate.

Conversely, for some applications, culture media have been developed to deliberately select for the survival of specific cell types of interest. Low-calcium, serum-free media developed for many epithelial cell types (Sato et al., 1994) are selective in that they promote the proliferation of epithelial cells but inhibit the outgrowth of fibroblasts in primary cultures; surviving fibroblasts are eliminated by dilution as the cultures are repeatedly passaged (UNIT 1.1). Selective media are generally designed to exploit differences in susceptibility to metabolic inhibitors or differences in metabolic pathways. An example of a metabolic inhibitor as a selection agent is the use of the antibiotic G418 (geneticin) to kill nontransfected mammalian cells while allowing the growth of transfectants containing the bacterial gene for aminoglycoside phosphotransferase (*neo^r*; Southern and Berg, 1982).

Endogenous synthesis of purines and pyrimidines, which are essential components of nucleosides and nucleotides, can be blocked in mammalian cells by the folic acid analogue aminopterin (A). Such a block is circumvented in normal cells by salvage pathways that use hypoxanthine (H) and thymidine (T). However, cells deficient in the salvage pathway enzymes hypoxanthine-guanine phosphoribosyltransferase (HGPRT⁻) and thymidine kinase (TK⁻) cannot produce purines or pyrimidines by means of the salvage pathways in the presence of aminopterin, and die. Thus, HAT medium (Littlefield, 1964) selects for cells that contain functional HGPRT and TK enzymes, and it selects against cells that are HGPRT⁻ or TK⁻. This selection system has been made applicable to the selection of hybridomas by the generation of HGPRT⁻ mouse myeloma cell lines. When these cells are fused with HGPRT⁺ spleen cells from an immunized mouse, the resulting HGPRT⁺ hybridomas survive in HAT medium, and the parental myeloma cells die.

Another example of the use of selective media takes advantage of cell line deficiencies. Most of the mouse myeloma cell lines that are commonly used to generate hybridomas (P3-X63-Ag8, NS-1-Ag4-1, X63-Ag8.653, and NS-0) are clonally derived from the MOPC21 tumor cell line P3 (Horibata and Harris, 1970), and they are all unable to synthesize cholesterol (Sato et al., 1984, 1987), which is an essential component of the plasma membrane. This trait is not a common characteristic of murine lymphoid cells or of mammalian cells in general, and it may have arisen from prolonged exposure to the antifungal agent amphotericin B (Sato et al., 1987). In all of these related myeloma cell lines, the defect in cholesterol biosynthesis has been traced to the enzyme 3-ketosteroid reductase (Sato et al., 1988). In cholesterol-free medium, NS-1 hybridomas are selected for while the cholesterol auxotrophic NS-1 parent cells are selected against (Myoken et al., 1989).

One must bear in mind that it is usually only after a population of cells has been expanded in culture that they can be characterized with respect to their physiological or differentiated properties. At one time it was thought that cells normally dedifferentiated in vitro and that culture techniques could not be used to study differentiated cellular functions. Subsequently, it was found that the dedifferentiation phenomenon resulted from the overgrowth of differentiated cells by contaminating fibroblasts. Once this problem was recognized, the first

differentiated animal cell lines were established (Buonassisi et al., 1962; Yasumura et al., 1966). Thus, it is incumbent upon the cell culturist to choose culture media and culture conditions that not only support the viability and proliferation, if possible, of the cells of interest, but that allow those cells to manifest some or all of their differentiated properties in vitro.

From a historical viewpoint, it is of interest that early culture media consisted of undefined mixtures of biological fluids, tissue extracts, and simple salt solutions. The first continuous mammalian cell line, the mouse L cell fibroblast line (Sanford et al., 1948), was established from 20-methylcholanthrene-treated C3H mouse tissue explants grown in chicken plasma clots in 40% horse serum, 20% chick embryo extract, and 40% saline. The first continuous human cell line, the HeLa cervical adenocarcinoma line (Gey et al., 1952), was isolated in a mixture of chicken plasma, bovine embryo extract, and human placental cord serum. At about the same time, attempts were being made to make culture media more defined by creating synthetic nutrient media and by determining the nutritional requirements of cells in culture. An early synthetic nutrient medium, medium 199 (Morgan et al., 1950), was created to increase the longevity of primary chicken muscle cell cultures that were started as tissue explants in Earle's salt solution with 40% horse serum and 1% chick embryo extract. Medium 199 consisted of a salt solution with amino acids, vitamins, purines, pyrimidines, pentose sugars, adenosine triphosphate (ATP), adenylic acid, Tween 80 as a source of oleic acid, cholesterol, antioxidants, and iron in the form of ferric nitrate. Although medium 199 promoted the outgrowth of "large, flat and spindle-shaped" cells from the original tissue fragments, none of the medium components except glutamine clearly enhanced the life span of the cultures. The pioneering work of Eagle (Eagle, 1955) showed that L cells and HeLa cells had similar, demonstrable nutritional requirements for the thirteen essential amino acids, seven vitamins, glucose or other carbohydrates, and electrolytes. Strikingly, neither L cells nor HeLa cells would grow under these minimal essential conditions without the further addition of a small amount of dialyzed serum protein. Nonetheless, this research marked the beginning of concerted efforts to optimize basal nutrient media and to determine the growth requirements of cells in vitro.

Culture media for a number of normal, immortalized, and transformed cells have been

improved through the application of two complementary strategies. The approach of Ham and his colleagues has been to optimize the compositions of basal nutrient media for individual cell lines or cell types (Ham and McKeehan, 1979; Ham, 1984; Bettger and McKeehan, 1986) in the presence of ever-decreasing concentrations of dialyzed serum protein. These efforts gave rise to Ham's F-12 nutrient medium and the MCDB media, which are commonly used today. MCDB media are optimized basal media developed for specific cell types by R. Ham and colleagues. In studying hormonally responsive cell lines in culture, Sato and his colleagues realized that a major role of serum in culture medium was to provide hormones and hormone-like growth factors that were required for cell proliferation and expression of differentiated functions (Bottenstein et al., 1979; Barnes and Sato, 1980; Barnes, 1987). This understanding led them to replace serum with purified hormones, growth factors, transport proteins, and attachment factors as supplements for preexisting nutrient media. In combination, these two experimental approaches demonstrated (1) that basal nutrient media could be optimized for individual cell types, but optimal cell proliferation required additional hormones or growth factors, transport proteins, and attachment mediators in the absence of serum, and (2) that the combinations of purified medium supplements for individual cell lines could be simplified when using an optimized basal medium.

General conclusions that can be drawn from the work of Ham and Sato are: (1) individual cell types require quantitatively balanced sets of nutrients, of which some are cell type specific; (2) cell proliferation and differentiated properties are regulated by hormones, growth factors, protein-bound nutrients, and attachment factors, of which many are present in serum and tissue extracts; (3) most cells in culture are growth stimulated by the serum components insulin and insulin-like growth factors, the iron-transporting protein transferrin, and unsaturated fatty acids or lipoproteins; and (4) because different cell types have similar but different growth requirements, it is unlikely that a single medium formulation will prove optimal for all cells.

Critical Parameters

When choosing or developing a cell culture medium, the single most important parameter is cell viability. This holds true whether the medium is used to maintain a population of

differentiated cells, to stimulate cell proliferation, or to optimize the yield of a cellular product. An adequate serum-containing, serum-reduced, or serum-free medium formulation should promote a high degree of cell viability. Conversely, poor cell viability is a good indication that the culture medium or culture environment is deficient in one or more essential components. Suggestions for improving culture media are provided in Basic Protocols 1 and 2, and the reader is referred to *UNIT 1.1* for methods of assessing cell viability. On occasion, changes in culture conditions, such as a switch from serum-supplemented to serum-free medium, may cause the majority of cells in a culture to die, followed by the outgrowth of a surviving subpopulation of cells. Although this phenomenon has been referred to in the literature as adaptation or weaning, it is more likely to be a selective process in which the surviving cells differ phenotypically from the parental population. The investigator can reduce the chances of phenotypic changes becoming fixed in a population of cells by maintaining cells in culture medium that supports a high level of viability, by using low split ratios when passaging cells, and by periodically returning to cryopreserved stocks of low-passage-number cells.

The first choice for a basal nutrient medium should be one that other investigators have used successfully to culture the cells of interest and have reported in the literature. If for some reason that medium is not adequate for the purposes at hand, a number of basal media should be tested for the ability to support the proliferation of the cells of interest and to maintain their phenotypic properties. It is useful to start with basal media that have been used with similar or related cell types and in similar culture conditions (e.g., clonal or high-density cultures), but basal media developed for unrelated cell types may also yield good results (Ham, 1984). Commercially available basal media commonly used for continuous cell lines are DMEM; Ham's F-12 medium; a 1:1 mixture (v/v) of DMEM and Ham's F-12 medium (DMEM/F-12); and RPMI 1640, which was originally developed for lymphoid cells. The MCDB media were developed by Ham and his colleagues for individual types of normal cells, but they may also be effective on continuous cell lines. Most of these basal media are qualitatively similar but differ quantitatively. The basal medium selected based on empirical testing can be used as a starting point for further optimization. For excellent discussions on cel-

lular nutrition and procedures for optimizing basal nutrient medium see Ham and McKeehan, 1979; Ham, 1984; and Bettger and McKeehan, 1986.

The most commonly used serum supplement is fetal bovine serum (FBS). Less expensive alternatives to FBS are calf serum, newborn calf serum, calf serum fortified with transferrin or growth factors (available from Hyclone and Sigma), and horse serum. For normal human lymphocytes, the use of commercial human serum treated at 56°C for 30 min to inactivate complement may be appropriate. The most suitable serum supplement for the cells of interest should be determined empirically. Although variability in efficacy between batches of FBS owing to variations in composition is not as problematic as it once was, it is advisable to test batches of FBS at several concentrations for the ability to support the proliferation of cells of interest at low and high densities. Clonal growth assays are the most stringent tests of the efficacy of batches of serum, but acceptable batches of serum should also be able to support, at reasonable concentrations, the viability of high-density cultures.

A number of reduced-serum or serum-free media have been developed for continuous cell lines and nontransformed cells (Bottenstein et al., 1979; Jacoby and Pastan, 1979; Barnes et al., 1984; Freshney, 1987, 1992; Baserga, 1989; Davis, 1994; Sato et al., 1994), and most are optimized for a single cell line or cell type. However, the similarities between media developed for related cells are increasing the understanding of the nutritional and growth factor requirements of individual cell types, which in turn is making the development of serum-free medium a more rational process (Sato et al., 1994). As in choosing a basal nutrient medium for serum-supplemented medium, the best choice for a reduced-serum or serum-free medium is one that has been used by other investigators for the same or a related cell line or cell type. It is desirable to use a defined, serum-free medium whenever possible, as this affords the investigator the greatest degree of control over an *in vitro* culture-based experiment.

Proprietary serum-free media for specific cell types are commercially available from companies such as Clonetics and Cell Systems (see Table 1.2.1). If an optimized, preexisting serum-free medium is not available, then a serum-free or serum-supplemented medium that supports cell viability and suboptimal proliferation can be used as a starting point for

further medium development. As described above (see Background Information), two complementary strategies for improving culture media are to optimize the components of the basal nutrient medium (Ham and McKeehan, 1979; Ham, 1984) and to replace serum or other undefined medium components with defined, purified protein and nonprotein supplements (Bottenstein et al., 1979; Barnes and Sato, 1980; Barnes, 1987; Sato et al., 1994). Under both strategies, concentrations of serum, dialyzed serum, or other undefined supplements are lowered stepwise to reduce the rate of cell proliferation, and the concentrations of defined medium components are individually manipulated until cell proliferation is restored. A completely defined culture medium or a much reduced serum-supplemented medium should be attained after a number of rounds of optimization. See Basic Protocol 2 for description of both these approaches.

Clues as to which defined supplements are likely to be growth stimulatory for the cells of interest are provided by the following sources: hormones or growth factors that act on the cell type of interest *in vivo*; autocrine factors that the cells have been found to produce *in vitro*; and defined supplements that have been included in serum-free media developed for similar or related continuous cell lines or cell types. Thus, the first place to search for potentially useful supplements is the literature. The following general suggestions are based on the serum-free media that have been developed over the past 20 years.

1. Most cells are growth stimulated by insulin or insulin-like growth factor I and require iron obtained by the iron-transporting protein transferrin.

2. Fatty acid-free BSA is a useful carrier protein for unsaturated fatty acids, sterols, and steroid hormones, which are insoluble in an aqueous solvent.

3. Cells of epithelial origin often respond to epidermal growth factor (EGF), acidic fibroblast growth factor (aFGF or FGF-1), and dexamethasone or hydrocortisone.

4. Mesenchymal cells respond to EGF, aFGF, basic FGF (bFGF or FGF-2), and platelet-derived growth factor (PDGF).

5. In the absence of serum-derived attachment mediators, treating tissue culture plastic with attachment factors, such as type I collagen (*UNIT 10.3*), fibronectin, vitronectin, and laminin (*UNIT 10.2*), or with an incompletely defined, natural extracellular matrix (e.g., Matrigel, from Becton Dickinson Labware; *UNIT 10.2*) may

enhance the plating efficiencies and growth rates of adherent cells.

It is important to use highly pure water in preparing media and medium additives. Double-distilled water was a standard ingredient in medium for many years, but it has been superseded by purification systems that incorporate reverse osmosis. In the Milli-Q system, locally supplied water is subjected to deionization, reverse osmosis, and filtration through activated charcoal and a sterilizing filter. Use a vessel that is thoroughly rinsed with water after use but never washed with detergent.

Anticipated Results

As the survival and growth requirements of individual cell lines and cell types become better understood, the routine culture conditions for cells of interest become more defined. When all of the growth requirements of cells of interest are understood, any undefined medium supplements that were previously required can be completely eliminated. Defined culture conditions afford the investigator the greatest degree of control over in vitro culture experiments, and they provide more accurate insights into cellular physiology in vivo.

Time Considerations

Optimizing a basal nutrient medium or developing a serum-free medium formulation is not a trivial undertaking, and it can be very time-consuming with no guarantee of success. Thus, the investigator should carefully consider how important defined culture conditions are to the experimental goals before taking on either task. However, as optimized and defined media are developed for a wider array of cell types, it is becoming easier and less time-consuming to create defined media for additional types of cells. The amount of time required to mix and sterilize 5- to 20-liter batches of medium should not exceed 2 to 4 hours.

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Aseptic Technique for Cell Culture

This unit describes some of the ways that a laboratory can deal with the constant threat of microbial contamination in cell cultures. Microorganisms are ubiquitous. Bacteria can be isolated from nearly any surface including inanimate objects and human skin. Fungal spores and bits of vegetative hyphae drift into a laboratory from air conditioning ducts and open doors. Mycoplasma infections most frequently originate from improperly sterilized media or serum. At the risk of eliciting paranoia in the novice cell culture user who has no training in microbiological techniques, the possibility for microbial contamination exists everywhere. Inherent with successful manipulation of cell cultures is the basic understanding that everything that comes into contact with the cells must be sterile or noncontaminating. This includes media, glassware, and instruments, as well as the environment to which the cultures are briefly exposed during transfer procedures. Because cleaning up a contaminated culture is too frequently a disheartening and unsuccessful experience, the best strategy is to employ procedures to prevent microbial contamination from occurring in the first place.

This unit begins with a protocol on aseptic technique (see Basic Protocol 1). This catch-all term universally appears in any set of instructions pertaining to procedures in which noncontaminating conditions must be maintained. In reality, aseptic technique cannot be presented in one easily outlined protocol, but rather encompasses all aspects of environmental control, personal hygiene, equipment and media sterilization, and associated quality control procedures needed to ensure that a procedure is, indeed, performed with aseptic, noncontaminating technique. Although cell culture can theoretically be carried out on an open bench in a low-traffic area, most cell culture work is carried out using a horizontal laminar-flow clean bench (see Basic Protocol 2) or a vertical laminar-flow biosafety cabinet (see Alternate Protocol). Subsequent units within this chapter address these diverse considerations—e.g., sterilization and disinfection, use of antibiotics, and quality control. Where applicable, use presterilized, disposable labware and other equipment. The wide availability and reliability of these products has simplified cell culture, particularly for small-scale laboratory needs.

ASEPTIC TECHNIQUE

This protocol describes basic procedures for aseptic technique for the novice in cell culture technology. One basic concern for successful aseptic technique is personal hygiene. The human skin harbors a naturally occurring and vigorous population of bacterial and fungal inhabitants that shed microscopically and ubiquitously. Most unfortunately for cell culture work, cell culture media and incubation conditions provide ideal growth environments for these potential microbial contaminants. This procedure outlines steps to prevent introduction of human skin flora during aseptic culture manipulations.

Every item that comes into contact with a culture must be sterile. This includes direct contact (e.g., a pipet used to transfer cells) as well as indirect contact (e.g., flasks or containers used to temporarily hold a sterile reagent prior to aliquoting the solution into sterile media). Single-use, sterile disposable plastic items such as test tubes, culture flasks, filters, and pipets are widely available and reliable alternatives to the laborious cleaning and sterilization methods needed for recycling equivalent glass items. However, make certain that sterility of plastic items distributed in multiunit packages is not compromised by inadequate storage conditions once the package has been opened.

BASIC PROTOCOL 1

Ideally, all aseptic work should be conducted in a laminar cabinet (see Basic Protocol 2 and Alternate Protocol). However, work space preparation is essentially the same for working at the bench. Flame sterilization is used as a direct, localized means of decontamination in aseptic work at the open bench. It is most often used (1) to eliminate potential contaminants from the exposed openings of media bottles, culture flasks, or test tubes during transfers, (2) to sterilize small instruments such as forceps, or (3) to sterilize wire inoculating loops and needles before and after transfers. Where possible, flame sterilization should be minimized in laminar-flow environments as the turbulence generated by the flame can significantly disturb the sterile air stream.

Materials

Antibacterial soap
70% ethanol or other appropriate disinfectant
95% ethanol

Clean, cuffed laboratory coats or gowns
Latex surgical gloves
Clean, quiet work area
Shallow discard pans containing disinfectant
Bunsen burner or pilot-activated burner (e.g., Touch-o-Matic, VWR)

Take personal precautions

1. Just prior to aseptic manipulations, tie long hair back behind head. Vigorously scrub hands and arms at least 2 min with an antibacterial soap.

Superficial lathering is more prone to loosening than removing flaking skin and microbial contaminants. Loosely adhering skin flora easily dislodge and can potentially fall into sterile containers.

2. Gown appropriately. For nonhazardous sterile-fill applications, wear clean, cuffed laboratory coats and latex gloves.

Greater stringencies may be necessary depending upon laboratory regulatory requirements. Work with potentially hazardous agents certainly mandates additional considerations for safety. Front-closing laboratory coats are not recommended for work with hazardous biological agents. Safety glasses should be worn by laboratory personnel when manipulating biological agents outside the confines of a biosafety cabinet.

3. Frequently disinfect gloved hands with 70% ethanol while doing aseptic work.

Although the gloves may initially have been sterile when first worn, they will no doubt have contacted many nonsterile items while in use.

Note that 70% ethanol may not be an appropriate agent for latex glove disinfection when working with cultures containing animal viruses, as studies have shown that ethanol increases latex permeability, reducing protection for the wearer in the event of exposure. In this case, quaternary ammonium compounds are more appropriate.

4. Dispose of gloves by autoclaving after use. Do not reuse. Bag and autoclave single-use laboratory coats after use. Bag, autoclave (if necessary), and wash other laboratory coats within the laboratory facility or send out for cleaning at a laundry certified for handling biologically contaminated linens.

Never take laboratory clothing home for washing.

5. Thoroughly wash hands after removing protective gloves.

Prepare and maintain the work area

6. Perform all aseptic work in a clean work space, free from contaminating air currents and drafts. For optimal environmental control, work in a laminar-flow cabinet (see Basic Protocol 2 and Alternate Protocol).
7. Clear the work space of all items extraneous to the aseptic operation being performed.
8. Wipe down the work surface before and after use with 70% ethanol or other appropriate disinfectant.
9. Wherever feasible, wipe down items with disinfectant as they are introduced into the clean work space. Arrange necessary items in the work space in a logical pattern from clean to dirty to avoid passing contaminated material (e.g., a pipet used to transfer cultures) over clean items (e.g., flasks of sterile media).
10. Immediately dispose of any small contaminated items into a discard pan.
11. When the aseptic task has been completed, promptly remove any larger contaminated items or other material meant for disposal (e.g., old culture material, spent media, waste containers) from the work space and place in designated bags or pans for autoclaving. Disinfect the work space as in step 8.

Flame sterilize the opening of a vessel

12. For a right-handed person, hold the vessel in the left hand at ~45° angle (or as much as possible without spilling contents) and gently remove its closure. Do not permit any part of the closure that directly comes in contact with the contents of the vessel to touch any contaminating object (e.g., hands or work bench).

Ideally, and with practice, one should be able to hold the closure in the crook of the little finger of the right hand while still being able to manipulate an inoculating loop or pipettor with the other fingers of the hand.

Holding the vessel off the vertical while opening will prevent any airborne particulates from entering the container.

13. Slowly pass the opening of the vessel over the top of (rather than through) a Bunsen burner flame to burn off any contaminating matter.

Be careful when flaming containers of infectious material. Any liquid lodged in the threads of a screw cap container will spatter as it is heated. Aerosols thus formed may actually disseminate entrapped biological agents before the heat of the flame is hot enough to inactivate them.

14. While still holding the vessel at a slant, use a sterile pipet and pipettor to slowly add or remove aliquots to avoid aerosol formation.
15. Flame-sterilize again as in step 13, allow the container to cool slightly, and carefully recap the vessel.

Flame sterilize small hand instruments

16. Dip critical areas of the instrument (i.e., those that come into contact with the material of concern) in 95% ethanol.

Make certain that the alcohol is in a container heavy enough to support the instrument without tipping over.

CAUTION: 95% ethanol is flammable; keep the container at a safe distance from any open flame.

17. Remove the instrument from the alcohol, being careful not to touch the disinfected parts of the instrument. Allow excess ethanol to drain off into the container.
18. Pass the alcohol-treated part of the instrument through the flame of a Bunsen burner and allow residual alcohol to burn off.
19. Do not let the sterilized portion of the instrument contact any nonsterile material before use. Let the heated part of the instrument cool for ~10 sec before use.
20. After use, return the instrument to the alcohol disinfectant until needed again.

Flame sterilize inoculating loops and needles

21. Hold the inoculating wire by its handle and begin in the center of the wire to slowly heat the wire with the flame of a Bunsen burner. Proceed back and forth across the wire's full length until it glows orange.
22. While still holding the handle, allow the inoculating wire to cool back to room temperature (~10 sec) before attempting any transfer of material.

If transfers are made while the inoculating wire is hot, cells will be killed by the hot wire, and aerosols created from spattering material can disperse biological material throughout the work space.
23. After the transfer is made, reheat the inoculating wire as in step 21 to destroy any remaining biological material. Let cool to room temperature before putting aside for next use.

**BASIC
PROTOCOL 2**

USE OF THE HORIZONTAL LAMINAR-FLOW CLEAN BENCH

Laminar-flow cabinets (hoods) are physical containment devices that act as primary barriers either to protect the material being manipulated within the hood from worker-generated or environmental sources of contamination, or to protect the laboratory worker and laboratory environment from exposure to infectious or other hazardous materials that are present within the hood. Cell culture applications utilize two types of laminar-flow hoods: (a) the horizontal-flow clean bench (described here) and (b) the biological safety cabinet (see Alternate Protocol). Both types of hoods use a high-efficiency particulate air (HEPA) filter and blowers that generate a nonmixing stream of air.

The horizontal laminar-flow clean bench is used to provide a near-sterile environment for the clean (i.e., noncontaminating) handling of nonhazardous material such as sterile media or equipment. Because the air stream pattern directs the flow of air within the hood directly back to the hood operator and the room (Fig. 1.3.1), horizontal flow hoods are never to be used with infectious agents or toxic chemicals.

Materials

- 70% ethanol or other disinfectant
- Horizontal laminar-flow hood, certified for use
- Swabs (e.g., cheesecloth, paper towels)
- Pilot light-activated Bunsen burner (e.g., Touch-o-Matic, VWR)

1. Completely clear the bench of the laminar-flow hood and disinfect the bench working surface and the left and right sides of the hood with 70% ethanol or other disinfectant. Do not spray the back (gridded) wall where the HEPA filter is housed.

Resist the urge to leave frequently used items (e.g., pipet canisters or a bag of disposable plastic tissue culture flasks) in the hood between uses. Their presence makes thorough disinfection of the work space difficult.

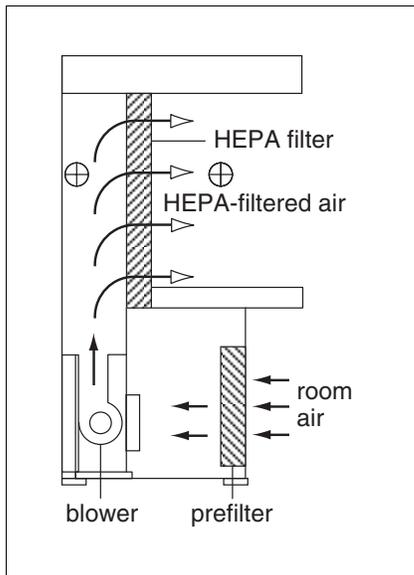


Figure 1.3.1 Horizontal laminar-flow clean cabinet. Solid arrows, dirty room air; open arrows, clean HEPA-filtered air; circled +, positive pressure with respect to room air.

2. Turn the hood blower and lights on and let the air circulate within the hood 10 min before use.
3. Place items needed for the specific procedure into the hood, wiping each item with 70% ethanol or other disinfectant just before introducing it into the laminar environment.

Do not overcrowd the work space. For horizontal laminar-flow effectiveness, maintain a clear path between the work area and the back wall of the cabinet where the HEPA filter is located.

4. Wash hands well before working in the hood and wear a clean laboratory coat and surgical gloves to further protect the work from shedding of skin flora that can contaminate any product (see Basic Protocol 1).
5. While working in the hood, perform all work at least 4 in. back from the front opening, and avoid rapid movements that might disrupt the laminar air flow. Avoid moving materials or hands in and out of the cabinet as much as possible.
6. If flame sterilization is needed in the hood for a particular application, use a burner that can be activated by a pilot light when needed, rather than one that burns constantly.

The open flame of a Bunsen burner causes turbulence that disrupts the unidirectional laminar air flow.

7. When work is completed, remove all material from the laminar work bench, clean any spills, and disinfect the bench working surface by wiping with 70% ethanol or other disinfectant.
8. Turn off hood blower and lights.

USE OF THE VERTICAL LAMINAR-FLOW BIOSAFETY CABINET

Biological safety cabinets provide a clean, safe environment for both the worker and the product. The Class II, Type A biosafety cabinet (Fig. 1.3.2) is frequently encountered in cell culture laboratories, and this protocol describes the use of this type of barrier device. The Class IIA biosafety cabinet is suitable for work with low- to moderate-risk biological agents in the absence of toxic or radioactive chemicals.

Materials (also see Basic Protocols 1 and 2)

Class II, Type A Biosafety Cabinet (BSC), certified for use
Pilot light-activated Bunsen burner (e.g., Touch-o-Matic, VWR) or electronic incinerator (e.g., Bacti-Cinerator III, VWR)
Closed-front laboratory gowns (for personnel working with biological agents)

1. Turn the hood blower on and verify air flow by feeling (by hand) the current near the front grill of the work surface. Turn the germicidal UV light off if it is on. Turn the fluorescent light on.

Before use, the cabinet should already be empty and clean from prior activity. The view window should be lowered to the proper operating height (normally 8 in.) or as specified by the cabinet manufacturer.

UV light is effective only for decontaminating clean, solid surfaces with which it comes in contact. It is not effective in decontaminating the cabinet air flow. UV light is not effective against bacterial spores. UV germicidal light tubes should be replaced frequently (at least every 6 months for biosafety cabinets in use on a daily basis) to assure that they are emitting light at 254 nm and at an intensity appropriate for decontamination.

CAUTION: *UV light is harmful to the eyes. Laboratory personnel should not be near the cabinet or looking at the UV light when it is in use.*

2. Wash and gown as required for the operation (see Basic Protocol 1, steps 1 to 5).
3. Wipe down the entire interior cabinet work surface area with 70% ethanol or other appropriate disinfectant.
4. Let blower run for 10 min to filter the cabinet air of any particulates.

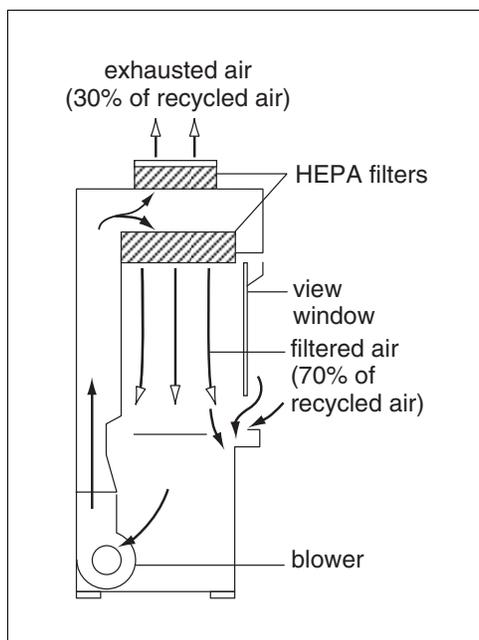


Figure 1.3.2 Biological safety cabinet, Class II, Type A. Note that filtered air is contaminated after passing through the work space, and is filtered again whether it is recycled to the workspace (70%) or exhausted (30%). Solid arrows, dirty (room/contaminated) air; open arrows, filtered air.

5. Raise the front view window as needed to bring necessary items into the cabinet. Wipe each item with 70% ethanol or other disinfectant as it is placed in the cabinet.

Do not crowd the work space and make sure no air vents are blocked by supplies or equipment. Do not position material so that it obscures any of the air vents at the front edges of the laminar hood. One frequent source of air flow restriction in biosafety cabinets is "lost" paper towels that have been drawn into the air ducts at the back of the work surface.
6. Organize the work surface for a clean-to-dirty work flow. Place clean pipets, flasks, and sterile media bottles at one side of the cabinet; place discard pans, spent cultures, and other wastes on the other side.
7. Return the view window to the 8-in. operating level. Wait ~10 min for the blowers to filter the disturbed cabinet air before starting work.
8. While working, keep all material and perform work ≥ 4 in. back from the front opening of the cabinet, and minimize rapid movements or activity. Keep the view window opening as close to 8 in. as allows reasonable access to the work surface and equipment.

These precautions assure that any drafts caused by arm movements will not disrupt air flow or churn room air currents into the clean work area.
9. If direct flame sterilization of items within the cabinet is necessary, use an electric burner or pilot light-activated flame burner located at the back of the work space.

A constant open flame in the cabinet can disturb the laminar air flow.
10. At the end of the procedure, enclose all contaminated materials. Clean the cabinet work surface with 70% ethanol or other disinfectant, being especially careful to wipe any spills of culture suspensions or media that can serve as future contamination points. Clear all material from the cabinet.
11. Let the blower run for ≥ 10 min with no activity to remove any aerosols that were generated. During this period, turn off the fluorescent light and turn on the germicidal UV light. Allow the UV light to operate ≥ 30 min.

COMMENTARY

Background Information

Aseptic technique

The dictionary definition of asepsis simply implies freedom from pathogenic organisms. However, the practical definition of the term for cell biologists, as well as other biotechnologists working with pure cultures, has come to be synonymous with sterile or noncontaminating conditions. The successful manipulation of cell cultures under any circumstance inherently relies upon the ability to maintain rigorous aseptic (i.e., noncontaminating) working conditions. The concept of aseptic technique is simple in theory: prevention of sterile or uncontaminated material and objects from coming into contact with any nonsterile or contaminated material.

Practical application of the theory is often illusive for beginning students. However,

breaches in aseptic technique can also cause significant problems for even well-experienced laboratories, particularly when the source of contamination is not readily evident. A single incident of culture contamination is frustrating in its own right, but repeated contamination (particularly by the same type of organism) invariably results in expensive losses and delays until the localization and source are identified.

The critical areas of concern with respect to successful aseptic technique include environmental conditions (laboratory or work space), source material (cell lines, media, and reagents), equipment (labware, instruments, and apparatuses), sterilization procedures and equipment (autoclave, dry heat, filtration), and human (laboratory personnel) considerations. Budgetary constraints aside, technological aids exist to greatly simplify the hardware needed

for aseptic work. Laminar-flow cabinets create clean working environments (see below); clean, certified cell lines are available from cell repositories; media manufacturers and biotechnology supply companies provide sterile media, sera and reagents; and presterilized disposable labware to satisfy most cell culture needs is available from any large distributor of scientific supplies.

Despite all the technological advances, the one weak link remaining in successful laboratory applications of aseptic technique is the human factor. Too frequently, contamination occurs because of the desire to work a little too quickly, the urge to eliminate an “unimportant” step, or lapses in concentration during mundane procedures. The only advice to offer as protection against the human factor is to work slowly and deliberately when performing procedures under aseptic conditions, don’t eliminate procedural steps, and pay attention! Establishing a standard routine of procedures and of placement of materials can help prevent the omission of steps.

Laminar-flow cabinets

Laminar-flow cabinets or hoods have replaced the open laboratory bench for aseptic work in almost all cell culture and microbiology laboratories. Their effectiveness as physical barriers to contamination relies on a cabinet design incorporating high-efficiency particulate air (HEPA) filters to trap airborne contaminants, and blowers to move the filtered air at specified velocities and in a nonmixing (laminar) stream across a work surface.

As noted in each protocol for the particular type of laminar-flow application, the proper choice of cabinet is imperative. Horizontal laminar-flow cabinets are never used with biological or toxic chemical agents as they are not containment devices but rather serve to provide a strong stream of near-sterile air for particle-free working conditions. As this air is blown directly from the HEPA filter (at the back of the cabinet) across the work surface and out of the cabinet (directly into the operator’s face and the room), the restricted use of the horizontal flow cabinet to nonhazardous material is obvious.

The Class IIA biosafety cabinet is a laminar containment device that (1) protects the material being manipulated within the cabinet by HEPA-filtered incoming air and (2) protects the operator and room environment from potentially hazardous material in the cabinet with an air curtain at the front of the cabinet (the view screen) and HEPA-filtered cabinet exhaust air.

As Class IIA biosafety cabinets are not totally leak-proof, they cannot be used for high-risk biological agents (see current Center for Disease Control and NIH guidelines for the status of any biological material used in the laboratory; Richmond and McKinney, 1993). Because Class IIA cabinets operate with ~70% recirculated air within the cabinet (Fig. 1.3.2), the potential for accumulation of chemicals within the laminar work space limits use to low-level toxic or radioactive material.

Laminar-flow cabinets are not replacements for good microbiological aseptic technique and must be used in conjunction with standard concerns for asepsis if full efficiency of the equipment is expected. Similarly, there is a limit to the protection a laminar cabinet can provide if it is operated in an environment not conducive to clean work conditions. The cabinets should be installed and operated in a relatively clean, quiet laboratory environment. Laboratory doors should be kept closed while the cabinet is in use to minimize strong room air currents that could break the laminar air stream within the cabinet. The units should not be located directly near room air ducts or anywhere a strong environmental air flow exists. Additionally, air flow disturbance by personnel or equipment, particularly within a few feet in front of the cabinets, should be limited when the laminar device is in use.

Because of the critical nature of their function (particularly for the biosafety laminar cabinet), these devices must be certified at installation by professional laminar flow technicians in accordance with National Sanitation Foundation Standard No. 49 for Class II (laminar flow) Biohazard Cabinetry (NSF International, 1992) or other applicable regulatory and safety guidelines. As HEPA filters are brittle and will crack with normal usage of the unit, laminar cabinets must also be recertified annually or after 1000 hr use, and whenever they are moved.

Critical Parameters and Troubleshooting

Human sources of contamination

As noted above, bacterial shedding from human skin is a natural occurrence. However, under times of physiological or psychological stress, a human may shed so excessively that routine gowning procedures are inadequate. A clue to this condition can be the veteran technician who suddenly can’t seem to transfer anything without contaminating it, especially when contamination is repeatedly bacterial and

by species of *Staphylococcus*, *Micrococcus*, or coryneforms.

Alleviation of the problem may be achieved by simply controlling the temperature of the laboratory. Gowned personnel sweat in 27°C (80°F) rooms, and people who sweat shed more than people who don't. Rigorous attention to gowning details as well as liberal washing of hands and arms with an antimicrobial soap just prior to aseptic work may alleviate the situation. If the problem involves psychological stress or physiological stress due to illness or medication, more rigorous gowning procedures may help. Use fresh, clean laboratory coats for each round of aseptic work and make sure laboratory coat sleeves are tucked inside gloves to prevent exposed wrists. Use disinfectants liberally. For worst-case incidences of excessive shedding, the only recourse may be to move the individual to nonaseptic procedures until the condition clears.

Decontamination of a laminar-flow cabinet

Any mechanical failure of a laminar cabinet must be evaluated by qualified, trained personnel. Increased incidences of microbial contamination (particularly by the same organism) could originate from (1) poor cleaning and disinfection of the cabinet work space, (2) a source of contamination lodged in the ducts within the cabinet (e.g., media or culture material spilled into the cabinet ducts), or (3) a crack in the HEPA filter.

Disinfect the catch basin if culture material has spilled through the vents in the work surface into the catch basin below. Use a strong disinfectant (such as 5% to 10% bleach in a sufficient volume to thoroughly contact the spilled material) and allow the disinfectant to stay in contact with the spill for 30 min. Drain the contents of the catch basin into a container suitable for final sterilization by autoclaving.

Visually inspect the working interior of the laminar cabinet for evidence of dried culture material or media, especially in the corners of the cabinet. Clean the interior of the cabinet with a laboratory detergent, rinse with water, dry, and treat the area with an appropriate disinfectant. Be very careful not to wet the exposed HEPA filter located on the back wall of horizontal flow cabinets, as this can compromise the filter integrity. Be careful not to let cleaning solutions enter any vents of the cabinet.

After thorough cleaning of the cabinet work surface, operate the cabinet (as detailed in Basic Protocol 2 or Alternate Protocol) using a control procedure for localizing the source of any

remaining contamination. This can be achieved with a series of opened plates of trypticase soy agar and Emmons' modification of Sabouraud's agar systematically coded and placed across the work surface. Leave the media plates open and the cabinet operating for 30 min. Close the lids of the agar plates and incubate them at 26°C for 5 days. If significant microbial contamination appears in the plates, consult with a qualified laminar technician. The resolution to the problem will require either caulking leaks in the HEPA filter or sealing the cabinet for total interior decontamination of filter and ducts with formaldehyde gas.

A final source of frequent contamination in a laminar working condition can be the "sterile" equipment, labware, or solutions used. A poorly filter-sterilized phosphate-buffered saline solution can give rise to significant numbers of pseudomonad bacteria within weeks when stored at room temperature. Insufficiently processed autoclaved or dry heat-sterilized labware frequently results in contamination of cell culture material by spore-forming bacteria.

Anticipated Results

When proper aseptic techniques are used, it should be possible to maintain cell cultures without contamination.

Time Considerations

It takes ~1/2 hr to properly prepare oneself and the cell culture area for culture procedures and a similar amount of time to properly clean up afterward.

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Chapter provides an overview of general concerns for working with biological agents, from a classic publication on general methods in bacteriology that often overlaps to satisfy the technical needs of cell biologists.

Chatigny, M.A. 1986. Primary barriers. *In* Laboratory Safety: Principles and Practices (B.M. Miller, D.H.M. Gröschel, J.H. Richardson, D.Vesley, J.R. Songer, R.D. Housewright, and W.E. Barkley, eds.) pp. 144-163. American Society for Microbiology, Washington, D.C.

Offers detailed considerations on the types and uses of laminar-flow barrier technology. The main publication is well worth its price for anyone (staff, supervisors, administrators) responsible for safety in a biological laboratory.

Freshney, R.I. 1994. Culture of Animal Cells: A Manual of Basic Technique, 3rd ed., pp. 51-69. Wiley-Liss, New York.

Offers suggestions for maintaining aseptic conditions while working with cell cultures. A classic cell culture publication that surveys the field while providing enough detail for an individual with intermediate knowledge of microbiology and cell biology.

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Sterilization and Filtration

This unit describes conditions and procedures for the use of the autoclave and the convection or gravity oven for sterilization of heat-stable laboratory materials, for depyrogenation by heat, and for decontamination of biological waste. Sterilization is not an absolute but rather a probability function. Terminal sterilization processes such as autoclaving or dry heat should have a 10^{-6} or less probability that an organism will survive treatment. The proper choice of sterilization method, well-maintained equipment, validated procedures, and adherence to protocol are all necessary to keep the statistics in one's favor.

This unit includes protocols for a variety of sterilization and decontamination methods. Moist-heat or steam sterilization is used for liquids, dry goods, and decontamination of biological wastes. Dry heat or depyrogenation is used to sterilize laboratory glassware and equipment. The efficacy of sterilization using these methods should be monitored using biological indicators. Disinfectants such as ethanol, quaternary ammonium compounds, and sodium hypochlorite are used for decontamination of facilities and equipment, and for clean up of certain spills. Vacuum or positive-pressure filtration is an alternative method for sterilization of liquids that do not withstand steam sterilization.

AUTOCLAVING LIQUIDS

The autoclave is used for sterilization by moist heat. The standard conditions for moist-heat sterilization are exposure to saturated steam under pressure at 121°C for 15 min, although other temperature/time specifications can be utilized for specialized needs. In general, materials suitable for autoclaving as a nondestructive sterilization process must meet the following criteria: (1) stable to the temperature and time of the autoclave cycle, (2) unaffected by moisture, (3) packaged to permit exposure to steam, and (4) hydrophilic, if liquid. Materials are sterilized by autoclaving only if they are wetted with the steam; thus, sealing gaskets on certain types of laboratory equipment may not be effectively sterilized if tightened in place during the autoclave cycle. Materials can be decontaminated by autoclaving providing criteria (3) and (4) are met.

The autoclave cycle is based on the time it takes the material being sterilized to be in contact with saturated steam at 121°C for 15 min, and not the time the autoclave itself has been selected to run at that temperature. As autoclave efficiency is machine specific, and steam penetration is container and volume dependent, autoclave cycles and load configurations should ideally be validated to assure that sterilization conditions are achieved. For many small laboratories, the purchase expense of temperature-monitoring thermocouples might be prohibitive. However, frequent use of biological indicators to monitor autoclave conditions (see Support Protocol 1) is strongly recommended for all laboratories, particularly when the machine is used for decontamination of biological waste. Table 1.4.1 lists suggested autoclave times for load configurations in an autoclave with a 20 × 20 × 38-in. (51 × 51 × 97-cm) chamber.

Materials

- Heat-resistant containers and vessels (e.g., borosilicate glass, high-grade stainless steel, noncytotoxic plastic)
- Liquid to be autoclaved
- Moisture-resistant labels
- Paper or aluminum foil
- Autoclave indicator tape

BASIC PROTOCOL 1

Autoclave
Autoclavable discard pans

1. Use a heat-resistant vessel that can hold twice the volume of the liquid to be autoclaved to assure that boiling encountered during the heating and cooling periods of the autoclave cycle do not result in a boil-over of the vessel contents.

The most frequent laboratory frustrations with autoclaves and sterilization of liquids are boil-over of material or blow-off of closures. When volume-to-container relationships are acceptable, and when the solution is not overly viscous, the problem most often occurs because the autoclave's slow exhaust or liquid cycle is not properly functioning and the machine is exhausting the chamber pressure too quickly. Adjustments or repairs to the unit should be made by qualified personnel.

2. Fill the vessel to desired volume with liquid to be autoclaved. Indicate the contents of the container with permanent ink and a moisture-resistant label.
3. Loosely cover any opening to the vessel. Do not overtighten screw-cap closures, as this prevents adequate pressure/steam exchange. Overwrap and secure cotton-plugged flasks with paper or aluminum foil to prevent plugs from blowing off if the autoclave is exhausted too rapidly.
4. Affix a piece of autoclave indicator tape to each item or package, as a visual reference (following the autoclave cycle) that the material has been processed.

The color change of autoclave tape indicates only that the tape has been exposed to a 121°C temperature, and not how long it has been held at that temperature. Thus, it is not an indicator of successful sterilization.

5. Load the autoclave with vessels of similar size, volume, and configuration. Place all liquid-containing vessels inside autoclavable discard pans inside the autoclave. Close and lock autoclave door.

The discard pans should be large enough to contain all fluid or glass in the event of boiling over or breakage during autoclaving.

6. Set autoclave controls for liquids or slow exhaust.
7. Select and set autoclave controls for appropriate sterilization time (see Table 1.4.1).

Table 1.4.1 Suggested Autoclave Run Times and Configurations for 121°C Sterilization in a 20 × 20 × 38-in. Autoclave^a

Vessel	Size	Volume (ml)	Minimum time (min) ^b	Maximum time (min) ^b
Test tubes	13 × 100 mm	4-6	18	20
	16 × 125 mm	5-10	18	20
	20 × 150 mm	12-20	18	20
Flasks	100 ml	25-50	20	26
	250 ml	75-100	24	28
	500 ml	250	26	30
	1000 ml	500	28	32
	2000 ml	1000	30	32
Media bottles	125 ml	50	20	22
	500 ml	250-500	30	32
Empty glassware	All	All	35	90

^aChamber dimensions (metric equivalent, 51 × 51 × 97 cm).

^bSterilization times indicated are actual autoclave timer settings. Material within the vessels will be exposed to 121°C for 15 min using these process times. See text for further explanation.

8. Start and run autoclave cycle to completion.

Any interruption in the cycle (e.g., a sudden drop in chamber pressure) invalidates the run, and the sterilization cycle must be rerun to assure efficacy of the process. This is problematic if the liquid has limited stability to prolonged or repeated autoclaving, such as the microbiological media used for quality control in cell culture work. In such cases, it is best to start over with new media.

9. Open the autoclave door only when chamber pressure registers 0 lb/in² (100°C or less).

CAUTION: Never stand in the path of escaping steam when opening an autoclave.

10. Remove flasks or containers only when all bubbling has stopped.

CAUTION: Superheated liquids can easily boil violently if even slightly jostled. Resulting boil-over can badly scald laboratory personnel.

11. Cool vessels to ambient or other prescribed temperature in a relatively clean area not subject to excessive air currents.

This helps avoid suctioning of heavily contaminated environmental air into the container, which can sometimes occur when a vacuum forms within the container as the liquid cools.

AUTOCLAVING DRY GOODS

Heat-stable dry materials (including stainless steel instruments, glassware, fabrics, and plasticware) can be effectively sterilized by autoclaving, providing all surfaces of the dry material come in contact with the saturated steam at 121°C. This can become problematic for small items (such as forceps) that must be packaged in an outer container or wrapping that impedes the flow of steam, or for folded fabrics that tend to harbor pockets of cooler air. For this reason, autoclaving times for dry goods sterilization often rely on overkill, as these materials generally have much higher heat resistance. As noted above, validation studies should be done to determine the most effective times and configurations for a given autoclave. For further information on general autoclaving considerations, see Basic Protocol 1 introduction. For default times for an autoclave with a 20 × 20 × 38-in. (51 × 51 × 97-cm) chamber, see Table 1.4.1.

Additional Materials (also see Basic Protocol 1)

Items to be autoclaved
Shallow heat-resistant container

1. Loosely arrange small items in a shallow, heat-resistant outer container. Loosely cover the outer container's opening with paper or aluminum foil. If container has its own lid, apply it loosely so that steam and pressure can penetrate. Cap larger items such as bottles or flasks, making sure that all closures (e.g., screw caps) are loose enough to permit penetration of pressurized steam.

Small items can also be individually wrapped in paper or foil.

2. Code each item or package with permanent ink and a moisture-resistant label identifying its contents. Affix a piece of autoclave indicator tape to each item or package as a visual reference (following the autoclave cycle) that the material has been processed.

The color change of autoclave tape indicates only that the tape has been exposed to a 121°C temperature, and not how long it has been held at that temperature. Thus, it is not an indicator of successful sterilization.

ALTERNATE PROTOCOL 1

3. Where feasible, add a small amount of deionized or distilled water to the outer container or the individual items to assure adequate moisture for effective sterilization.
4. Arrange material in the autoclave to avoid dense overpacking that will impede effective sterilization. Where possible, arrange items to permit downward displacement of cooler, heavier air (e.g., place empty bottles or flasks on their sides rather than upright in the autoclave).

This prevents pockets of cool air from being trapped in the bottom of the containers as the hot, pressurized steam flows into the vessels.

5. Set autoclave for a fast exhaust cycle.

If the sterilizer is so equipped, a drying cycle that removes moisture from the dry goods under vacuum at the end of the timed sterilization run can also be used.

6. Select and set autoclave controls for appropriate sterilization time (see Table 1.4.1).
7. Start and run autoclave cycle to completion.

Note that any interruption in the cycle (e.g., a sudden drop in chamber pressure) invalidates the run, and the sterilization cycle must be rerun to assure efficacy of the process.

8. Open the autoclave door to remove items only when chamber pressure registers 0 lb/in² (100°C or less).

CAUTION: *Never stand in the path of escaping steam when opening an autoclave.*

ALTERNATE PROTOCOL 2

AUTOCLAVING FOR DECONTAMINATION OF BIOLOGICAL WASTE

Biological laboratory waste is most frequently decontaminated by autoclaving unless it contains hazardous chemical materials that can volatilize in the sterilization process. In many mid- to large-sized laboratories, biological waste includes varying combinations of spent media, discarded cultures, and solid material. An autoclave load size or configuration can vary dramatically with each run. For this reason, autoclave cycles for decontamination most often employ the overkill approach. Validation studies prior to actual-use procedures must be performed to assure that selected operation procedures are adequate to achieve the desired conditions for successful decontamination. For further information on general autoclaving considerations, see Basic Protocol 1 introduction.

CAUTION: Do not dispose of biological material containing hazardous chemicals or radioactive isotopes in the waste stream designated for autoclaving.

Additional Materials (also see Basic Protocol 1)

Items for decontamination

Plastic (polyethylene or polypropylene) autoclavable bags for biohazardous waste

1. Place items for decontamination into plastic autoclavable bags clearly labeled as containing biohazardous material.

For greater ease with postautoclaving cleanup procedures, segregate plastic disposable material from reusable labware in separate bags.

CAUTION: *For the safety of laboratory personnel who sort the autoclaved waste for washing or disposal, do not dispose of sharps or pipets as loose items in the bags. These items must be segregated in their own containers (containing a disinfectant, if appropriate).*

2. Add ~500 ml water to bags containing only dry items (such as empty glassware or contaminated lab coats) to supply sufficient moisture for steam generation.

- Support bags by placing them in large, shallow, leak-proof, autoclave-resistant discard pans to prevent tearing of the bags and release of contents.

For the safety of personnel carrying the discard pans, use a maximum weight limit of 25 lb per pan.

- Securely seal each bag to prevent leakage of material. Transport biological waste only in closed containers.
- Code each bag with permanent ink and a moisture-resistant label to permit general identification of its contents or source-lab should an accident occur (e.g., mycoplasma testing lab; QC lab).
- Affix a piece of autoclave indicator tape to each item or package as a visual reference (following the autoclave cycle) that the material has been processed.

The color change of autoclave tape indicates only that the tape has been exposed to a 121°C temperature, but does not indicate the time held at that temperature. Thus, it is not an indicator of successful sterilization.

- Transport the waste using a sturdy laboratory cart or autoclave carriage and dolly.
- Load the discard pans loosely into the autoclave to allow steam to flow over and around the material. While loading, slit each individual bag open in several spots to allow direct exposure of the contents of the bags to the steam from the autoclave.

Do not stack pans directly on top of each other.

- Close and lock autoclave door.
 - Run autoclave cycle for 90 min at 121°C on a fast-exhaust, gravity cycle.
- The cycle time listed here is for a large (24 × 36 × 48-in.; 61 × 91 × by 122-cm) autoclave. With small loads and smaller-chamber autoclaves, 45 min may be sufficient. Any interruption in the cycle (e.g., a spurious drop in autoclave steam pressure) invalidates the run.*
- Open the autoclave door to remove items only when chamber pressure registers 0 lb/in² (100°C or less).

CAUTION: *Never stand in the path of escaping steam when opening an autoclave.*

USE OF BIOLOGICAL INDICATORS FOR MONITORING AUTOCLAVE PROCESSES

Biological indicators are used to effectively monitor the efficacy of moist- or dry-heat sterilization processes. The indicators contain standardized preparations and concentrations of resistant endospores of specific strains of bacteria that will survive suboptimal sterilization conditions, and proliferate when subsequently incubated under normal growth conditions. For greatest control of sterilized material, biological controls should be included with every load. Under general laboratory conditions, biological indicators should be used for validation studies in conjunction with thermocouple temperature-sensing probes, and they should be used for frequently scheduled monitoring of the performance of sterilization equipment and procedures. Bioindicator sources listed in this unit are examples only; other products by other manufacturers can work well. Specific manufacturer instructions for product use supercede general instructions described in this protocol.

This protocol describes the use of biological indicator ampules for monitoring sterilization. For monitoring dry-heat sterilization, see Support Protocol 2.

SUPPORT PROTOCOL 1

Cell Culture

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Additional Materials (also see Basic Protocol 1; see Alternate Protocols 1 and 2)

Biological indicator ampules: standardized concentration of *Bacillus stearothermophilus* (ATCC #7953) spores suspended in growth medium containing bromocresol purple as a pH indicator (e.g., Prospore from Raven Biological Laboratories)
55° to 60°C incubator

1. Label the desired number of biological indicator ampules with permanent ink to indicate location and autoclave run number.
2. Place one or more ampules in the most difficult locations to sterilize in the autoclave, including (1) near the front drain of the autoclave, located on the chamber floor at the door of the autoclave, and (2) suspended within the item being sterilized. For liquids, suspend the ampule in the container of liquid by a string tied around the ampule neck and secured around the opening of the container. For dry goods, tuck the ampule within the samples being autoclaved.

Manipulating test ampules in loads containing biohazardous waste must be performed by personnel trained for dealing with the potential hazards of the material.

3. Run autoclave cycle at prescribed conditions (see Basic Protocol 1; see Alternate Protocols 1 and 2) and retrieve ampules.

CAUTION: After sterilization, handle ampules with care if still hot, as they are under pressure and might burst if strongly jostled.

Validation studies should never be performed in routine process cycles; unfortunately, they are too often used interchangeably in small laboratories. If validation and processing are used simultaneously, the sterilized material must be quarantined until the results of the sterilization monitoring tests are confirmed.

4. Place autoclaved test ampules and a labeled, unautoclaved positive control ampule in a vertical position in a 55° to 60°C incubator.
5. Incubate 48 hr.
6. Analyze results by noting the color of the test ampules and positive control ampule.

Growth of the positive control confirms lack of sterilization. The positive-control ampule exhibits a color change from purple (prior to sterilization) to yellow (postincubation), with or without turbidity.

Growth of the test sample indicates failed sterilization, and is seen as a color change from purple (prior to sterilization) to yellow (postincubation). Positive sterilization is indicated by a purple-colored test ampule (postincubation). An intermediate yellowish color is suspicious and necessitates additional testing of the autoclave parameters. An intermediate grayish color, without turbidity, usually indicates heat destruction of the bromocresol pH indicator, resulting from prolonged autoclaving conditions.

DRY-HEAT STERILIZATION AND DEPYROGENATION

Dry heat is used for components and materials that are resistant to the 140° to 180°C temperatures needed for effective dry sterilization; it is most often used for the sterilization of laboratory glassware and stainless steel instruments. It is also used for sterilization of nonaqueous, heat-stable liquids such as mineral oil. Depyrogenation of heat-tolerant materials is done with ovens capable of operating at the required processing temperatures of 220° to 350°C. As with autoclaving, standard dry-heat sterilization and depyrogenation times refer to the time the material is held at the prescribed temperature and not to the time the oven has been set to run. Dry-heat sterilization using gravity ovens generally requires a longer time than does sterilization with convection ovens, which evenly

BASIC PROTOCOL 2

Sterilization and Filtration

1.4.6

distribute the heated air throughout the chamber with blowers. In all instances, process validation of any dry-heat sterilization protocol is required. The frequent use of bacterial spore strips (see Support Protocol 2) is advantageous for routine monitoring of the efficacy of an established sterilization process.

Materials

Items to be sterilized

Heat-resistant outer containers (borosilicate glass or stainless steel) for small items

Aluminum foil

Heat-resistant labels or tape

Dry-heat indicator tape

Laboratory oven (operating temperature of 140° to 180°C for sterilization; 220° to 350°C for depyrogenation)

1. Place small items to be sterilized into heat-resistant outer containers. Use aluminum foil to cover any openings to larger, individually sterilized items, or to cover any openings to large items that do not have their own closures.
2. Code each item or package as to its contents with permanent ink and a heat-resistant label.
3. Affix a piece of dry-heat indicator tape to each item or package as a visual reference (following the oven cycle) that the material has been processed.

The color change of indicator tape shows only that the material has been exposed to a prescribed sterilization temperature, but does not indicate the time held at that temperature. It is not an indicator of successful sterilization.

4. Loosely arrange material in the oven. Do not overpack, as this prevents efficient heat penetration to all items.
5. Close and secure oven door.
6. Select operating temperature and time (see Table 1.4.2 for general guidelines).

Note that times designated in Table 1.4.2 do not include temperature buildup time, as this is equipment specific. The actual sterilization time begins when the oven chamber reaches the prescribed temperature. Heating times are long for dry-heat sterilization, and can actually be longer than the sterilization time itself. Thus, a load of material might require 2 hr to reach 180°C, while needing only 0.5 hr at that temperature to be effectively sterilized.

7. Run dry-heat sterilization cycle to completion.

Any interruption in the cycle (e.g., opening the door to add just one more item to the load) invalidates the run.

Table 1.4.2 Time-Temperature Relationships for Dry-Heat Sterilization

Oven temperature (°C)	Sterilization time (hr) ^a
180	0.5
170	1.0
160	2.0
150	2.5
140	3.0

^aSterilization time indicated is the amount of time for which material should be raised to a given temperature and does not include heating time.

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8. Turn oven heating element off and allow material to cool to room temperature before removing items from the oven.

This can take several hours for large loads in gravity ovens.

**USE OF BIOLOGICAL INDICATORS FOR MONITORING DRY-HEAT
STERILIZATION**

Biological indicator strips are used to monitor dry-heat sterilization. For a general discussion of biological indicators, see Support Protocol 1.

Additional Materials (also see Basic Protocol 2)

Biological indicator strips containing standardized concentrations of *Bacillus subtilis* (ATCC #9372) spores (e.g., Spore-O-Chex; PyMaH, or VWR)

Trypticase soy broth (see recipe)

30°C incubator

1. Label the appropriate number of biological indicator strips with location and cycle number or date.
2. Place one or more strips in the most difficult-to-sterilize areas of the load.
3. Run sterilization cycle at prescribed conditions (see Basic Protocol 2) and retrieve strips when cool.
4. Aseptically open the outer wrapping of the indicator, remove the spore strip, and insert it into an appropriately labeled tube of trypticase soy broth (6 to 10 ml per tube).
5. Prepare a positive control by aseptically inserting an unsterilized spore strip into a separate tube of trypticase soy broth. Prepare an uninoculated tube of trypticase soy broth as a negative control.
6. Incubate tubes at 30°C for 4 days.
7. Analyze results by noting turbidity of the broth. Compare test samples with positive and negative control tubes. Resterilize any material in loads with positive test samples.

Bacterial growth in the positive control, indicated by cloudy medium, confirms lack of sterilization. Growth should be absent in the negative control; the medium should remain clear, with no precipitate.

**BASIC
PROTOCOL 3**

USE OF DISINFECTANTS: 70% ETHANOL

Ethanol is widely used in many laboratories for benchtop or laminar-space disinfection. The antimicrobial activity of the alcoholic solution is very much dependent upon the working concentration of the solvent, proper preparation, storage, and conditions of its use. Ethanol is an effective disinfectant against vegetative bacterial and fungal cells, but is totally ineffective in germicidal activity against bacterial spores. Ethanol is suitable for spraying or swabbing, but is not recommended for large-volume applications. Ethanol is highly flammable, and spills near the flame of a Bunsen burner are always a possible safety hazard. Similarly, an elevated concentration of vaporized ethanol in a liberally disinfected biosafety cabinet could ignite in the presence of a flame or spark. Furthermore, 70% ethanol is not recommended for use in discard pans or for decontamination of biological spills in the catch basins of biological safety cabinets.

**Sterilization and
Filtration**

1.4.8

Materials

100% denatured ethanol
Ethanol-resistant spray-type storage container

1. Add 700 ml of 100% denatured ethanol to 300 ml deionized or distilled water. Mix well by stirring.

Denatured ethanol is absolute ethyl alcohol to which small amounts of chemicals have been added to render it unsuitable for human consumption. This does not interfere with most industrial uses.

2. Store working solution in a tightly closed container to retard evaporation.

Choose a spray bottle rather than a squirt bottle to retard the evaporation of solvent that occurs with the larger opening of a squirt bottle neck.

3. *For lab benches or laminar-flow cabinets:* Liberally spray the alcoholic solution in a crisscross pattern over the work surface, making certain that the entire area is wetted. Let disinfectant remain in contact with the surface for ≥ 10 min. Wipe away excess solution with absorbent towels
4. *For objects (e.g., media bottles, culture flasks):* Wet absorbent towels (cheesecloth or paper towels) with the alcoholic solution. Thoroughly swab the object, being careful not to introduce any of the liquid into the threads of screw caps or other container closures. Let disinfectant remain in contact with the object for 3 to 5 min. Wipe away excess solution with absorbent towels

USE OF DISINFECTANTS: QUATERNARY AMMONIUM COMPOUNDS

The discovery of the antimicrobial activity of quaternary ammonium compounds during the early 20th century was a major advancement in the development of effective germicides. The inherent antimicrobial activity of these compounds was soon shown to be significantly improved by the addition of long-chain alkyl groups to the nitrogen moiety of the quaternary compound. The various quaternary ammonium compounds commercially used as disinfectants today are chemical modifications of this original concept. The mode of action of quaternary ammonium compounds is as cationic surface-active agents, although this chemical property does not fully explain the germicidal activity of the compounds. All have broad-based antimicrobial activity and have proven effectiveness against algae, gram-positive bacteria, some gram-negative bacteria, fungi, and certain viruses, when used at the manufacturer's recommended concentrations (0.1% to 2.0% active ingredient, or 200 to 700 ppm). They are relatively nontoxic to humans when used according to manufacturer's instructions and are not chemically destructive to equipment under normal use. They can be autoclaved without formation of toxic vapors and thus are frequently used as disinfectants in discard pans.

The limitations of quaternary ammonium compounds include lack of effectiveness at low concentrations against some commonly encountered gram-negative bacteria (e.g., *Pseudomonas* sp.). Like many other disinfectants, they are quickly inactivated by the presence of heavy organic burden.

Materials

Quaternary ammonium compound disinfectant of choice: e.g., Roccal (Sterling Winthrop), Micro-Quat (Ecolab), Zephirel (Bayer)
Tightly closed containers
Spray bottles
1-gallon jugs

**ALTERNATE
PROTOCOL 3**

Cell Culture

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1. Dilute concentrated quaternary ammonium compound according to manufacturer's instructions in deionized or distilled water and stir well to mix.

Depending upon use, normal working concentrations of 235 ppm active ingredient are used for sanitization purposes such as floor mopping, whereas concentrations of 470 to 700 ppm active ingredient are used for disinfection of laminar-flow cabinet work surfaces or for use in discard pans.

2. Store working solution in tightly closed containers to retard evaporation.

Prepare fresh working solutions frequently (e.g., once a month; follow manufacturer's instructions).

3. *For work surfaces:* Liberally spray the working solution in a crisscross pattern over the work surface, making certain that the entire area is wetted. Let disinfectant remain in contact with the surface for >10 min. Wipe away excess solution with absorbent towels.

4. *For use in discard pans:* Prepare a solution containing 700 ppm quaternary ammonium compound. Fill discard pan about half full with disinfectant solution. Carefully place used pipets into the pans to avoid splashing. Securely cover pan when moving it.

Volume of disinfectant must be enough to fully cover the pipets placed into the pan, but not so full that spilling could occur when the pan is filled with pipets or when it is moved.

ALTERNATE PROTOCOL 4

USE OF DISINFECTANTS: SODIUM HYPOCHLORITE

Chlorine, in various forms, has a long history of use as a powerful disinfectant, yet the exact mode of germicidal action is unclear. Hypochlorites are the most widely used chlorine compounds for disinfection. Commercial liquid bleach products (e.g., Clorox) are solutions containing 5.25% (w/v) sodium hypochlorite. Sodium hypochlorite is effective against vegetative microbial cells, most spores, and many viruses. It has some residual effect after the treated surface dries. It can be used in sanitization procedures for laboratory floors and in laboratory coat washing. It is strongly germicidal and can be used to decontaminate small- to mid-volume spills of biological material.

Despite their germicidal effectiveness, chlorine solutions are limited in their use as laboratory disinfectants because of their corrosiveness to metals and their human toxicity. They should not be routinely used in discard pans or in any solutions that are autoclaved, as the chlorine fumes liberated are significant skin and respiratory irritants. Frequent autoclaving of chlorine solutions will corrode the chamber interior of the sterilizer. One exception to this autoclave ban is the need to sterilize any biological spill material in which bleach was used as a disinfectant during the cleanup process. A solution of 10% (v/v) household bleach is a strongly germicidal, containing ~0.52% (w/v) sodium hypochlorite. Excess hypochlorite is needed in mopping up spills of biological agents, to supply additional chlorine to replace that consumed by the large amount of organic matter associated with the spill.

Sodium hypochlorite solutions can be inactivated by organic matter (which consumes the available free chlorine that constitutes microbiocidal activity), by exposure to UV light, and by inorganic chemical reducing agents (such as ferrous or manganese cations and hydrogen sulfide). Hypochlorite solutions should be stored away from heat to avoid deterioration.

Materials

Household liquid bleach (e.g., Clorox, Dazzle)
5% (w/v) sodium thiosulfate solution

1. Add 100 ml household bleach to 900 ml water to give a 10% (v/v) solution. Mix well. Store in the dark away from heat.
2. *For cleanup of open spills:* Soak paper towels with bleach and gently cover the spill, being careful not to enlarge the area of the spill. Let bleach stay in contact with the spill for ≥ 20 min. During this time, decontaminate any nearby areas that may have been subject to spatters from the original spill by swabbing with additional 10% bleach. Collect used paper towels in a suitable container and place in an autoclave bag.
3. *For autoclaving bleach-containing waste:* Add ~ 1 vol of 5% sodium thiosulfate solution to the bleach solution to help neutralize the chlorine. Seal the bag and autoclave. Prominently label the autoclave room door to warn laboratory personnel of the potential for irritating vapors. When the autoclave cycle is finished, crack the autoclave door slightly to allow remaining chlorine fumes to dissipate before removing the bags.
4. *For sanitizing solution:* Add 14.8 ml household bleach to 3.78 liter water (0.4%). Mix well.

Solutions containing 0.4% (v/v) household bleach (200 ppm available chlorine) are suitable for soaking lab coats without being so strong as to harm the fabric. The solution is acceptable for routine floor maintenance.

5. *For disinfection solution:* Add 44.4 ml household bleach to 3.78 liter water (1.2%). Mix well. Liberally apply to surface with clean absorbent towels and let stand for >10 min. Dry surface with a separate clean towel.

This concentration of bleach (600 ppm available chlorine) is suitable for disinfecting biosafety cabinets during scheduled weekly maintenance. More frequent use on stainless steel may not be recommended because of the corrosiveness of the bleach.

FILTER STERILIZATION OF SOLUTIONS

All solutions that come in contact with cell cultures must be sterile in order to prevent microbial contamination. This includes non-nutritive preparations such as distilled/deionized water and reagents (e.g., dimethyl sulfoxide used as a cryoprotectant). Although heat-stable solutions can be sterilized by autoclaving, many solutions used in cell culture contain one or more heat-labile components (e.g., antibiotics), or are chemically formulated with ingredients that will form deleterious precipitates if subjected to steam sterilization temperatures (e.g., phosphate-buffered salines). Membrane filtration is the most common cold sterilization method for these types of solutions.

Filter membranes with 0.2- μm pore size are used for general sterilization purposes; however, some environmentally stressed bacteria (e.g., *Pseudomonas* sp.) as well as mycoplasma can pass through filters of this porosity. To provide a greater degree of assurance for complete removal of these common tissue culture contaminants, cell culture media and sera should be sterilized using 0.1- μm filter membranes. Filter manufacturers offer many different types of membranes. With respect to cell culture applications, membranes fabricated from cellulose acetate or cellulose nitrate are used for general purpose filtration of aqueous solutions such as media and buffers, but may need prewashing with hot distilled water to remove extractable substances that may be cytotoxic. Nylon membranes are very low in extractable substances such as surfactants or wetting agents; polyethersulfone membranes are low in extractables and have very low protein binding.

The availability of presterilized, ready-to-use, disposable filter systems has eliminated much of the labor and risk of failure inherent with earlier filtration methods. Filter units come in a wide variety of sizes to handle small (≤ 10 -ml) to large (≥ 20 -liter) volumes. Disposable systems are available for either vacuum or positive-pressure filtration. Many manufacturers have filter systems designed specifically for cell culture applications: the sterilization membranes, housings, and receiving vessels are certified noncytotoxic and nonpyrogenic.

This section outlines selection of filters and filter-sterilization procedures for various types of liquids encountered in cell culture laboratories. The most common small-volume filtration technique is positive pressure using a syringe to force the liquid through the filter membrane (see Basic Protocol 5). Volumes ranging from 50 ml to 1 liter are most efficiently processed with a vacuum (see Basic Protocol 4). Larger volumes should be filter sterilized with positive pressure (see Alternate Protocols 5 and 6). The primary use of membrane filtration is in the preparation of tissue culture media; this topic is treated in depth in *UNIT 1.2*. This unit focuses on problematic filtration needs that often appear in cell culture applications, such as the need to filter sterilize a hazy solution like the serum/yeast extract additives used in mycoplasma media, or the chemically aggressive reagent dimethyl sulfoxide (DMSO). The methods outlined in this section are equally adaptable for the preparation of tissue culture media or stock solutions of additives such as glutamine or puruvate. For media preparation, use noncytotoxic cellulose acetate/nitrate membranes, or similar membranes specific to the application, and food-grade silicon tubing. For further details on the use of vacuum versus positive-pressure filtration, see Background Information.

BASIC PROTOCOL 4

Vacuum Filtration

Solutions that are initially clean preparations, in that they are free of particulate debris and are not proteinaceous, can be directly filter sterilized with no difficulty. Solutions with high particle load require centrifugation and/or nonsterile prefiltration through depth filters (see Background Information) and larger-porosity membranes prior to sterile 0.2- μm filtration. This protocol uses 200-ml to 1-liter disposable systems designed for the final vacuum filtration of media, sera, and other aqueous solutions. The protocol also describes nonsterile prefiltration for particulate removal from 200-ml to 2-liter volumes of filtrate.

Materials

- Solution to be filtered
- 47-mm funnel/support assembly (optional; e.g., Kontes, Millipore) attached to a 1- to 2-liter vacuum filtration flask (Fig. 1.4.1)
- 47-mm glass fiber depth filters (optional; Gelman, Millipore)
- 47-mm membrane filters (optional; 0.45- μm and 0.2- μm pore sizes)
- Disposable, sterile filter unit (e.g., Corning, Nalgene) including:
 - Filter funnel, housing an integrally sealed 0.2- μm filter membrane
 - Funnel dust cover
 - Removable receiver bottle and cap
 - Barbed tubing adapter
 - Nonsterile depth prefilters (included by most manufacturers)
- Vacuum source

NOTE: Perform all procedures using aseptic technique (*UNIT 1.3*).

1. If the solution to be sterilized is a hazy suspension or has a noticeable precipitate, centrifuge 30 min at $10,000 \times g$ to clarify. Alternatively, use a funnel/filter assembly

to process the solution through a series of nonsterile prefilters: depth filter, followed by 0.45- μm membrane, followed by 0.2- μm membrane.

Depending upon the particulate load, the filter membranes may have to be replaced if they clog before all the solution is processed.

If a water aspirator is used as the vacuum source, include an in-line catch flask or hydrophobic filter to prevent any water from accidentally being drawn into the vacuum port and into the filtration flask.

2. Remove a disposable, sterile filter unit, barbed tubing adapter, and individual wrapped sterile receiver cap from the plastic bag.
3. Check to make certain that the filter funnel is firmly attached to the receiver. Hand tighten if necessary.
4. Attach barbed tubing adapter to the side vacuum port on the neck of the filter funnel. Attach the vacuum tubing to the adapter.

If a water aspirator is used, include an in-line catch flask (see step 1).

5. Set the filter upright and provide support to avoid tipping the unit when it is top-heavy with liquid in the funnel.
6. Remove the funnel dust cover and slowly add solution (centrifuged or prefiltered if necessary) to the funnel. Slowly apply a slight vacuum—5 pounds per square inch gravity (psig)—to prevent excessive foaming of proteinaceous solutions.
7. When filtration is complete, turn the vacuum source off. Carefully disconnect the filter unit from the vacuum tubing.

If the tubing is pulled off while the vessel is still under full vacuum, the room air rushes into the receiving vessel at a much higher velocity.

8. Using aseptic technique, carefully remove the filter funnel from the receiver bottle and seal the bottle with the sterile screw-cap closure provided with the filter unit.

If the total volume of solution to be filtered exceeds the capacity of the receiver bottle supplied with the filter unit, the initial volume of sterile filtrate can be aseptically transferred to a secondary sterile storage vessel, and the filter funnel can be reattached to the original receiver to process a second volume of solution.

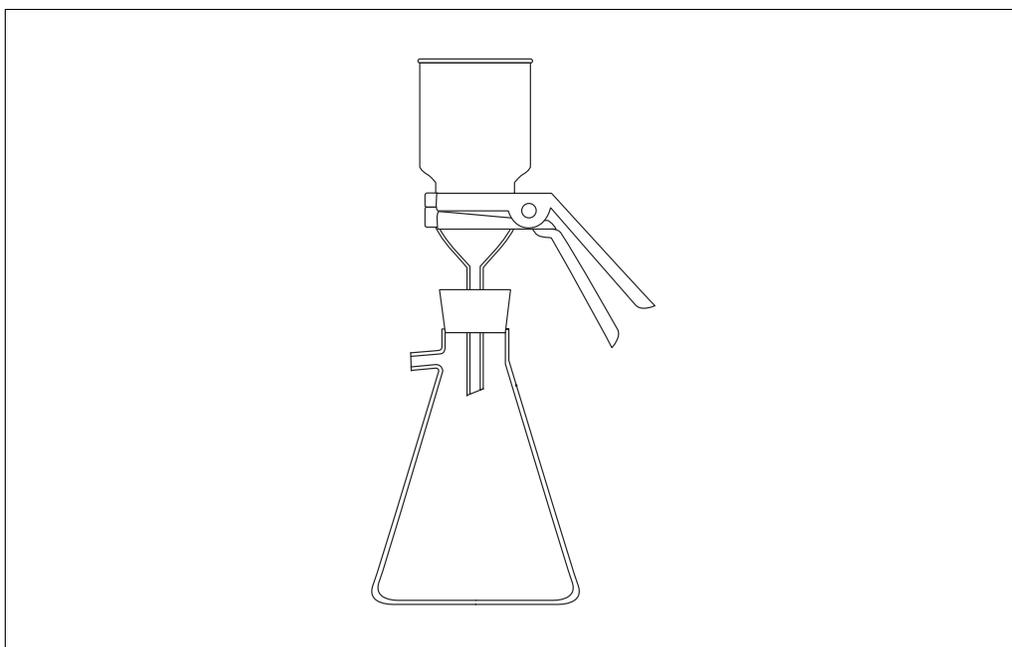


Figure 1.4.1 Funnel/support assembly for vacuum prefiltration.

Small-Volume Positive-Pressure Filtration of Nonaqueous Solutions

Dimethyl sulfoxide (DMSO) is used as the cryoprotectant for liquid-nitrogen preservation of cell cultures. The reagent is not stable to autoclaving conditions and must be filter sterilized. DMSO is an aggressive solvent that dissolves general-use filter membranes (such as cellulose acetate or cellulose nitrate) as well as the polystyrene filter units themselves. This protocol describes small-volume positive-pressure filtration using DMSO-resistant syringe-type filter units. For large volumes, see Alternate Protocols 5 and 6.

Materials

Dimethyl sulfoxide (DMSO)
Glass 25-ml syringe with Luer-lok tip
Sterile syringe filter: 25-mm-diameter nylon membrane, 0.2- μ m pore size, polypropylene housing (Nalgene or equivalent)
Laminar-flow cabinet
Sterile amber glass storage vessels with polytetrafluoroethylene (Teflon, PTFE)-lined screw-cap closure

NOTE: Perform all procedures using aseptic technique (*UNIT 1.3*).

1. Load a glass 25-ml syringe with deionized or distilled water.
2. Aseptically remove a sterile syringe filter from its blister-package wrapper, being careful not to touch the outlet nipple.
3. Attach the inlet end of the filter to the syringe and finger tighten the Luer-lok connection.
4. Apply a firm, but not forceful, pressure to slowly discharge the water through the filter into a waste container.

This initial step is necessary to wet the filter to permit flow of the DMSO through the nylon membrane.

5. Carefully remove the filter from the syringe, and rest the filter on the laminar-flow cabinet work surface, being careful to keep the outlet nipple facing up (i.e., not touching any surface).
6. Load the syringe with ~25 ml DMSO and replace the filter as in step 3.
7. Apply a firm, but not forceful, pressure to slowly discharge this first volume of DMSO into a waste container.

This step clears any water remaining in the syringe and filter.

8. Reload the syringe with ~25 ml DMSO and discharge the filtrate into a suitable sterile amber glass storage vessel. Cap immediately with a PTFE-lined screw-cap closures. Store up to 6 to 9 months at room temperature.

Large-Volume Positive-Pressure Filtration of Nonaqueous Solutions

This protocol uses a peristaltic pump to provide positive pressure for large-volume filtration. The setup is shown in Figure 1.4.2.

Materials

Dimethyl sulfoxide (DMSO)
Sterile filter capsule: 400-cm²-surface-area nylon membrane, 0.2- μ m pore size, polypropylene housing (Whatman Polycap 36AS or equivalent)
Glass 25-ml syringe with Luer-lok tip

Worm drive clamps

PTFE tubing: polytetrafluoroethylene (PTFE or Teflon) with 0.25-in. (6.4-mm) i.d., 0.06-in. (1.6-mm) wall thickness, 0.38-in. (9.5-mm) o.d. (Norton or equivalent)

Peristaltic pump assembly capable of providing an operating pressure of 15 to 20 lb/in.²

Sterile amber glass storage vessels with PTFE-lined screw caps

NOTE: Perform all procedures using aseptic technique (*UNIT 1.3*).

1. Carefully remove a sterile capsule filter from its plastic bag.
2. Remove the nipple cover from the inlet barb.
3. Hold the filter over a waste container, and use a glass 25-ml syringe to carefully add water to the filter housing through the inlet barb. Fill the syringe as necessary and continue to flush the housing until water begins to drip from the sterile outlet side.

This step wets the nylon membrane to allow the DMSO to pass through the filter.

4. Attach a piece of PTFE tubing to the inlet barb from the nonsterile DMSO reservoir. Using worm drive clamps, secure the tubing at all connections when working with positive pressure to prevent sudden blowing off of tubing in case of accidental overpressurization.
5. Secure the capsule filter to an upright support at a height with sufficient clearance to accept any receiving vessels.
6. Connect the tubing as shown in Figure 1.4.2, to the peristaltic pump head according to the pump manufacturer's instructions. Apply power to the pump and begin pumping.
7. Discharge the first 200 ml of DMSO filtrate to a waste container.

This step clears any water remaining in the filter and tubing.

8. Collect sterile DMSO in suitable sterile amber glass storage vessels and cap immediately with PTFE-lined screw-cap closures. Store up to 6 to 9 months at room temperature.

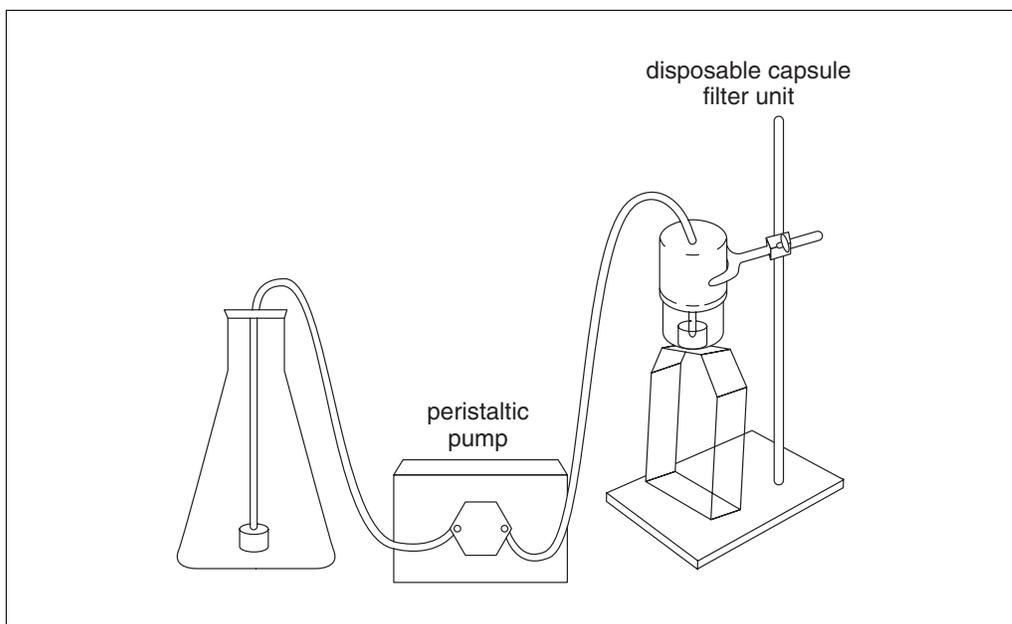


Figure 1.4.2 Positive-pressure filtration assembly for use with a peristaltic pump.

Large-Volume Positive-Pressure Filtration Using Pressurized Nitrogen

This alternative method for large-volume filtration uses pressurized nitrogen to force the solvent through the filter membrane. It offers an advantage for filtration of DMSO in that it saturates the solvent with an oxygen-free gas phase that helps retard oxidation of the material during storage. The setup is shown in Figure 1.4.3.

Additional Materials (also see Alternate Protocol 5)

- 5-liter pressure vessel (or size to fit application; Gelman or Millipore)
- Pressurized nitrogen tank
- Additional tubing to fit pressure vessel and nitrogen tank

1. Prepare a capsule filter (see Alternate Protocol 5, steps 1 to 3).
2. Attach a piece of tubing to the filter inlet barb from the outlet barb of a 5-liter pressure vessel. Using worm drive clamps, secure the tubing at all connections when working with positive pressure to prevent sudden blowing off of tubing in case of accidental overpressurization.
3. Fill pressure vessel with DMSO. Close and secure pressure vessel lid. Open pressure relief valve on vessel.
4. Attach another piece of tubing from a pressurized nitrogen tank to the inlet barb on the pressure vessel and secure with a worm drive clamp.
5. Slowly open nitrogen feed valve and wait until gas can be heard escaping from the pressure relief valve.
6. Close pressure relief valve.

Pressure will begin to rise in the vessel as indicated either on the pressure gauge included with the vessel, or by the gauges on the nitrogen tank regulator.

To prevent bursting of the filter membrane, keep the operating pressure below the maximum rated pressure specified by the filter's manufacturer.

7. Collect sterile DMSO filtrate (see Alternate Protocol 5, steps 7 and 8).

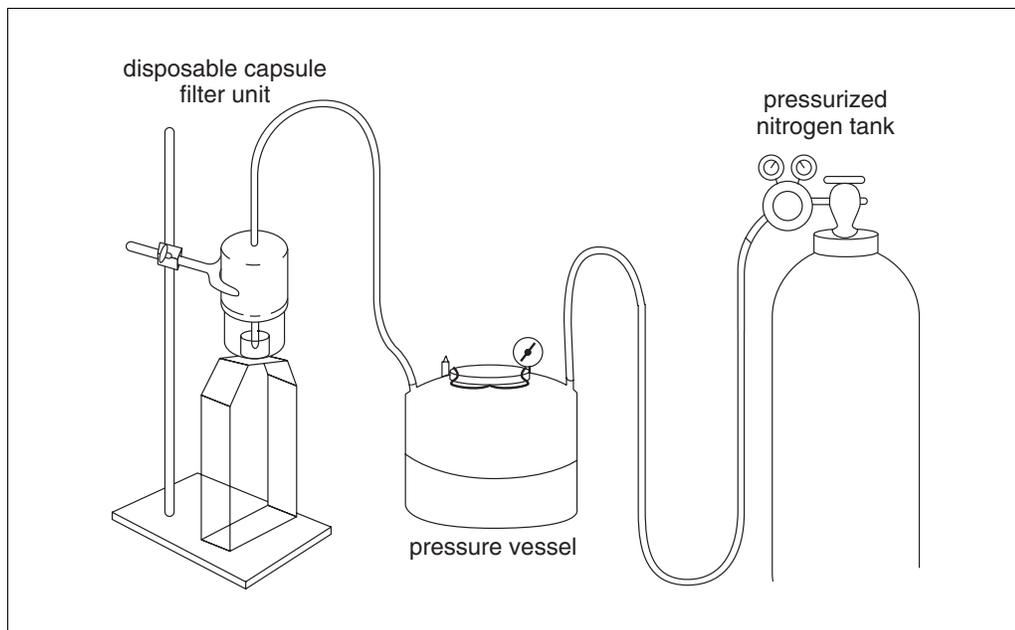


Figure 1.4.3 Positive-pressure filtration assembly for use with pressurized nitrogen.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Trypticase soy broth

Suspend 30.0 g trypticase soy broth powder (BBL) in 1.0 liter water and stir to dissolve. Dispense 6 to 10 ml per 16 × 125-mm tube and cap loosely. Sterilize by autoclaving at 121°C for 15 min. Store prepared tubes at 4° to 8°C for up to 6 to 9 months.

COMMENTARY

Background Information

Autoclaving

Steam (autoclave) and dry heat are destructive terminal sterilization processes in which the effectiveness of the method is characterized by the rate of the microbial killing. As the order of death in a terminal sterilization process is a logarithmic function, mathematical calculations will never result in a zero survival rate. Thus, theoretically, complete sterilization is impossible. Therefore, rather than expecting an absolute, the effectiveness of terminal sterilization processes is mathematically expressed in terms of the decimal reduction time (*D* value), which is the time required at a given temperature to destroy 90% of survivors. A corollary term used in discussions of terminal sterilization is the *Z* value, which is the temperature at which a survival curve decreases by one log. Factors influencing these values include the concentration and type of microbial contaminants initially in the material to be sterilized, the physical nature of the material undergoing sterilization, and the performance characteristics of the sterilization equipment.

Disinfectants

The use of disinfectants in the cell culture laboratory is directed both to issues of personal safety as well as quality control. In most instances the distinction between the two considerations blurs; however, this unit discusses the use of disinfectants primarily as a means for prevention of microbial contamination from the standpoint of quality control (i.e., maintenance of noncontaminating conditions for cell culture manipulations). Human safety considerations are limited to brief notations regarding potential effects of misuse of specific disinfectants (e.g., chemical incompatibilities, lack of effectiveness against certain biological agents) rather than a reiteration of the need for disin-

fectants as a means of personal defense against etiologic agents.

The list of common disinfectants is lengthy: alcohols (ethanol or isopropanol), chlorine compounds (bleach), hydrogen peroxide, phenolics, and iodophores (povidone-iodine), to name a few. Yet from this broad list, there is no universal disinfectant solution that can work effectively for all laboratory situations. Thus, the concern for any laboratory is deciding upon its different needs for disinfection (e.g., spill cleanup, work surface disinfection, discard pan disinfection, routine floor cleaning, lab coat laundering), and then selecting the appropriate disinfectant and concentration for each purpose. It is beyond the scope of this unit to provide a lengthy treatise on all the commercially available liquid germicides. Focus is directed, instead, to the three most commonly encountered disinfectants utilized in tissue culture: 70% ethanol, quaternary ammonium compounds, and sodium hypochlorite (bleach).

Filtration

Filtration as a method of sterilization has been in use for over one hundred years. Early filters were designed to trap contaminants within the depths of a thick, tortuous maze of filter material. Scintered glass filters and the asbestos (later cellulose) Seitz filters commonly used in the 1950s and 1960s worked on the entrapment principle. These depth filters have significant loading capacity and can retain much particulate matter before clogging. The limitation of depth filters is the structural nature of the filter matrix. At best, the loose matrix of a depth filter permits only a nominal designation of particle size retention. Because the pores of a depth filter are of random size and shape, there is a real probability that small-sized contaminants will successfully channel through the filter through interconnecting pores. In addition, the effects of moisture and pressure associated with autoclave sterilization and/or use of

depth filters tends to result in a shifting of the filter material (media migration) that compromises the integrity of the fibrous filter matrix.

By the mid 1970s membrane filters had essentially replaced depth filters for sterile applications. Membranes are classified as screen filters: they are thin and the pores are uniformly sized and spaced across the sheet. This structural consistency allows predictable retention characteristics. Thus, membrane filters can be rated according to the minimal diameter of the smallest particle they will retain (e.g., 1.2- μm , 0.45- μm , 0.2- μm). Membrane filters have limited loading capacity (i.e., they quickly clog). For this reason, they are most effectively used in tandem with depth filters and membrane filters of larger porosity to conserve the filter surface area on the final sterilization membrane.

The type of filtration system, as well as the type of membrane used in the system, depends upon the nature of the filterable material and the volume of material being filtered. A wide variety of configurations is available in both presterilized disposable units containing integrated filter membranes, as well as membrane discs that can be used in conjunction with reusable glass or stainless steel filter housings. In the past, positive pressure was the preferred filtration method for cell culture media because it reduced the foaming and concomitant protein denaturation associated with vacuum filtration systems. Unfortunately, positive-pressure filtration systems are efficient only for small (syringe filtration) or large (pressure vessel) volumes. With recent technological advances in new membrane matrices and improved membrane supports for filter housings that eliminate excessive foaming, vacuum filtration with presterilized disposable units is now the easiest and most effective sterilization method for intermediate volumes (0.1 to 2 liters) of tissue culture media.

Filter membranes are made from a number of different materials. The most common filters featured in presterilized, disposable filtration units are those fabricated from esters of cellulose acetate or cellulose nitrate. These general-purpose hydrophilic membranes are suitable for aqueous solutions such as tissue culture basal media and supplements. These membranes can, however, bind proteins and may be of concern for certain critical applications. With the huge cell culture market as a direct target, filter manufacturers have, in recent years, begun to offer presterilized filtration units customized for cell culture, featuring noncyto-

toxic, low-protein-binding membranes (nylon or polyethersulfone).

Not every solution utilized in cell culture applications is truly aqueous or hydrophilic. Nonpolar liquids, such as DMSO, or chemically aggressive solutions, such as concentrated acids or bases, demand special chemically resistant membrane filters. Quite often, filters resistant to nonpolar solvents will need pretreatment with an appropriate wetting agent to quickly render the membrane filterable to a particular nonpolar liquid. For example, PTFE (Teflon) membrane filters require prewashing with methanol prior to use with DMSO. This poses no problem providing all traces of the cytotoxic alcohol are removed by washing prior to collection of any sterile, unadulterated DMSO filtrate. Where residual toxic wetting agents are a concern, nylon filters may be a more appropriate consideration. Nylon membranes, when wetted with water, readily accept DMSO.

With wide recognition of the detrimental effects of insidious mycoplasma contamination and the concern about absolute removal of these contaminants from a major point of entry (i.e., serum) into a cell culture system, much emphasis is now placed on 0.1- μm filtration of cell culture media and sera. Unfortunately, this pore size is not widely available in disposable filtration units. Gelman Sciences does, however, offer presterilized filter units with 0.1- μm polyethersulfone (Supor) membranes for small- to large-volume cell culture media sterilization. Expect slower flow rates with 0.1- μm filtration.

When lacking presterilized filtration units, a laboratory can turn to individual membrane discs of specified porosity and membrane type, available from major filter manufacturers. These can be sterilized by autoclaving as part of an integrated unit in a small-volume filter assembly (e.g., Fig. 1.4.1), or for large-volume needs in a 142- or 293-mm filter holder (Gelman or Millipore). If this route for sterile filtration is chosen, follow the manufacturer's instructions precisely with respect to membrane sterilization times, and use a slow exhaust cycle to avoid the membrane cracking that can occur with rapid pressure changes.

This unit outlines procedures that are suitable for any number of variations. Large volumes of tissue culture media can be processed with the peristaltic pump or pressurized nitrogen procedures providing a hydrophilic filter is used. Similarly, a hydrophilic syringe filter can be used to sterilize small volumes of media. Sterilization of DMSO is, however, limited to

those filter membranes and filter unit housings fabricated of materials resistant to the reagent.

Critical Parameters

Autoclaving

Successful autoclave sterilization is dependent upon the contents of a load coming into full contact with saturated steam at 121°C for 15 min. Many operational factors tend to work against these criteria. With respect to the sterilizer itself, if an autoclave is improperly maintained, problems can occur with inadequate removal of air from the chamber or with excessive moisture buildup. Either condition compromises sterilization parameters. Most autoclaves have cool spots that can move about in the chamber like a current depending upon load pattern and configuration. The nature of the load content also influences the time necessary to reach the time-temperature relationship. Small volumes will heat to sterilization temperatures faster than large volumes. Agar solutions that have solidified prior to sterilization will take longer than those that are loaded into the autoclave while still molten. Dry materials take longer to sterilize than those that are moist.

For any laboratory investing in as expensive a pursuit as tissue culture, sterilization equipment and procedures should be validated to assure that conditions for sterilization are met. Ideally a laboratory should invest in a thermocouple to monitor temperatures within areas of autoclave loads, in order to determine exactly how long it takes the material of concern to reach sterilization temperature. In many cases, the lag between the time an autoclave temperature gauge indicates 121°C in the autoclave chamber and the time the contents of a large flask or discard pan within the chamber reaches the same temperature is sobering. Where thermocouples are not available, the use of biological indicators can yield useful information about autoclave procedures and machine performance, although the time needed for incubation and interpretation of results is a drawback.

A final, but most important, consideration for autoclaving is the source of steam generation. Autoclaves used in the preparation of media or for sterilization of materials that come into contact with cell cultures must be supplied with clean steam (i.e., steam generated from purified water). Steam generated directly from general building physical plant sources (e.g., the building heating system) is frequently produced from water treated with potentially cy-

totoxic boiler amines and/or chemical softening agents.

Dry-heat sterilization

Hot dry air is an inefficient means of sterilization and should be reserved for those materials that cannot effectively be exposed to saturated steam in an autoclaving process. Sterilization by dry heat can be accomplished with temperatures as low as 140°C, but 170° to 180°C are more routine operating temperatures because of the difficulty in controlling the rate of heat penetration into the load. The time-temperature relationships for dry-heat sterilization noted in Table 1.4.2 indicate a requirement for longer times and higher temperatures than for autoclaving, because dry heat is less efficient than moist heat. Lag time for conduction of heat into the materials to be sterilized can be significant, as can cool-down periods for large, dense objects. Dry-heat sterilization is applicable only for materials resistant to 140° to 180°C, and thus is unsuitable for paper, many plastics, or rubber. As noted for autoclaving, a dry-heat sterilization procedure should be validated with thermocouples and biological indicators.

Disinfectants

The term disinfection refers to the treatment of surfaces with chemical solutions to reduce microbial presence. Exposure to a disinfectant may result only in bacteriostatic or fungistatic rather than microbiocidal conditions. Disinfection does not imply sterilization, and it should never be used as an alternative to appropriate sterilization methods (e.g., autoclaving, dry heat, ethylene oxide, incineration). Similarly, disinfectants are not detergents and they should not be used as the sole method for cleaning solid surfaces. Indeed, many disinfectants are quickly inactivated when burdened with organic matter. Thus, routine disinfection of work surfaces in laminar-flow cabinets first requires that the surface be washed with a good detergent to remove dried media or other dirt before application of the germicide.

Ethanol. A solution of 70% ethanol is not germicidal against bacterial spores. While freshly prepared solutions are normally free of spores, the working solutions can contain bacterial spores from cross-contamination and poor aseptic technique. Once the spores are separated from the physical presence of the disinfectant (i.e., when the ethanol volatilizes from a surface, leaving the dried spores behind), they can germinate in suitable growth conditions. Thus, fresh working solutions of

70% ethanol should be prepared frequently (e.g., weekly).

The efficacy of ethanol as a disinfectant is highly concentration dependent. The mode of action of alcohol as a disinfectant is protein denaturation. Thus, 95% ethanol is a poor disinfectant because there is not enough water in the preparation to permit effective denaturation of contaminant proteins. As ethanolic concentration drops below 70%, there simply isn't enough of the solvent present to adequately react with large concentrations of proteinaceous matter. Because of the dilution effect, 70% ethanol is not effective in disinfecting large spills of culture material.

Quaternary ammonium compounds. Quaternary ammonium compounds are not effective against spores and, in this aspect, should be used with the same cautions noted for ethanol. Quaternary ammonium compounds are quickly inactivated by organic matter. They must not be used for disinfection of large spills of culture material, nor should they be used for kill pans (see below).

Bleach. Chlorine compounds, most frequently sodium hypochlorite, are strongly germicidal. Yet their potential for human toxicity and strong corrosiveness limits their use.

Biological spills. Strong disinfectant solutions are the first line of defense in decontaminating small to moderate spills of biological agents in both open areas and biosafety cabinets. The choice and concentration of disinfectant is particularly critical in this application. Use of 70% ethanol would be a poor choice as its germicidal activity is highly concentration dependent and dilution effects associated with spill cleanup would diminish its effectiveness (see above). Quaternary ammonium compounds are germicidal against a broad range of microbes, but they are quickly chemically overwhelmed and inactivated by organic matter (e.g., the culture material or medium in a spill). Chlorine compounds are strongly germicidal.

Kill pans. A final note of caution regarding disinfectants is their use in discard pans or pipet pans. Too many laboratories consider these containers kill pans and use them as a convenient way to dispose of excess liquid cultures or other contaminated solutions. Discard pans should never be used in such a manner. Most general disinfectants are inactivated by excess organic matter and/or exhibit diminished germicidal effects with dilution. Kill pans should contain 10% (v/v) household bleach (0.525% sodium hypochlorite).

Filtration

When using disposable filtration units, replace the filter funnel immediately with a permanent receiving vessel closure once filtration is complete. If the funnel is left on the receiver, the filter membrane will crack as soon as it dries, thereby compromising the sterility of the filtrate.

Be prepared and willing to prefilter any hazy or precipitated suspension. The additional steps will much repay the effort when balanced against the significant monetary expense, time, and frustration spent dealing with prematurely clogged sterilization filters. If one repeatedly filter sterilizes the same type of suspension, a prefiltration scheme can be tailored according to the nature of the particulates. For example, if particulates are retained only by 0.2- μm porosity filters, then omit prefiltration with depth filters and 0.45- μm filters.

Process large volumes of slow-filtering liquids (such as serum or other proteinaceous substances) in a cold room, if possible, to retard proliferation of microbial growth during the sometimes time-consuming, nonsterile prefiltration steps.

Anticipated Results

If sterilization and disinfection procedures are effective and proper aseptic technique is used, it should be possible to initiate and maintain cell cultures without any incidence of contamination.

Time Considerations

Preparation of materials for autoclaving and the autoclaving itself should take 1 to 2 hr; cooling solutions and equipment may require several hours. Dry-heat sterilization should take less than half a day plus cooling time. Monitoring the efficacy of sterilization requires several days to allow time for contaminants to grow. Disinfection of space and equipment requires a variable amount of time. The time required for filter sterilization can be a few minutes to hours depending on the solution.

Key References

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This filter manufacturer's catalog and reference guide provides a wealth of background, practical, and technical information to assist a user in appropriate choices for filters and filtration equipment.

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Assessing and Controlling Microbial Contamination in Cell Cultures

UNIT 1.5

This unit describes procedures for the detection of bacterial, fungal, and mycoplasma contaminants in cell cultures. Bacterial and fungal contaminants are detected by direct culture under conditions that specifically favor bacteria, mycelia, and yeast (see Basic Protocol 1). The direct method for detecting mycoplasma contamination similarly involves screening with microbiological media designed to encourage proliferation of mycoplasma (see Basic Protocol 2). The two indirect methods presented are (1) a slight modification of Barile's adaptation on the use of the Hoechst stain to detect mycoplasma by DNA fluorescence (see Alternate Protocol 1), and (2) the use of polymerase chain reaction in conjunction with a commercially available mycoplasma detection kit (see Alternate Protocol 2 and Support Protocol 1). In addition, a procedure is described for controlling microbial contamination through the use of antibiotics (see Basic Protocol 3).

Testing for microbial contamination should be integrated into a cell culture program as part of routine quality control. Microbial and mycoplasma testing should be performed upon arrival of all incoming cell lines and on lot samples of ampules prepared for master or working cell banks and seed stocks. Testing for microbial contamination should also be done whenever contamination is suspected (e.g., unusually slow growth rates for a particular cell line, aberrant appearance of cells). Indirect mycoplasma screening methods should also be done on new lots of serum used in media preparation when first received by the laboratory. Testing for microbial contamination should be performed after the cells have been cultured in the absence of antibiotics for several weeks.

TESTING FOR BACTERIAL AND FUNGAL CONTAMINANTS

The media and methods described in this protocol are suitable for detection of most bacteria and fungi that would be expected to survive as contaminants in cell lines. Brain heart infusion and trypticase soy agar with sheep blood are used for the cultivation of nutritionally fastidious bacteria of clinical origin that may be present in primary tissue cultures or in material contaminated by bacterial flora from human skin and poor aseptic technique. Fluid thioglycollate supports the growth of bacteria that require reduced oxygen tension; these microaerophilic or slightly anaerobic contaminants are frequently spore formers that originate from inadequately autoclaved or heat-sterilized materials. Soybean/casein digest broth is a general-purpose medium that supports the growth of a wide range of bacteria of human or environmental origin. HEPES/trypticase/yeast extract (HTYE) broth is also a general bacterial growth medium, but has the advantage of supporting growth of nutritionally or physiologically stressed bacteria not easily culturable with other media. These general types of bacteria are primarily environmental in origin and can be found in distilled water carboys, in fouled deionization systems, or as air-borne contaminants. Emmons' modification of Sabouraud's agar and YM agar are used for detection of filamentous fungi (molds) and yeasts, respectively. Molds are frequently environmental contaminants that can thrive under a wide variety of conditions. They often take residence in air-handling ducts to generate a constant microscopic rain of spores into a laboratory. Similarly, molds can often be found as films colonizing the dispensing tubing from distilled water reservoirs. The types of yeasts normally found as cell culture contaminants are human in origin.

Many of the media listed below may be purchased in their final configurations as sterile plates or test tubes from microbiological media manufacturers such as BBL or Difco, as well as other suppliers. Be that as it may, the cell culture user should carefully evaluate

**BASIC
PROTOCOL 1**

Cell Culture

1.5.1

Contributed by Rosalie Coté

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Supplement 1

the configurations, performance, and cost of any prepared quality control medium before commencing full-scale use in a cell culture laboratory.

Materials

Medium for bacterial detection: e.g., brain heart infusion (BBL, Difco), fluid thioglycollate medium (BBL, Difco), HTYE broth (see recipe), soybean/casein digest broth USP (e.g., trypticase soy broth, BBL; tryptic soy broth, Difco), or trypticase soy agar (BBL)

Medium for mycelial and yeast fungal detection: e.g., Sabouraud's dextrose agar (Emmon's modified; BBL, Difco), or YM agar (Difco)

Sterile, defibrinated sheep blood (e.g., Colorado Serum, Waltz Farm)

Cell culture test samples

Antibiotic-free culture medium (optional)

Conductivity meter (Corning model 162 or equivalent), if not integrated with the laboratory water purification system

50°C water bath

16 × 125-mm borosilicate screw-cap test tubes with rubber-lined caps

100 × 15-mm sterile plastic disposable petri dishes

Semiautomated repeat-volume filling unit to accurately dispense 5- to 24-ml aliquots (optional)

Incubators: 26°C, 35° to 37°C, and 37°C with 5% (v/v) CO₂

NOTE: To avoid inadvertent contamination of clean cell lines, bacterial and fungal testing should be segregated to a laboratory not used for general cell culture work.

Prepare media

For liquid (broth) media:

- 1a. Reconstitute brain heart infusion, HTYE broth, soybean/casein broth, and fluid thioglycollate medium per manufacturer's instructions, or per specific recipe instructions, in 10-megaohm (or higher) distilled or deionized water. Heat to ~50°C with frequent stirring to dissolve components. Heat to boiling with frequent stirring to dissolve any medium containing even small amounts of agar (e.g., fluid thioglycollate).
- 2a. Dispense medium into 16 × 125-mm borosilicate screw-cap test tubes at 10 ml/tube for fluid thioglycollate medium, and a 5 ml/tube for all other media.
- 3a. Cap tubes loosely, threading caps securely enough to prevent them from blowing off during autoclaving, but loosely enough to permit pressure exchange within the tube head space during the sterilization process.

Because of the large (10-ml) volume required with fluid thioglycollate medium, one should anticipate significant tube blow-outs upon autoclaving, and should prepare ~25% more tubes than required to compensate for the rejected material.

- 4a. Sterilize tubes by autoclaving at 121°C for 15 min under slow exhaust or liquid cycle.

Autoclaving times indicate the time necessary to hold the medium at the 121°C temperature for 15 min, not the time selected to run an autoclave cycle (UNIT 1.4; Table 1.4.1). Autoclave efficiency is very much machine and maintenance specific. However, an autoclave cycle of 20 min for a single 6 × 12 test tube rack of bacteriological medium, and a 32-min cycle for a 2-liter flask containing 1 liter of bulk medium, provide general guidelines for achieving the 121°C for 15 min sterilization criteria for these medium/vessel configurations.

- 5a. Remove tubes of medium from autoclave immediately after sterilization cycle is completed and/or when autoclave gauges indicate atmospheric pressure in the auto-

clave chamber. Allow medium to cool to ambient room temperature in a location not subject to excessive air currents or temperature fluctuations.

Oversterilization or prolonged holding of bacteriological media at elevated temperatures will severely affect performance. Cooling of just-autoclaved media in laboratory areas with significant temperature fluctuations or personnel movement can cause environmental contamination of otherwise sterile media.

- 6a. When tubes of medium reach ambient temperature, fully tighten screw caps and store tubes at 4° to 8°C until further quality control checks or until use (up to 6 to 9 months).

Fluid thioglycollate is a medium formulated to detect slightly anaerobic bacteria and contains a small amount of agar to retard atmospheric oxygen diffusion into the medium, cysteine as a reducing agent, and methylene blue as an oxygen indicator. Freshly prepared fluid thioglycollate will have a very small zone of aerobiosis at the medium surface/head space interface, as indicated by a slight purple to orange band of oxidized methylene blue. As oxygen slowly continues to permeate with prolonged storage of the medium, the pigmented, oxidized band will enlarge. Do not use the medium if the color has changed to orange in greater than the top 25% of the medium. Oxidized fluid thioglycollate may be rejuvenated, but only once, by steaming the tubed medium in a boiling water bath to purge gaseous oxygen from the medium, and then cooling just prior to use. Other bacteriological media cited here have a shelf life of 6 to 9 months when stored in the dark at 4° to 8°C.

Any of the liquid media cited above may be adapted for agar plate or test tube slant use by the addition of agar as the solidification agent as noted below.

For bulk agar media for plates:

- 1b. Reconstitute trypticase soy agar, Sabouraud's dextrose agar, and YM agar per manufacturer's instructions in 10-megohm (or higher) distilled or deionized water. Use an autoclavable container capable of holding at least twice the volume of the medium being prepared (e.g., use a 2-liter Erlenmeyer flask to autoclave 1 liter of medium) to avoid boiling over during the autoclave cycle.
- 2b. Sterilize the bulk medium by autoclaving at 121°C for 15 min under slow exhaust or liquid cycle (see step 4a annotation).
- 3b. Cool in a water bath to ~50°C.
- 4b. Add 50 ml/liter (5%) defibrinated sheep blood aseptically to trypticase soy agar.

It is critical that medium be cooled to 45° to 50°C before sheep blood is added.

- 5b. Dispense medium aseptically in 24-ml aliquots to 100 × 15-mm sterile plastic disposable petri dishes.

A semiautomated repeat-volume filling unit fitted with a weight on the inlet line to sink the tubing to the bottom of the flask of medium will greatly alleviate drawing of surface foam from the bulk flask to the petri dishes. Surface bubbles most often occur when postautoclaving agitation of the bulk flask is necessary to incorporate heat-labile additives such as sheep blood. A small number of media bubbles can be removed from the plates by lightly passing the flame of a Bunsen burner across the surface of the plated medium as soon as it is dispensed.

- 6b. Place plates in stacks of 10 to 20 and allow to cool and solidify overnight at room temperature. Store in vented plastic bags at 4° to 8°C until quality control checks or use (up to 12 weeks).

Cooling the plates in stacks of 10 to 20 retards formation of excessive condensation on the lids of the plates.

Prepare test samples

- 7a. *For lot sample preparation of cryopreserved ampules:* Use a 1-ml serological pipet to pool and mix the contents of ~5% of the cell culture ampules prepared from each freeze lot.
- 7b. *For cell culture vessels:* Examine cell culture vessels individually under low power, preferably with phase contrast, using an inverted microscope. Look for aberrant growth or appearance of the cells. Aseptically remove 5-ml aliquots from suspect cultures to use for further examination and testing. Quarantine any suspect cultures or containers to ensure that they will not be inadvertently mixed with and cross-contaminate clean cultures.

Unless the cultures are heavily contaminated, microbial growth will not be readily evident under low-power magnification. Mycelial fungal contamination sometimes is first noticed macroscopically by the appearance of small “cottony” or “lint-like” debris in the culture vessel.

8. Prepare wet mounts for microscopic evaluation of test samples and examine under oil immersion with high-power objectives ($\geq 1000\times$ magnification).

Bacterial contamination is recognized by the presence of small, uniformly sized spheres, rods, or spirals scattered throughout the field. The organisms may be individual, in clusters, or in chains. Rod-shaped bacteria may contain bright, refractile spores. The bacteria may be motile. Fungal yeast contamination appears as ovoid, fairly regularly sized nucleated cells scattered throughout the microscopic field. The yeast may be individual, in the process of budding off smaller daughter cells, or in short chains. Mycelial fungal contamination is characterized by the presence of long filaments or pieces of broken filaments in the microscopic field. Fungal spores, frequently appearing as spherical objects covered with spines or other protrusions, might be observed. Low-level contamination may not be detected under the microscope even by a trained microbiologist, particularly if the cell culture sample contains much debris.

9. If culture contains antibiotics, wash prior to inoculation of microbiological test media by centrifuging at $2000 \times g$ for 20 min (at room temperature or 4° to 8°C), removing the supernatant, and resuspending the pellet in an equal volume of antibiotic-free medium. Repeat for a total of three washes to eliminate traces of antibiotics that might interfere with microbial cultivation.

Inoculate microbiological media with test samples

10. For each test sample, inoculate each of the following with 0.3-ml aliquots of cell suspension:

- 2 tubes of brain heart infusion
- 2 tubes of fluid thioglycollate medium
- 2 tubes of HTYE broth
- 2 tubes of soybean/casein digest broth
- 2 plates of trypticase soy agar with 5% sheep blood
- 2 plates of Sabouraud’s dextrose agar, Emmon’s modified
- 2 plates of YM agar.

11. Incubate one plate of trypticase soy agar (with 5% sheep blood) at 37°C aerobically and the other at 37°C under 5% CO_2 .
12. Incubate one sample each of the other media at 26°C and the other sample at 35° to 37°C .
13. Examine all inoculated media daily for 14 days.

Usually, visual evidence of bacterial growth appears within 72 hr; fungal growth within 96 hr. Low-level contamination, or proliferation of contaminants previously stressed by the presence of antibiotics or other adverse culture conditions, may take longer to appear.

Positive bacterial and yeast growth appears as turbidity or the formation of a precipitate in liquid media. In an undisturbed test tube, growth may be restricted to a pellicle of growth at the surface of the liquid. On solid media, these bacteria and yeast appear as distinct, slightly convex, discretely isolated circular or confluent areas of microbial colonial proliferation, most often off-white or yellow in color. Filamentous fungal colonial growth is characterized by the appearance of typical cottony, whitish-gray to green to black mold on plates.

14. Autoclave and discard any general-use cell culture preparations that are positive for contamination.

If a cell culture that has tested positive is critical to maintain, repeat the microbial evaluation. If still positive, autoclave and discard the culture. If it is necessary to attempt to clean a microbially contaminated critical cell culture, see Basic Protocol 3.

Reusable glassware from discarded contaminated cultures should be decontaminated by autoclaving, cleaned, and depyrogenated by dry heat (UNIT 1.4).

TESTING FOR MYCOPLASMA CONTAMINATION BY DIRECT CULTURE

BASIC PROTOCOL 2

This protocol describes the direct detection of mycoplasma contamination by screening with microbiological media designed to encourage proliferation of mycoplasma. Total incubation time for this method is ~35 days. This schedule is necessary to detect low levels of mycoplasma contamination that might otherwise be scored as false negatives.

Materials

Cell line for testing

Mycoplasma broth medium (see recipe): 6 ml medium in 16 × 125–mm screw-cap test tubes

Mycoplasma agar plates (see recipe): 10 ml solidified medium in 60 × 15–mm petri dishes

37°C incubators: one without CO₂ and one humidified with 5% (v/v) CO₂

Inverted microscope with 100 to 300× magnification

NOTE: To avoid inadvertent contamination of clean cell lines, mycoplasma testing should be segregated to a laboratory not used for general cell culture work.

- 1a. *For adherent cultures:* Select a cell culture that is near confluency and has not received a fluid renewal within the last 3 days. Remove and discard all but 3 to 5 ml of the culture medium. Scrape a portion of the cell monolayer into the remaining culture medium using a sterile disposable scraper.
- 1b. *For suspension cultures:* Take the test sample directly from a heavily concentrated culture that has not received a fresh medium supplement or renewal within the last 3 days.

Samples can also be taken directly from thawed ampules that have been stored frozen.

2. Inoculate 1.0 ml of the test cell culture suspension into 6 ml mycoplasma broth medium in a 16 × 125–mm screw-cap test tube. Also inoculate 0.1 ml of the test sample onto the center of a 60 × 15–mm mycoplasma agar plate.
3. Incubate the broth culture aerobically at 37°C. Incubate the agar plate in a humidified 37°C, 5% CO₂ incubator. Observe broth culture daily for development of turbidity and/or shift in pH (medium becomes redder for alkaline shift, yellower for acid shift).

As an alternative, a self-contained anaerobic system such as the GasPak equipment (Becton Dickinson Microbiology Systems) can be used in conjunction with a standard 37°C incubator to provide proper CO₂ levels.

Cell Culture

1.5.5

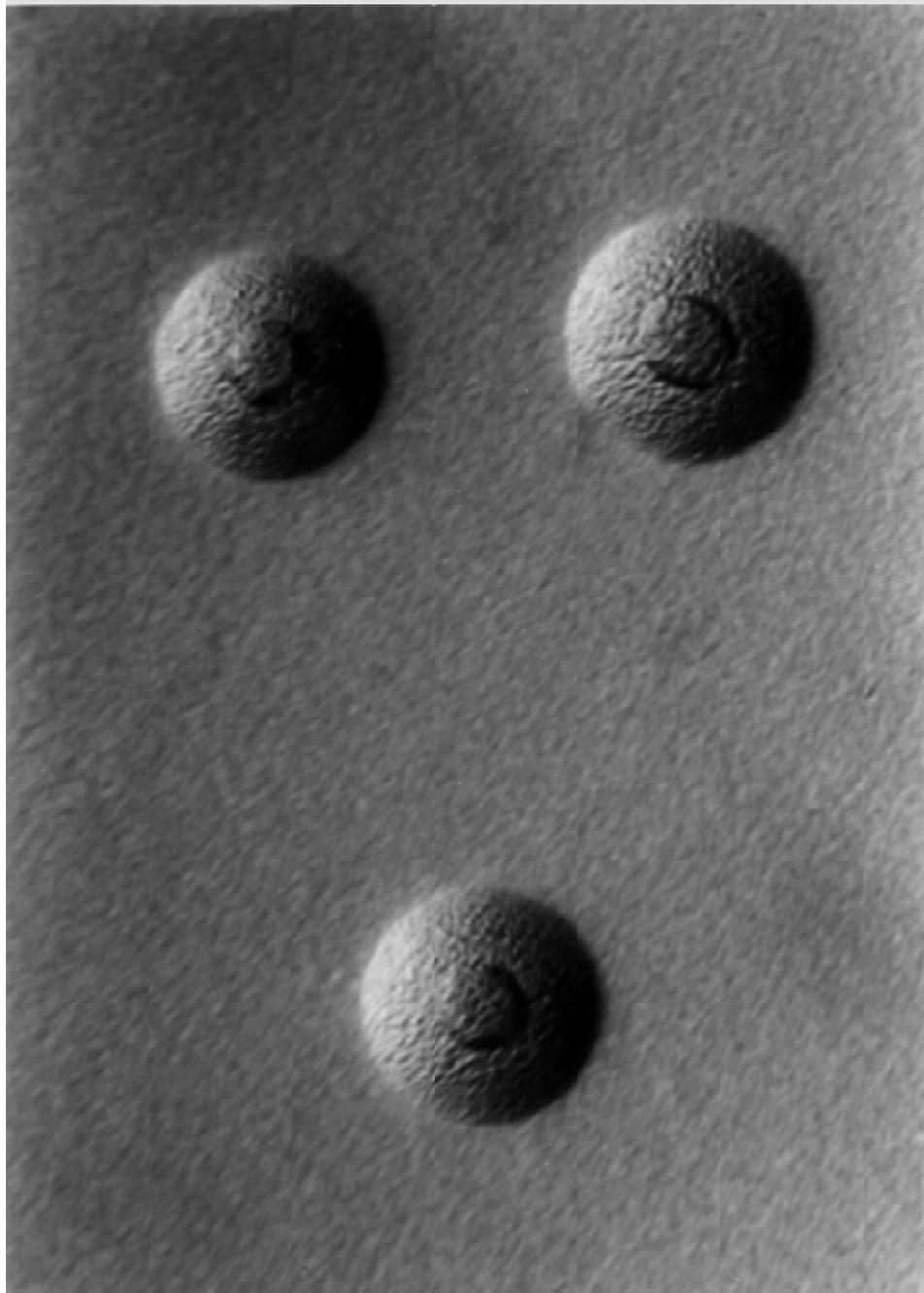


Figure 1.5.1 Mycoplasma colonies at 230 \times magnification. Figure provided by W. Siegel, Bio-Whittaker, Inc.

4. After 5 to 7 days of incubation and again after 10 to 14 days, remove a 0.1-ml sample from the broth culture and inoculate a fresh mycoplasma agar plate. Incubate these plates as in step 3.
5. Using an inverted microscope at 100 to 300 \times magnification, examine the agar plates weekly for at least 3 weeks for mycoplasma colony formation and growth.

Mycoplasma colonies range from 10 to 55 μm in diameter and classically look like a fried egg, with the dense center of the colony embedded in the agar and the thinner outer edges of the colony on the surface of the substrate (Fig. 1.5.1). However, much variation in colony morphology occurs between species and culture conditions. Although colonies typically

appear within 4 days after inoculation, plates should be kept for the full incubation period before scoring them as negative.

6. To confirm presumptive mycoplasma colonies, subculture a small (~1 cm²) section of the suspicious area of the agar plate into a tube of mycoplasma broth medium, incubate up to 14 days, and observe as in step 3.

When other detection methods are not available, the ability to subculture presumptive mycoplasma colonies will help to differentiate authentic mycoplasma presence from artifacts such as air bubbles, tissue culture cells, or pseudocolonies.

INDIRECT TESTING FOR MYCOPLASMA BY STAINING FOR DNA

The total time for this protocol, which includes the use of an indicator cell culture, is 6 days. The staining procedure itself takes ~1 hr. The use of an indicator cell culture provides a number of advantages. The indicator cell line supports the growth of more fastidious mycoplasma species. Both positive and negative controls are thus readily available for direct comparison with the culture samples being tested. Selection of a proper indicator cell is important to the success of this procedure. It must first have good viability. Transformed cell lines are not recommended as indicators as they produce significant nuclear background fluorescence, which interferes with interpretation of results. Cell lines that produce much debris, such as hybridomas, are also not recommended as indicator cells because of the amount of positive staining artifacts that confuse interpretation of results.

Materials

Complete EMEM-10: Eagle's minimum essential medium (EMEM) with Earle's salts (Life Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% (v/v) bovine calf serum (see *UNIT 1.2* for media preparation methods)

Indicator cell line: e.g., African green monkey cell line Vero (ATCC #CCL81) or 3T6 murine cell line (ATCC #CCL96)

Cell culture for testing

Mycoplasma hyorhinis (ATCC #29052) or a known mycoplasma-infected cell line to use as a positive control, actively growing

Fixative: 3:1 (v/v) absolute methanol/glacial acetic acid

Hoechst stain (see recipe)

Mounting medium (see recipe)

60 × 15-mm culture dishes, sterile

No. 1 or no. 1½ coverslips, sterilized by autoclaving (*UNIT 1.4*)

37°C, 5% (v/v) CO₂/95% air incubator

NOTE: To avoid inadvertent contamination of clean cell lines, mycoplasma testing should be segregated to a laboratory not used for general cell culture work.

Prepare indicator cell cultures

1. Aseptically place a sterile glass no. 1 or 1½ coverslip into each sterile 60 × 15-mm culture dish.

Use two culture dishes for the positive control, two dishes for the negative control, and two dishes for each test sample.

2. Aseptically dispense 3 ml complete EMEM-10 into each culture dish.

Make certain that each coverslip is totally submerged and not floating on top of the medium.

3. Prepare a single-cell suspension of the indicator cell line in complete EMEM-10 at a concentration of 1.0×10^5 cells per ml.

ALTERNATE PROTOCOL 1

Cell Culture

1.5.7

4. Inoculate 1 ml indicator cell suspension into each culture dish.
5. Incubate overnight at 37°C in a 5% CO₂/95% air incubator.
6. Microscopically examine cultures to verify that the cells have attached to the glass coverslip. Code the top of each culture dish for identification purposes (to record the test samples to be inoculated).

Inoculate test samples

7. Add 0.5 ml/dish complete EMEM-10 to two culture dishes for negative controls.
- 8a. *For adherent cultures:* Select a test cell culture that is near confluency and has not received a fluid renewal within the last 3 days. Remove and discard all but 3 to 5 ml of the culture medium. Scrape a portion of the cell monolayer into the remaining culture medium using a sterile disposable scraper.
- 8b. *For suspension cultures:* Take the test sample from a heavily concentrated culture that has not received a fresh medium supplement or renewal within the last 3 days.
Samples can also be taken directly from thawed ampules from frozen stocks.
9. For each test sample, add 0.2 to 0.5 ml/dish test sample to two culture dishes.
10. Add 0.5 ml/dish actively growing *Mycoplasma hyorhinis* to two culture dishes for positive controls.

CAUTION: To prevent spread of mycoplasma, infected strains should be destroyed and removed as quickly as possible. Benches and incubators should be cleaned. For safety of the investigator, BSL 2 laboratory conditions are appropriate.

Alternatively, a known mycoplasma-infected cell line can be used.

11. Return the cultures to the CO₂ incubator and allow to incubate undisturbed for 6 days.
Cultures should be at 20% to 50% confluent. Confluence can interfere with microscopic examination for mycoplasma.

Fix, stain, and mount coverslips

12. Remove cultures from incubator. Aspirate medium and immediately add 5 ml fixative to each culture dish. Incubate for 5 min.
Do not allow the culture to dry between removal of the culture medium and addition of the fixative.
13. Aspirate fixative from each culture dish and repeat fixation for 10 min.
These fixing times are minimal. Additional fixation time will not harm the procedure and can be beneficial for some preparations.
14. Aspirate the fixative and let the cultures air dry.
Dry completely and store in a 60-mm petri or tissue culture dish if samples are to be accumulated at this stage for later staining.
15. Add 5 ml Hoechst stain to each culture dish, cover, and let stand at room temperature for 30 min.
16. Aspirate the stain and rinse each culture three times with 5 ml distilled water.
17. Aspirate well so that the glass coverslip is completely dry. Let air dry if necessary.
18. Place a drop of mounting medium on a clean glass microscope slide.
19. Use forceps to remove the glass coverslip containing the fixed cells from the culture dish and place face up on top of the mounting medium, being careful to eliminate air bubbles.

20. Add a second drop of mounting medium onto the top of the specimen coverslip and cover with a larger clean coverslip, being careful to eliminate air bubbles.
21. Label each slide to identify the specimen.
22. Observe each specimen by fluorescence microscopy at 500× using immersion oil. Use a blue glass excitation filter (330/380 nm) in combination with a 440-nm barrier filter (see UNIT 4.2). Compare test samples to positive and negative controls.

The nuclei of the indicator cells appear as large (~20 μm), ovoid fluorescing bodies. Mycoplasma will appear as small fluorescing particles (0.1 to 1.0 μm) that are regular in shape and size in the cytoplasm or in intercellular spaces. If infection is heavy, the particles may be tightly clustered in some areas. With low-level contamination, not all cells will be infected. Thus, all of the slide should be examined.

INDIRECT TESTING FOR MYCOPLASMA BY PCR

Kits are now available from a number of manufacturers for the detection of mycoplasma in cell cultures or other material using PCR. The kits, although expensive and requiring the expertise and equipment needed for molecular procedures, provide advantages with quick results (1 day) and the ability to speciate the contaminant and thus potentially identify its source. The PCR kits are also useful for detecting mycoplasma in cultures prone to forming artifact debris that often obscures definitive interpretations with staining detection methods. The procedure described here uses primers from a commercially available kit and a nested PCR assay that amplifies the spacer region between the 16S and 23S rRNA genes of mycoplasmas. This specific protocol details procedures using cell cultures; however, instructions provided with the kit also explain how to make modifications for analyzing test samples of serum or frozen cells. The novice practitioner of PCR methodologies is strongly urged to consult Sambrook et al. (1989) for specific details (also see APPENDIX 3).

Materials

- Cells for testing
- 10× PCR buffer (usually provided with *Taq* polymerase)
- 2.5 mM 4dNTP mix: 2.5 mM each dGTP, dCTP, dTTP, and dATP
- 25 mM MgCl₂
- 5 U/μl *Taq* DNA polymerase
- Mineral oil (if needed for thermal cycler)
- Mycoplasma detection kit (ATCC), containing first- and second-stage primer mixtures (total 7 primers), as well as two positive control mycoplasma DNAs (*Mycoplasma pirum*, *Acholeplasms laidlawii*)
- Thin-wall microcentrifuge tubes
- Aerosol-preventive micropipettor tips, sterile
- Positive-displacement micropipettors
- Picofuge
- Thermal cycler
- Additional reagents and equipment for agarose gel electrophoresis (see Support Protocol)

NOTE: To avoid inadvertent contamination of clean cell lines, mycoplasma testing should be segregated to a laboratory not used for general cell culture work.

NOTE: To avoid amplification of contaminating DNA from laboratory workers, room contaminants, or previous mycoplasma DNA amplifications, all PCR should be performed using aseptic technique (also see special considerations for PCR experiments in APPENDIX 2A).

ALTERNATE PROTOCOL 2

Prepare test sample

- 1a. *For confluent monolayers:* Detach cell monolayer from flask surface with a cell scraper. Gently agitate the flask to dispense the cells in the medium. Transfer 0.5 ml cell suspension ($\sim 5 \times 10^4$ cells/ml) to a sterile 1.5-ml microcentrifuge tube.
- 1b. *For suspension cultures:* Mix the suspension culture by gently pipetting to obtain an even dispersal of cells in the medium. Transfer 0.5 ml of the suspension ($\sim 5 \times 10^4$ cells/ml) to a sterile 1.5-ml microcentrifuge tube.
2. Centrifuge at $12,000 \times g$ for 10 min at 4°C .
3. Discard 400 μl supernatant and use a pipet to resuspend the cell pellet in the remaining 100 μl medium.

Pipet gently to avoid formation of bubbles. The 100- μl cell suspension is ready for PCR.

Run first-stage PCR

4. Using a permanent ink marker, label each thin-wall microcentrifuge tube (reaction tube) with appropriate test sample or control DNA codes.
5. Prepare a master mix of PCR reagents in sufficient quantity for all samples to be tested ($n + 1$ or 2 reactions) plus a minimum of two positive DNA controls and one negative control. Use the following volumes per reaction:

5 μl 10 \times PCR buffer
1 μl first-stage primer mixture
1 μl 2.5 mM 4dNTP mix
1 μl 25 mM MgCl_2 (see annotation)
0.2 μl 5 U/ μl *Taq* DNA polymerase
Deionized water to 45 μl .

Store the mix on ice until it is aliquotted.

*The optimal reaction conditions for this procedure are 10 mM Tris·Cl (pH 8.3 to 8.8), 50 mM KCl, 2.0 mM MgCl_2 , 50 μM of each dNTP, and 1 U *Taq* polymerase. Check for the inclusion and final concentration of MgCl_2 in the 10 \times PCR buffer supplied with the *Taq* polymerase and adjust the volume of MgCl_2 in the reagent mix, if necessary, to give a final concentration of 2.0 mM. Adjust the amount of water in the mix accordingly for a final reaction mix volume of 45 μl .*

NOTE: In this and all subsequent steps, use positive-displacement micropipettors and sterile aerosol-preventive micropipettor tips to prevent contamination of the amplification reaction.

6. Pipet 45 μl reaction mix into each sample and control reaction tube.
7. If the thermal cycler used requires mineral oil to minimize sample evaporation, add 40 to 60 μl of mineral oil to each tube.

Keep the reaction tubes closed, except when aliquotting into them, to avoid possible cross-contamination.

8. Add 5 μl test sample (step 3) to the appropriate reaction tube containing reagent mix (final reaction volume 50 μl). If reaction tubes contain mineral oil, pipet the samples directly into the mix below the mineral oil layer (final tube volume 90 to 110 μl).
9. Add 5 μl of each positive control mycoplasma DNA into separate positive control tubes.
10. Add 5 μl sterile deionized water into the negative control tube.

11. Mix each tube thoroughly by flicking it with a finger, and centrifuge the tube briefly in a microfuge.
12. Place all the tubes into a thermal cycler and perform amplification using the following program:

Initial step:	30 sec	94°C	(denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	2 min	55°C	(annealing)
	2 min	72°C	(extension)
Final step:	5 min	72°C	(extension)
Chill:	indefinitely	4°C	(hold).

Store PCR products at 4°C or on ice until further use (2 to 3 weeks). For longer periods, store at -20°C.

Run second-stage PCR

13. Using a permanent ink marker, label each second-stage reaction tube with appropriate test sample or control DNA codes.
14. Prepare a master mix of reagents in sufficient quantity for all samples ($n + 1$ or 2 reactions), plus positive and negative controls, using the following volumes per reaction:

5 μ l 10 \times PCR buffer
1 μ l second-stage primer mixture
1 μ l 2.5 mM 4dNTP mix
1 μ l 25 mM MgCl₂ (see step 5 annotation)
0.2 μ l 5 U/ μ l *Taq* DNA polymerase
Deionized water to 49 μ l.

Store the mix on ice until it is aliquotted.

15. Pipet 49 μ l reaction mix to each sample and control tube.
16. If the thermal cycler used requires mineral oil to minimize sample evaporation, add 40 to 60 μ l of mineral oil to each tube.

Keep the reaction tubes closed, except when aliquotting into them, to avoid possible cross-contamination.

17. Carefully pipet 1 μ l from the first-stage PCR reaction tube (step 12) into the second-stage reaction tube (final reaction volume 50 μ l). If mineral oil was used, add the sample to the reagent mix below the oil layer (final tube volume 90 to 110 μ l).
18. Mix each tube thoroughly by flicking it with a finger, and centrifuge the tube briefly in the microfuge.
19. Place all the tubes in the thermal cycler and run the program as above (step 12). Store PCR products at 4°C or on ice until further use (2 to 3 weeks). For longer periods, store at -20°C.
20. Analyze reaction products by agarose gel electrophoresis (see Support Protocol).

AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCTS

Agarose gel electrophoresis is used to analyze the products of the second-stage PCR for the presence of mycoplasma sequences. The protocol is written for one 10-lane gel, which can accommodate up to four unknown samples, two positive controls, one negative PCR control, one negative electrophoresis control, and two molecular weight marker lanes. For additional samples, the procedure can be scaled by using a comb with additional wells, or additional gels can be run.

Materials

Agarose (e.g., NuSieve, FMC Bioproducts)
1× TBE electrophoresis buffer (*APPENDIX 2A*)
10 mg/ml ethidium bromide solution
Second-stage PCR products from test samples and controls (see Alternate Protocol 2)
6× electrophoresis sample buffer (see recipe)
Molecular weight marker (100-bp DNA ladder)

Electrophoresis apparatus with a 10 × 14-in. gel tray and a 1-mm, 10-tooth comb
Power supply
UV light box

Prepare 2.5% agarose containing ethidium bromide

1. Seal a 10 × 14-in. gel tray from an electrophoresis apparatus according to manufacturer's instructions and place on a level surface.
2. Weigh 2.25 g agarose and place in a 250-ml Erlenmeyer flask.
3. Add 90 ml of 1× TBE electrophoresis buffer, swirl to mix, and heat to boiling to completely dissolve agarose.
4. Add 5.4 µl of 10 mg/ml ethidium bromide solution, swirl to mix, and cool to ~55°C.

CAUTION: Ethidium bromide is a mutagen and a potential carcinogen. Gloves should be worn and care should be taken when handling ethidium bromide solutions.

Cast gel

5. Pipet 80 ml agarose solution into the center of the gel tray. Remove any bubbles.
6. Gently place a 1-mm, 10-tooth comb into the gel mold. Allow gel to harden until it becomes milky and opaque in appearance (~1 hr).
7. Remove tape or sealers from the gel mold. Place gel into electrophoresis tank.
8. Pour ~950 ml of 1× TBE electrophoresis buffer into the electrophoresis tank.

Gel should be totally submerged in buffer, but not covered more than 1 cm.

9. Gently remove the gel comb.

Prepare sample

10. Add 10 µl of each second-stage PCR product to a separate microcentrifuge tube containing 2.0 µl of 6× electrophoresis sample buffer. Mix well.
11. Add 5 µl of 100-bp DNA ladder to 2 µl of 6× electrophoresis sample buffer. Mix well.

Load and run the gel

12. Add 7 µl DNA ladder to each of the first and last wells (lanes 1 and 10) of the gel.
13. Add 12 µl of 6× electrophoresis sample buffer to one lane as a negative control.

14. Add 12 μ l unknown test samples and positive and negative PCR controls to the remaining wells.
15. Connect a power supply and run the gel at 75 V for ~1 hr and 40 min.
16. View the gel on a UV light box.

CAUTION: UV light is damaging to eyes and exposed skin. Protective eyewear should be worn at all times while using a UV light source.

*With the primers used in Alternate Protocol 2, the mycoplasmas commonly encountered as cell culture contaminants should generate a second-stage PCR DNA amplicon that ranges in size from 236 to 365 bp. In contrast, the *A. laidlawii* positive control should generate two amplicons of 426 bp and 219 bp, and the *M. pirum* positive control should generate a single 323-bp amplicon. No discrete amplicon band should be seen in the negative controls. Fuzzy bands <100 bp are sometimes observed in control and test samples and are probably primer multimer artifacts.*

USE OF ANTIBIOTICS TO CONTROL MICROBIAL CONTAMINATION

The best way to deal with a contaminated cell culture is to autoclave it. Be that as it may, situations will undoubtedly occur when there is a need to attempt to salvage a microbially contaminated cell culture. This brief protocol describes the use of antibiotics in cell culture and cautions against misuse of these potent antimicrobial agents.

Materials

Contaminated cell culture

Sterile antibiotic stock solution(s) as appropriate (Table 1.5.1)

Additional reagents and equipment for identifying microbial contamination (see Basic Protocols 1 and 2; see Alternate Protocols 1 and 2)

1. Immediately quarantine the contaminated culture to prevent possible cross-contamination with other cultures in the laboratory.
2. Identify the microbial contaminant as to type: bacterial, fungal, or mycoplasmal (see Basic Protocols 1 and 2; see Alternate Protocols 1 and 2).

If bacteriological expertise is available, identification of a bacterial contaminant to genus can help narrow antibiotic selection to one that is particularly effective against the contaminant.

- 3a. *For an identified microbial contaminant:* Select an appropriate antibiotic from the Table 1.5.1, and prepare and filter sterilize a stock solution.
- 3b. *For an unknown bacterial contaminant:* Prepare and filter sterilize the following 10 \times antibiotic cocktail:

2500 U/ml penicillin
2.5 mg/ml streptomycin sulfate
2.5 mg/ml neomycin
25 U/ml bacitracin

- 4a. *For monolayers:* Remove contaminated medium by suction and add fresh medium containing antibiotics. Add specific antibiotic stock solutions (step 3a) to fresh medium to give the appropriate working concentration (Table 1.5.1). Alternatively, add 1 vol of 10 \times antibiotic cocktail (step 3b) to 9 vol fresh medium.

Do not add greater than the recommended amount of antibiotic. All of these chemical reagents are cytotoxic in that they have essentially the same metabolic effect on cells as they do on microorganisms if the concentration is great enough.

**BASIC
PROTOCOL 3**

Cell Culture

1.5.13

Table 1.5.1 Antibiotics^a

Organism	Antibiotic	Solvent	Stability (days at 37°C)	Working concentration
Bacteria (gram-positive only)	Ampicillin	Water	3	100 mg/liter
	Erythromycin	2 M HCl	3	100 mg/liter
	Gentamicin sulfate	Water	5	50 mg/liter
	Kanamycin sulfate	Water	5	100 mg/liter
	Neomycin sulfate	Water	5	50 mg/liter
	Penicillin-G, K ⁺ salt	Water	3	10 ⁵ U/liter
	Streptomycin sulfate	Water	3	100 mg/liter
	Tetracycline HCl	Water	4	10 mg/liter
Fungi (molds and yeasts)	Amphotericin B	DMSO; DMF ^b	3	2.5 mg/liter
	Nystatin	DMF	3	2.5 × 10 ⁶ U/liter
Mycoplasma	Gentamicin sulfate	Water	5	50.0 mg/liter

^aAll antibiotics must be filter sterilized in the solvent noted. Antibiotics are meant only for short-term use; if contamination is not cleared after 14 days of treatment or two subcultures, discard culture.

^bAbbreviations: DMF, dimethylformamide; DMSO, dimethyl sulfoxide.

4b. *For suspensions:* Centrifuge culture 10 min at 125 × g. Remove supernatant and resuspend cells in fresh medium containing antibiotics as described in step 4a.

5. Add additional antibiotic solution every 3 to 5 days to maintain the working concentration (note stability times at 37°C in Table 1.5.1). Continue treatment for 14 days.

Change medium or passage cells as required during this time, being sure to maintain the antibiotic concentration.

6. Examine the contaminated culture microscopically during the treatment period; look for evidence of cytotoxicity as well as elimination of the contaminant.

7. If the antibiotic treatment is unsuccessful, destroy the culture by autoclaving. If culture appears to have been cleaned of the contaminant, inoculate into fresh, antibiotic-free medium.

It is not recommended that cultures be maintained in media containing antibiotics. The chemicals may have adverse effects on cell characteristics and will also create the risk of selecting for a population of antibiotic-resistant contaminants that could pose a human health hazard.

8. Test for microbial and mycoplasma contamination (see Basic Protocols 1 and 2; see Alternate Protocols 1 and 2).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Dextrose/arginine supplement

50.0 g dextrose

10.0 g L-arginine HCl

Dissolve ingredients with stirring and heating at 37°C in ~800 ml deionized, distilled water. Bring final volume of solution to 1.0 liter with water. Filter sterilize and dispense in convenient aliquots (e.g., 100-ml aliquots for making 1 liter of mycoplasma agar or broth). Store frozen (at -20° to -70°C) until needed (up to 12 months).

Electrophoresis sample buffer, 6×

- 2.5 ml 1% (w/v) bromphenol blue
- 2.5 ml 1% (w/v) xylene cyanol
- 5.0 ml glycerol
- Store up to 6 months at 4°C in glass container

Hoechst stain

Stock concentrate:

- 5.0 mg bisbenzamide fluorochrome stain (Calbiochem)
- 10.0 mg thimerosal (Sigma)
- 100 ml 1× HBSS (*APPENDIX 2A*) without sodium bicarbonate or phenol red
- Prepare in a brown bottle wrapped in aluminum foil. Mix thoroughly 45 min at room temperature using a magnetic stirrer. Store 1-ml aliquots up to 1 year at –20°C in the dark.

The stock concentrate is heat and light sensitive.

The concentrate can support microbial growth and should be examined periodically for contamination. Filter sterilization of the concentrate is not recommended as it diminishes fluorescence.

Working solution: In a brown bottle, dilute 1.0 ml stock concentrate in 100.0 ml HBSS without sodium bicarbonate or phenol red. Mix thoroughly 20 to 30 min at room temperature using a magnetic stirrer. Prepare fresh immediately before use.

HTYE (HEPES/trypticase/yeast extract) broth

- 5.0 g trypticase peptone (BBL)
- 2.0 g yeast extract
- 4.0 g HEPES acid
- 1.0 liter H₂O
- Adjust to pH 7.1 ± 0.1 with NaOH
- Dispense as required
- Autoclave at 121°C for 15 min
- Store up to 6 to 9 months at 4° to 8°C

Mounting medium

- 22.2 ml 0.1 M citric acid
- 27.8 ml 0.2 M Na₂HPO₄
- 50.0 ml glycerol
- Adjust to final pH 5.5
- Store up to 6 months at 2° to 8°C

Periodically check pH of prepared mounting medium, as pH is critical for optimal fluorescence.

Mycoplasma agar plates

- 23.8 g mycoplasma agar base (BBL)
- 600 ml deionized, distilled H₂O
- Heat to boiling with constant stirring to dissolve all ingredients. Sterilize by autoclaving at 121°C for 15 min (*UNIT 1.4*, Basic Protocol 1). Cool to 50°C and aseptically add the following sterile solutions which have been equilibrated to 37°C:
 - 200 ml horse serum (Life Technologies)
 - 100 ml fresh yeast extract solution (Life Technologies)
 - 100 ml dextrose/arginine supplement (see recipe)
- Aseptically adjust to pH 7.2 to 7.4 and dispense in 10-ml aliquots to sterile 60 × 15-mm petri dishes. Store up to 6 weeks at 4°C to 8°C.

continued

Solutions should be prewarmed to 37°C before adding to the agar solution to avoid immediate gelling.

The sterile fresh yeast extract solution used in the preparation of the mycoplasma broth and agar media frequently contains large amounts of sediment that must be removed before it is used in media preparation. Sediment can be removed by coarse filtration followed by filter sterilization (UNIT 1.4) with a 0.2- μ m membrane.

The formation of bubbles in agar plates should be avoided as it can confuse examination. When preparing medium, add and mix heat-labile supplements carefully to avoid formation of bubbles. As most of the bubbles are at the surface of the medium, they can be avoided during dispensing by using a semiautomatic dispensing apparatus, such as a peristaltic pump with a sinker attached to the inlet tubing to assure that the medium is being drawn from below the surface of the flask. If a few bubbles form when the medium is dispensed into the plates, they may be removed by lightly passing the flame of a Bunsen burner across the agar surface before the agar has solidified.

***Mycoplasma* broth medium**

14.7 g mycoplasma broth base (BBL)

20.0 mg phenol red

600.0 ml deionized, distilled H₂O

Mix well to dissolve all ingredients. Sterilize by autoclaving at 121°C for 15 min (UNIT 1.4, Basic Protocol 1). Cool to room temperature and aseptically add the following sterile solutions:

200 ml horse serum (Life Technologies)

100 ml fresh yeast extract solution (Life Technologies)

100 ml dextrose/arginine supplement (see recipe)

Aseptically adjust to pH 7.2 to 7.4 and dispense in 6-ml aliquots to sterile 16 × 125-mm test tubes. Store up to 12 weeks at 4° to 8°C.

The sterile fresh yeast extract solution used in the preparation of the mycoplasma broth and agar media frequently contains large amounts of sediment that must be removed before it is used in media preparation. Sediment can be removed by coarse filtration followed by filter sterilization (UNIT 1.4) with a 0.2- μ m membrane.

COMMENTARY

Background Information

Bacterial and fungal contamination

Bacterial and fungal contamination of cell cultures can originate from many sources. Lapses in aseptic technique at any stage of culture manipulation can introduce low-level contamination that may take days or weeks to proliferate to noticeable levels. Mechanical or operational failures with air handling, primary barriers (e.g., biosafety cabinets), water purification and storage, or sterilization systems utilized by the laboratory can give rise to contamination in spite of adequate aseptic practices during culture transfers. Commonly encountered contaminants of human origin include *Escherichia coli*, *Micrococcus* and *Staphylococcus* sp., and the fungal yeast *Candida* sp. Common contaminants from environmental sources include *Bacillus* sp., *Escherichia coli*, *Pseudomonas* sp. and allied genera, and the ubiquitous filamentous fungi *Aspergillus* sp.,

Cladosporium sp., and *Penicillium* sp. Testing procedures described in this unit detect most of the common organisms cited above as well as some less-frequently encountered microbial contaminants of cell cultures. None of the techniques listed, however, will detect all potential microbial cell culture contaminants. If microbial contamination is suspect but not culturable with these media, consultation with a reputable microbiology laboratory should be the course of action.

As an integral part of quality control in a cell culture lab, microbial testing should be performed on culture media, on incoming cultures, and on all seed stock and working cell banks.

***Mycoplasma* contamination**

Mycoplasma contamination of cell cultures is a frequent occurrence that often goes unobserved because, unlike bacterial or fungal contamination, it is rarely visible to the naked eye. The presence of mycoplasma in cultures can

inhibit cell metabolism and growth, alter nucleic acid and protein syntheses, affect cell antigenicity, induce chromosomal alterations, interfere with virus replication, and mimic viral actions. Bovine serum (used in media preparation), laboratory personnel, original tissue samples, and other infected cell lines can be sources of mycoplasma contamination.

No one method, either direct or indirect, will detect all mycoplasma, particularly at low levels of infection. A good mycoplasma screening program will include both direct and indirect methods. Direct culture involves a long test period of over a month and may not detect mycoplasmal strains with nutritional requirements not satisfied by the growth medium. DNA fluorescence is an efficient method for screening mycoplasma contamination, but sometimes it takes experience to recognize the presence of contaminants amid background debris and artifacts. PCR is very fast and sensitive, but expensive in terms of both basic equipment and reagents.

Commercial mycoplasma testing services may be an option for laboratories without the experience or specialized equipment needed to fully screen a cell line for contamination with these microorganisms. A good testing service should be able to provide direct culture, indirect staining, and molecular (DNA/RNA hybridization or PCR) methods. Many of the larger reference culture collections with cell line holdings—e.g., American Type Culture Collection (ATCC), European Collection of Cell Cultures (ECACC), or German Collection of Microorganisms and Cell Culture (DSMZ)—offer mycoplasma testing services. Expect to pay approximately \$100 U.S. per sample for direct culture methods and staining. PCR or other molecular techniques will increase the testing fee because of the cost of the reagents involved.

Mycoplasma testing, along with testing for bacteria and fungi, should be included in the routine quality control procedures for all cell culture laboratories. Tests should be performed on sera used in culture media, on incoming cultures, and on all seed stock and working cell banks.

Antibiotic treatment

Antibiotics are not substitutions for good aseptic technique and should not be routinely used as prophylactics against possible contamination. Antibiotics work by disrupting metabolic processes (e.g., protein synthesis) and the chemical mode of action does not always dif-

ferentiate between microbial contaminants and cultured cells. Antibiotics can be cytotoxic, sometimes at working concentrations. The use of combined antibiotics can exert cytotoxic effects at lower concentrations than that of an individual antibiotic. Do not exceed the suggested working concentration for any antibiotic. As cytotoxicity is cell line dependent, it is necessary to monitor the effects of antibiotic use on cell characteristics as well as on the contaminant.

Critical Parameters

Because of the insidious nature of infection, mycoplasma testing should be segregated from rooms where routine cell culture work is conducted to avoid cross-contamination of clean cultures with mycoplasma-infected cell cultures or mycoplasma cultures being grown with the direct culture screening method. Any mycoplasma-infected material should be immediately destroyed by autoclaving.

It is important to note the stability of antibiotics at 37°C (Table 1.5.1). Antibiotic potency diminishes within days of incubation and additional antibiotic should be added during the 2-week treatment to ensure that working concentrations are maintained, in order to prevent development of antibiotic-resistant contaminants.

Anticipated Results

If contamination of cultures is suspected, careful application of these testing procedures should allow identification of most common microbial, fungal, and/or mycoplasma contaminants. Success rates for treatment of contaminated cultures cannot be generalized, as they depend on the type of contaminant and the severity of the infection.

Time Considerations

Testing for contamination can take a few minutes for visual inspection to several weeks for identification of contaminants such as mycoplasma. Treating contaminated cultures with antibiotics requires 14 days for treatment itself, plus additional days to weeks to verify that the culture is no longer contaminated.

Literature Cited

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Key References

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Provides invaluable information on the mode of action of, for instance, antibiotics and antagonisms.

Budavari, S., O'Neil, M.J., Smith, A., Heckelman, P.E., and Kinneary, J.F. (eds.) 1996. The Merck Index. Merck Research Laboratories Division of Merck & Co., Whitehouse Station, N.J.

A technical reference providing information on physical characteristics, solubilities, and stabilities of most chemicals (including antibiotics) used in a tissue culture laboratory.

Freshney, R.I. 1994. Contamination. *In* Culture of Animal Cells: A Manual of Basic Technique, 3rd ed., pp 243-252. Wiley-Liss, New York.

This chapter of the classic text on cell culture discusses various types of contamination and methods for detection and eradication.

Hay, R.J., Caputo, J., and Macy, M.L. 1992. ATCC Quality Control Methods for Cell Lines, 2nd ed. American Type Culture Collection, Rockville, Md.

A manual of procedures used in this cell culture collection's quality control program.

Sigma-Aldrich Co. 1998. Cell Culture Catalogue. Sigma-Aldrich Co., St. Louis, Mo.

Most specialty chemical companies feature a wealth of useful, technical information with their catalogues. This particular edition can be most helpful to the novice user of cell cultures.

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This unit describes basic aspects of preparing media for and growing *Saccharomyces cerevisiae* cells. It also covers some aspects of handling yeast strains, such as growth, storage, and shipment. *Saccharomyces* has been one of the most extensively studied organisms in the laboratory, and an extensive repertoire of genetic and molecular biology methods have been developed, as described in this volume and elsewhere (see Key References). A large number of yeast strains with useful characteristics have been documented, and many can be obtained for a nominal cost from public collections including the Yeast Genetics Stock Center (YGSC; Rebecca Contopoulou, Curator; e-mail: ygsc305@violet.berkeley.edu) or the American Type Culture Collection (ATCC). For contact information for these collections, see Internet Resources and *SUPPLIERS APPENDIX*.

Culturing *Saccharomyces* is relatively straightforward and can be accomplished with readily available and inexpensive materials. Wild-type yeast are prototrophic for most nutrients; that is, they are capable of synthesizing most metabolites from inorganic salts and a carbon source for energy. However most laboratory strains carry mutations that make them auxotrophic for one or more metabolites, such as amino acids or nucleotides, that must be supplied in the growth medium. The auxotrophic mutations, or *markers*, are used in the maintenance of plasmids and in other genetic experiments.

The first part of this unit will discuss the preparation of media. General aspects of media preparation will be described followed by specific recipes. The second part of the unit will discuss the growth and handling of yeast strains.

NOTE: All incubations of yeast cells are performed at 30°C unless otherwise noted.

PREPARATION OF MEDIA

Yeast media are referred to as either *liquid* or *solid*. Liquid medium is self-explanatory. A solid medium typically contains 2% (w/v) Bacto Agar. Other comparable gelling agents can be used in special circumstances, but they are not discussed in this unit.

Liquid Media

Liquid media are prepared in the same way as solid media (described below) except that agar is not added. As a result, liquid media can be sterilized either by autoclaving or by filter sterilizing with a 0.22- μ m filter. Filter sterilization is faster and eliminates the risk of heat inactivation of any medium ingredients. Liquid medium is prepared in either bottles or growth flasks. In flasks, medium should never exceed one-fifth the flask volume, so that maximum aeration can be achieved during shaking.

Solid Media

Solid media can be prepared in Erlenmeyer or Fernbach flasks; however, the most convenient container for pouring plates is the Fleaker (Corning). Heat-stable ingredients are mixed in water until completely dissolved (with the exception of the agar, which is added but will not dissolve). It is convenient to add a magnetic stir bar to the flask or Fleaker prior to autoclaving. The medium is autoclaved at 121°C on the liquids or slow exhaust program/setting at 15 psi for 15 min, after which it is placed on a stir plate at room temperature and stirred as it is allowed to cool to 60° to 65°C. Alternatively, the flasks or Fleakers can be placed in a 55°C water bath. Filter-sterilized, heat-sensitive ingredients are added at this point, and the medium is mixed completely prior to plate pouring. At this point the agar should be fully suspended within the medium.

Most yeast medium recipes, including those described in this unit, should result in a pH between 5.5 and 7.0. If a more acid medium is prepared, care should be taken to minimize or avoid autoclaving agar at $\text{pH} \leq 4.8$, as the agar will hydrolyze and fail to gel when cooled.

A pouring area should be prepared in a quiet place with minimal dust and airflow, and the bench surface should be wiped with 70% ethanol or another sterilizing agent. The appropriate number of bags of petri dishes (typically $15 \times 100\text{-mm}$ or $15 \times 95\text{-mm}$) should be opened by carefully cutting off the top of the bags. The bags are overturned, allowing the plates to slide out in a stack. The bags are saved for storing the filled plates. The bottom of the plates are labeled, and the plates overturned and divided into stacks of eight. When the medium has cooled to 55° to 60°C , it can be carefully poured into the plates. When pouring, the Fleaker or flask of medium should be held in one hand, allowing the other hand to grab the stack of plates. The top seven plates are lifted as a stack, the medium is poured into the eighth (bottom) plate, and the stack is lowered. This process is then repeated by picking up the top six plates, pouring the seventh plate, and so on. Although this system of pouring is awkward at first, it has the advantages of being faster and taking up less space than pouring plates in stacks of one.

One liter of medium should generate 40 to 50 plates. Sometimes bubbles will be formed while pouring medium. These can be removed by quickly passing a bunsen burner flame over the surface of the molten agar. The lid should be placed on the plates as soon as possible to minimize the risk of contamination. After the agar hardens (20 to 45 min), the plates should be flipped so that the lids are on the bottom. Plates should be allowed to dry at room temperature for 2 to 3 days. Because they are more prone to collecting condensation, media for yeast are left to dry at room temperature for a longer time than are media for bacteria. Once dried, the plates are placed in plastic bags, and the bags are taped closed and stored at room temperature or at 4°C for extended periods of time. Most media will be stable for ≥ 3 months; if excessive drying and contamination can be avoided, most media are stable for up to one year. Sometimes it is helpful to make small slits in the plastic bags to let them “breathe.” This reduces condensation, which in turn leads to the appearance of fewer contaminating organisms.

Defined Versus Complex Media

Yeast media are often referred to as either *defined* or *complex*. A defined medium is made with chemically defined components, such as salts, sugars, and amino acids. A complex, or rich, medium includes the addition of a complex lysate or hydrolysate, such as yeast extract or peptone. For the purpose of these definitions, agar-containing medium may still be referred to as defined. The following sections provide details about materials for media, and give recipes for specific complex and defined media. A number of special-purpose media are detailed.

Materials

Media should be prepared using distilled or deionized water. Most materials for media preparation should be of high grade, although the specific vendor is usually not important. However, yeast extract, peptone, agar, and yeast nitrogen base (YNB) should all be Bacto brand, sold by Difco. Difco products are carried by several scientific distributors.

Best results are obtained when the sugar component of a yeast medium (e.g., dextrose, sucrose, raffinose, galactose) is not autoclaved with the other components of the medium. If dextrose is included with other ingredients during the autoclaving process, it can caramelize. The standard alternative method is to autoclave or filter sterilize a concentrated stock solution of the sugar and add it to the medium after the other components have been autoclaved. For dextrose, a sterilized 40% (w/v) stock solution is added at 50

ml/liter to the autoclaved mixture of other medium components. Other sugars are typically made as 20% (w/v) stock solutions and added at 100 ml/liter.

Complex Media

Yeast extract/peptone/dextrose (YPD or YEPD) medium

This complex medium is the one most commonly used for the routine growth of yeast when no auxotrophic selection is required.

10 g Bacto yeast extract (1% w/v final)
20 g Bacto Peptone (2% w/v final)
20 g agar (2% w/v final)
950 ml H₂O
Autoclave
Add 50 ml 40% (w/v) dextrose (2% final; sterilized separately by autoclaving or filtering)

*Many researchers prefer to add adenine to YPD medium, especially when working with adenine auxotrophs. Red-colored strains of Saccharomyces almost always lack either *ade1* or *ade2* gene function. To make YPAD, add 20 mg/liter adenine sulfate to YPD prior to autoclaving.*

Similar media can be made by substituting other sugars for dextrose. Generally, Saccharomyces grows best on dextrose, but specific experiments may require replacement of dextrose with another sugar. The most common substitutes are YPF (2% w/v fructose), YPGal (2% w/v galactose), YPSuc (2% w/v sucrose), YPRaf (2% w/v raffinose), or YPMal (2% w/v maltose). Except for fructose, not all yeast strains are capable of growing on these alternative sugars. Growth on raffinose is sometimes facilitated by adding 0.05% (w/v) dextrose.

Yeast extract/peptone/glycerol (YPG) medium

Nonfermentable glycerol is the only defined carbon source in this complex medium. Because there is no fermentable carbon source, YPG does not support the growth of *petite* yeast strains (mutant strains that lack functional mitochondria).

10 g Bacto yeast extract (1% w/v final)
20 g Bacto Peptone (2% w/v final)
50 ml glycerol (5% v/v final)
20 g agar (2% w/v final)
950 ml H₂O
Autoclave

Some recipes call for 3% glycerol, but 5% gives better growth. Glycerol can be autoclaved with the medium. Other nonfermentable carbon sources can be used, including ethanol, acetate, or lactate, or combinations thereof. Glycerol is the most commonly used nonfermentable carbon source.

Yeast extract/peptone/dextrose/glycerol (YPDG) medium

This complex medium is used to determine the proportions of wild-type and *petite* cells. The different cells form large and small colonies respectively.

10 g Bacto yeast extract (1% w/v final)
20 g Bacto peptone (2% w/v final)
30 ml glycerol (3% v/v final)
20 g agar (2% w/v final)
970 ml H₂O
Autoclave
Add 2.5 ml 40% (w/v) dextrose (0.1% final; sterilized separately by autoclaving or filtering)

Special-Purpose Complex Media

Galactose indicator medium

Several gene expression systems used in yeast research rely on a *GAL* (galactose) promoter for regulated expression. When such a system fails to work as expected, one source of trouble is the use of yeast strains that are unable to respond to galactose. Strains that respond to galactose will ferment it and release acid into the medium. Acidification causes the indicator dye to change color from blue to yellow.

- 10 g Bacto yeast extract (1% w/v final)
- 20 g Bacto Peptone (2% w/v final)
- 20 g agar (2% w/v final)
- 930 ml H₂O
- Autoclave
- Add 50 ml 40% (w/v) galactose (2% final; sterilized separately by autoclaving or filtering)
- Add 20 ml 0.4% (w/v) bromthymol blue (0.08% final; sterilized separately by filtering)

Sporulation medium

Saccharomyces strains can be maintained as either haploids or diploids. Most diploid strains will sporulate when grown on a poor source of carbon and nitrogen.

- 10 g potassium acetate (1% w/v final)
- 1 g Bacto yeast extract (0.1% w/v final)
- 0.5 g dextrose (0.05% w/v final)
- 20 g agar (2% w/v final)
- 1 liter H₂O
- Autoclave

For this recipe dextrose can be added prior to autoclaving.

Presporulation (PSP) medium

Sporulation of some *Saccharomyces* strains is facilitated by growth on a rich medium prior to plating on sporulation medium.

- 8 g Bacto yeast extract (0.8% w/v final)
- 3 g Bacto Peptone (0.3% w/v final)
- 20 g agar (2% w/v final)
- 750 ml H₂O
- Autoclave
- Add 250 ml warm 40% (w/v) dextrose (10% final; sterilized separately by autoclaving or filtering)

The dextrose solution should be warmed to ~40° to 55°C before it is added, so that the agar does not harden prematurely.

Dissection agar

Preparation of dissection agar is frequently tailored to the configuration of the dissection apparatus. In general, thin slabs of YPD medium (10 ml per standard 15 × 100–mm petri plate) are used. Best results are obtained with light-colored (most transparent) medium, which can be facilitated by minimizing the length of time it is heated and by autoclaving the dextrose separately.

Defined Media

It is generally best to add inositol to all synthetic media. Although wild-type yeast strains are prototrophic for inositol, some laboratory strains bear an *ino1* mutation. Unfortunately, this mutation is sometimes forgotten in reporting the genotype because yeast nitrogen base (YNB) contains enough inositol that the effect of the mutation is not easy to see. However, the amount of inositol in YNB is not enough to fully supplement the auxotrophy, and selection for compensating mutations can occur.

Salts and dextrose (SD) medium

This minimal medium contains enough nutrients to support growth of prototrophic yeast strains (strains having no nutritional requirements).

6.7 g Bacto yeast nitrogen base (YNB) without amino acids (0.67% w/v final)
20 g agar (2% w/v final)
950 ml H₂O
Autoclave
Add 50 ml 40% (w/v) dextrose (2% final; sterilized separately by autoclaving or filtering)

Similar media can be made by substituting other sugars for dextrose. Generally, Saccharomyces grows best on dextrose, but specific experiments may require replacement of dextrose with another sugar. The most common substitutes are SGal (2% galactose) and SRaf (2% raffinose). Not all yeast strains are capable of growing on these alternative sugars. Growth on raffinose is sometimes facilitated by adding 0.05% (w/v) dextrose.

Vendors carry several similar types of YNB, e.g., with or without amino acids or ammonium sulfate. YNB in this recipe includes ammonium sulfate. Although ammonium sulfate is the preferred nitrogen source, Saccharomyces will grow on other nitrogen sources, albeit more slowly. For these media, YNB without ammonium sulfate and without amino acids is used at 1.7 g/liter. Nitrogen sources include ammonium sulfate (0.5% w/v), arginine (0.1% w/v), asparagine (0.1% w/v), or proline (0.1% w/v).

Most yeast strains require one or more nutrients that are not included in SD medium. These nutrients can be added in various ways (see supplemented SD medium and SC medium).

Supplemented SD medium

Add supplements (see Table 1.6.1) individually or in groups to SD medium. Alternatively, add supplements to individual plates by spreading, and allow one day for supplement(s) to permeate the medium. Add most supplements before autoclaving; add tryptophan and histidine, which are sensitive to heat, after autoclaving. Store tryptophan in the dark, as it is also sensitive to prolonged exposure to light.

Synthetic complete (SC) medium

As an alternative to adding individual nutrients to SD medium, add a dry mixture of the most common supplements. Combine the dry reagents listed in Table 1.6.1 and mix thoroughly using a coffee grinder or a mortar and pestle. Then add the mixture before sterilizing the medium, without tryptophan and histidine, which should be added after autoclaving.

Alternative mixtures can be constructed (e.g., SC minus tryptophan) by omitting specific ingredients. These mixtures are sometimes referred to as “dropout” media—e.g., SC –Trp dropout medium.

5-Fluoroorotic acid (5-FOA) medium

Selection for *URA3* function is used for the stable maintenance of many yeast plasmids. For some experiments, however, loss of the *URA3* gene is desired. The *URA3* gene product converts 5-FOA into a toxin. Therefore, strains that have lost the *URA3* gene can be identified by their resistance to 5-FOA.

continued

Solution 1:

1 g 5-FOA (0.1% w/v final)

500 ml H₂O

6.7 g yeast nitrogen base (YNB) – amino acids (0.67% w/v final)

50 mg uracil (50 µg/ml final)

20 g dextrose (2% w/v final)

Appropriate supplements (see Table 1.6.1)

Add 5-FOA, H₂O, and a stir bar to a 1-liter flask. Autoclave or stir over a low to medium heat ~1 hr to dissolve completely. Add YNB, uracil, glucose, and supplements. Choose supplements from Table 1.6.1 based on the genotype of the strain, but always add uracil. If supplements are added in liquid form, be sure to adjust the volume of H₂O accordingly. Dissolve completely (warming to 55°C is useful) and filter sterilize.

Solution 2:

20 g agar (2% w/v final)

500 ml H₂O

Mix agar and H₂O in a 2-liter flask, autoclave, and cool to ≤80°C.

5-FOA medium:

Mix solution 1 into cooled solution 2

Cool to 55°C

Pour into petri dishes

Table 1.6.1 Common Supplements Used In Defined Media

Nutrient	Stock concentration (g/100 ml)	Stock volume per liter medium (ml)	Final concentration (mg/liter) ^a	Stock volume to spread on plate (ml)
Adenine sulfate ^b	0.2	10	20	0.2
L-Arginine·Cl	1	2	20	0.1
L-Aspartic acid	1	10	100	0.2
L-Glutamic acid	1	10	100	0.2
L-Histidine·Cl	1	2	20	0.1
L-Isoleucine	1	3	30	0.1
L-Leucine	1	3	30	0.1
L-Lysine·Cl	1	3	30	0.1
L-Methionine	1	2	20	0.1
Myo-inositol ^c	3.6	1	36	0.02
L-Phenylalanine	1	5	50	0.1
L-Serine	8	5	400	0.1
L-Threonine	4	5	200	0.1
L-Tryptophan	1	2	20	0.1
L-Tyrosine	0.2	15	30	0.2
Uracil	0.2	10	20	0.2
L-Valine	3	5	150	0.1

^aMixtures of supplements can be made in advance by weighing out individual components in the ratio indicated by this column; mixing the dry reagents in a coffee grinder, mill, or mortar and pestle; and then weighing out the appropriate amount of the mixture.

^bWhen working with *ade1* or *ade2* mutants, the amount of adenine should be increased three fold to prevent accumulation of red color.

^cThe traditional recipe for SC medium does not include inositol. Inositol is added as a precaution because some common laboratory strains contain the *ino1* mutation, which is not always documented. YNB contains enough inositol for significant, albeit incomplete, supplementation, which makes recognizing the presence of an *ino1* mutation difficult.

Xgal medium

Wild-type *Saccharomyces* strains lack endogenous β -galactosidase activity, which facilitates use of the *E. coli lacZ*-encoded β -galactosidase enzyme as a reporter activity. This plate assay provides a semi-quantitative assay for activity.

- 6.7 g yeast nitrogen base (YNB) – amino acids (0.67% w/v final)
- Appropriate supplements (see Table 1.6.1)
- 20 g agar (2% w/v final)
- 850 ml H₂O
- Autoclave
- Cool to 65°C
- Add 100 ml 0.7 M potassium phosphate, pH 7.0 (70 mM final; best if warmed prior to adding to agar)
- Add 50 ml 40% (w/v) dextrose (2% final; sterilized separately by autoclaving or filtering)
- Add 2 ml 20 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Xgal) in 100% *N,N*-dimethylformamide (DMF)

Xgal can be prepared in advance, aliquoted, and stored as a frozen stock. Supplements are chosen from Table 1.6.1 based on the genotype of the strain. If supplements are added in liquid form, the volume of H₂O should be adjusted accordingly.

Xgal plates are frequently made as above, but without the substrate Xgal. Approximately 1 to 2 days prior to using the plates, 0.1 ml of 20 mg/ml Xgal stock is spread across the surface of the plate and allowed to diffuse through the agar.

GENERAL CONSIDERATIONS FOR CULTURING YEAST

For the most part, culturing *Saccharomyces* is fairly straightforward. Wild-type strains are typically grown at 30°C, although they are capable of growing at a wide range of temperatures. Many strains will grow to some extent at temperatures as low as 4°C and at least as high as 37°C. Liquid or solid media can be used. Liquid cultures grow better with aeration, but this is not required. For the most consistent results, it is important to maintain a reproducible level of aeration with each experiment by marking or taping the speed dial of the shaker.

Culturing Yeast in Liquid Media

Yeast strains are readily cultured in liquid media. For most experiments it is best to grow them with agitation, as this prevents them from settling to the bottom of the liquid. When cultures are grown in flasks, the liquid volume should be limited to 20% of the flask volume, although this guideline is sometimes exceeded. Another standard method is to grow 1- to 5-ml cultures in 18 × 150-mm glass tubes that are rotated on a tube roller (e.g., New Brunswick). Metal or plastic caps are readily available for these tubes. Many brands of tabletop or preparative centrifuges accommodate these tubes directly.

Yeast cells can be transferred from liquid or solid cultures to a new liquid culture by many methods. One popular method is to use sterile disposable 15-cm-long applicator sticks. The sticks can be autoclaved in capped culture tubes. Another popular method is to use nonflavored, noncolored wooden toothpicks, which can be autoclaved in their original boxes.

For any given set of conditions—namely, yeast strain, medium, temperature, and agitation—a generation time and saturation density can be readily determined. Most strains grown with agitation in YPD medium at 30°C will double every 80 to 100 min. An overnight culture seeded with $\sim 3 \times 10^4$ to 1×10^5 cells or a ~ 1 -mm colony will typically

grow to $\sim 3 \times 10^7$ cells/ml. After two days the culture should reach $\sim 5 \times 10^8$ cells/ml. When grown in SD or SC medium, the generation time will be on the order of 120 to 150 min. An overnight culture will typically grow to $\sim 5 \times 10^6$ cells/ml.

The number of cells per milliliter of culture can be estimated using a spectrophotometer. With a standard 1-cm-path-length cuvette at 600 nm, an optical density (OD_{600}) of 1.0 is equal to $\sim 2 \times 10^6$ cells/ml. Because this method relies on measuring light scattering and not true absorbance of the culture, this rule of thumb should be confirmed for each specific spectrophotometer. The conversion factor can vary widely depending on the configuration of the instrument. The spectrophotometer can be calibrated by comparing the OD_{600} reading with an accurate cell count obtained with a hemacytometer or by counting the number of colonies that can be grown from a diluted sample. Once an instrument is calibrated, however, the value should not change from day to day.

Culturing Yeast on Solid Media

Yeast strains are readily cultured on solid media. For most experiments it is best to use a 30°C incubator. While growth rates are hard to establish on solid media, colonies formed from single cells are generally visible to the unaided eye after 2 or 3 days and patches of cells are visible after one day. There are five common methods for transferring yeast cells to solid media: (1) spread plating a suspension of cells, (2) streaking cells with a toothpick or inoculating loop, (3) patching cells with a toothpick or inoculating loop, (4) replica plating from an existing plate, and (5) suspending cells in top agar and pouring them over an agar plate. Each of these is described below.

Spread plating cells

In this method, an appropriate number of yeast cells are calculated and suspended in a small volume of liquid, typically 0.05 to 0.25 ml. The appropriate cell number will depend on the nature of the experiment. It is generally desirable to produce 20 to 200 colonies per plate.

A glass rod can be fashioned into a spreader as follows. A roughly 30-cm length of 3-mm glass rod is cut and both ends are smoothed in a flame. Hold the rod over a bunsen burner flame ~ 5 cm from one end. When the glass is pliable, bend it $\sim 45^\circ$. Hold the rod over the flame ~ 10 cm from the same end and bend it another 45° , so that one end has now formed a triangle. Optionally, the point at which the triangle section meets the shaft can be bent 30° from the plane of the triangle to make the spreader more ergonomic. Prior to use the spreader is sterilized by dipping the triangle end in 70% to 95% ethanol and igniting it in a flame. Before applying to a cell suspension, the spreader should be cooled by touching it onto the surface of the agar plate or onto the condensation that frequently collects on the lid of the plate. Alternatively, an unmodified sterile glass pipet can be used to spread cells, but this is more difficult. Plastic pipets are not recommended as they tear the agar.

Although not required, spread plating works better if an inoculating turntable (available from Fisher Scientific and other laboratory suppliers) is used. An inoculation turntable consists of a solid base and a freely rotating platform that is the approximate size of a petri plate.

The cells should be spread across the plate immediately after pipetting them onto the agar. If a turntable is used, give it a gentle push. If it is not used, the plate can be rotated frequently by hand. The flat part of the triangle is used to push the cells back and forth across the agar. The technique of spread plating yeast cells, especially with a turntable, differs from spread plating bacteria in one notable respect: yeast cells are more likely to be pushed to the periphery of the plate. To avoid this problem, gently push the “elbow” of the spreader into edge of the plate for a few rotations and then, while the plate is still

rotating, push the elbow back and forth across the plate in a chord that is roughly half way between the center and the edge of the plate.

Streaking cells

The streaking method is used for isolating single colonies of yeast. Single isolated colonies are desirable because, when the procedure is done properly, each colony is derived from a single cell, so that every cell in the colony is genetically identical.

In this method, the plate is divided into four pie-shaped sections and one sample of yeast cells is streaked for single colonies in each section. With practice and increasing skill, the plate can be divided into six or more sections. This method is best performed with sterile toothpicks. A minimal amount of yeast cells are picked with the toothpick and placed in a spot near the edge of the plate. A clean toothpick is used to smear the cells back and forth in a 4 × 10-mm patch with the long axis of the patch running roughly parallel to the lip of the plate. A third toothpick is drawn once across the short axis of the patch and for another ~15 mm towards the center of the plate. A fourth toothpick is dragged on the agar in an arc that parallels the plate edge and crosses the line made by the third toothpick; the toothpick is then drawn in a series of shorter and shorter arcs that parallel the plate edge and move consecutively towards the center of the plate. The plate is then incubated for 2 to 4 days until individual colonies are visible.

Patching cells

Once pure strains of yeast have been isolated, it is frequently useful to compile a set of the strains on a single agar plate, either for short-term storage or in preparation for testing them in parallel by replica plating. Between 30 and 100 patches can be arranged on a standard 100-mm petri plate.

Before patching yeast from one plate to another, an ordered grid is prepared on a piece of paper and is inserted in a clear plastic sleeve. A standard sheet of paper (A4 or 8 × 10-in.) can accommodate four identical grids for 100-mm petri plates. The agar plates are set on the grid with the lids facing up. The plates can be held in place with a piece of double-sided tape or with tape that has been rolled into a circle. Two pieces of tape are much more effective than one in holding the plates in place. Either sterile toothpicks or inoculating loops can be used for the patch technique, but toothpicks work better. The patches are made by barely touching a source colony with the end of the toothpick and then gently dragging the toothpick over a ~2-mm diameter spot on the recipient plate.

Replica plating cells

Replica plating is used for testing multiple strains or isolates in parallel. This technique requires three specialized pieces of equipment: a replica block, a locking ring, and velveteen cloth squares. All three are available commercially from Fisher Scientific, Cora Styles, and other suppliers. The diameter of the block must be designed for the diameter of the petri plates being used; 95-mm diameter plates will not work with a block designed for 100-mm plates. Having the correct type of cloth is also essential for replica plating to work.

Prior to the first use and after each subsequent use, the cloth squares are laundered in any standard washer and dryer with standard laundry detergent. If recombinant organisms are used, it is prudent to soak the contaminated squares in a disinfectant prior to laundering them. Care should be taken to monitor the lint screen during drying because a significant amount of lint is produced; failure to do this can damage the clothes dryer. After the squares are dried, excess lint can be removed with a standard lint brush, although this is not always necessary. The squares are sterilized by wrapping them in heavy-gauge aluminum foil and autoclaving them. Depending on usage patterns, 5 to 50 squares can be stacked, soft-side down, on the center of a large piece of foil. The foil is then folded

around the stack to make a securely closed package that is held with a piece of tape. The package is then autoclaved on the dry cycle.

A *master* plate is made by spread plating, streaking, or patching cells. Typically, the master plate is incubated for 1 to 3 days before use. The master plate works best if the colonies are not extremely large and overgrown. Each good-quality master can be used to produce up to eight replicas.

The replica block and locking ring can be cleaned by squirting them with 70% ethanol and drying them with a paper towel. The package of velvets is carefully opened with the soft side of the cloth squares facing down. One velvet is picked up by the corner, turned over, and placed on the block. The package should then be partially closed to prevent contamination. The locking ring is pushed down so that the velvet is drawn tight. The master plate is gently but firmly pushed onto the surface of the velvet and then lifted up again. Each test plate is then pushed onto the velvet in the same manner.

Suspending cells in top agar

This is probably the least preferred method unless the yeast cell wall has been compromised and an osmotic support is needed. A stock of sterile Bacto agar is prepared in water or medium. The agar should be prepared so that the final concentration is between 0.5% and 1.0% after adding the yeast suspension. Once it has been sterilized, the agar stock solution can be melted any number of times in a boiling water bath. For a 100-mm plate, 3 to 10 ml of the melted agar is transferred to a sterile tube and cooled to 37°C. A small volume of yeast cells are added, and the suspension is mixed and poured over the surface of an agar plate. After a few minutes, the plates can be inverted and incubated for 2 to 4 days at 30°C.

Storage of Yeast Strains

Short-term storage of yeast strains

Most yeast strains will survive extended periods of storage at room temperature or 4°C; however, this type of storage should not be relied upon. Storage at these temperatures may result in loss of viability, or worse, selection of uncharacterized mutants that survive better under these conditions.

Long-term storage in glycerol

Two methods are commonly used for long-term storage of yeast strains: glycerol/water vials at –80°C and agar slants at room temperature or 4°C. The glycerol vials are the preferred method of storage.

Yeast strains can be stored indefinitely at –80°C in 15% (v/v) glycerol in water. Any glass vial may be used; however, 2-ml Wheaton vials (in a lab file) are an ideal size. These vials can be stored in standard freezer storage boxes (100 per box).

To prepare the vials, 1 ml of 15% glycerol solution is dispensed per vial, lids are placed loosely on the vials, and the vials are autoclaved. As soon as the vials are cool enough to handle, the lids are tightened. Prior to use, the vials may be stored at room temperature indefinitely.

To store the strain, the vials are labeled and a single colony is transferred using a sterile toothpick or inoculating loop from a fresh plate into each vial. Each vial is shaken until the colony is evenly distributed in the liquid. The vials are then transferred to a –80°C freezer. The best colonies are 1 to 3 days old, although older ones will work.

Labeling the vials can be difficult because some brands of label fall off at such cold temperatures and some inks run when they come in contact with freezer frost. One method

that works is to use good quality office labels and to cover the labels with one wrap of cellophane tape. The tape sticks to itself very well and protects the writing.

A sample of a liquid culture may be preserved by this method, although it is not recommended. If liquid cultures are to be preserved, the concentration of glycerol should be adjusted accordingly to allow for dilution by the liquid culture.

Long-term storage in YPAD slants

YPAD medium is prepared and the agar is dissolved into the medium in a boiling water bath. Portions of 1.5 ml are dispensed into 3-ml vials, screw cap lids are applied loosely, and the medium is autoclaved. After autoclaving, the vials are inclined so that the agar is just below the neck of the vial. After drying for 1 to 2 days, the lids are tightened.

A small sample of a fresh culture is transferred to the agar slant using a sterile toothpick or inoculating loop. The culture may be spread across the agar surface or it may be pushed into the agar. The screw-cap lid is tightened. Parafilm can be wrapped around the lid to improve the seal. The slants are stored in a cool (23°C) dark place. Viable yeasts can be recovered from the slants for ≥ 3 years.

Shipping Yeast Strains

Yeast strains can be shipped by any of several different methods. The shipped cultures may be dried, on agar, or in liquid as long as sterility is maintained. The simplest method is to use sterile swabs. Other methods generally involve more preparation time or are more vulnerable to damage in the mail.

Shipping yeast strains in sterile swabs

One convenient and safe method is to use sterile swabs for taking throat cultures, such as the Culturette system from Becton Dickinson. These systems contain a sterile swab and a saline solution housed in a leak-proof container that is resistant to rough handling. To use this system, the package is opened, a colony is swabbed from a fresh culture plate, the swab is inserted back into the package, and the package is labeled and taped or stapled shut. To revive the strain, the swab is removed and dabbed on a small area of a petri plate containing YPD or another appropriate medium. Toothpicks are then used to streak for single colonies and the plate is incubated.

Shipping yeast strains on filter paper

Yeast cultures can be dried onto filter paper and stored or transported. This method was developed by at the YGSC by John Bassel, and is best used for strains that are frequently sent out, because multiple filters may be prepared once and stored up to several years at 4°C. Sterile filter paper squares (Whatman no. 4, 1 × 1 cm) are prepared by packaging them in a sheet of heavy-duty aluminum foil (7 × 6 cm) and autoclaving the packages. The strain to be shipped is grown exponentially in YEPD and a 5- μ l sample of culture is added to 0.2 ml evaporated milk. The milk is handled aseptically but is not sterilized. The filter paper squares are immersed in the milk suspension of cells and returned to the aluminum foil packet. The packets are folded only once and stored in a desiccator at 4°C for 2 to 3 weeks or until the liquid congeals into a hard, dry lump. Following desiccation, the packets are folded tightly and stored at 4°C in plastic boxes. To revive the strain, the filter paper is swiped on a petri plate of YPD or other appropriate medium and returned to its packet. Toothpicks are used to streak for single colonies and the plate is incubated.

Shipping yeast strains by other methods

Other methods include sending agar slants (described above) or petri plates. Care should be taken in packaging petri plates because they are easily broken in the mail.

KEY REFERENCES

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A compendium of technical methods in yeast cell culture, genetics, biochemistry, molecular biology, and cell biology.

Rose, M.D, Winston, F., and Hieter, P. 1990. *Methods in Yeast Genetics, A Laboratory Course Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

The laboratory manual from a Cold Spring Harbor laboratory source on yeast molecular biology.

INTERNET RESOURCES

<http://www.atcc.org/>

Web site for the ATCC.

http://www.atcc.org/hilights/sc_info.html

Web site for the ATCC Saccharomyces page.

<http://dgm2ibm.nihs.go.jp/ygsc.htm>

This Web page provides access to YGSC catalogs, though it is not official.

<http://genome-www.stanford.edu/Saccharomyces/>

This Web site contains an extensive amount of information and links related to Saccharomyces.

<http://www.tiac.net/users/cstyles/>

This Web site contains specific information about Saccharomyces and is a source for some harder-to-find equipment.

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BY-2 Cells: Culture and Transformation for Live Cell Imaging

This unit describes the production of stable transformants of tobacco Bright Yellow 2 (BY-2) cells with such fluorescent markers as the green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* to study subcellular dynamics. These markers are increasingly used in plant cell biology, and the number of fluorescent proteins available for plant cell imaging and physiology is increasing almost exponentially (Brandizzi et al., 2002; Table 1.7.1).

BY-2 cells were initially established for mass production of raw material for cigarettes, but they have lately been described as the HeLa cell of the biology of higher plants (Nagata et al., 1992). This title is well deserved, as these cells are amenable to cell culture, transformation, and manipulation. BY-2 cells have a high synchronization efficiency and growth rate, and they are homogeneous. Because of their good size and the absence of autofluorescent chloroplasts, they are also easily transformed and imaged. When BY-2 cells are grown on solid medium they form calli. Calli are aggregated cells that arise from multiple divisions of a single cell—similar to a bacterial colony. The BY-2 calli are used as a way to keep a stock of cells that can be used to start suspension cultures as required.

Table 1.7.1 Examples of BY-2 Cellular Components Highlighted with Fluorescent Proteins^a

Construct	Cellular location	Fluorescent protein	Reference
Korrigan (KOR1) and mutants	Golgi apparatus, growing cell plates	GFP	Zuo et al., 2000
Soybean α -1,2 mannosidase (Gm-Man1)	Golgi apparatus	GFP	Nebenführ et al., 2000
Rat sialyltransferase 1 (ST)	Golgi apparatus	GFP	Saint-Jore et al., 2002
N-acetylglucosaminyl transferase I (Nag)	Golgi apparatus and NE	RFP	Dixit and Cyr, 2002
ER-targeted GFP-HDEL	ER	GFP	Nebenführ et al., 2000
<i>Arabidopsis thaliana</i> RAN GTPase activating protein 1 (AtRanGAP1) and deletion clones	NE and other structures	GFP	Rose and Meier, 2001
Truncated lamin B receptor (LBR)	NE	GFP	Irons et al., 2003
Matrix attachment filament-like protein (MAF1)	Outer NE and other cellular structures	GFP	Rose and Meier, 2001
Actin-binding domain of a mouse talin	Actin	CFP GFP	Dixit and Cyr, 2002 Kost et al., 1998
Tubulin	Microtubules	GFP	Kumagai et al., 2001
Microtubule-binding domain (MBD)	Microtubule-binding domain	GFP	Granger and Cyr, 2000 Dixit and Cyr, 2002
Cyclin B1 and mutants	Cytoplasm and nucleus	GFP	Criqui et al., 2001

^aAbbreviations: CFP, cyan fluorescent protein; ER, endoplasmic reticulum; GFP, green fluorescent protein; NE, nuclear envelope; RFP, Red fluorescent protein.

Within a month a callus can grow to several centimeters in diameter, at which point a small piece (~2 to 5 mm² lump) should be transferred to fresh solid medium to keep the cells supplied with nutrients. If cells are already in suspension culture, stock plates can be made by plating 1 ml of suspension cells on solid medium. BY-2 calli are easily broken up by agitation in liquid medium. The establishment of a BY-2 suspension culture is described in Basic Protocol 1; Basic Protocol 2 describes their routine culturing.

Transient transformation of BY-2 cells may be achieved after protoplasting and electroporation (F.B. and C.H., unpub. observ.; *UNIT 20.5*) or directly by particle bombardment (An, 1985) and electroporation (Koscianska and Wypijewski, 2001; *UNIT 20.5*). Co-cultivation with *Agrobacterium tumefaciens* also easily leads to stable transformation.

BY-2 cells are readily susceptible to inducible expression systems such as the tetracycline repressor and, even more tightly, the glucocorticoid-inducible system, neither of which suffers from leakage of gene expression in the absence of the inducer (Criqui et al., 2000; David and Perrot-Rechenmann, 2001; Nishihama et al., 2001; Geelen et al., 2002). Besides chemical inducers, gene expression in the BY-2 cell system may be induced by heat shock. In this respect, the heat-sensitive promoter HSP18.2 may be used as an alternative to chemical inducers with fast kinetic responses (Yoshida et al., 1995; Shinmyo et al., 1998).

BY-2 cells can easily be used for following the cell cycle and for studying the effect of test compounds on its progression. As the authors describe in this unit, aphidicolin synchronization (Basic Protocol 3) may produce a good mitotic index. The current availability of cellular fluorescent markers opens the exciting possibility to follow the dynamics of organelles and the cytoskeleton during the cell cycle.

The transformation of BY-2 cells with *Agrobacterium* that contains a reporter construct in a suitable binary vector is an easy, quick, and very reliable method (Basic Protocol 4). In comparison with protoplast-based protocols, this method offers several advantages over transient transformation. These include working with a relatively unperturbed cellular environment, little variability of DNA expression among cells, longer imaging periods, amenability to synchronization of mitosis, and ease of handling. BY-2 cells can also be stably transformed with two constructs, each expressing a different fluorescent marker (Alternate Protocol).

When GFP is expressed as a chimeric protein either with selected peptide targeting sequences or with complete proteins that are resident in subcellular locations, expression of the chimeric constructs can be observed in living cells using conventional epifluorescence or, preferably, confocal laser scanning microscopes.

CAUTION: BY-2 cells are unlikely to survive outside the laboratory culture environment. It is, however, good laboratory practice to treat transformed BY-2 cells as biological hazards and therefore to dispose of them accordingly. The cells in culture should be killed by autoclaving.

NOTE: All procedures should be carried out under sterile conditions in a laminar flow hood sprayed with 70% (v/v) ethanol (and allowed to stand for 20 min before use). A heat bead sterilizer or flame should be used to sterilize metallic tools such as forceps and razor blades (tools must be cool before using on biological materials). All items placed in the hood should be sprayed with 70% (v/v) ethanol.

ESTABLISHING A BY-2 SUSPENSION CULTURE

This protocol describes the method to establish a BY-2 cell suspension from a BY-2 or another tobacco callus. Subculturing the cells is technically termed passaging (also see Basic Protocol 2). It is advisable to work in duplicate or even triplicate when establishing a suspension culture. This allows initiation of fresh suspensions of the same transformed cell line should any contamination occur.

Materials

Calli of wild-type and/or transformed BY-2 cells (available from various laboratories) grown on solid BY-2 medium (see recipe) in petri dish
50-ml conical flasks containing 20 ml liquid BY-2 medium (see recipe), covered with aluminum foil and autoclaved
Suitable filter-sterilized antibiotics, for culturing transformed BY-2 cells only
Razor blades, sterile
Packet of aluminum foil squares for covering flasks, sterile
Shaking incubator, 25°C

1. Cut a 2-cm² piece of wild-type or transformed BY-2 callus with a sterile razor blade directly on the petri dish on which the calli are growing.
2. Loosen aluminium foil from top of a sterile 50-ml conical flask containing 20 ml liquid BY-2 medium. Lightly flame neck of flask. Add suitable filter-sterilized antibiotics if needed. Open a sterile packet of aluminum foil squares so that they are readily accessible.

This will aid the speed at which the items in the hood can be handled and hence will reduce the possibility of contamination.

The flasks should be open for as short a time as possible to avoid contamination. Avoid passing anything (e.g., hands or sleeves) over the open flasks.

The preparation and storage of antibiotics should be according to manufacturer's instructions. Antibiotics should be filter sterilized and handled as sterile material. To filter sterilize the antibiotic solution, a stock solution of the required concentration (usually 100×) is prepared. The antibiotic solution is aspirated into a sterile syringe without a needle. A 0.2-μm syringe filter is attached to the syringe, and the solution is pressed through the filter into a sterile tube.

3. Transfer the cut callus to the 50-ml flask with medium.

When establishing suspension cultures it is best to begin in a small volume as the cells grow better. To encourage growth the authors have found that subculturing newly established suspensions 1 to 2 weeks after placing calli in liquid medium, the culture should be quite thick—like runny tomato ketchup. Passaging cultures at lower dilution ratio (1:10 instead of 1:20) can also encourage good suspension culture growth. Once the cells are growing well in the small volume they can be subcultured into larger volumes of medium.

4. Gently pipet the culturing medium up and down to break up callus.
5. Reseal the flask with a new square of aluminium foil.
6. Place cells in a shaking incubator set at 130 rpm and 25°C with illumination of choice.

A flat-bed orbital platform in a 25°C culture room may also be used.

BY-2 cell suspensions may be kept in the dark. An illumination regime of 16 hr light and 8 hr dark can also be used.

Cells should be subcultured after 7 days (see Basic Protocol 2).

ROUTINE CULTURE OF BY-2 CELLS

This protocol describes the routine method to culture BY-2 cells and to maintain suspension and callus cultures. As described in Figure 1.7.1, the protocol requires liquid and solid media.

Materials

- 50-ml conical flasks containing 20 ml liquid BY-2 medium (see recipe), covered with aluminum foil and autoclaved
- Suitable filter-sterilized antibiotics, for transformed BY-2 cells only
- Wild-type or transformed stationary-phase BY-2 cells (i.e., 7-day-old cultures) grown in suspension (see Basic Protocol 1)
- Packet of aluminum foil squares for covering flasks, sterile
- Trimmed 1-ml pipet tips (i.e., 4 to 5 mm cut off from narrow end), sterile
- Shaking incubator, 25°C

1. Loosen aluminium foil from top of a sterile 50-ml conical flask containing 20 ml liquid BY-2 medium. Lightly flame neck of flask. Add suitable filter-sterilized antibiotics if needed. Open a sterile packet of aluminum foil squares so that they are readily accessible.

This will aid the speed at which the items in the hood can be handled and hence will reduce the possibility of contamination.

The flasks should be open for as short a time as possible to avoid contamination. Avoid passing anything (e.g., hands or sleeves) over the open flasks.

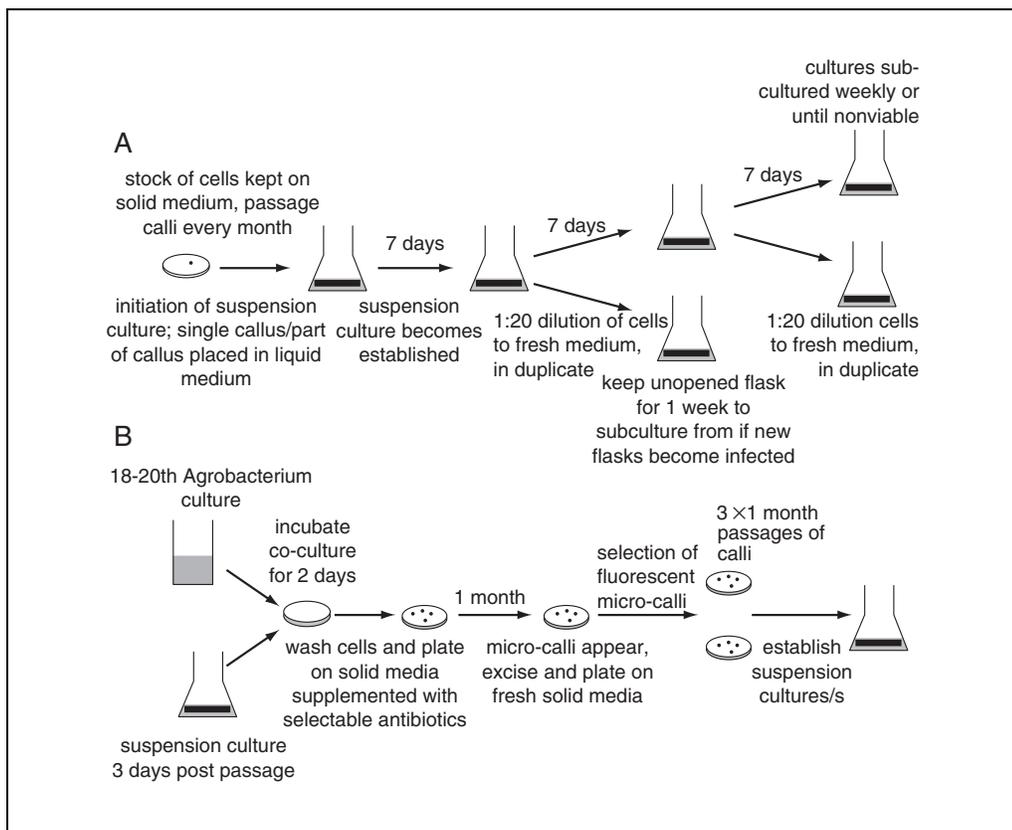


Figure 1.7.1 (A) Routine culture of BY-2 cells (see Basic Protocol 2). (B) Production of stable transformants (see Basic Protocol 4).

The preparation and storage of antibiotics should be according to manufacturer's instructions. Antibiotics should be filter sterilized and handled as sterile material. To filter sterilize the antibiotic solution, a stock solution of the required concentration (usually 100×) is prepared. The antibiotic solution is aspirated into a sterile syringe without a needle. A 0.2- μ m syringe filter is attached to the syringe, and the solution is pressed through the filter into a sterile tube.

2. Use a trimmed 1-ml pipet tip to take a 1-ml aliquot of wild-type or transformed stationary-phase BY-2 cells from their flask.

The ends of uncut tips will be too narrow to allow easy entry of cells. A razor blade can be used to cut the tips, and cut tips are autoclaved as normal.

For larger volumes of cells, use 100 ml BY-2 medium in a 250-ml flask. For 100-ml suspension cultures, passage 10 ml cells into 100 ml fresh medium.

3. Remove foil from the new flask and pipet the cell aliquot directly into the fresh medium.
4. Cover the new flask with a new square of aluminum foil and seal firmly.
5. Place cells in a shaking incubator set at 130 rpm and 25°C with illumination of choice for 7 days.

A flat-bed orbital platform in a 25°C culture room may also be used.

BY-2 cell suspensions may be kept in the dark. An illumination regime of 16 hr light and 8 hr dark can also be used.

After 7 days, cells should be passaged again.

6. Passage cells every 7 days.

APHIDICOLIN-MEDIATED SYNCHRONIZATION OF CELL CULTURES

Synchronization of cultures can be achieved by a variety of methods, which include mineral and hormone starvation, hydroxyurea treatment, heat shock, light-dark cycle control, or chemical inducers, such as aphidicolin. Aphidicolin is a toxin produced by the fungus *Cephalosporium aphidicola*, and it acts as a specific and reversible inhibitor of DNA polymerase α (Ikegami et al., 1978). In the presence of this compound, cells are blocked in G1; when aphidicolin is washed out of the suspension, the cells are released from G1 and the cell cycle progresses to S phase (see Fig. 1.7.2; Menges and Murray, 2002).

The following describes how to synchronize BY-2 cells with aphidicolin. With this method it is possible to achieve a 40% mitotic index. Higher synchrony is possible using treatments such as propyzamide (1.6 mg/liter) 6 hr after aphidicolin release (Nagata et al., 1992). However, as this compound may perturb microtubule structure, its use may not be desirable if subcellular dynamics, such as the cell cycle, are being studied.

With the method described below, cells generally reach M phase 10 to 11 hr after the aphidicolin wash.

CAUTION: Aphidicolin is very toxic and should be handled with great care. Aphidicolin should be handled wearing gloves and in accordance with general good lab practice (i.e., with lab coat and eye protection). Inhalation of dust should be avoided. The chemical should be diluted in at least 1 liter of water and flushed down the drain.

BASIC PROTOCOL 3

Cell Culture

1.7.5

Materials

50-ml conical flasks containing 20 ml liquid BY-2 medium (see recipe), covered with aluminum foil and autoclaved

5 mg/ml aphidicolin (Fisher) in dimethyl sulfoxide, store up to 1 yr at 4°C

Suitable filter-sterilized antibiotics, for transformed BY-2 cells only

Wild-type or transformed stationary-phase BY-2 cells (i.e., 7-day-old cultures) grown in suspension (see Basic Protocol 1)

Five 100-ml beakers, covered with aluminum foil and autoclaved

20- μ m nylon filter mounted on a cut plastic autoclavable 50-ml beaker, sterile

Packet of aluminum foil squares for covering flasks, sterile

Trimmed 1-ml pipet tips (i.e., 4 to 5 mm cut off from narrow end), sterile

Shaking incubator, 25°C

1-liter liquid waste container

1. In a laminar flow hood, aliquot 80 ml BY-2 medium to each of five sterile 100-ml beakers.
2. Loosen aluminium foil from top of a sterile 50-ml conical flask containing 20 ml liquid BY-2 medium. Lightly flame neck of flask. Add 20 μ l of 5 mg/ml aphidicolin and suitable filter-sterilized antibiotics, if needed. Open a sterile packet of aluminium foil squares so that they are readily accessible.

This will aid the speed at which the items in the hood can be handled and hence will reduce the possibility of contamination.

The flasks should be open for as short a time as possible to avoid contamination. Avoid passing anything (e.g., hands or sleeves) over the open flasks.

The preparation and storage of antibiotics should be according to manufacturer's instructions. Antibiotics should be filter sterilized and handled as sterile material. To filter sterilize the antibiotic solution, a stock solution of the required concentration (usually 100 \times) is prepared. The antibiotic solution is aspirated into a sterile syringe without a needle. A 0.2- μ m syringe filter is attached to the syringe, and the solution is pressed through the filter into a sterile tube.

3. Use a trimmed 1-ml pipet tip to take a 1-ml aliquot of wild-type or transformed stationary-phase BY-2 cells from their flask and transfer them to aphidicolin-supplemented medium.

The ends of uncut tips will be too narrow to allow easy entry of cells. A razor blade can be used to cut the tips, and cut tips can be autoclaved as usual.

4. Cover the new flask with a new square of aluminium foil and seal firmly.
5. Place cells in a shaking incubator set at 130 rpm and 25°C and incubate 24 hr in the dark.
6. Hold a sterile 20- μ m nylon filter mounted on a cut plastic 50-ml beaker over a sterile 1-liter liquid waste container and gently pour aphidicolin-treated cells onto the filter. Discard medium in liquid waste container.

To construct the filter apparatus, the bottom end of a plastic beaker is cut off and discarded. A 2-cm section is cut from the bottom of the beaker. This ring is placed on top of the nylon filter, and then the filter and ring are inserted in the upper part of the beaker. In this way, the filter will be secured to the cut end of the beaker (see Fig. 1.7.2). The apparatus is wrapped in foil before autoclaving.

7. Quickly transfer filter with cells to a sterile 100-ml beaker containing 70 to 80 ml liquid BY-2 medium. Gently agitate cells in filter for 5 min.

The transfers must be carried out quickly to prevent the cells from drying out.

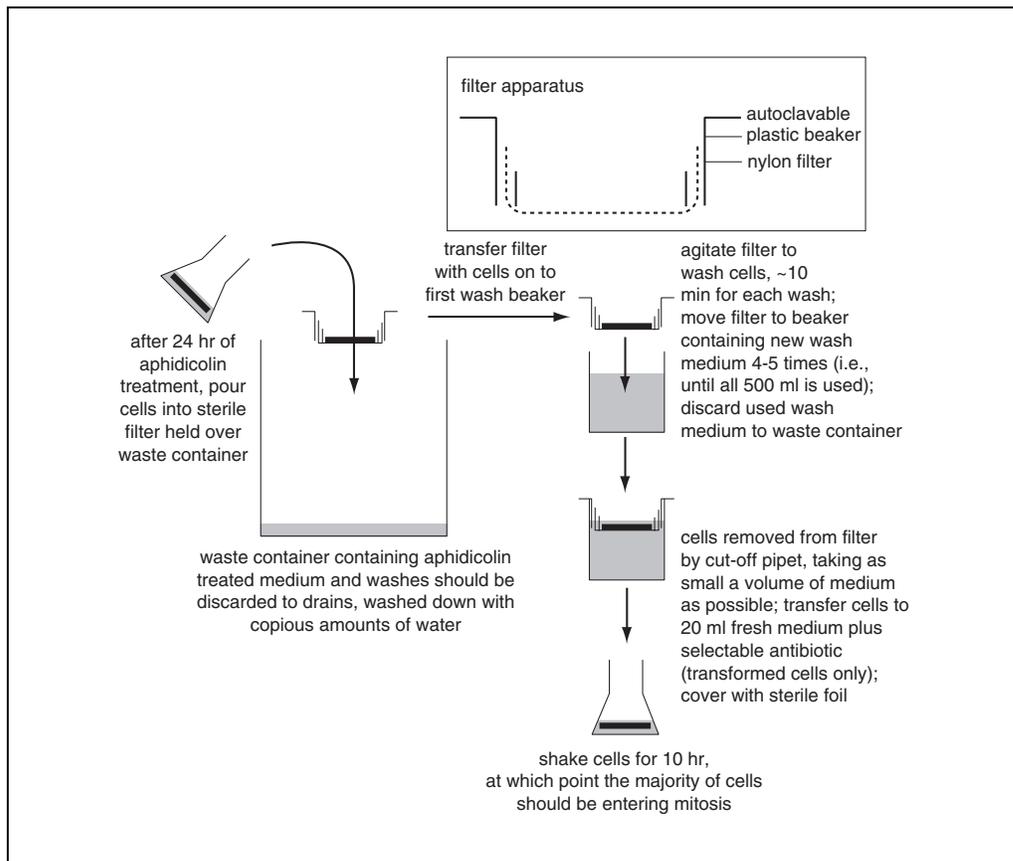


Figure 1.7.2 Aphidicolin synchronization of BY-2 suspension cultures.

8. Repeat transfer four more times, using a beaker with fresh medium each time. Discard used medium in liquid waste container.
9. Resuspend cells in new 50-ml flask containing 20 ml medium (with antibiotic if needed).
10. Shake cells 10 to 11 hr at 25°C.

At this point the majority of cells are entering into M phase.

STABLE TRANSFORMATION OF BY-2 CELLS MEDIATED BY *AGROBACTERIUM* FOR VISUALIZATION OF SUBCELLULAR ORGANELLES

This protocol involves the production of BY-2 lines that stably express GFP targeted to subcellular structures for subsequent live cell imaging (see Fig. 1.7.3). The procedures are suitable for other tobacco cell lines and may be modified for *Arabidopsis thaliana* suspension cells as well (Foreiter et al., 1997).

Stably transformed calli may be identified and selected directly on a petri dish with the aid of a portable UV lamp if the fluorescent protein used to transform cells has a UV peak excitation (such as the green fluorescent protein GFP5; Haseloff et al., 1997). Alternatively, a small portion of the callus may be analyzed with a conventional fluorescence microscope using the appropriate excitation-emission filter set to establish the positive transformation. In Figure 1.7.1B, a scheme of the production of stable transformants is given.

**BASIC
PROTOCOL 4**

Cell Culture

1.7.7

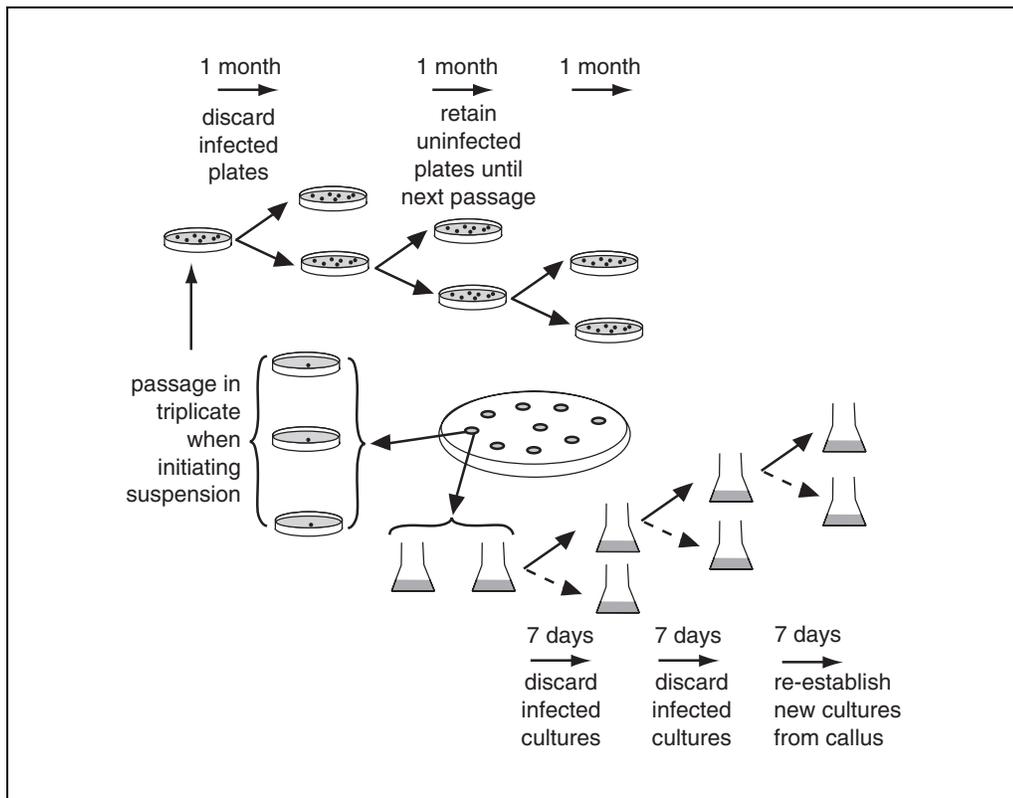


Figure 1.7.3 Passaging of stably transformed BY-2 cells.

NOTE: To transform BY-2 cells, a binary vector that can be transformed into agrobacteria is needed and can be the same as is used to transform tobacco and *Arabidopsis* plants. The binary vector, in which the fluorescent protein marker is subcloned, must carry two resistance markers (antibiotic resistance). One is a selectable marker for bacteria—to select positive transformants after bacterial transformation—and the other is a selectable marker for plants, which will allow the growth only of transformed plant cells. When working with these selection markers, it is very important to follow the manufacturer’s instructions for the specific antibiotic in use. Factors such as light and pH, for example, may alter the properties of the antibiotics and thus affect the yield of stable transformants.

Materials

YEB medium (see recipe) containing appropriate filter-sterilized bacterial selection antibiotic

Agrobacterium tumefaciens transformed with vector containing appropriate GFP construct (e.g., strain GV3101::pMP90; Konez and Schell, 1986) transformed with another plasmid (e.g., pVKHI8En6, pBII21) which contains GFP and the insert of interest.

3-day-old wild-type BY-2 suspension culture (see Basic Protocol 1)

Liquid BY-2 medium (see recipe), sterile

Solid BY-2 medium (see recipe) plates with plant selectable antibiotic, 100 $\mu\text{g/ml}$ carbenicillin (see recipe), and 20 $\mu\text{g/ml}$ timentin (see recipe)

Shaking incubator, 25°C

Trimmed 1-ml pipet tips (i.e., 4 to 5 mm cut off from narrow end), sterile

5- and 10-cm petri dishes, sterile

1.5-ml microcentrifuge tubes, sterile

Forceps, sterile

Grow *agrobacteria*

1. Inoculate 5 ml YEB medium containing appropriate filter-sterilized bacterial selection antibiotic with a single colony of *Agrobacterium tumefaciens* transformed with a binary vector containing the appropriate GFP construct. Incubate 18 to 20 hr at 130 rpm and 25°C.

Transform BY-2 cells

2. Using a trimmed 1-ml pipet tip, transfer 1 ml of a 3-day-old wild-type BY-2 suspension culture to a sterile 5-cm petri dish.
3. Add 50 μ l overnight *Agrobacterium* culture (step 1) and mix very gently with pipet tip.
4. Seal plate with Parafilm and incubate 2 days at 25°C in the dark without shaking.

Alternatively, the plates may be wrapped in aluminium foil or placed in sealed boxes.

5. Add 1 ml fresh liquid BY-2 medium and collect the cells with a trimmed 1-ml pipet tip. Transfer cells to a sterile 1.5-ml microcentrifuge tube.

Because the cell medium may have dried out during the 2-day incubation, 1 ml medium is added to facilitate collection of the cells.

It may be necessary to divide cells between two tubes at this stage.

6. Allow BY-2 cells to settle to bottom of tube by gravity (2 min).
7. Remove excess medium and add 1 ml fresh liquid BY-2 medium. Resuspend cells by gently flicking the tube.
8. Repeat steps 6 and 7 two more times for a total of three washes.

Plate BY-2 cells

9. Resuspend cells with 1 ml fresh liquid BY-2 medium and use trimmed 1-ml pipet tip to transfer cells to solid BY-2 medium plates with plant selectable antibiotic, 100 μ g/ml carbenicillin and 20 μ g/ml Timentin.
10. Gently rotate plate to spread cells over the surface of the solid medium.
11. Seal plate with Parafilm and incubate in the dark at 25°C without shaking until microcalli appear (~1 month).
12. Use sterile forceps to excise individual microcalli and plate them onto fresh solid BY-2 medium plates with plant antibiotic. Place a maximum of nine calli per 10-cm plate.

Calli can be screened, after excision and before passaging, for fluorescence using an UV lamp for constructs based on GFP5 (Haseloff et al., 1997); calli that have been successfully transformed will fluoresce.

13. Passage calli three times, allowing a 1-month interval between each passage, before establishing suspension cultures.

Each passaging step except the first (when the microcalli are too small to divide) should be carried out in duplicate to ensure that, should one plate become contaminated, there is still another stock plate available. These passages are carried out to ensure that the cells are stably transformed.

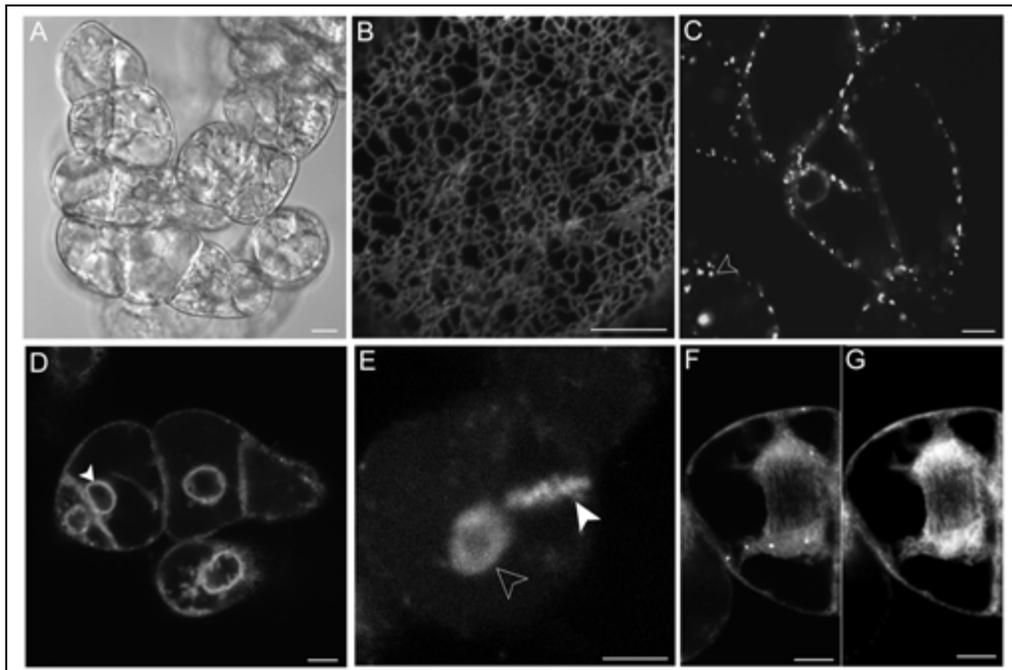


Figure 1.7.4 ((A) DIC image of BY-2 suspension culture. Membranous structures, nuclei, and vacuole are clearly visible. (B) Cortical endoplasmic reticulum (ER) visualized with yellow fluorescent protein (YFP) targeted and retained in a BY-2 cell stable transformant. This construct, SpYFP-HDEL, is composed of a sporamin signal peptide (to target the protein to the ER lumen), YFP, and the tetrapeptide HDEL to retain the protein in the lumen of the organelle (Irons et al., 2003). (C) To visualize the Golgi apparatus (arrowhead), the signal anchor of a rat sialyl transferase has been fused to green fluorescent protein (GFP) and expressed stably in BY-2 cells (Saint-Jore et al., 2002). (D) Nuclear envelope (arrowhead) of BY-2 cells stably expressing the amino terminus of the human lamin B receptor fused to GFP (LBR-GFP; Irons et al., 2003). (E) A histone-YFP (Boisnard-Lorig et al., 2001) construct highlights the chromatin in stably transformed cells. Cell in interphase (white arrowhead) and at metaphase (black arrowhead). (F, G) Stable BY-2 transformant co-expressing LBR-GFP (F) and SpYFR-HDEL (G) during cell division. Scale bars, 10 μ m.

ALTERNATE PROTOCOL

STABLE TRANSFORMATION OF BY-2 CELLS WITH TWO CONSTRUCTS

BY-2 cells are also an amenable system for stably co-expressing two constructs (Fig. 1.7.4F, G). To achieve this, the fluorescent markers should be subcloned independently into a bacterial plasmid bearing the same antibiotic resistance. For the transformation, Basic Protocol 4 is carried out with the following changes. In step 1, two separate overnight cultures are grown for the two fluorescent marker constructs. In step 3, 50 μ l of each bacterial population is added to 1 ml untransformed BY-2 cells. Following the transformation, calli are screened for co-expression of the two fluorescent markers using a fluorescent microscope with adequate excitation and emission filters to discriminate the two fluorochromes.

BASIC PROTOCOL 5

PREPARATION OF BY-2 CELLS FOR FLUORESCENCE MICROSCOPY

BY-2 cells are generally mounted in half-strength agarized medium to prevent excessive movement of the cells and are maintained in the culture medium to limit cellular stress. For longer observation (up to several days) of immobilized suspension culture cells, a chamber covered with the gas-permeable foil bioFOLIE (Vivascience) can be used as an alternative to the agar medium (J.W. Vos and A.M.C. Emons, Plant Cell Biology, Wageningen University; pers. comm.). BioFOILE allows gas exchanges at the junction of the gas and liquid phase and gently immobilizes cells.

BY-2 Cells: Culture and Transformation for Live Cell Imaging

1.7.10

Materials

Solid BY-2 medium (see recipe), for short-term observation only
Wild-type or transformed BY-2 suspension culture (see Basic Protocol 1)
70% (v/v) ethanol, for long-term observation only
Valap: 1:1:1 (w/w/w) Vaseline/lanolin/paraffin (*UNIT 13.1*)
Electrical tape, cut in 0.5-cm-wide strips, for short-term observation only
76 × 26-mm glass slides and 51 × 20-mm O thickness coverslips, for short-term observation only
Microwave oven, for short-term observation only
Trimmed 200- μ l pipet tips (i.e., 4 to 5 mm cut off from narrow end), sterile
76 × 51-mm glass slides with 18-mm hole in the center and 24 × 24-mm O thickness coverslips, for long-term observation only
50°C oven, for long-term observation only
Double-sided tape, for long-term observation only
Scalpel, for long-term observation only
2.5 × 2.5-cm piece of bioFOLIE film (Vivascience), for long-term observation only
Sterile tissues, for long-term observation only

For short-term observation:

- 1a. Place two 0.5-cm-wide strips of electrical tape on a clean 76 × 26-mm glass slide perpendicular to the long sides of the slide. Place the strips of tape ~10 mm apart.

The electrical tape will prevent excessive compression of the cells.

- 2a. Warm a small amount of solid BY-2 medium in a microwave oven until melted but not boiling. Place 100 μ l medium on the glass slide between the strips of tape and allow to cool.
- 3a. At the critical point at which the medium solidifies, use a trimmed 200- μ l pipet tip to transfer 50 to 100 μ l wild-type or transformed BY-2 suspension culture to the medium. Use the pipet tip to gently mix the cells and medium.
- 4a. Place a 51 × 20-mm O thickness coverslip over the sample prior to observing at the microscope.

The cells will be held in place by the pressure of the coverslip.

Cells should be observed within up to 4 hr. Discard after observing; autoclave the slide before discarding if the cells are transformed.

For long-term observation:

- 1b. Clean a 76 × 51-mm glass slide with an 18-mm hole in the center and a 24 × 24-mm coverslip with soap and hot tap water, rinse in distilled water, and dry in an oven at 50°C.
- 2b. Place two 3-cm-long strips of double-sided tape on the slide, to cover the surface of the slide, including the hole.
- 3b. Use a scalpel to remove the tape covering the hole.
- 4b. Clean a 2.5 × 2.5-cm piece of bioFOLIE film in 70% ethanol and allow to air dry.
- 5b. Mount the bioFOLIE film on the tape on the glass slide and remove excess tape and foil.

The bioFOLIE film is mounted on the underside of the glass slide.

- 6b. Clean with soap and water, rinse with 70% ethanol, and air dry in a laminar flow hood.
- 7b. Working under sterile conditions, use a trimmed 200- μ l pipet tip to transfer 20 μ l of a wild-type or transformed BY-2 suspension culture onto the bioFOLIE in the hole of the glass slide.
- 8b. Cover the hole with the coverslip. Blot excess fluid with a sterile tissue while gently pressing on the coverslip.
- 9b. Seal the slide with Valap to prevent drying and infection.

Cells should be observed within 2 to 3 days. Store the slides at 25°C during observations. Discard slides, autoclaving first if the cells are transformed.

REAGENTS AND SOLUTIONS

Use ultrapure distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Carbenicillin, 100 mg/ml

Dissolve 100 mg/ml carbenicillin disodium (Melford Laboratories) in ultrapure water. Filter sterilize with a 0.2- μ m filter and store up to 6 to 8 weeks at -20°C .

To use at 100 $\mu\text{g/ml}$, add 100 μl to 100 ml medium.

2,4-Dichlorophenoxyacetic acid (2,4-D), 1 mg/ml

Dissolve 1 mg 2,4-D in 1 ml 70% (v/v) ethanol. Store up to 6 to 8 weeks at 4°C .

Liquid BY-2 medium

Place 750 ml water in a 1-liter beaker and add 30 g sucrose (AnalaR grade, BDH Chemicals). Stir until dissolved. Add 4.3 g Murashige and Skoog medium (without sucrose, indole acetic acid, kinetin, agar; ICN Biomedicals), 200 μ l 1 mg/ml 2,4-D (see recipe), and 3.4 μ l 100 mg/ml KH_2PO_4 (potassium dihydrogen orthophosphate; AnalaR grade, BDH Chemicals). Adjust pH to 5.8 with 0.1 M KOH (not NaOH), make up to 1000 ml with water, and mix well. Pour into flasks, cover with foil and autoclave 15 min at 121°C . Allow to cool and store up to 2 weeks at 4° to 8°C .

The final concentration of KH_2PO_4 in BY-2 medium is 540 mg/liter. BY-2 cells have a high phosphate usage, greater than that supplied in Murashige and Skoog medium alone (Nagata et al., 1992).

Solid BY-2 medium

Add 1% (w/v) low-melting-temperature agar (Bacto Agar; Difco) to liquid BY-2 medium (see recipe) in suitable flask. Autoclave 15 min at 121°C and allow to cool until flask can be held for pouring. Pour medium into sterile petri dishes (~25 ml/9-cm dish) in laminar flow hood. Allow plates to cool and store at 4° to 8°C .

If working with stable transformants, antibiotics should be added before pouring, and the flask should be swirled to mix them into the medium.

Timentin, 20 mg/ml

Dissolve 20 mg/ml Timentin (tricarcillin disodium salt/potassium clavunculate; Melford Laboratories) in ultrapure water. Filter sterilize with a 0.2- μ m filter and store up to 6 to 8 weeks at -20°C .

An alternative to Timentin is ticarcillin/potassium clavulanate.

To use at 20 $\mu\text{g/ml}$, add 100 μl /100 ml medium.

YEB medium

In a 1-liter beaker dissolve 5 g beef extract (Difco), 1 g yeast extract (Merck), 5 g peptone (Difco), 5 g sucrose (BDH Laboratory), and 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 0.8 liter water. Add water, while stirring continuously, up to 1 liter. Autoclave and store up to 4 to 6 weeks at room temperature.

The components may take some time to dissolve completely.

COMMENTARY

Background Information

The reader may find different protocols for BY-2 cell culture and transformation being used in different laboratories. This is because researchers tend to optimize a general protocol based on their experience with handling BY-2 cell lines.

The medium used for the same cell type sometimes differs between labs as a result of different working practices and preferences. Different suspension plant cell types have differing medium requirements. For example in the authors' experience carrot suspension cells grow optimally in M and S media supplemented with sucrose, coconut milk, zeatine, and 2,4-dichloro-phenoxyacetic acid (2,4-D), in comparison to BY-2 cells which require the addition of sucrose, KH_2PO_4 , and 2,4-D only. In turn, *Arabidopsis* suspension cells proliferate in M and S supplemented with sucrose, NAA (naphthaleneacetic acid), and kinetin. The cells are all grown at the same temperature, with the same light and shaking regimes and are subcultured every 7 days.

There are several factors that have to be taken into consideration when working with transformed BY-2 cells. The expression of a fluorescent construct may vary among calli from the same transformation. Mistargeting of a protein may well be due to overexpression. Therefore, transgenic calli should be analyzed with a fluorescence microscope to ascertain the fidelity of the expression pattern. The authors have also found that levels of fluorescence of transformed BY-2 cells of a single line and patterns of expression may change after repeated passages. This may be due to aging of the culture, degradation of the fluorescent construct, and/or gene silencing.

Critical Parameters

The efficiency in obtaining transgenic calli may vary between experiments. This may be related to the quality of the BY-2 cells. In particular, cells that are in M and early G1 phase are more susceptible to transformation via *A. tumefaciens* than cells residing in G2 (Geelen

and Inzé, 2001). Similarly, the strain of *Agrobacterium* used may affect the yield. For example, the *Agrobacterium* strain LBA4404, which constitutively expresses the *virG* gene (van der Fits et al., 2000), has been found to be superior in generating transgenic calli (Geelen and Inzé, 2001).

Contamination of cultures may easily occur even if appropriate sterile procedures are followed. Therefore, to avoid wasted time and effort, it is preferable to work in duplicate or, in certain situations, in triplicate. This is to ensure that there is a backup stock of sterile material should infection occur at any point. The authors found that when routinely working with suspensions and calli (for the weekly and monthly passages, respectively), one should work in duplicate, but, when initiating a suspension culture, the calli should be passaged in triplicate. This is illustrated in Figure 1.7.3. When passaging calli and suspension cultures, the operator should discard infected plates. At each passage, new subcultures should be generated from one plate or flask only. It is advisable to retain the uninfected and unopened plates or flasks until the next passage to ensure a backup of material should contamination occur.

Once transformed lines are established, they can be passaged in a 7- to 14-day cycle. It is advisable, however, to maintain the line as calli on solid medium so that if expression levels decrease with time, a new suspension can be established. It is also the authors' experience that with some transformed lines, correct targeting of constructs is lost after three to four passages, and regular renewal from calli is then necessary (Fig. 1.7.3).

Troubleshooting

General rules for troubleshooting that apply in culturing and transforming of any plant cell line (Evans et al., 2003) apply for BY-2 cells.

In general, to enhance the possibility of a successful transformation, the operator should produce at least three initial plates of mixed *agrobacteria* and BY-2 cells.

There may be an absence of fluorescence in the transformed BY-2 cells. This may be due to several factors, including gene silencing, toxicity of the construct, mutation in the sequence of the fluorescent protein, degradation of gene product, or even misuse of the selectable markers. When cells are stably transformed via *Agrobacterium*, the DNA is integrated into the genome, hence resulting in an absence of a defined position effect. This may lead to silencing. The fidelity of a construct should always be checked by transient expression before attempting any stable transformation. Very often constructs may prove toxic to cells so that only lines with low levels of expression survive.

The gene product may not be tolerated by the cells and may be degraded. Nonspecific overproliferation of untransformed cells in calli may also occur. This may arise because of an impairment of antibiotic activity, for instance by temperature, light, or pH inactivation of the selective marker.

Anticipated Results

Transformation and culturing of BY-2 cells is a straightforward approach to set up an *in vivo* system to analyze cellular dynamics. This system is particularly well suited for investigation into cell cycle progression.

BY-2 cells are present in variable shapes from isodiametric to elongated and as single cells or concatamers (Fig. 1.7.4A). The large nucleus is suspended in a highly vacuolated cytoplasm. Numerous plastids are present in these cells, but they are not autofluorescent.

A variety of cellular organelles have been highlighted by *in vivo* fluorescent protein markers thanks to the ease of transformation of BY-2 cell and their ability to tolerate heterologous constructs based on green fluorescent protein (GFP) and its fluorescent derivatives (Table 1.7.1), including the actin cytoskeleton (Kost et al., 1998), microtubules (Granger and Cyr, 2000; Kumagai et al., 2001), and the Golgi apparatus (Nebenführ et al., 1999; Saint-Jore et al., 2002).

Figure 1.7.4B shows the endoplasmic reticulum (ER) of BY-2 cells stably expressing the yellow fluorescent protein targeted to the ER by an N-terminal sporamin signal peptide and retained by means of a C-terminal tetrapeptide, HDEL (SpYFP-HDEL). The Golgi apparatus can be visualized with a rat sialyl transferase membrane-anchoring domain GFP construct (Fig. 1.7.4C; Saint-Jore et al., 2002), and the nuclear envelope by the N-terminal domain of a mammalian lamin B receptor–GFP fusion (Fig. 1.7.4D; Irons et al., 2003). Chromatin can

be visualized by a histone-YFP fusion (Fig. 1.7.4E, G; Boisnard-Lorig et al., 2001), which allows the visualization of chromosome dynamics in interphase and mitotic cells.

BY-2 cells are also an amenable system for stable co-expression of two constructs (Fig. 1.7.4F, G). Thus BY-2 cells may be used to express a double combination of fluorochromes for the analysis of organelle-organelle and protein-protein interactions.

Time Considerations

As shown in Figure 1.7.1B, production of stable cell lines of BY-2 cells may take about 4 months. Detection of fluorescence in calli may be possible after 1 month from transformation with *agrobacteria*. This may be useful to distinguish fluorescent calli from nonfluorescent ones and to propagate the former.

After inoculation of liquid medium with callus, it is advisable to let the culture establish for a minimum of 1 week before microscopy. This gives time for cells to divide and separate from clumps of callus.

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Key References

Geelen and Inze, 2001. See above.

Reviews the applications of BY-2 cells in plant cell biology studies. Includes a comparison of Arabidopsis and BY-2 cells as model plant cells.

Kodama and Komamine, 1995. Synchronization of cell cultures of higher plants. *Methods Cell Biol.* 49:315-329.

Summarizes the techniques used in synchronization in different plant cultures including determination

of cell number, mitotic index, and percentage of living cells.

Nagata et al., 1992. See above.

Comprehensively reviews the history, growth, characteristics, and applications of BY-2 cells.

Internet Resources

<http://www.bch.msu.edu/pamgreen/bv2.htm>

Describes useful hints for transforming BY-2 cells and culturing.

<http://botany1.bio.utk.edu/cellbiol/default.htm>

Illustrates GFP fluorescent BY-2 cells, with most of the images from BY-2 cells expressing a GmMan1-GFP fusion protein to label Golgi stacks. Also shows combinations with other fluorochromes to simultaneously highlight other cellular components in interphase and cell division and in the presence of different test compounds.

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CHAPTER 2

Preparation and Isolation of Cells

INTRODUCTION

Chapter 1 describes the techniques of cell culture, but, of course, to culture cells one must have cells. Many cell biologists, if asked where cells come from, would probably give the American Type Culture Collection (ATCC) as their answer. As important as such repositories are as a resource, cells really come from organisms, and Chapter 2 deals with the preparation and isolation of cells from their true source.

As indicated in the introduction to Chapter 1, 1998 marked the golden anniversary of the first continuous mammalian cell line. That line was the L cell fibroblast line established from a mouse tissue explant by Sanford et al. in 1948 (*J. Natl. Cancer Inst.* 9:229-246). Fibroblast lines continue to be important now, half a century after the establishment of the L cell line. Fibroblasts can be obtained from humans without sacrificing the individual (human skin fibroblasts are indeed available from the ATCC), and fibroblast cultures established from patients with inherited disorders have often been used to identify genetic abnormalities. Skin fibroblasts are also instrumental in characterization of functional abnormalities in transgenic or knockout animals. The methodologies of preparing and maintaining skin fibroblasts are described in *UNIT 2.1*.

Blood represents a “renewable resource” for obtaining human cells. *UNIT 2.2* describes procedures for the isolation and maintenance of human lymphocytes from peripheral blood. Relatively large numbers of these cells (1 to 2 million per milliliter of blood) can be isolated by techniques that are moderately simple and inexpensive. In addition to the basic protocol for lymphocyte preparation by density-gradient centrifugation, protocols by which various subfractions of the lymphocyte population can be separated are provided. Monocytes/macrophages are isolated (or depleted) by utilizing their tendency to adhere to plastic. Monoclonal antibodies recognizing the specific surface molecules are utilized together with magnetic beads to select positively or negatively for populations of T cells or B cells. These cells can also be used to generate continuously growing B cell lines via transformation with Epstein Barr virus.

In *UNIT 2.3*, we move from blood to the blood vessels, from which endothelial cells can be isolated. Endothelial cells have been used extensively to explore cell-cell and cell-matrix interactions. Endothelial cell function (or dysfunction) participates in pathological processes as diverse as coronary artery disease and tumor invasion, as well as in the inflammatory response. The expression of surface proteins and secretion of soluble mediators by the endothelium controls vascular tone and permeability, regulates coagulation and thrombosis, and directs the passage of leukocytes into areas of inflammation. Endothelial cells play a central role in angiogenesis, the formation of new blood vessels that is necessary for tumor growth. As such, endothelial cells are being extensively studied in the quest for antiangiogenic agents that might be used in cancer therapy. A basic protocol for the preparation of endothelial cells from human umbilical vein is provided in *UNIT 2.3*, along with alternative protocols for isolation of endothelial cells from retroperitoneal adipose tissue, nasal mucosa, and human foreskins.

UNIT 2.4 provides guidance for preparing immortalized B cell lines via transformation by Epstein-Barr virus. This methodology allows for the establishment and maintenance of

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Preparation and
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2.0.1

Supplement 22

cell lines derived from resting human B lymphocytes obtained from blood samples, including those obtained from individuals with genetic changes leading to disease conditions (e.g., sickle cell anemia and cystic fibrosis).

Animal tissues are heterogenous because they are composed of mixtures of cell types. Analysis of the functions of these cells necessitates obtaining pure samples of the cells of interest. One recent approach to the daunting task of isolating homogenous morphologically identified cell populations is laser capture microscopy (LCM). The technique as described in *UNIT 2.5* is based on selective adherence of visually identified cells within a tissue section to a plastic membrane following activation by low-energy infrared laser. Although initially developed at the National Cancer Institute for separation of malignant or premalignant cells from surrounding normal tissue, LCM is being applied to a wide variety of cell types. Hundreds of papers have already appeared in the literature wherein DNA, RNA, and/or proteins have been extracted and analyzed from laser-captured cells.

UNIT 2.6 describes a method for establishing cultures of human keratinocytes. Although the source for the protocol described is human newborn foreskin from circumcision, the method may also be used to prepare keratinocyte cultures from skin biopsies, from other surgical procedures, or from cadavers.

In future supplements to Chapter 2, protocols for the isolation of additional cell types will be provided.

Joe B. Harford

Establishment of Fibroblast Cultures

UNIT 2.1

This unit describes methods for establishing fibroblast cultures from skin. Because fibroblasts can be expanded to relatively large numbers from a small skin sample, they have been widely used to study basic aspects of cell biology as well as genomic, biochemical, and/or functional abnormalities in human patients and in transgenic or knockout animals. Two protocols are described for the development of fibroblast lines from human and mouse skin samples; the same protocols are also applicable, with slight modification, to other animals including rats and rabbits. The first technique (see Basic Protocol) employs a “skin explant” culture system in which fibroblasts grow out of skin specimens. The second technique (see Alternate Protocol) employs a dissociated fibroblast culture system in which fibroblasts are first released from skin specimens by enzymatic digestion and then placed in culture. In general, the explant culture system is technically simpler, requiring almost no special experience or reagents, whereas the dissociated fibroblast culture is more suitable for obtaining relatively large numbers of fibroblasts in a short period.

NOTE: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly. Surgical equipment may be sterilized by simply soaking in 70% ethanol; however, it is important to rinse in PBS before use since ethanol will “fix” the tissue.

SKIN EXPLANT CULTURE

When skin specimens are “transplanted” onto culture plates, fibroblasts (in dermis) and keratinocytes (in epidermis) migrate over the plastic surfaces, as they do in an ordinary skin graft. Because fibroblasts will eventually overgrow keratinocytes in conventional culture media, relatively pure fibroblast cultures can be obtained by simply placing small pieces of skin on tissue culture dishes. On the other hand, the optional enzymatic separation of the epidermis will ensure the absence of epidermal components from the resulting fibroblast cultures; it will also allow investigators to establish keratinocyte cultures from the same skin samples.

Materials

- Skin specimen (see Commentary)
- Phosphate-buffered saline (PBS; see recipe)
- 0.5% (w/v) dispase II (Boehringer-Mannheim) in PBS (store up to 3 months at –20°C; optional)
- 0.3% (w/v) trypsin (from bovine pancreas; Sigma) in PBS (store up to 3 months at –20°C; optional)
- Complete growth medium (DMEM or RPMI; see recipe)
- Trypsin/EDTA solution (see recipe)
- 0.4% (w/v) trypan blue in PBS (store up to 6 months at room temperature)
- Freezing medium: 10% DMSO/90% FBS or 10% DMSO/90% complete DMEM
- 15-ml and 50-ml polypropylene centrifuge tubes
- Lids from 100-mm tissue culture dishes
- Eye forceps (2 pairs)
- Surgical scalpel with disposable no. 22 blade

BASIC PROTOCOL

Preparation and Isolation of Cells

Contributed by Akira Takashima

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2.1.1

35-mm tissue culture dishes or 6-well plates
22-mm glass coverslips (wrap several coverslips in aluminum foil and sterilize by autoclaving)
Hemocytometer
25-cm² tissue culture flasks
1.5-ml cryotubes (e.g., Nunc)
Liquid nitrogen freezer

CAUTION: When working with human blood, cells, or infectious agents, appropriate biosafety practices must be followed.

NOTE: Use Milli-Q water or equivalent in all protocol steps and for preparing all solutions.

Prepare skin sample

1. Wash skin samples in PBS by gently shaking or agitating in a 50-ml polypropylene centrifuge tube.

When handling skin samples from mice, rats, or rabbits, it is easier to remove hair before excision. This can be achieved by applying 70% ethanol on skin and shaving with a single-edged razor blade (e.g., Personna Prep).

- 2a. *For relatively large skin samples (e.g., foreskin, surgically removed samples, or cadaver skin):* Place sample on the lid of a 100-mm tissue culture dish and spread it out with the epidermal side down. Remove the subcutaneous tissue by scraping the dermal side using two pairs of eye forceps. Cut skin into strips of ~0.5-cm width using a surgical scalpel.
- 2b. *For smaller skin samples (e.g., punch-biopsy samples):* Place the sample on a 100-mm tissue culture dish and excise the subcutaneous tissue using a surgical scalpel and a pair of forceps.

Remove epidermis (optional)

- 3a. *For human skin samples:* Incubate 45 min to 4 hr with 0.5% dispase/PBS in a 37°C water bath, then separate as an intact sheet mechanically by gentle agitation for 10 sec or by using two pairs of forceps.

The incubation time required for epidermal separation varies depending upon the thickness of the skin specimen and the completeness of the subcutaneous tissue removal.

- 3b. *For mouse, rat, and rabbit skin samples (optional):* Incubate with 0.3% trypsin/PBS for 30 to 60 min in a 37°C water bath or overnight at 4°C. Place the sample on the lid of a 100-mm tissue culture dish with the epidermal side up and scrape off the epidermis mechanically using two pairs of forceps.

In general, trypsin works better than dispase for skin with deep hair follicles.

4. Wash the dermal sample in PBS by gently shaking or agitating in a 50-ml polypropylene centrifuge tube.

Culture fibroblasts

5. Place the dermal sample on the lid of a 100-mm tissue culture dish and cut it into small (2- to 3-mm) squares.

Fibroblast outgrowth occurs only from sharply cut edges. Thus, it is crucial to use a new, fine surgical scalpel; disposable surgical blades (no. 22) can be used for this purpose.

6. Place 5 to 10 skin pieces in the center of a 35-mm tissue culture dish or 6-well plate.

The 6-well plates are more convenient for handling multiple fibroblast cultures in parallel.

- Place a sterile 22-mm glass coverslip gently over the skin specimens (Fig. 2.1.1).

Skin specimens need to be attached physically to the plate. This can be achieved most effectively by making a sandwich using a coverslip (Fig. 2.1.1). The coverslip also assists the growth of fibroblasts by maintaining the microenvironment.

- Add a few drops of 4°C complete growth medium (DMEM or RPMI) into the space below the coverslip (by applying at the edge of the coverslip so that it is drawn under), then add 1 to 2 ml of 4°C complete medium to the dish or well, gently so as to avoid disturbing the skin specimens.

Either complete DMEM or complete RPMI can be used for growing fibroblasts.

- Place the culture in a humidified 37°C, 5% CO₂ incubator. Check the fibroblast outgrowth every 3 to 4 days under an inverted phase-contrast microscope and change medium every 3 to 4 days, taking care not to agitate the coverslip.

Initial outgrowth should be detectable within 3 to 4 days (Fig. 2.1.2B). The coverslip should be left on the skin specimens until the culture becomes confluent. The original skin specimens may come off the plate spontaneously during culture and will be removed in step 10.

- Upon confluency, remove the coverslips and wash the dish or wells twice with 4°C PBS.

Fibroblasts sometimes migrate over the surface of the coverslip instead of the tissue culture plate. If this is the case (as judged by focusing up and down with the microscope) transfer the coverslip into a new dish, wash it with PBS as in this step, then harvest cells by trypsin treatment as in step 11.

Washing with PBS is essential because trypsin (see step 11) does not work effectively in the presence of the FBS present in the culture medium.

In the absence of epidermal separation, the cultures are often contaminated with outgrowing keratinocytes, which are readily distinguishable by their cobblestone-like appearance. Contaminating keratinocytes can be removed selectively by incubating 10 to 30 min with 0.5% dispase/PBS, because keratinocytes are much more sensitive than fibroblasts to this treatment. Keratinocytes will be detached, leaving the majority of fibroblasts attached to the plate.

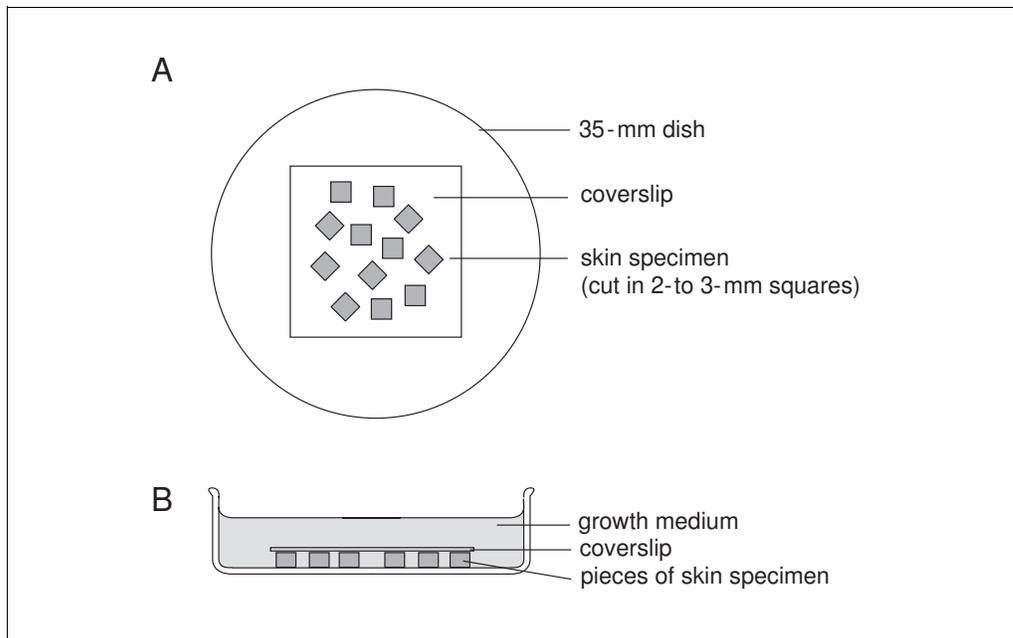


Figure 2.1.1 Skin explant culture: (A) top view; (B) side view.

11. Add 1 ml of 4°C trypsin/EDTA solution and incubate 3 to 5 min at room temperature while examining periodically under the inverted phase-contrast microscope. As soon as the fibroblasts round up, add 1 ml ice-cold complete growth medium (DMEM or complete RPMI) to inactivate trypsin and harvest cells by gentle pipetting.

Avoid trypsinization for excessive periods of time and vigorous pipetting, which are the two common causes of the “low viability” problem (see Troubleshooting). Do not pipet the cells before the trypsin has been diluted by addition of complete culture medium.

Harvest fibroblasts before they become overconfluent and form bundle-like alignments (Fig. 2.1.3B).

The original skin specimens may be removed using forceps.

12. Collect fibroblast suspensions into a 15-ml polypropylene centrifuge tube and centrifuge 10 min at $150 \times g$, 4°C. Aspirate the supernatant, tap the pellet to dissociate the cells, and resuspend them in 100 to 200 μ l of fresh 4°C complete growth medium.
13. Mix a 10- to 20- μ l sample of the cell suspension with an equal volume of 0.4% trypan blue/PBS, then count total and viable cells under a microscope using a hemacytometer.

The cell viability as measured by trypan blue exclusion should be >90%.

14. Plate 3–10 $\times 10^4$ viable cells in 5 ml of fresh complete growth medium in a 25-cm² tissue culture flask.

The cells will attach to a new flask within 2 to 3 hr and begin to exhibit the characteristic spindle shape in 24 hr.

Contamination occurs less frequently in flasks than in dishes or multiwell plates

15. Change medium every 3 to 4 days until the culture becomes confluent. Harvest fibroblasts as described in steps 10 and 11.

See Figure 2.1.4 for the typical growth rate of human fibroblasts; their doubling time varies from 24 to 72 hr, depending upon the culture conditions. Human fibroblasts can be passaged up to 10 times without significant changes in morphology or growth rates. It is recommended, however, that frozen stocks be prepared after the second or third passage (see step 16).

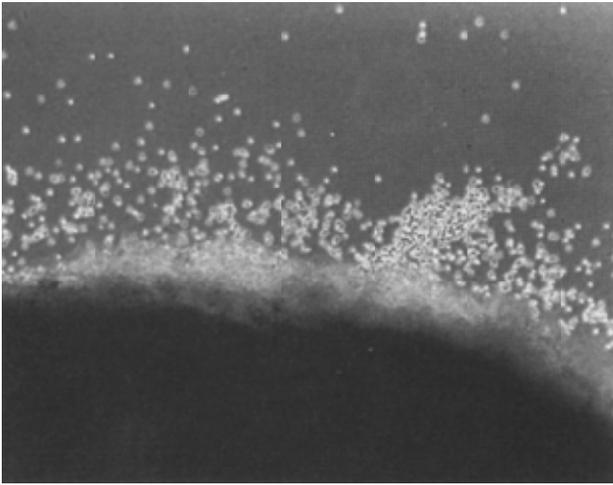
Maximal fibroblast growth requires 5% to 10% FBS (Fig. 2.1.5A). It is technically challenging to grow fibroblasts in the absence of added serum. On the other hand, there is minimal, if any, variation among FBS batches purchased from different vendors in their capacity to promote the growth of human fibroblasts (Fig. 2.1.5B). Human fibroblasts also grow well in the presence of heat-inactivated human serum, which may be used instead of FBS (Fig. 2.1.5C).

16. To freeze cells, resuspend in ice-cold 10% DMSO/90% FBS or 10% DMSO/90% complete DMEM at $0.3\text{--}1 \times 10^6$ cells/ml. Dispense into 1.5-ml cryotubes at 1 ml/tube and freeze first at –20°C, then move on to –80°C, and finally place in liquid nitrogen.

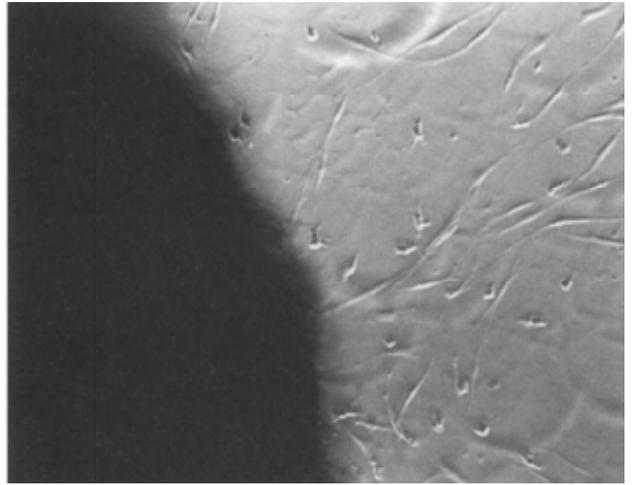
The –20°C and –80°C freezing steps may be performed in a styrofoam box to promote a gradual drop in temperature. Commercially available cell-freezing instruments may also be used for this purpose.

Figure 2.1.2 (at right) Microscopic appearance (magnification, 40 \times) of fibroblast cultures established from a newborn foreskin sample using the skin explant culture system (panels A to C) or the cell dissociation culture system (panels D to F). (A) Skin explant, day 1; (B) skin explant, day 5; (C) skin explant, day 14. (D) Dissociation culture, day 1; (E) dissociation culture, day 5; (F) dissociation culture, day 14. Note that fibroblasts migrate out from the edge of a skin specimen (panel B) and become confluent, except for the area where the original skin specimen was located (panel C). In dissociated-cell cultures, fibroblasts attach (panel D), spread on culture plates (panel E), and become confluent (panel F).

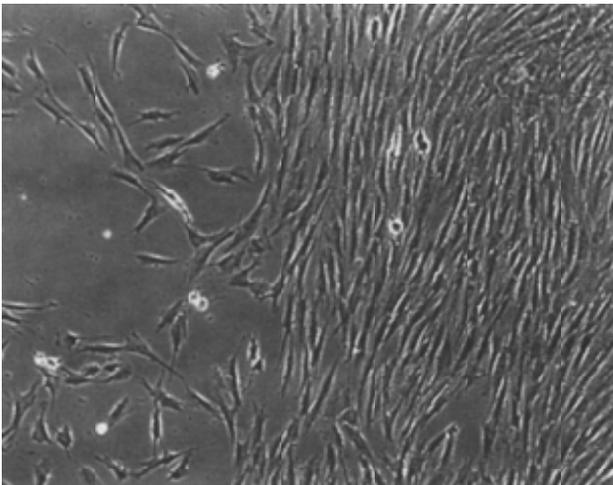
A



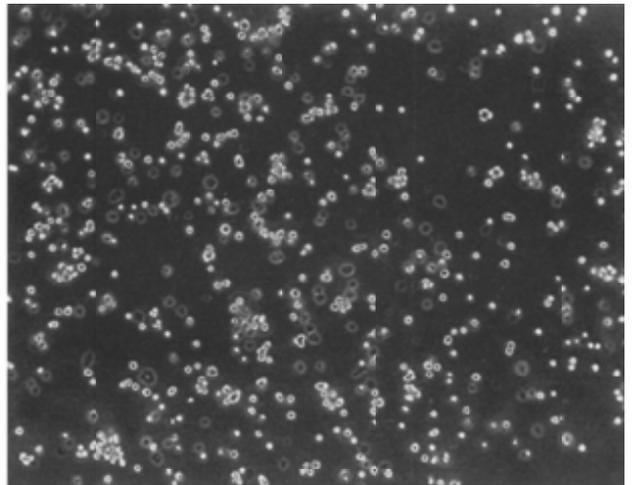
B



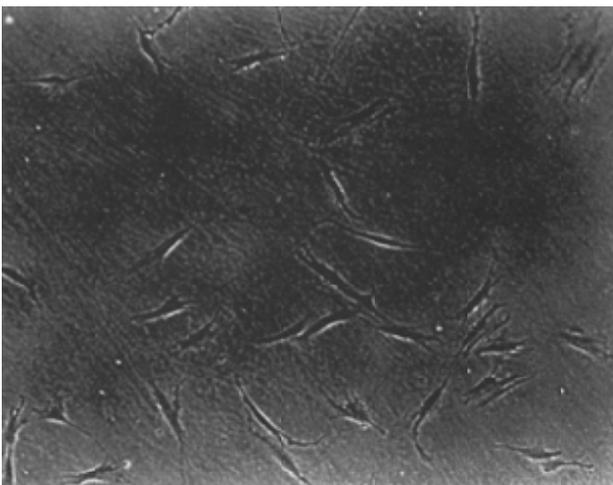
C



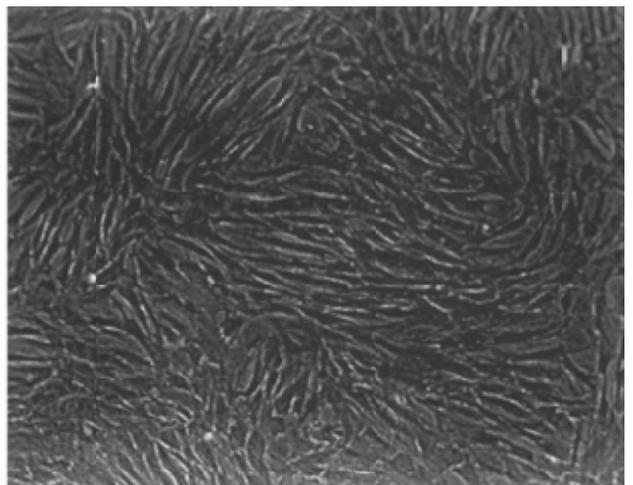
D



E



F



**Preparation and
Isolation of Cells**

2.1.5

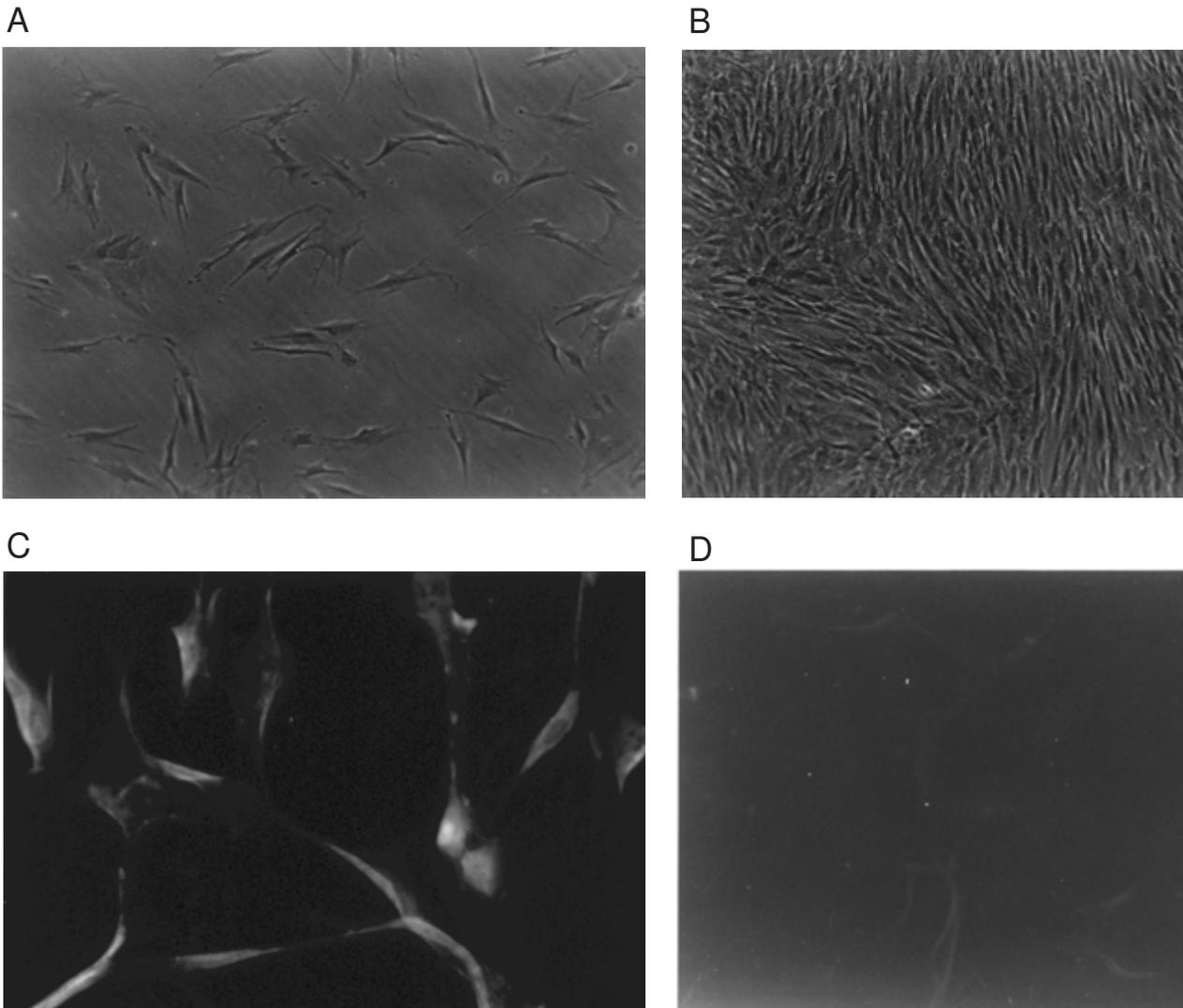


Figure 2.1.3 Identification of fibroblasts. Fibroblasts change their morphology depending upon the extent of confluency (or cell density). **(A)** Morphology at low density (10% to 20% confluence; magnification, 40 \times); **(B)** morphology at high density (100% confluence; magnification, 40 \times). **(C)** Indirect immunofluorescence staining of human fibroblast cultures with antibodies against type I collagen (magnification, 100 \times). **(D)** Indirect immunofluorescence staining of human fibroblast cultures with control antibodies (magnification, 100 \times). Briefly, fibroblasts were cultured for 2 days on LabTek chamber slides, fixed in 3% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, and then subjected to immunofluorescence staining (*UNIT 4.3*) with rabbit anti-type I collagen (Chemicon), followed by labeling with FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch).

**Establishment of
Fibroblast
Cultures**

2.1.6

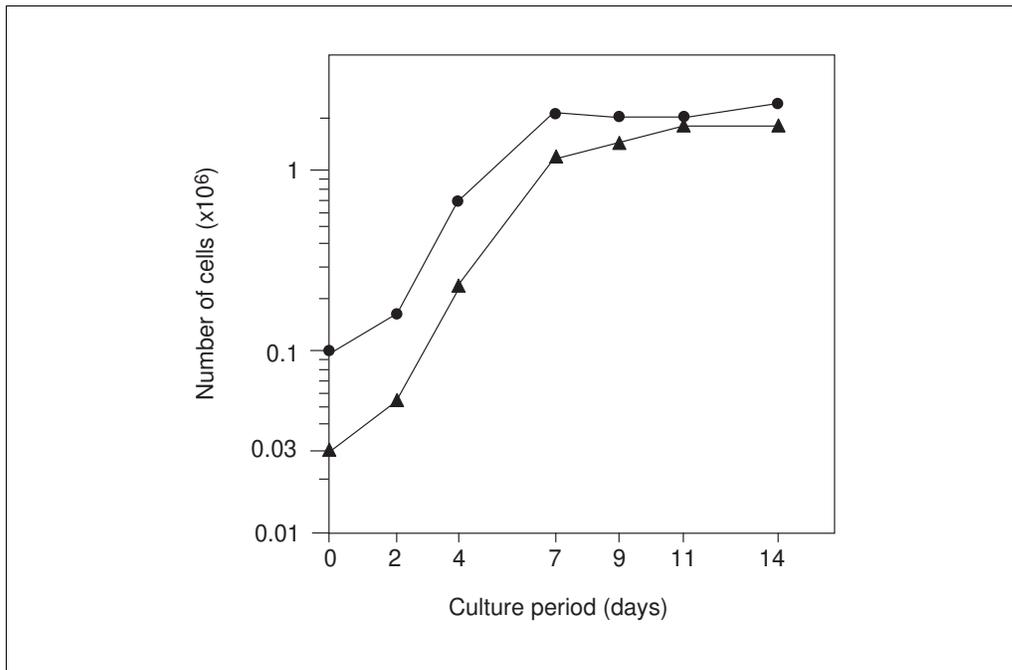


Figure 2.1.4 Fibroblast growth curve. A third passage of human fibroblast culture was plated on 35-mm dishes at either 30,000 (triangles) or 100,000 (circles) cells per dish, and cultured in complete RPMI. At the indicated time points, cultures were harvested by incubation with 0.3% trypsin/25 mM EDTA and counted to determine cell number. Note that cells grow relatively rapidly, with an approximate doubling time of 24 hr, and then stop dividing as they reach confluency.

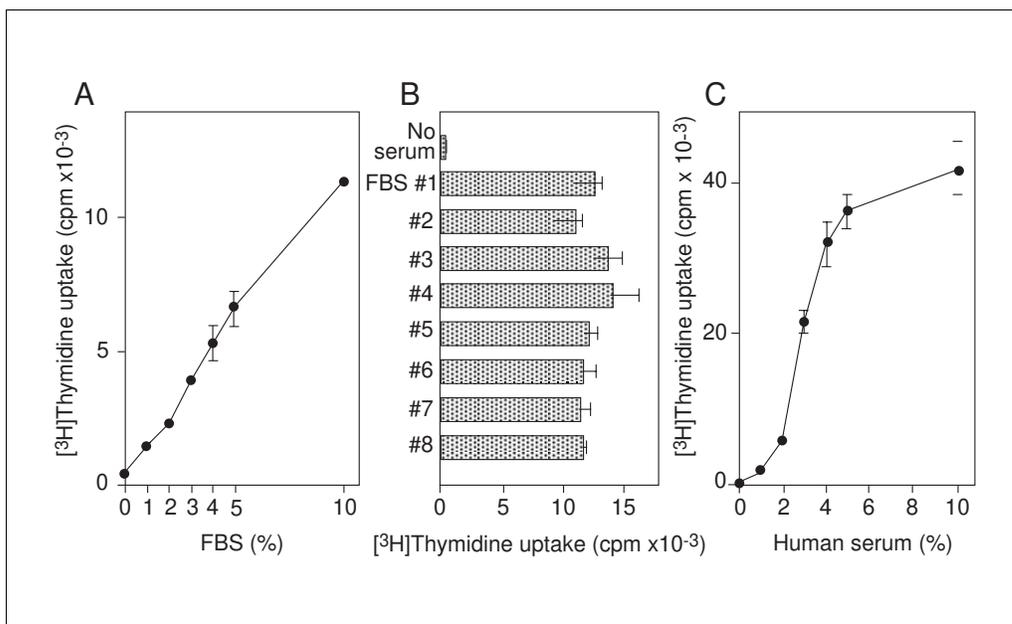


Figure 2.1.5 Serum requirement for fibroblast growth. A third passage of human fibroblasts (3000 cells/well) was cultured in flat-bottom 96-well plates, pulsed with [³H]thymidine on day 3, and harvested on day 4 using an automated cell harvester. The culture media were supplemented with (A) different concentrations of FBS; (B) 10% FBS from different vendors; and (C) different concentrations of human serum. Data shown are the means and standard deviations from triplicate cultures.

DISSOCIATED FIBROBLAST CULTURE

Although more complicated than the skin explant culture (see Basic Protocol), the dissociated fibroblast culture described in this protocol is more suitable for those experiments that require relatively large numbers of fibroblasts. After removal of the epidermis by dispase treatment, fibroblasts are released from the remaining dermis by enzymatic treatment with trypsin. The resulting dermal cells are then plated in suspension onto tissue culture plates. The most tricky step is the enzymatic digestion of dermal tissues. Investigators may need to compare various conditions (e.g., batches of trypsin, trypsin concentrations, and incubation periods) to maximize the cell yield while maintaining the cell viability. Alternatively, collagenase, which is less cytotoxic than trypsin, can be used for the same purpose.

Additional Materials (also see Basic Protocol)

- 1000 U/ml collagenase type IA in PBS (see recipe for PBS; store enzyme solution up to 3 months at -20°C)
- Nylon mesh (85- μm mesh; Tetko; cut into 5-cm square, wrap in aluminum foil, and sterilize by autoclaving)

CAUTION: When working with human blood, cells, or infectious agents, appropriate biosafety practices must be followed.

NOTE: Use Milli-Q water or equivalent in all protocol steps and for preparing all solutions.

Prepare dissociated cell suspension

1. Wash skin samples in PBS, remove the subcutaneous tissues, remove the epidermis by enzymatic digestion, wash the dermal sample in PBS, and cut the sample into small squares (see Basic Protocol, steps 1 to 5).

Since the trypsin that is used to digest the dermal connective tissue also dissociates epidermal cells, the epidermis must first be separated from dermal layer. Otherwise, the resulting cultures will be heavily contaminated by epidermal keratinocytes.

2. Place 10 to 20 dermal pieces in a 15-ml polypropylene tube with 3 ml of 0.3% trypsin/PBS and incubate 10 min in a 37°C water bath, inverting the tube several times every 2 to 3 min. Alternatively, incubate 10 to 20 dermal pieces 1 to 2 hr with 3 ml of 1000 U/ml collagenase at 37°C , agitating every 20 to 30 min.
3. Add 3 ml of ice-cold complete growth medium (DMEM or RPMI containing 10% FBS) to stop the reaction. Vortex the tube vigorously several times.

Although fibroblasts detach from collagen fibers after treatment with trypsin or collagenase, mechanical agitation is required for releasing them into the solution. Do not vortex before the addition of complete medium.

4. Pass the fibroblast suspension through 85- μm nylon mesh (placed over the top of a tube) to remove dermal debris.
5. Centrifuge 10 min at $150 \times g$, 4°C . Aspirate the supernatant, then resuspend the pellet in 100 to 200 μl of complete growth medium.
6. Count total and viable cells (see Basic Protocol, step 13).

The cell viability varies depending upon the conditions used for enzymatic digestion. Cutting skin sample into smaller sizes usually increases cell recovery as well as cell viability.

Culture fibroblasts

7. Plate $3\text{--}10 \times 10^4$ cells in 5 ml of complete growth medium in a 25-cm² tissue culture flask and begin incubation.

Viable fibroblasts will attach to the flask within 24 hr and begin to exhibit the spindle-shape in 2 to 3 days (Fig. 2.1.2D and E).

8. Gently remove the medium containing nonadherent cells and add fresh medium on day 2.

Because the presence of dead cells in culture affects the growth of viable fibroblasts, nonadherent, dead cells must be removed from the culture.

9. Change medium every 3 to 4 days until the culture becomes confluent.

As fibroblasts become overconfluent, they appear as bundle clusters instead of spindle-shaped cells (Fig. 2.1.2 and Fig. 2.1.3). Harvest the cells before they reach this level.

10. Harvest fibroblasts by washing with PBS followed by incubation with trypsin/EDTA solution. Passage fibroblasts and prepare frozen stocks (see Basic Protocol, steps 10 to 16).

REAGENTS AND SOLUTIONS

Use Milli-Q water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Complete growth medium

- 500 ml DMEM or RPMI 1640 (Life Technologies or Sigma)
 - 60 ml FBS (heat-inactivated 60 min at 56°C; APPENDIX 2A)
 - 5 ml 1 M HEPES buffer solution (Life Technologies)
 - 5 ml 100× nonessential amino acid mixture (Life Technologies)
 - 5 ml 100× L-glutamine (Life Technologies)
 - 5 ml 100× penicillin/streptomycin (Life Technologies)
 - 5 ml 100× sodium pyruvate (Life Technologies)
- Store up to 1 month at 4°C

Phosphate-buffered saline (PBS)

- 4 liters distilled water
 - 32 g NaCl (140 mM final)
 - 0.8 g KH₂PO₄ (1.5 mM final)
 - 8.7 g Na₂HPO₄·7H₂O (8.1 mM final)
 - 0.8 g KCl (2.7 mM final)
- Adjust the pH to 7.4 with 1 N NaOH
Store indefinitely at room temperature

Trypsin/EDTA solution

- Prepare the following stock solutions:
- 0.3% (w/v) trypsin (from bovine pancreas; Sigma) in PBS (see recipe for PBS)
 - 1% (w/v) tetrasodium EDTA in PBS (see recipe for PBS)
- Store stock solutions up to 3 months at –20°C
- Combine 97.5 ml 0.3% trypsin/PBS and 2.5 ml 1% EDTA/PBS. Store trypsin/EDTA solution up to 1 week at 4°C.

COMMENTARY

Background Information

Fibroblasts are the major cellular component of connective tissues, where they play an important role in maintaining structural integrity. They produce and secrete a wide array of extracellular proteins, including proteinases, thereby regulating the biochemical composition and remodeling of tissues. Because biopsy samples can be easily obtained from skin, this tissue serves as a most convenient source of fibroblasts. Skin is composed of continually renewing multilayered squamous epithelium (the epidermis), connective tissue (the dermis), and subcutaneous (adipose) tissue. Human epidermis contains, in addition to keratinocytes (epithelial cells producing keratin intermediate filaments), relatively small numbers of Langerhans cells (antigen-presenting cells of the dendritic cell lineage) and melanocytes (which produce pigment granules called melanosomes). By contrast, mouse epidermis contains keratinocytes, Langerhans cells, and resident $\gamma\delta$ T cells called “dendritic epidermal T cells.” The dermis, in both human and mouse skin, is a fibrous and filamentous connective tissue that contains fibroblasts, endothelial cells, mast cells, macrophages, and occasionally other leukocyte populations. Despite the complexity of cellular composition, relatively pure fibroblast cultures can be obtained from skin specimens without sophisticated purification processes. This is primarily due to the fact that fibroblasts grow rapidly and continuously when cultured in the presence of serum, whereas other cell types require additional growth factors (e.g., epidermal growth factor or keratinocyte growth factor for keratinocytes), or show very little mitotic activity *in vitro* (Schuhmachers et al., 1995).

Morphological features—e.g., elongated cell bodies, oval nuclei, and linear or bundle-like alignment of cellular distribution—serve as conventional markers of fibroblasts in culture (Fig. 2.1.2 and Fig. 2.1.3). It is important to emphasize, however, that fibroblasts change their morphology dramatically depending upon the culture conditions, especially the extent of confluency (compare Fig. 2.1.3 panels A and B). Unfortunately, there is no antibody available that recognizes fibroblasts selectively. On the other hand, the absence of specific markers that are expressed by other dermal components (e.g., cytokeratin in keratinocytes, VCAM-1 on endothelial cells, IgE receptor on mast cells, and CD14 on macrophages) serves as a pheno-

typic marker of fibroblasts (Xu et al., 1995). Production of large amounts of type I collagen, as detected by immunofluorescence staining (Fig. 2.1.3C and D), can be used as a functional marker (Schuhmachers et al., 1995). Nevertheless, because the fibroblast cultures established by the standard protocols described in this unit are rarely “contaminated” by other cell types, especially after a few passages, it is generally accepted that they can be used as “fibroblasts” without further characterization.

Fibroblasts grow rapidly, with a doubling time of 24 to 72 hr (Fig. 2.1.4), and can be passaged successfully >10 times. Because of this outstanding mitotic potential, fibroblasts have been used for a variety of investigative purposes. For example, they serve as useful tools for studying the function and metabolism of extracellular matrix proteins as well as other fundamental aspects of cell biology. Fibroblast cultures established from patients with inherited disorders have often been used to identify genetic abnormalities. Moreover, autologous fibroblasts can be used as a “vector” in gene therapy to deliver transgenes into patients (Suhonen et al., 1996; Nolte and Kohn, 1997). Fibroblast lines generated from healthy human volunteers can be purchased from American Type Culture Collection (ATCC).

The protocols described in this unit are also applicable to other animal species. For example, in the author’s laboratory, several fibroblast lines from rats and rabbits have been developed using the same protocols as described for mouse fibroblasts. After enzymatic separation of the dermal compartment, the remaining epidermal portion can be used to grow epidermal cells, such as keratinocytes and melanocytes.

As sources of human fibroblast cultures, newborn foreskin (obtained in circumcision), skin samples excised during surgical operations, cadaver skin (obtained from the transplantation unit), or skin biopsies are routinely used in the author’s laboratory. Fibroblast cultures can be established from relatively small skin specimens; the author routinely uses 4-mm punch biopsies for this purpose (Pandya et al., 1995). It is also practical and feasible to establish fibroblast cultures without sacrificing experimental animals; mouse “ear punch” samples are used for this purpose by the author.

Critical Parameters

Because fibroblast outgrowth occurs predominantly from sharp edges of skin speci-

mens, it is crucial to use fine razor blades or surgical scalpels for cutting skin into small pieces. The author routinely uses disposable no. 22 surgical blades for this purpose. Drying of skin specimens is another common cause of poor fibroblast outgrowth. This can be avoided by adding a few drops of PBS while cutting skin specimens. If the surgical blades and forceps are to be soaked with 70% ethanol for sterilization, they should be rinsed well in PBS before use. When performed appropriately, outgrowing fibroblasts should become detectable within 3 to 4 days in skin explant cultures (Fig. 2.1.2B). In cell-dissociation cultures (see Alternating Protocol), the enzymatic digestion process is the most critical; if the viable cell count of resulting suspensions is <70% by trypan blue exclusion, this indicates overdigestion. When performed appropriately, the plated cells should attach firmly to culture plates within 24 hr and begin to spread in 2 to 3 days (Fig. 2.1.2D and E).

Fibroblasts can be frozen safely in 10% DMSO/90% FCS or 10% DMSO/complete DMEM and stored for >10 years in liquid nitrogen. Thus, it is suggested that several aliquots be frozen at a relatively early phase in culture (e.g., after the second or third passage). Because the original features of the cells may be altered during extended culture periods, it is not recommended that they be cultured continuously without experimental usage. If the cells suddenly stop dividing, or if the growth rate accelerates, the cultures need to be replaced. In the author's laboratory the original cultures are routinely discarded after the sixth passage and new cultures are started from a frozen stock.

Care should be taken in harvesting fibroblasts from culture plates. Although fibroblasts are more resistant to contact inhibition than other cell types (e.g., keratinocytes), it is suggested that cells be harvested during their exponential growth phase (Fig. 2.1.4). In the author's laboratory, after removal of culture medium, culture plates are routinely washed briefly with PBS, and then minimal amounts of 0.3% trypsin/25 mM EDTA are added. These plates can be incubated at room temperature under a microscope; as soon as the cells become rounded (before being released spontaneously from plates), the enzymatic reaction is stopped by the addition of ice-cold growth medium containing 10% FBS, and cells are harvested by tapping the culture vessel or gentle pipetting. These cells need to be centrifuged immediately

to remove trypsin and EDTA. The cell viability should be >90% by trypan blue exclusion.

Troubleshooting

Bacterial or fungal contamination

Clean the skin well with 70% ethanol before taking a biopsy. Check all the culture media and reagents, including PBS, dispase, trypsin, collagenase, and complete growth medium. Sterilize surgical blades and forceps with 70% ethanol. Always keep the incubator clean.

Contamination by keratinocytes

Remove the epidermis before setting up the fibroblast cultures. Treat the contaminated cultures with 0.5% dispase for 10 to 30 min at 37°C to remove keratinocytes. Because keratinocytes usually require special growth factors for continuous growth, they will eventually disappear in the first or second passage.

Low cell yields or low cell viabilities after enzymatic digestion

Optimize the concentrations, batches of enzymes, and incubation periods. Cut skin into smaller pieces. Use freshly prepared trypsin (or collagenase) solutions; they will lose enzymatic activity gradually when kept at 4°C. Alternatively, prepare enzyme solutions in large quantities, divide them into 5- to 10-ml aliquots, and freeze them at -20°C.

Low cell viabilities during passage

Care must be taken not to overtrypsinize cultures. Wash the culture with PBS before trypsin treatment. Use freshly prepared trypsin solutions. Add ice-cold complete growth medium to stop the enzymatic reaction immediately after fibroblasts round up as determined by examination under a microscope. Avoid excessive pipetting.

Slow fibroblast growth

Increase FBS concentrations (up to 10%); most of the commercially available FBS batches work well for fibroblast cultures (Fig. 2.1.5). Check the temperature, CO₂ level, and humidity of the incubator. The growth rates of fibroblasts often slow down after >10 passages; thaw a frozen stock and start new cultures to avoid this.

Anticipated Results

In skin explant cultures, outgrowing fibroblasts become detectable within 3 to 4 days

and continue to grow thereafter. When skin pieces from one 4-mm punch-biopsy sample are plated in a 35-mm tissue culture dish, it usually takes 3 to 5 weeks to obtain a confluent culture. Starting from one newborn foreskin sample, one can obtain 5 to 10 confluent 35-mm dishes in 3 to 5 weeks. Approximately $1-2 \times 10^6$ cells can be harvested from a confluent 35-mm dish. Cell yields are usually higher in the dissociated fibroblast preparation (see Alternate Protocol); after enzymatic digestion with trypsin, $\sim 1-3 \times 10^5$ cells can be harvested from a 4-mm punch-biopsy sample and $1-3 \times 10^6$ cells can be harvested from a newborn foreskin sample. The use of collagenase typically increases the yield up to 2-fold. When $1-2 \times 10^5$ cells are originally seeded in a 25-cm² flask, they will become confluent within 2 to 3 weeks, producing $2-3 \times 10^6$ cells. After the first passage to new culture plates, fibroblasts begin to grow much faster, with a typical doubling time of 24 to 72 hr (Fig. 2.1.4).

Time Considerations

Skin explant cultures can be set up in 30 to 60 min (without epidermal separation) or 2 to 4 hr (with epidermal separation). Dissociated fibroblast cultures take 3 to 4 hr, depending upon the extent of enzymatic digestion. Although it has been possible in the author's laboratory to establish fibroblast cultures from 1- to 2-day-old skin samples kept in complete growth medium at 4°C, it is highly recommended that cultures be set up immediately after taking biopsy samples. Subsequent passages can be made in 15 to 30 min.

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Preparation and Culture of Human Lymphocytes

This unit describes procedures for preparation and culture of human lymphocytes and lymphocyte subpopulations obtained from peripheral blood. Because of ease of access, peripheral blood is the primary source of human lymphocytes (mononuclear leukocytes) used in most studies of lymphocyte function. Peripheral blood is a mixture of cells including lymphocytes, granulocytes, erythrocytes, and platelets. Density gradient centrifugation (see Basic Protocol 1) has proven to be an easy and rapid method for separation of lymphocytes from these other peripheral blood cell populations. Lymphocytes and platelets can be separated from granulocytes and erythrocytes according to their lower densities—they will float on top of a density gradient of Ficoll-Hypaque, whereas granulocytes and erythrocytes will traverse this fluid and collect at the bottom of the tube (Fig. 2.2.1). Monocytes/macrophages can then be separated from the other lymphoid-cell populations by adherence to plastic tissue culture vessels (see Basic Protocol 2). The procedures described in this section can be applied to the isolation of peripheral blood lymphocyte populations obtained either from whole blood or via a leukapheresis procedure.

Human lymphocyte subpopulations can be purified based on their cell-surface display of specific distinguishing molecules that can be recognized by monoclonal antibodies. The physical basis for such separation procedures involves the coupling of antibody reagents to magnetic beads, which permit the rapid sequestration of cells that have been bound by the specific antibodies. T and B cells can be positively selected using monoclonal antibody-coated magnetic beads (see Basic Protocol 3) or by exposing the cells to monoclonal antibody and then purifying cells that have bound the antibody using magnetic beads coated with anti-immunoglobulin G (anti-IgG; see Alternate Protocol 1).

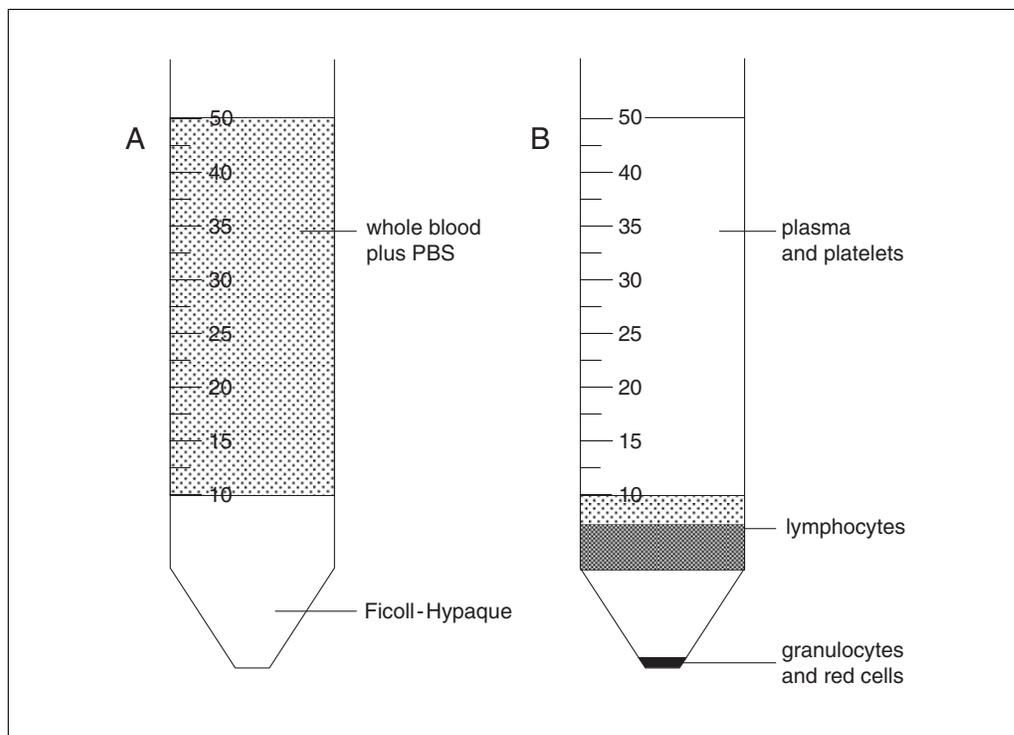


Figure 2.2.1 Isolation of human lymphocytes on a Ficoll-Hypaque gradient. (A) Before centrifugation; (B) after centrifugation.

In addition, specific subpopulations can be isolated by negative selection, in which all unwanted subpopulations are removed using monoclonal antibodies and anti-IgG-coated magnetic beads (see Alternate Protocol 2).

CAUTION: When working with human blood, cells, or infectious agents, appropriate biosafety practices must be followed.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

**BASIC
PROTOCOL 1**

**PREPARATION OF LYMPHOCYTES BY FICOLL-HYPAQUE GRADIENT
CENTRIFUGATION**

In this procedure, whole blood or white blood cells from leukapheresis donors are centrifuged in the presence of a density gradient medium to separate lymphocytes from other peripheral blood cell populations.

Materials

- Anticoagulated whole blood or white blood cells from leukapheresis donor
- Phosphate-buffered saline (PBS), without calcium or magnesium (Bio-Whittaker), room temperature
- Ficoll-Hypaque solution (see recipe), room temperature
- Lymphocyte culture medium (LCM; see recipe), room temperature
- Iscove's modified Dulbecco's medium (IMDM; Life Technologies) containing 20% heparinized human plasma
- Freezing medium (see recipe)
- 50-ml conical centrifuge tubes
- Sorvall RT-6000B centrifuge with H-1000 rotor (or equivalent)
- 1.5-ml cryotubes (e.g., Nunc)
- Controlled-rate freezer (e.g., CryoMed from Forma Scientific)
- Liquid nitrogen freezer
- Additional reagents and equipment for counting cells and determining cell viability (*UNIT 1.1*) and flow cytometry (Robinson et al., 1998)

Prepare Ficoll-Hypaque gradient

For whole blood

- 1a. Pipet 15 ml of whole blood into a 50-ml conical centrifuge tube and add 25 ml room temperature PBS
- 2a. Using a 10-ml pipet, underlay with 10 ml room temperature Ficoll-Hypaque solution.

For leukapheresis preparation

- 1b. Pipet 10 ml of cell suspension from a leukapheresis preparation into a 50-ml conical centrifuge tube and add 30 ml room temperature PBS.
- 2b. Using a 10-ml pipet, underlay with 7.5 ml room temperature Ficoll-Hypaque solution.

Separate cells

3. Centrifuge 20 min at 800 × g (2000 rpm in H-1000 rotor), 20°C, with the brake off.

4. Aspirate most of the plasma- and platelet-containing supernatant above the interface band (granulocytes and erythrocytes will be in red pellet; Fig. 2.2.1). Aspirate the interface band (which includes the lymphocytes) along with no more than 5 ml of fluid above the pellet into a 10-ml pipet, then transfer to a new 50-ml conical centrifuge tube, combining the bands from 2 to 3 Ficoll-Hypaque gradients into one 50-ml tube. Add PBS to 50-ml mark.
5. Centrifuge 10 min at $600 \times g$ (1500 rpm in H-1000 rotor), 20°C , with the brake on.
6. Aspirate supernatants and resuspend the pellet in each tube with 10 ml room temperature PBS. Combine resuspended pellets into as few 50-ml tubes as possible. Add PBS to 50-ml mark in each tube used.
7. Centrifuge 15 min $300 \times g$ (750 rpm in H-1000 rotor), 20°C , with brake on.

This low-speed centrifugation permits as many platelets as possible to remain above the pellet of lymphocytes.

Process cell pellet

8. Aspirate platelet-containing supernatant and resuspend lymphocyte pellet in room temperature LCM. Count cells and determine number of viable cells by trypan blue exclusion (UNIT 1.1).

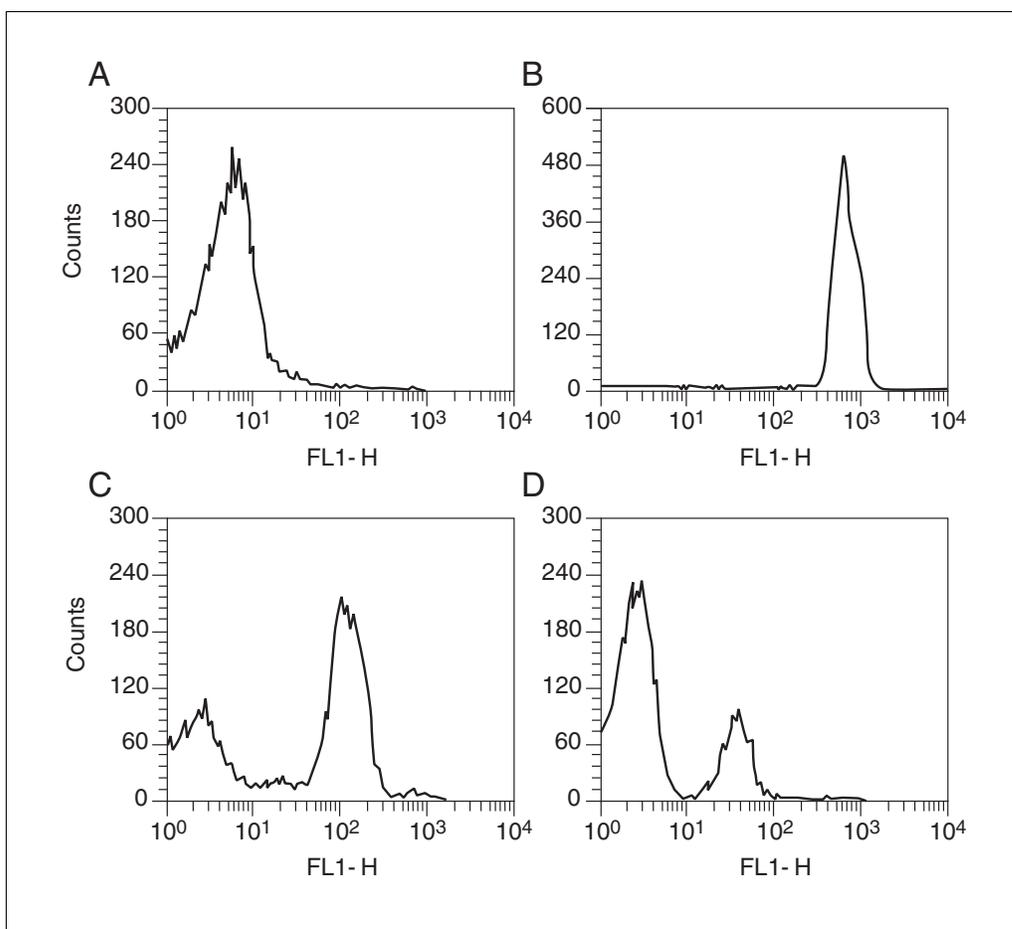


Figure 2.2.2 Flow cytometric analysis of human lymphocytes isolated by Ficoll-Hypaque density gradient centrifugation stained with (A) fluorochrome-labeled anti-mouse Ig antibody alone (negative control), (B) fluorochrome-labeled anti-CD45 monoclonal antibody, (C) fluorochrome-labeled anti-CD3 monoclonal antibody, and (D) fluorochrome-labeled anti-CD19 monoclonal antibodies. FL1-H refers to the pulse height for the fluorochrome.

9. Determine purity of lymphocyte preparation by flow cytometry (see, e.g., Robinson et al., 1998) using an anti-CD45 (anti-leukocyte common antigen) antibody (see Fig. 2.2.2 for typical results).
10. To cryopreserve cells, resuspend lymphocytes at twice the concentration desired in the freezing vials using MDM/20% human plasma. Add an equal volume of freezing medium, in increments, over a period of 1 to 2 min, mixing after each addition. Divide the cell suspension into 1-ml aliquots in 1.5-ml cryotubes, place cryotubes in a precooled 4°C controlled-rate freezer and freeze at -1°C/min until -50° or -60°C is attained. Finally, place cryotubes in the gaseous phase of a liquid nitrogen freezer.

PREPARATION OF MONOCYTES/MACROPHAGES AND “DENDRITIC-LIKE” CELLS FROM LYMPHOCYTE POPULATIONS

Monocytes/macrophages comprise 5% to 20% of the lymphocytes prepared by density gradient centrifugation (see Basic Protocol 1). To isolate this cell population, or to deplete these cells from the population of lymphocytes, the most expedient mechanism is to exploit the adherence property of monocytes/macrophages. By coating plastic tissue culture vessels with serum as a source of fibronectin and other components of the extracellular matrix, monocytes/macrophages, but not T and B cells, will adhere to these surfaces. This adherence can be significantly enhanced by the presence of recombinant human interleukin 3 (rhIL-3; Biddison et al., 1997). Cells with properties of dendritic cells and a markedly enhanced capacity for antigen processing and presentation can be generated from the monocyte/macrophage population by differentiation in the presence of rhIL-4 and granulocyte/macrophage colony stimulating factor (GM-CSF; Sallusto and Lanzavecchia, 1994).

Materials

- Lymphocyte culture medium (LCM; see recipe), 37°C
- Lymphocyte population (see Basic Protocol 1)
- Recombinant human interleukin 3 (rhIL-3), interleukin 4 (rhIL-4), and GM-CSF (PeproTech)
- 5 mM tetrasodium EDTA in PBS (also available as Versene from Life Technologies), filter-sterilized using 0.22- μ m Nalgene filter, prewarmed to 37°C
- 175-cm² tissue culture flasks
- 50-ml conical centrifuge tubes
- Sorvall RT-6000B centrifuge with H-1000 rotor (or equivalent)
- Additional reagents and equipment for counting cells and determining cell viability (*UNIT 1.1*) and flow cytometry (Robinson et al., 1998)

Allow monocyte/macrophage population to attach to plastic

1. Add 20 ml of 37°C LCM to 175-cm² tissue culture flasks. Incubate 30 min.
2. Add 500×10^6 lymphocytes in 10 ml LCM to each flask plus rhIL-3 to a final concentration of 200 U/ml.
3. Incubate 3 hr, gently rocking flasks every hour.
4. Remove nonadherent cells by aspirating the medium. Wash flasks twice, each time with 20 ml 37°C LCM.

Isolate macrophages/monocytes or “dendritic-like” cells

To isolate monocytes/macrophages

- 5a. Add 20 ml of 5 mM EDTA/PBS to each flask and incubate 20 min to release adherent monocytes/macrophages.
- 6a. Vigorously pipet medium up and down to detach adherent cells, then transfer to 50-ml centrifuge tube.

To generate and isolate “dendritic-like” cells

- 5b. Add 30 ml LCM containing 200 U/ml rhIL-4 and 200 U/ml GM-CSF to each flask and incubate 60 hr.
- 6b. Remove nonadherent cells and place in 50-ml centrifuge tubes, then add 20 ml of 5 mM EDTA/PBS to each flask and incubate 20 min to release adherent cells. Vigorously pipet medium up and down to detach adherent cells, then combine with nonadherent cells in 50-ml centrifuge tubes.

Separate and analyze isolated cells

7. Centrifuge 10 min at $600 \times g$ (1500 rpm in Sorvall H-1000 rotor), room temperature. Aspirate supernatant and resuspend pellet in LCM.
8. Count cells and determine number of viable cells by trypan blue exclusion (*UNIT 1.1*).
9. Determine purity of the monocyte/macrophage or “dendritic-like” cell populations by flow cytometry (see, e.g., Robinson et al., 1998).

Monocytes/macrophages can be distinguished from T and B cells by the absence of cell-surface CD3 and CD19 and by the presence of CD14 or CD35. “Dendritic-like” cells can be distinguished from monocytes/macrophages by the presence of cell-surface CD1 molecules (Sallusto and Lanzavecchia, 1994).

POSITIVE SELECTION OF T AND B CELLS BY MONOCLONAL ANTIBODY-COATED MAGNETIC BEADS

In this procedure, T cells and B cells are positively selected based on their differential cell-surface expression of CD3 (T cells) or CD19 (B cells). Monoclonal anti-CD3 and anti-CD19 antibodies coupled to magnetic beads are commercially available. Aliquots of a lymphocyte population purified as described in Basic Protocol 1 are incubated in separate tubes with anti-CD3-coupled beads and anti-CD19-coupled beads. T cells and B cells will be bound by their specific antibody-coupled beads and are then physically separated from unbound cells by exposure of the tubes to a strong magnetic field. Unbound cells are removed and washed away, and the specifically bound cells are released by incubation with a soluble antiserum specific for mouse Fab fragments (Detachabead solution from Dynal) which competes with the bead-coupled monoclonal antibody that is bound to the surface of the cells and thus causes the cells to come off. The procedure here describes specific reagents for separation of T cells and B cells, but is directly applicable to separation of any lymphocyte subpopulation that can be distinguished by monoclonal antibody-coated beads—e.g., purification of CD4⁺ and CD8⁺ T cell populations with anti-CD4- and anti-CD8-coated magnetic beads.

Materials

- Lymphocyte population (see Basic Protocol 1)
- Anti-CD3 and anti-CD19 antibodies for flow cytometry (Becton-Dickinson or Coulter)
- Anti-CD3- and anti-CD19-coated magnetic beads (Dynabeads M-450; Dynal)
- PBS without calcium and magnesium (Bio-Whittaker)

BASIC PROTOCOL 3

Preparation and Isolation of Cells

2.2.5

PBS/HSA: PBS without calcium and magnesium (Bio-Whittaker) containing 0.5% (w/v) human serum albumin (American Red Cross Blood Services)
IMDM/HSA: Iscove's modified Dulbecco's medium (Life Technologies) containing 0.5% (w/v) human serum albumin
Polyclonal anti-mouse Fab antiserum (Detachabead; Dynal)
15-ml conical centrifuge tubes
Magnetic separation device (e.g., Dynal MPC-I or Advanced Magnetics Biomag Separator)
Platform rocker (e.g., Clay Adams Nutator, Becton Dickinson Primary Care Diagnostics)
Sorvall RT-6000B centrifuge with H-1000 rotor (or equivalent)
Additional reagents and equipment for flow cytometry (Robinson et al., 1998) and for counting cells and determining cell viability (*UNIT 1.1*)

Prepare and wash magnetic beads

1. Determine the approximate number of T cells and B cells in the starting population of lymphocytes by flow cytometry (or indirect immunofluorescence staining) using anti-CD3 and anti-CD19 antibodies, then determine the number of antibody-coated magnetic beads that will be required to purify the required number of T cells and B cells (5 to 10 beads will be needed for each specific target cell).
2. Based on the bead concentration supplied by the manufacturer, remove the required volume of anti-CD3- and anti-CD19-coated beads from the source vials and place each in a 15-ml conical centrifuge tube. Add PBS to the 14-ml mark and resuspend the beads.
3. Place tubes on magnetic separation device (vertical magnet) for 2 min, then gently aspirate supernatant, leaving beads clinging to one side of each tube.
4. Remove tubes from magnet. Add 10 ml PBS to each tube, resuspend beads, then place tubes on vertical magnet for 2 min. Aspirate PBS as in step 3.

Perform magnetic separation

5. Resuspend each tube of beads in 2 ml PBS/HSA. Put tubes on ice for 15 min.
6. Resuspend lymphocyte population ($\leq 200 \times 10^6$ cells) in 10 ml PBS/HSA. Place on ice for 15 min.
7. Add 5 ml of lymphocyte suspension to the tube with anti-CD3 beads and 5 ml to the tube with anti-CD19 beads.
8. Incubate 45 min with gentle tilting and rotation on a platform rocker at 4°C.
9. Place tubes on vertical magnet for 2 min. Aspirate nonadherent cells, taking care not to disturb the beads that are clinging to one side of each tube.
10. Add 5 ml IMDM/HSA to each tube. Gently resuspend beads, then place tubes on vertical magnet for 2 min. Aspirate nonadherent cells as in step 9.
11. Add 3 ml IMDM/HSA to each tube. Gently resuspend beads, then place tubes with their conical bottoms on the horizontal magnet. Incubate 2 min at room temperature.

Release T cells and B cells from beads

12. Carefully remove 2.5 ml of supernatant. Tap tubes gently to resuspend beads.
13. Add 200 μ l Detachabead solution. Incubate 30 min at room temperature with gentle resuspension every 5 min.

Smaller volumes of Detachabead solution may be used when starting with smaller numbers of lymphocytes; see manufacturer's instructions.

14. Add 3 ml PBS/HSA to each tube and resuspend beads. Place tubes on vertical magnet for 2 min, then aspirate and save supernatant containing detached cells, taking care not to disturb the beads clinging to the side of each tube. Repeat this step four times, saving and combining detached cell-containing supernatants from each separation.
15. Centrifuge detached cells 10 min at $600 \times g$ (1500 rpm in H-1000 rotor), room temperature.
16. Aspirate supernatants and resuspend cells in IMDM/HSA. Count cells and determine number of viable cells by trypan blue exclusion (*UNIT 1.1*).
17. Determine purity of the T cell and B cell populations by flow cytometry using anti-CD3 and anti-CD19 antibodies (see, e.g., Robinson et al., 1998).

POSITIVE SELECTION OF T AND B CELLS BY MONOCLONAL ANTIBODIES AND ANTI-IgG-COATED MAGNETIC BEADS

ALTERNATE PROTOCOL 1

This procedure differs from Basic Protocol 3 in that it does not require the acquisition of separate magnetic beads coupled with individual monoclonal antibodies. The procedure is described for T and B cell separation, but can be applied to any lymphocyte subpopulation that can be distinguished by monoclonal antibodies. The principle of the technique is that a human mixed lymphocyte population is separately exposed to saturating amounts of either anti-CD3 or anti-CD19 mouse IgG monoclonal antibodies, unbound antibodies are washed away, then the cells that have bound these antibodies are physically separated from unbound cells by magnetic beads coated with goat anti-mouse IgG. The cells that are specifically bound to the goat anti-mouse IgG-coated beads are then detached by exposure to soluble antiserum against mouse Fab fragments.

Additional Materials (also see *Basic Protocol 3*)

- Anti-CD3 and anti-CD19 IgG monoclonal antibodies (Becton Dickinson or Coulter)
- Goat anti-mouse IgG-coated magnetic beads (Dynabeads M-450; Dynal)

Prepare cells and antibodies

1. Determine the approximate number of T cells and B cells in the starting population of lymphocytes by flow cytometry using anti-CD3 and anti-CD19 antibodies (e.g., Robinson et al., 1998).
2. Determine the saturating concentration of the anti-CD3 and anti-CD19 antibodies to be used by flow cytometry. Prepare a solution of each antibody in PBS/HSA at ten times ($10\times$) the saturating concentration.

Treat cells with antibodies

3. Resuspend the lymphocyte population ($\leq 200 \times 10^6$ cells) in 9 ml PBS/HSA and add 4.5 ml of the suspension to each of two 15-ml conical centrifuge tubes. Add 0.5 ml of $10\times$ anti-CD3 antibody to one tube and 0.5 ml of $10\times$ anti-CD19 antibody to the other tube.
4. Incubate 45 min with gentle tilting and rotation on a platform rocker at 4°C .
5. Wash cells twice, each time by centrifuging 10 min at $600 \times g$ (1500 rpm in H-1000 rotor), room temperature, removing the supernatant, resuspending in 10 ml PBS/HSA, and removing the supernatant. Finally, resuspend each tube in 5 ml PBS/HSA and put on ice for 15 min.

Preparation and Isolation of Cells

2.2.7

Prepare magnetic beads

6. Based on the number of T and B cells estimated in step 1, determine the number of goat anti-mouse IgG-coated magnetic beads that will be required to purify the required number of T and B cells (5 to 10 beads will be needed for each specific target cell).
7. Based on the bead concentration supplied by the manufacturer, remove two aliquots of the required volume of goat anti-mouse IgG-coated beads from the source vial and place in 15-ml conical centrifuge tubes. Add PBS to the 14-ml mark and resuspend the beads.
8. Wash beads (see Basic Protocol 3, steps 2 to 4).

Perform separation

9. Add 5 ml of anti-CD3-coated cells to one tube of washed anti-IgG-coated beads and 5 ml of anti-CD19-coated cell suspension to the other tube of anti-IgG-coated beads.
10. Perform magnetic separation to isolate T and B cells (see Basic Protocol 3, steps 8 to 17).

ISOLATION OF T AND B CELL SUBPOPULATIONS BY NEGATIVE SELECTION

For certain experimental conditions, it is desirable to isolate lymphocyte subpopulations without antibody engagement of cell-surface molecules such as the immunoglobulin receptor on B cells and the CD3 complex on T cells. Therefore, negative selection procedures are followed that aim to maximize the elimination of all lymphocyte subpopulations except the desired one. For T cell isolation, the procedure involves elimination of B cells (CD19⁺), monocytes/macrophages (CD14⁺), and NK cells (CD16⁺); for B cell isolation T cells (CD3⁺), monocytes/macrophages, and NK cells are eliminated. The procedure is very similar to the above protocol for selection of T and B cells using anti-IgG-coated magnetic beads.

Additional Materials (also see Basic Protocol 3)

- Anti-CD3, anti-CD14, anti-CD16, and anti-CD19 IgG monoclonal antibodies (Becton-Dickinson or Coulter)
- Goat anti-mouse IgG-coated magnetic beads (Dynabeads M-450; Dynal)

Prepare cells and antibodies

1. Determine the approximate number of T cells, B cells, monocytes/macrophages, and NK cells in the starting population of lymphocytes by flow cytometry using anti-CD3, anti-CD19, anti-CD14, and anti-CD16 antibodies (e.g., Robinson et al., 1998).
2. Determine the saturating concentrations of the anti-CD3, anti-CD19, anti-CD14, and anti-CD16 antibodies to be used by flow cytometry. Prepare a solution of each antibody in PBS/HSA at ten times (10×) the saturating concentration.

Treat cells with antibodies

3. Resuspend the lymphocyte population ($\leq 200 \times 10^6$ cells) in 3.5 ml PBS/HSA in a 15-ml conical centrifuge tube.
- 4a. *For T cell isolation:* Add 0.5 ml each of 10× anti-CD19, 10× anti-CD14, and 10× anti-CD16 antibody preparations to the tube.
- 4b. *For B cell isolation:* Add 0.5 ml each of 10× anti-CD3, 10× anti-CD14, and 10× anti-CD16 antibody preparations to the tube.

5. Incubate 45 min with gentle tilting and rotation on a platform rocker at 4°C.
6. Wash cells twice, each time by centrifuging 10 min at $600 \times g$ (1500 rpm in H-1000 rotor), room temperature, removing the supernatant, resuspending in 10 ml PBS/HSA, and removing the supernatant. Finally, resuspend cells in 5 ml PBS/HSA and put on ice for 15 min.

Prepare magnetic beads

7. Based on the number of cells of each subpopulation estimated in step 1, determine the number of goat anti-mouse IgG-coated magnetic beads that will be required to bind the required number of antibody-coated lymphocytes (5 to 10 beads will be needed for each specific target cell).
8. Based on the bead concentration supplied by the manufacturer, remove the required volume of goat anti-mouse IgG-coated beads from the source vial and place in a 15-ml conical centrifuge tube. Add PBS to the 14-ml mark and resuspend the beads.
9. Wash beads (see Basic Protocol 3, steps 2 to 4).

Perform separation

10. Add 5 ml of washed antibody-coated cells (from step 6) to the tube with the washed anti-IgG-coated beads.
11. Incubate 45 min with gentle tilting and rotation on a platform rocker at 4°C.
12. Place tube on vertical magnet for 2 min. Aspirate and save supernatant containing nonadherent cells, taking care not to disturb the beads clinging to the side of the tube.
13. Add 5 ml IMDM/HSA to tube. Gently resuspend beads.
14. Repeat steps 12 and 13 twice, saving and pooling nonadherent cells from each wash.
15. Centrifuge nonadherent cells 10 min at $600 \times g$ (1500 rpm in H-1000 rotor), room temperature. Aspirate supernatant and resuspend cells in 5 ml IMDM/HSA. Count cells and determine number of viable cells by trypan blue exclusion (*UNIT 1.1*).
16. Determine purity of the T cell and B cell populations by flow cytometry using anti-CD3 and anti-CD19 antibodies (see, e.g., Robinson et al., 1998).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Ficoll-Hypaque solution (density 1.07 to 1.08 g/ml)

Dissolve 6.2 g Ficoll (mol. wt. 400,000; e.g., Sigma) in 75 ml distilled water with slow stirring. Add 10.4 g sodium diatrizoate and stir until solution is clear. Add water to 100 ml. Filter-sterilize using 0.22- μ m filter (Nalgene). Store up to 6 months at 4°C in the dark.

Alternatively, solutions can be purchased from commercial suppliers (e.g., Lymphocyte Separation Medium from Organon Teknika Cappel or equivalent product from Bio-Whittaker).

Freezing medium

Mix 20 ml Iscove's modified Dulbecco's medium (IMDM; Life Technologies) and 20 ml heparinized human plasma. Cool to 4°C. Slowly add 10 ml dimethylsulfoxide, mixing after each incremental addition. Cool to 4°C and filter sterilize using a 0.22- μ m filter.

Lymphocyte culture medium (LCM)

Iscove's modified Dulbecco's medium (IMDM; Life Technologies) containing:

100 U/ml penicillin

100 µg/ml streptomycin

10% heparinized human plasma

Store up to 2 weeks at 4°C

COMMENTARY

Background Information

Density-gradient separation of lymphocytes

Isolation of human lymphocytes from peripheral blood by density gradient centrifugation provides the following advantages: it is a simple technique, it is relatively inexpensive, it does not require special laboratory equipment, sterility is easily maintained, and high yields of lymphocytes are provided (Boyum, 1968). A typical yield of lymphocytes from whole blood of healthy adult donors is $1-2 \times 10^6$ cells/ml. Figure 2.2.2 shows a typical result from flow cytometric analysis of density gradient-purified lymphocytes in which 98% of cells are CD45⁺ (positive for common leukocyte antigen), 61% are CD3⁺ (T cells), and 21% are CD19⁺ (B cells). The non-T non-B cell population contains monocytes/macrophages and natural killer (NK) cells.

Monocytes/macrophages can be easily purified from the lymphocyte population based on their differential adhesiveness to immobilized components of the extracellular matrix contained in human serum. A typical yield of monocytes/macrophages from a lymphocyte population is 5% to 10%. Flow cytometric analysis shows that $\geq 95\%$ of these cells are HLA class I⁺, HLA-DR⁺, and CD35⁺, and $\leq 1\%$ are CD3⁺ T cells (Fig. 2.2.3). A subset of these monocytes/macrophages can be differentiated into immature antigen-presentation-competent "dendritic-like" cells by culture in IL-4 and GM-CSF (Sallusto and Lanzavecchia, 1994). These "dendritic-like" cells have proven to be extremely useful antigen-presenting cells for the generation and cloning of rare self-reactive T cells (Biddison et al., 1997).

Magnetic-bead separation of lymphocytes

Cell-separation procedures based on magnetic-bead technology have the advantages of easy manipulation, safety from contamination with microorganisms, and avoidance of high shear forces that affect cell viability. The most direct procedures involve positive selection with specific monoclonal antibodies directly

coupled to magnetic beads and the subsequent liberation of the bound cells by competing anti-Fab antibodies. This procedure also has the added advantage of providing a positively selected lymphocyte subpopulation free of any cell-bound selecting antibodies. A variation on this approach involves precoating the lymphocyte subpopulation of choice with a specific monoclonal IgG antibody and subsequent physical sequestration with anti-IgG-coated magnetic beads. In experimental situations in which specific antibody binding to cell-surface structures must be avoided, negative selection procedures are employed, wherein the lymphocyte subpopulations that are not desired are coated with specific IgG antibodies and are then removed by anti-IgG-coated magnetic beads. Each of these procedures has been used successfully to isolate human T cells, monocytes/macrophages, and B cells (Lea et al., 1986; Vartdal et al., 1987; Funderud et al., 1990).

Alternative procedures for positive selection are cell sorting of fluoresceinated antibody-coated cells with a flow cytometer and panning of antibody-coated cells on plastic surfaces containing immobilized anti-immunoglobulin reagents. This author's experiences with these techniques indicate that they present a much higher likelihood of contamination with microorganisms and subject the lymphocytes to conditions that can lead to more loss of viable cells than occurs with the magnetic-bead procedures. Cell sorting also has a major limitation in the number of cells that can be separated. Negative selection can also be provided by complement-fixing antibodies, but this approach involves the need to remove dead-cell debris before the remaining viable cells can be used for experimentation.

Critical Parameters and Troubleshooting

To maximize the yield and purity of lymphocytes using the density gradient protocol (see Basic Protocol 1), it is essential to harvest all of the cells at the interface between the

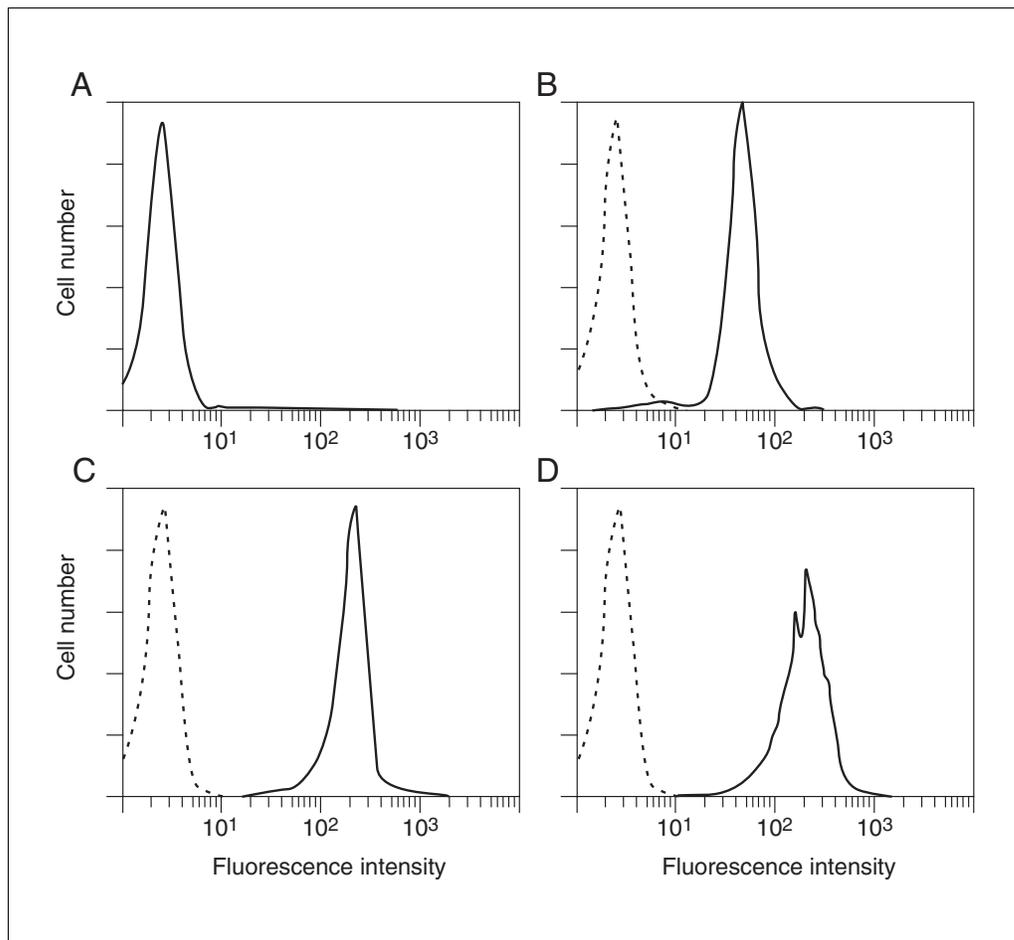


Figure 2.2.3 Flow cytometric analysis of isolated monocytes/macrophages. The solid lines represent cells stained with (A) fluorochrome-labeled anti-CD3 monoclonal antibodies, (B) fluorochrome-labeled anti-CD35 monoclonal antibodies, (C) fluorochrome-labeled anti-HLA class I monoclonal antibodies, and (D) fluorochrome-labeled anti-HLA-DR monoclonal antibodies. The dotted lines in panels B, C, and D represent cells stained with fluorochrome-labeled anti-mouse IgG alone.

Ficoll-Hypaque solution and the plasma/platelet layer. This may require harvesting up to 5 ml of the underlying Ficoll-Hypaque solution. This is why it is recommended that at least 7.5 ml of this solution be used as an underlay for each tube. Great care should be taken not to disturb or harvest any of the pelleted granulocytes and erythrocytes. One common problem is the accumulation of platelets at the Ficoll-Hypaque interface, which produces clumping and contamination with platelets and erythrocytes. This problem can be partially avoided by further dilution of blood with PBS and by not centrifuging the cells at temperatures $<20^{\circ}\text{C}$. The platelet-contamination problem can be alleviated in the subsequent washing steps. If significant cloudiness of the supernatant is observed (due to platelet contamination) following the 15-min centrifugation at $300 \times g$ (see

Basic Protocol 1, step 7), aspirate the supernatants, resuspend the cells in 50 ml PBS, and do another 15-min, $300 \times g$ centrifugation to remove as many remaining platelets as possible.

Isolation of monocytes/macrophages by adherence is significantly enhanced by precoating the tissue culture flasks with serum-containing medium and including IL-3 during the adhesion incubation. No diminution in antigen-presenting function has been observed in the author's laboratory by cells cultured in IL-3. However, if the presence of IL-3 may add an unknown variable to other types of experiments, especially those that will utilize the nonadherent lymphocyte fraction, it is recommended that IL-3 not be included during the adherence step.

The specificity and avidity of the antibody reagents used in these cell-separation procedures are clearly of critical importance. Care must be taken to utilize appropriate concentrations of coating monoclonal antibodies to ensure that all specific target cells are coated with antibody. It is also crucial that, when precoating cells with soluble antibodies, all unbound antibodies are adequately removed during the washing steps. Another critical factor is the use of adequate numbers of antibody-coated magnetic beads to completely engage all appropriate available cells. The use of 5 to 10 beads per specific target cell helps to ensure a complete saturation of the available target cells. Certain pitfalls are relatively easy to avoid. Where called for in the protocols, be sure to do manipulations on cells that have been precooled and do appropriate incubations at 4°C. This will ensure that the selecting antibodies will not be removed by capping. Use of a sufficiently strong magnet will improve cell yields and purity and reduce the amount of time for each magnetic-selection step. This author's experience is that the Biomag Separator available from Advanced Magnetics is more than adequate for the task of selecting the Dynal Dynabeads. Also note that the protocols do not include use of fetal bovine serum (FBS) in any step. FBS and other heterologous sera should be avoided in all steps because they can produce unwanted activation of lymphocytes.

At the conclusion of each of the cell-separation procedures, the purity of the cell populations produced should be assessed by flow cytometry.

Anticipated Results

Whole blood from healthy adult donors will typically yield $1\text{--}2 \times 10^6$ lymphocytes per ml of blood by the density gradient protocol. A typical leukapheresis in which lymphocytes are collected from 5 liters of blood will yield $4\text{--}6 \times 10^9$ lymphocytes when processed by the density gradient protocol. Isolated lymphocytes should be $\geq 95\%$ viable.

Isolation of monocytes/macrophages from 500×10^6 lymphocytes will usually yield $25\text{--}50 \times 10^6$ cells with $\geq 95\%$ viability. Generation of "dendritic-like" cells from the same number of starting lymphocytes will usually yield $5\text{--}10 \times 10^6$ cells with $\geq 90\%$ viability.

The lymphocyte subpopulations produced by the magnetic-bead techniques should be $\geq 95\%$ pure and $\geq 95\%$ viable by trypan blue dye

exclusion. The overall yield of T and B cells from the starting lymphocyte preparation from peripheral blood should be 40% to 60%.

Time Considerations

Isolation of lymphocytes from peripheral blood (see Basic Protocol 1) usually takes 2 hr, depending on the volume of blood obtained or the number of lymphocytes collected by leukapheresis. Monocyte/macrophage isolation (see Basic Protocol 2) requires a subsequent 3.5-hr time period, and culture of the isolated monocytes/macrophages with IL-4 and GM-CSF requires an additional 60 hr of incubation time.

Positive selection using monoclonal antibody-coated magnetic beads (see Basic Protocol 3) requires 2 to 3 hr. The method using anti-IgG coated-beads (see Alternate Protocol 1) requires an additional hour for precoating of the cells with selecting antibodies.

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Key References

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Comprehensive description of magnetic bead technology for lymphocyte separations.

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Preparation of Endothelial Cells

This unit presents various approaches to obtaining endothelial cells from tissues for cell culture. The isolation of these cells from umbilical vein (see Basic Protocol) is particularly useful because the tissue source is readily available and the cells can be obtained in quantity. The isolated cells are generally pure and grow well. Although umbilical veins are the most frequently employed source, cells can be obtained from other vessels and from tissues—e.g., adipose tissue, nasal mucosa, and skin.

The isolation of human umbilical vein endothelial cells (HUVEC) is first described (see Basic Protocol). Additional protocols describe the isolation of microvascular endothelial cells from tissues with few contaminating cells (retroperitoneal adipose tissue and nasal mucosa; see Alternate Protocol 1) and from tissues with a significant number of contaminating cells (skin; see Alternate Protocol 2).

CAUTION: When working with human blood, cells, or infectious agents, appropriate biosafety practices must be followed.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations should be performed in a 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

PREPARATION OF ENDOTHELIAL CELLS FROM HUMAN UMBILICAL VEIN

BASIC PROTOCOL

This protocol describes the isolation and culture of human umbilical vein endothelial cells (HUVEC).

Materials

- Fresh human umbilical cord
- RPMI 1640 medium, 4° and 37°C
- Phosphate-buffered saline (PBS; Life Technologies)
- 5 mg/ml collagenase CLSII (Worthington) in PBS (filter sterilized), 37°C
- HUVEC medium (see recipe), 37°C
- 250-ml wide-mouthed tissue culture flasks
- Sterile pads and gauze
- Sterile scissors, razors, hemostats, and non-toothed forceps
- Syringes and yellow 200- μ l pipet tips
- 50-ml conical centrifuge tubes
- Tabletop centrifuge
- Nunc T-75 tissue culture flasks *or* 75-cm² gelatin-coated flasks (see recipe)
- 150-mm-diameter Nunc tissue culture dishes
- Additional reagents and equipment for trypsinization of cells (UNIT 1.1)

1. Place the umbilical cord in a 250-ml wide-mouthed flask containing RPMI 1640 medium and store at 4°C for no more than 24 hr after delivery.

Cords older than 24 hr should be discarded. It is easiest to drop off the medium in a wide-mouth flask at the hospital ahead of time so the staff can deposit the cord as soon as it is available.

Preparation and Isolation of Cells

2.3.1

2. Working in a sterile tissue culture hood with sterile pads on the hood counter top, wash the cord with PBS. Using sterile gauze to hold the cord, cut the ends with a razor to yield a 10- to 15-cm segment.

Wear a lab coat and two pairs of gloves. The cord is human tissue and could be infectious or pathogenic.

3. Dilate vein using non-toothed forceps.

Dilate carefully to avoid tears.

4. Using a syringe with a yellow 200- μ l pipet tip attached, flush 5 to 10 ml 37°C RPMI 1640 through the vein.

5. Using a fresh syringe with a yellow 200- μ l pipet tip, flush 5 to 10 ml of 5 mg/ml collagenase through the vein.

6. Clamp one end of the cord with a hemostat and squeeze ~10 ml more collagenase solution into the vein. Clamp top of cord with a second hemostat and invert the cord several times to spread the collagenase solution.

7. Place the clamped umbilical cord in a 250-ml wide-mouthed flask with warm RPMI 1640 and incubate 15 min at room temperature.

8. Open one end of the cord over a 50-ml conical centrifuge tube to catch the effluent from the vein. Flush vein with 15 ml of 37°C HUVEC medium and collect the effluent.

The HUVEC medium reduces the collagenase activity and helps the cells to remain viable.

9. Add enough additional HUVEC medium to fill the tube and centrifuge 5 min at 170 \times g in a tabletop centrifuge, room temperature.

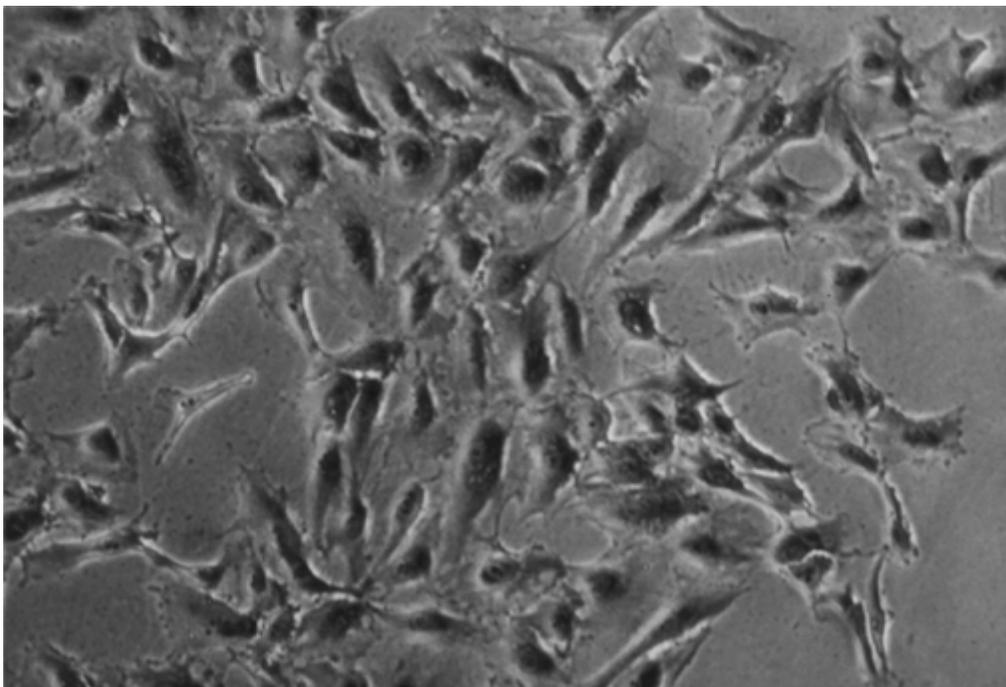


Figure 2.3.1 Culture of human umbilical vein endothelial cells 2 days after passaging. Passage 3 cells were at ~70% confluence and were fixed and stained, respectively, with Diff-Quik Fix and Solution II (Baxter). Cells were viewed using a Zeiss inverted microscope.

10. Decant and discard supernatant, tap tube to resuspend cell pellet, then add 15 ml 37°C HUVEC medium and transfer to two T-75 Nunc or 75-cm² gelatin-coated flasks. Incubate 1.5 hr.

This is based on an average yield from a 10- to 15-cm cord.

11. After 1.5 hr, remove medium and add 15 ml fresh medium. Incubate 2 to 3 days (until confluent).

The medium that is removed can be used to plate additional cells in another flask; after 1.5 hr, that medium should be removed and discarded, and fresh medium added. It is important with this second flask to be sure that there are not too many dead cells or fibroblasts (which can be detected microscopically on the basis of morphology).

12. When cells become confluent, split flask 1:4 into 150-mm Nunc tissue culture dishes.

These cells are considered passage 1. Cells should not be used beyond passage 6. At passage 2, there should be 32 dishes. Some of the passage 2 cells can be frozen and stored in liquid nitrogen for future use (UNIT 1.1). Figure 2.3.1 shows a culture of endothelial cells with typical elongated morphology.

ISOLATION OF ENDOTHELIAL CELLS FROM RETROPERITONEAL ADIPOSE TISSUE OR NASAL MUCOSA

ALTERNATE PROTOCOL 1

It is well recognized that not all endothelial cells behave in a similar manner or have identical phenotypes. Preparations of endothelial cells from different tissue sources are often needed. Retroperitoneal adipose tissue or nasal mucosa can be obtained at the time of surgery. Usually, small pieces are provided from the normal edges of removed lesions such as polyps in the nasal mucosa.

Additional Materials (also see *Basic Protocol*)

Retroperitoneal adipose tissue or nasal mucosa

0.02% (w/v) collagenase A (Boehringer Mannheim) in PBS (filter sterilized)

Cell scraper

Laminin-coated 6-well tissue culture plates (see recipe)

Additional reagents and equipment for trypsinization of cells (UNIT 1.1)

1. Cut tissue into 2- to 4-mm-square pieces and wash with sterile PBS.
2. Incubate 3 to 5 tissue pieces for 30 min with enough 0.2% collagenase at 37°C to just cover the pieces.
3. Gently aspirate collagenase solution and wash the tissue once in PBS, discarding the supernatants.
4. Add 3 ml RPMI 1640 medium, squeeze tissue with a cell scraper, and collect cells in a 50-ml conical tube.
5. Filter through sterile gauze to remove tissue debris.
6. Centrifuge 5 min at 170 × g in a tabletop centrifuge, room temperature.
7. Discard supernatant, gently tap tube to resuspend cell pellet, and add 15 ml of room temperature HUVEC medium.
8. Plate 3 ml of cell suspension onto each well of a laminin-coated 6-well plate. Incubate 1 hr, then remove medium (along with unattached cells) and add 3 ml fresh medium.
This step reduces the fibroblast contamination.
9. Incubate cells until confluent and passage as described above for HUVECs (see Basic Protocol).

Preparation and Isolation of Cells

2.3.3

ISOLATION OF MICROVASCULAR ENDOTHELIAL CELLS FROM DERMIS

Human microvascular endothelial cells can be isolated from neonatal foreskins (Kubota et al., 1988). These cells are relatively easy to prepare and are widely used.

Additional Materials (also see Basic Protocol and Alternate Protocol 1)

Dermal tissue (e.g., neonatal foreskins)

Discontinuous Percoll gradient (see recipe)

5 mM tetrasodium EDTA in PBS (also available as Versene from Life

Technologies), filter-sterilized using 0.22- μ m Nalgene filter, prewarmed to 37°C

PBS containing 5% FBS, room temperature to 37°C (store up to 1 month at 4°C)

Laminin-coated 6-well tissue culture plates (see recipe)

Anti-CD31-coated petri dishes (see recipe)

Gelatin- or fibronectin-coated 6-well tissue culture plates (see recipes)

Additional reagents and equipment for trypsinization of cells (*UNIT 1.1*)

1. Prepare a cell suspension from dermal tissue as described for retroperitoneal adipose tissue or nasal mucosa (see Alternate Protocol 1, steps 1 to 6). Resuspend the pellet in 1 ml of RPMI 1640 medium.
2. Carefully layer 1 ml of the resuspended pellet on top of the discontinuous Percoll gradient. Centrifuge 20 min at $670 \times g$ in a tabletop centrifuge, room temperature.
3. Collect each layer separately and transfer to individual tubes. Wash by centrifuging 5 min at $170 \times g$, room temperature, adding 10 ml RPMI 1640 medium, then centrifuging again and removing the supernatant.

Endothelial cell fractions are usually in the 32% and 34% layers.

4. Resuspend pellets in HUVEC medium and culture each fraction in a well of a laminin-coated 6-well plate (see Alternate Protocol 1, steps 7 to 9).

Usually the yield of cells is very low due to the small amounts of biopsy material available. All of the pellet obtained from each fraction should be placed in a well. The number of endothelial cell colonies will be small.

5. After 2 days, remove medium, wash cells with PBS, and detach cells by incubating ~5 min with 1.0 ml/well of 5 mM EDTA/PBS at room temperature.
6. Centrifuge 5 min at $170 \times g$, room temperature. Discard supernatant, tap tube to resuspend cell pellet, add 15 ml RPMI 1640 medium (prewarmed to 37°C), and incubate 1 to 3 hr on a CD31-coated dish at 37°C.
7. Gently aspirate the medium containing nonadherent cells and wash the dish twice gently with 15 ml of room temperature to 37°C PBS containing 5% FBS.
8. Wash dish gently with 15 ml of room temperature to 37°C PBS and release attached cells by trypsinization.

The cells usually detach within a few minutes.

9. Centrifuge cells 5 min at $170 \times g$, room temperature. Discard the supernatant, tap the tube gently to resuspend the cell pellet, add HUVEC medium and culture on gelatin- or fibronectin-coated 6-well tissue culture plates (see Basic Protocol).

Fibronectin is more expensive than gelatin.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Anti-CD31-coated petri dishes

Prepare 10 $\mu\text{g/ml}$ anti-CD31 monoclonal antibody in 50 mM Tris-Cl, pH 9.5 (store according to supplier's instructions). Add 10 ml of this solution to a 10-cm-diameter petri dish and incubate 1.5 hr at 37°C. Remove the solution and use it to coat two more dishes in the same way. Wash each dish three times with 0.15 M NaCl, then add 10 ml of 1 mg/ml bovine serum albumin (BSA) to each dish and incubate overnight at 4°C. Remove the liquid before adding the cells.

The anti-CD31 is available from several suppliers. Preparations without BSA work better. The coated plates should be used immediately.

Fibronectin-coated 6-well tissue culture plates

Prepare 333 $\mu\text{g/ml}$ fibronectin in PBS (Life Technologies) and filter sterilize (store up to 1 week at 4°C). Add 0.3 ml to each well and incubate 30 min at 37°C just prior to addition of cells. Remove the solutions containing unbound fibronectin prior to addition of the cells.

Gelatin-coated tissue culture flasks and plates

Prepare 0.1% (w/v) gelatin in PBS (Life Technologies) and filter sterilize (store up to 1 month at 4°C). Incubate flasks with the gelatin solution 30 min at 37°C, then remove gelatin and rinse with sterile PBS. Store coated flasks up to 1 day at 4°C or air dry and use within 1 week.

HUVEC medium

500 ml RPMI-1640 medium
100 ml defined iron-supplemented calf serum (HyClone)
100 mg endothelial cell growth supplement (ECGS; Collaborative Biomedical)
2500 U heparin
5 ml 100 \times glutamine
5 ml 100 \times penicillin/streptomycin
5 ml 100 \times Fungizone
0.5 ml 1000 \times gentamicin
Store up to 1 month at 4°C

Laminin-coated 6-well tissue culture plates

Prepare 333 $\mu\text{g/ml}$ laminin in PBS (Life Technologies) and filter sterilize (store up to 1 year at -80°C). Add 0.3 ml to each well and incubate 30 min at 37°C just prior to addition of cells. Remove the solutions containing unbound laminin prior to addition of the cells.

Percoll gradient, discontinuous

Prepare 30%, 34%, and 38% (v/v) Percoll in PBS
Prepare 32%, 36%, and 40% (v/v) Percoll in RPMI-1640 medium (with phenol red)

Pipet 2 ml of the 40% Percoll solution into a 15-ml round-bottom Nalgene centrifuge tube. Carefully layer 2 ml of the 38% Percoll on top of this, followed successively by the 36%, 34%, 32%, and 30% Percoll.

The Percoll solutions are prepared in an alternating fashion with PBS and (phenol red containing) RPMI 1640 to make it possible to distinguish the layers easily.

COMMENTARY

Background Information

Endothelial cells line blood vessels and are attached to each other (cell-to-cell) and to the underlying basement membrane matrix (cell-to-matrix). Various methods have been developed to isolate these cells in quantity for cell culture. Human umbilical vein is an ideal source because it is easy to obtain, cells can be isolated in quantity, and the cells are generally pure and grow well (Gimbrone, 1976; Jaffe, 1980). A disadvantage is the expensive medium needed to propagate the cells. The ECGS (endothelial cell growth supplement) is costly but can be prepared by the investigator if very large quantities are needed (Gordon et al., 1983). Newer media are under development and appear to maintain the survival of the cells. Previously, tumor-conditioned medium had been used (Folkman et al., 1979), but that material was somewhat variable and the results were not as reproducible as those observed with the HUVEC medium used in this unit (see Reagents and Solutions). Another disadvantage of HUVEC is that the cells cannot be used beyond passage 6, due to changes in the phenotype.

Other blood vessel and tissue sources have yielded endothelial cells for culture. For example, endothelial cells have been prepared from nasal mucosa (Fukuda et al., 1989), bovine capillary, pancreatic islet, mouse hemangi endothelioma (Folkman et al., 1979; Voest et al., 1995), adrenal cortex, and human dermis (Kubota et al., 1988).

Critical Parameters and Troubleshooting

Once the cells are isolated, their identity as endothelial cells can be confirmed by their characteristic cobblestone morphology or by specific markers such as factor VIII or uptake of acetylated LDL (Kubota et al., 1988; Kibbey et al., 1992). The type of dish used to culture the cells is important and the protocols should be followed exactly.

Because the cells are of human origin, full precautions for infectious and viral diseases must be employed. Gloves (at least two pair) should be used even after the cells have been in culture for several passages.

As compared with other types of primary cells, HUVEC are more “delicate” and problems with growth are observed with slight changes in the carbon dioxide or the temperature.

Umbilical cord or biopsy tissue should be stored at 4°C in medium and should not be used if it is >24 hr old.

Anticipated Results

One umbilical vein should yield two confluent T-75 flasks in 3 to 5 days. By passage 2 there should be 32 confluent 150-mm dishes with $\sim 1 \times 10^7$ cells per dish. The yield from tissues is less than that from umbilical vein.

Time Considerations

Preparation of the cells for all three methods should not take more than half a day.

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Generation of Continuously Growing B Cell Lines by Epstein-Barr Virus Transformation

UNIT 2.4

**BASIC
PROTOCOL**

Epstein-Barr virus (EBV) has been widely used to transform human B cells in vitro and to produce continuously growing B cell lines for use in a variety of in vitro studies. Most human B cell lines that are transformed by EBV secrete little or no infectious viral particles. For this reason, a marmoset line transformed with the human Hawley strain of EBV, which secretes active infectious EBV into the culture supernatant, is used as a source of transforming virus (Miller and Lipman, 1973). This protocol explains how to prepare the marmoset-derived EBV and transform human B cells obtained from peripheral blood.

CAUTION: EBV is a known human pathogen. Appropriate biosafety practices must be followed.

Materials

- Complete culture medium (see recipe), 37°C
- B95-8 EBV-transformed marmoset cell line (ATCC #CRL 1612)
- Anti-CD3 monoclonal antibody produced by OKT3 hybridoma (ATCC #CRL 8001)
- 25-cm² and 75-mm² tissue culture flasks
- Sorvall RT-6000B centrifuge with H-1000 rotor (or equivalent refrigerated centrifuge and rotor) and 50-ml centrifuge tubes
- 0.45- μ m sterile filter
- Additional reagents and equipment for growing cells, determining cell viability by trypan blue exclusion, and cryopreservation of cells (UNIT 1.1) and preparation of peripheral blood lymphocytes (UNIT 2.3)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

1. Resuspend B95-8 cells in complete culture medium at 1×10^6 cells/ml and incubate in 75-mm² tissue culture flasks at 50 ml of culture per flask for 3 days (until $\geq 95\%$ viable and in exponential growth phase; see UNIT 1.1 for basic culture technique and determination of cell viability).
2. Transfer cultures to 50-ml centrifuge tubes. Centrifuge 10 min at $600 \times g$ (1500 rpm in H-1000 rotor), 4°C. Filter supernatant through 0.45- μ m sterile filter, divide into 0.6-ml aliquots, and store at -70°C .

The culture supernatants should contain 10^2 to 10^3 infectious units/ml (Miller and Lipman, 1973). Determination of EBV titers can be done by quantitative assessment of transformation of umbilical cord leukocytes (Miller and Lipman, 1973); however, because of the difficulty in obtaining such cells, this determination is usually omitted.

3. Prepare peripheral blood lymphocytes as described in UNIT 2.3. Resuspend lymphocytes in 37°C complete culture medium at 1×10^6 cells/ml, then place 5 ml of the lymphocyte suspension in an upright 25-cm² tissue culture flask.
4. Add anti-CD3 antibody to final concentration of 10 μ g/ml. Incubate cells and anti-CD3 antibody for 1 hr with the flask in the upright position.

**Preparation and
Isolation of Cells**

2.4.1

5. Add 0.5 ml of the EBV-containing B95-8 supernatant (from step 2) to flask. Incubate with the flasks in the upright position for 1 to 2 weeks (until medium begins to turn orange/yellow and small clumps of cells become visible).
6. Add 5 ml of fresh 37°C complete medium, then incubate 2 to 3 days. After that period, remove 5 ml of the supernatant and add 5 ml of fresh 37°C complete medium and continue incubating. Repeat the feedings as described in this step until total cell number exceeds 5×10^6 .
7. Transfer growing cells to 75-cm² flask in 50 ml of 37°C complete medium and incubate until cell concentration is $\geq 1 \times 10^6$ /ml.
8. Cryopreserve aliquots of cells (*UNIT 1.1*) and maintain B cell line by splitting to 1×10^5 cells/ml in complete medium, incubating to 1×10^6 cells/ml, then splitting again.

The cells can be maintained this way indefinitely in the absence of any contamination with microorganisms.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

Complete culture medium

RPMI 1640 medium containing:

5% fetal bovine serum (FBS, heat-inactivated; *APPENDIX 2A*)

100 U/ml penicillin

100 µg/ml streptomycin

100 µg/ml gentamycin

2 mM L-glutamine

10 mM HEPES

Store up to 2 weeks at 4°C

COMMENTARY

Background Information

EBV is able to transform a subset of human resting B cells from peripheral blood (Sugden and Mark, 1977; Aman et al., 1984). The outgrowth of EBV-transformed B cells is prevented by the presence of EBV-immune T cells contained within the peripheral blood lymphocyte population (Rickinson et al., 1979). For this reason, soluble anti-CD3 antibody is included in the transformation process to inhibit the ability of T cells to respond to EBV antigens presented by the transformed B cells. Other procedures to eliminate T cell reactivity that could be utilized include T cell depletion of the lymphocyte population (*UNIT 2.3*) and functional T cell inactivation by cyclosporin A (Tosato et al., 1982).

EBV-transformed B cell lines have proven useful in studies of the cell biology of antigen presentation (Roche and Cresswell, 1990) and in the production of large-scale protein preparations for characterization of MHC antigens

(Orr et al., 1979) and MHC-bound peptides (Falk et al., 1991).

Critical Parameters

EBV-transformed B cells will not survive if there is an effective anti-EBV T cell response in the culture. It is essential that T cell functions be blocked with an adequate concentration of anti-CD3 antibody (10 µg/ml is sufficient). Also the number and viability of resting B cells in the culture must be sufficient to permit an efficient transformation by EBV (at least 5×10^6 peripheral blood lymphocytes are recommended). In addition, the titer of infectious EBV in the B95-8 culture fluid must be adequate for transformation. If transformation fails to occur, it will be necessary to obtain umbilical cord leukocytes to measure EBV infectivity (Miller and Lipman, 1973). Different batches of FBS have variable capacities for promoting the growth of EBV-transformed B cells. It is best to predetermine whether the available FBS can support the growth of existing EBV-trans-

formed B cell lines before using it for establishing new EBV-transformed lines. Great care should be taken to avoid contamination with mycoplasma (also see *UNIT 1.5*). Mycoplasma-containing EBV-transformed B cell lines will lose their capacity for continuous growth.

Anticipated Results

With the procedure described above, EBV-transformed B cell lines should be obtained which can maintain continuous growth indefinitely in the absence of contamination by microorganisms.

Time Considerations

Preparation of B95-8 culture supernatants requires a 3-day incubation. The establishment of the cultures for transformation takes 1 to 2 hr once the peripheral blood lymphocyte population has been obtained. Culture for 2 to 3 weeks is required to produce continuously growing EBV-transformed B cell lines.

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Key Reference

Miller and Lipman, 1973. See above.

Presents detailed description of B cell transformation and determination of EBV titers using umbilical cord leukocytes.

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Laser Capture Microdissection

UNIT 2.5

This unit describes a method for isolating pure populations of cells for biochemical and molecular analysis, called laser capture microdissection (LCM; see Basic Protocol). A second protocol outlines procedures for staining frozen or paraffin-embedded tissue samples for LCM (see Support Protocol).

ISOLATION OF A PURE CELL POPULATION FROM TISSUE SECTIONS

**BASIC
PROTOCOL**

Laser capture microdissection (LCM) provides the scientific community with a rapid and reliable method for obtaining pure cell populations from tissue sections under direct microscopic visualization. It incorporates both an inverted light microscope (with or without a fluorescent module) and a near-infrared laser to facilitate the visualization and procurement of cells. Briefly, a stained slide is placed under a microscope, and a specific adherence cap with an ethylene vinyl acetate (EVA) film is placed onto the tissue. The user moves the slide until the area of interest lies in the center of the microscope's field of view. When the cells of interest are located, a near-infrared laser is fired, which melts the EVA film in the targeted area. The EVA film expands into the void of the stained tissue and solidifies within 200 msec as it rapidly cools. The targeted tissue bonds to the EVA film, retaining the exact cellular morphology, DNA, RNA, and proteins intact. Both frozen and fixed tissue samples are successfully microdissected using LCM. Recovered cells can be analyzed for DNA, RNA, and protein content, and used to construct cell-specific cDNA libraries (Emmert-Buck et al., 1996; Krizman et al., 1996; Simone et al., 1998, 2000; Banks et al., 1999). LCM offers a quick means of procuring pure cell populations; however, it is necessary to follow strict protocols pertaining to fixation, preparation, and handling of tissue samples to be microdissected.

Materials

- Stained tissue samples, either frozen or formalin paraffin-embedded, cut into 2- to 10- μ m sections, and mounted on plain, uncharged microscope slides
- PixCell II Laser Capture Microdissection System (Arcturus Engineering)
- CapSure transfer film (Arcturus Engineering)
- Compressed gas duster
- CapSure pads (Arcturus Engineering)
- Cap removal tool (Arcturus Engineering)

Setup LCM apparatus

1. Load the CapSure cassette module with a CapSure cartridge.
2. Move joystick into vertical position to properly position the cap in relation to the capture zone. Place the microscope slide containing the prepared and stained tissue sample on microscope stage. When the target zone for microdissection appears in the viewing area, turn on Vacuum on front of controller to hold slide in place.
3. Slide CapSure cassette backward or forward to place cap at Load position. Slide transport arm over CapSure cap, then lift transfer film transport arm and place cap onto slide.
4. To enable the PixCell II laser, turn the keyswitch located on front of the controller and press the Laser Enable button.

Figure 2.5.1 shows components of the PixCell II.

**Preparation and
Isolation of Cells**

2.5.1

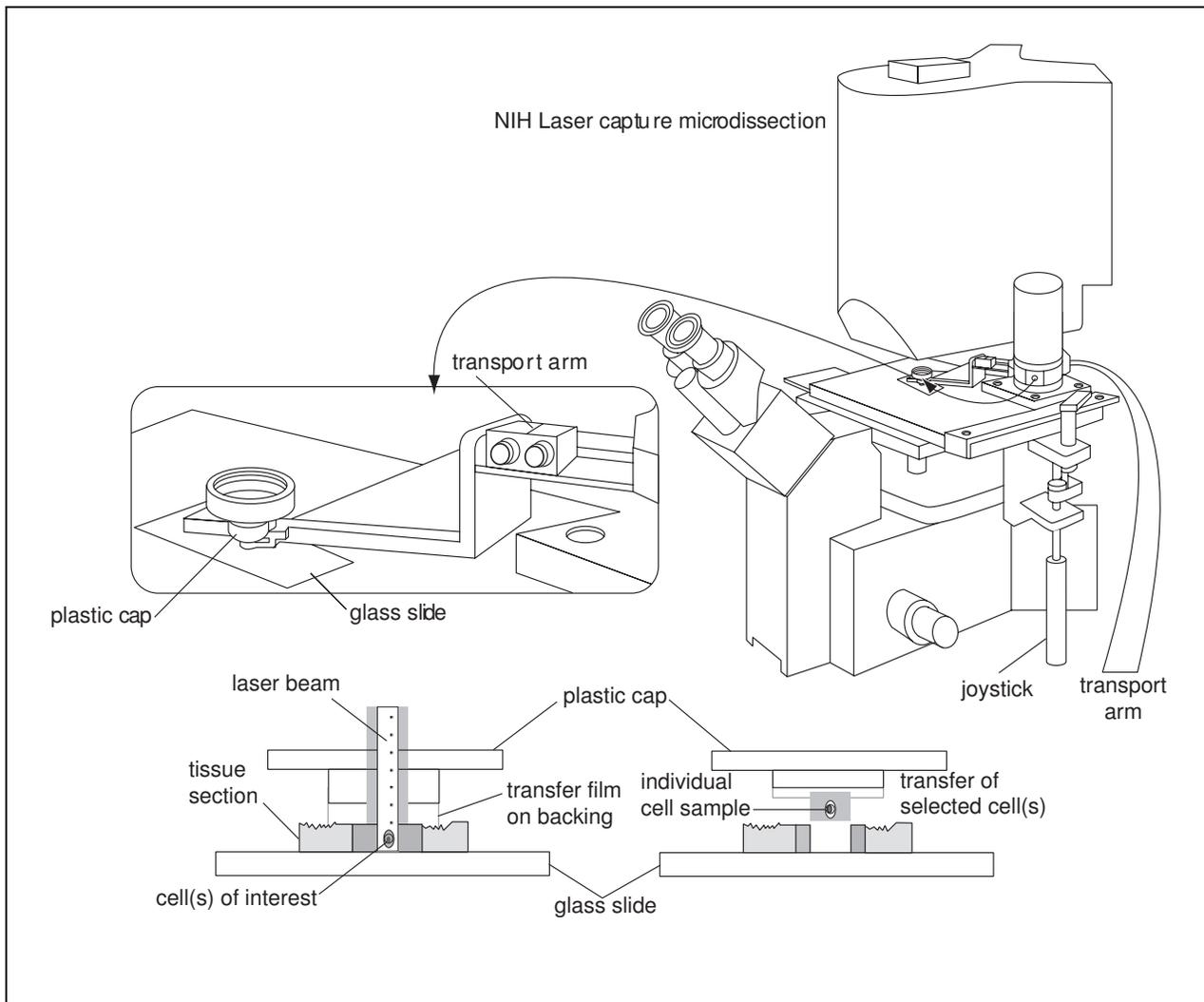


Figure 2.5.1 Schematic overview of the Laser Capture Microdissection Microscope. Through activation of a laser beam and under direct microscopic visualization, individual cells can be isolated for use in molecular analysis of any kind.

5. Verify that target beam is focused by selecting a spot size of 7.5 μm , using the Spot Size Adjust lever found on the left side of the microscope. Rotate microscope objectives until the 10 \times objective is in place. Reduce light intensity through the optics until field seen on the monitor appears almost dark and target beam is easily viewed. Using the Fine Focus Adjust located below the Spot Size Adjust lever, adjust target beam until it reaches the point of sharpest intensity and most concentrated light with little or no halo effect (Fig. 2.5.2).

The laser is now focused for any of the three laser spot sizes.

6. Select the laser spot size suitable to perform the microdissection.
7. Adjust Power and Duration of the laser pulse on front of the controller to vary the diameter of the “capture zone”, using target settings as a reference point. Adjust these settings up or down to customize the laser to the type and thickness of the tissue to be dissected. Suggested settings are listed in Table 2.5.1.

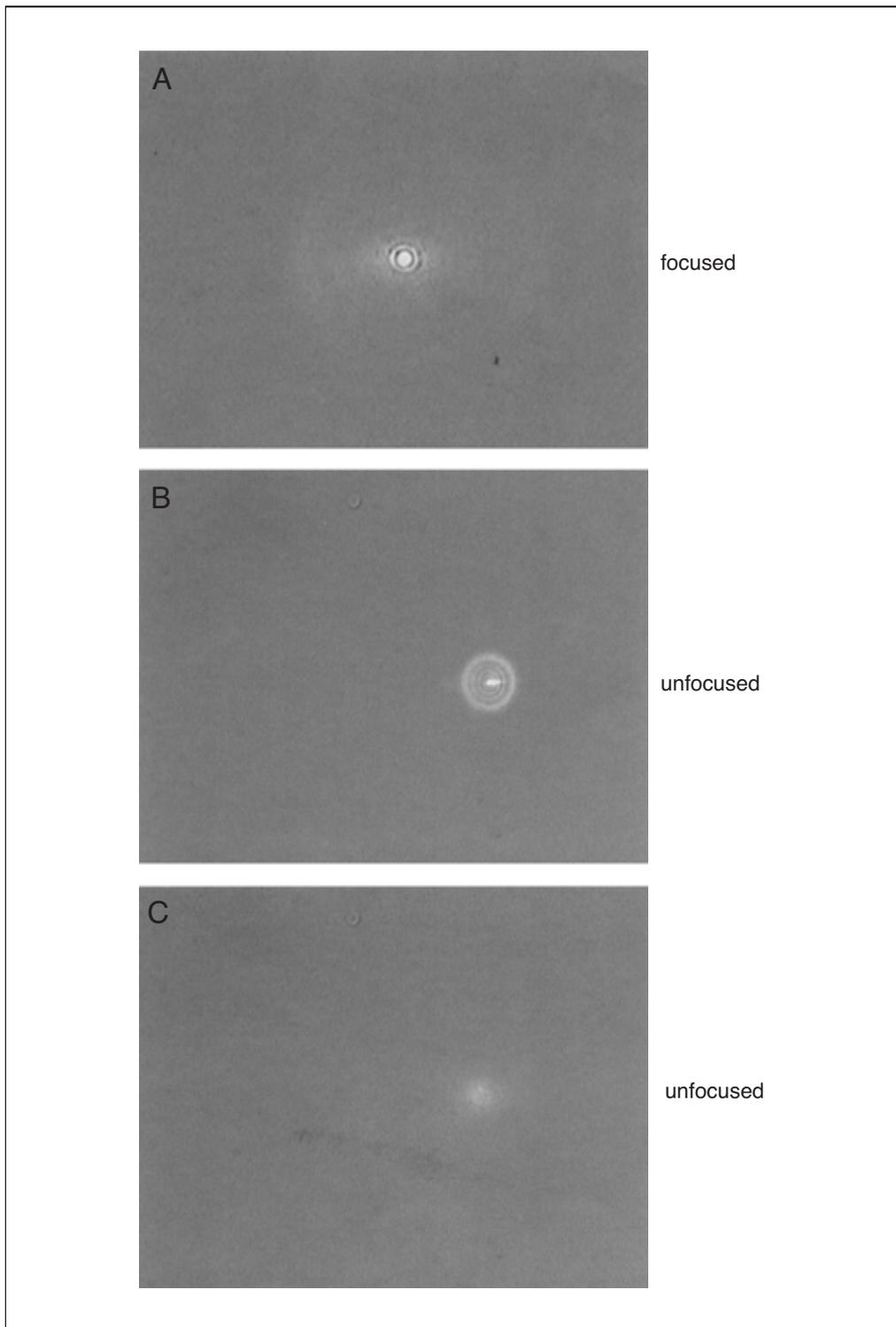


Figure 2.5.2 Focusing of the target beam. If transfer of cells to the cap is not adequate, improper focus may be the cause. Select a spot size of 7.5 μm using the Spot Size Adjust lever found on the left side of the microscope. Rotate the objectives of the microscope until the 10 \times objective is in place. Reduce the intensity of the light through the optics until the field viewed on the monitor is almost dark and the target beam is easily viewed. Compare the target beam with this figure. If the target is not focused, refocus the target beam as described (see Basic Protocol, step 2).

Table 2.5.1 Settings for LCM

Spot size	Power	Duration
7.5 μm	25 mW	3.0 msec
15 μm	30 mW	5.0 msec
30 μm	30 mW	8.0 msec

Perform LCM

- Microdissect cells of interest. Guide microdissection with target beam and press Pendant switch for a single microdissection. To microdissect multiple shots, hold Pendant switch down.

The tissue sample slide must contain no residual xylene, because it melts the transfer film, making microdissection impossible. Dry slide with a compressed gas duster before microdissection to remove residual liquid from staining procedure (see Support Protocol).

To adjust the frequency interval, select Repeat on controller, then choose the desired time between laser pulses.

- Observe wetting as the laser fires, maintaining a distinct clear circle surrounded by a dark ring (Fig. 2.5.3). If proper wetting is not observed, refocus the target beam (step 5).

Collect microdissected cells

- After collecting the desired number of cells, lift cap from slide using the transport arm. Lift and rotate transport arm until cap reaches the Cap Removal Site. Lower transport arm, then rotate it back toward slide, leaving the cap in place at the removal site. Remove cap using the provided cap tool.
- Blot polymer surface of CapSure transfer film with CapSure pads to remove nonspecific debris adhered to it.
- Inspect slide and cap for successful microdissection (Fig. 2.5.3).
- Insert polymer end of cap into top of a 500- μl microcentrifuge tube.

The sample is ready for extraction of desired components or can be frozen at -80°C for later analysis.

**SUPPORT
PROTOCOL****HEMATOXYLIN AND EOSIN STAINING OF TISSUES FOR LCM**

A reaction sequence for performing basic hematoxylin and eosin staining is outlined in Table 2.5.2. Although LCM is not limited to hematoxylin and eosin staining, it is probably the most versatile stain used. Staining can be done using Coplin jars or racks and dishes. DNA can be retrieved from frozen as well as paraffin-embedded tissue that has been fixed in formalin or ethanol. RNA and protein retrieval is best when frozen tissue is used or alternatively when ethanol-fixed paraffin-embedded tissue is used. RNA and protein are cross-linked when fixed in formalin. However, the morphology of formalin- or ethanol-fixed paraffin-embedded tissues is almost always better than that of frozen tissues, and this is often the deciding factor as to what tissue fixation to use.

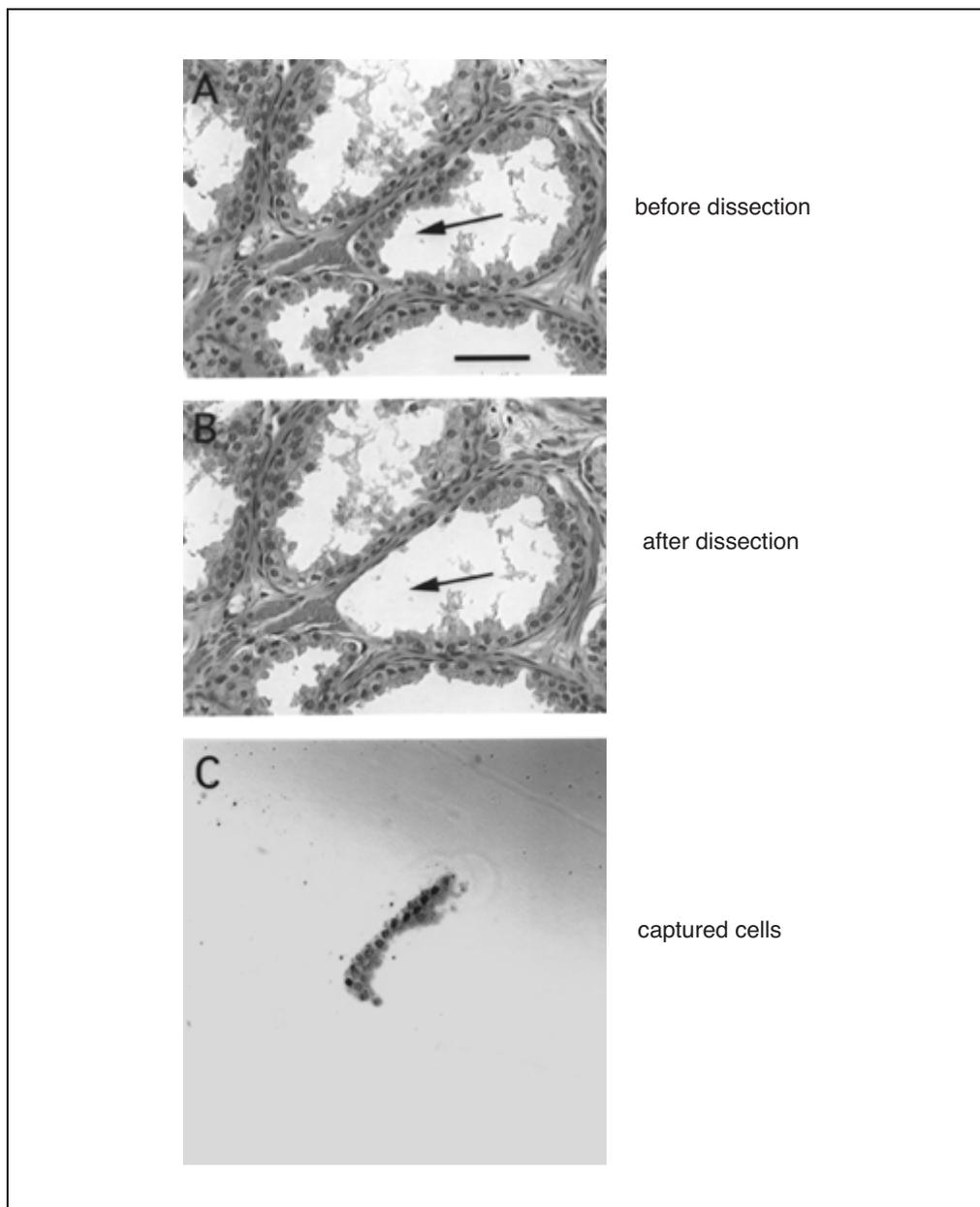


Figure 2.5.3 LCM capture of complex tissue structures. Successful transfer is achieved when nonspecific cells are absent on cap and when microdissection does not leave residual cells behind (see Critical Parameters and Troubleshooting). Example of good microdissection: (A) heterogeneous prostate tissue, (B) removal of normal epithelium, and (C) transfer to EVA film in <5 min.

COMMENTARY

Background Information

One of the problems encountered during the study of disease in actual pathologic lesions arises from the fact that cells in tissue do not exist in a vacuum, but rather interact with each other and neighboring cells in a complex environment. Components of this microenvironment—such as cell-to-cell contact, autocrine and paracrine growth factors, vascular and lymphatic circulation, among many others—influ-

ence growth and differentiation. Even more troublesome is the study of changes that occur during disease progression. Most solid tumors are comprised of a large variety of mixed cell populations. For example, a biopsy of breast tissue harboring a malignant tumor may also contain fat cells in the abundant adipose tissue surrounding the ducts, normal epithelium, myoepithelium in the branching ducts, fibroblasts and endothelial cells in the stroma and

Table 2.5.2 Reaction Sequence for Hematoxylin and Eosin Staining Frozen and Paraffin-Embedded Tissues for LCM

Reagent ^a	Frozen tissue	Paraffin-embedded tissue
Xylene	—	2 × 5 min
100% ethanol	—	30 sec
95% ethanol	—	30 sec
70% ethanol	5 sec	30 sec
Deionized water	10 sec	20 sec
Mayer's hematoxylin	30 sec	30 sec
Deionized water	10 sec	20 sec
Scott's tap water	10 sec	20 sec
70% ethanol	10 sec	20 sec
Eosin-Y	60 sec	60 sec
95% ethanol	2 × 10 sec	2 × 20 sec
100% ethanol	2 × 10 sec	2 × 20 sec
Xylene	30-60 sec	30-60 sec

^aMayer's hematoxylin and Eosin-Y are available from Sigma-Aldrich; Scott's tap water is available from Fisher.

blood vessels, premalignant carcinoma in situ lesions, and invasive carcinoma. Thus, one cannot simply grind up such tissue and hope to analyze molecular events taking place in premalignant lesions.

In the past, several approaches have been used to overcome this problem. The use of immunological techniques to visualize cells of interest or to deplete contaminating cells has been used to purify or at least enrich cell populations. Modern techniques, such as flow cytometry with cell sorting and affinity-labeled magnetic beads, allow separation of subpopulations from heterogeneous pools of single cells in suspension. However, these techniques are generally not practical when applied to tissue, as there is a requirement for the dissolution of intercellular adhesion and the formation of a suspension of individual cells. Alternatively, other approaches aim to imitate *in vivo* conditions such as cell cultures of specific cell populations. However, cultured cells may not accurately represent the molecular events taking place in the actual tissue they were derived from. Assuming methods are successful to isolate and grow the tissue cells of interest, the gene-expression pattern of the cultured cells is influenced by the culture environment and can be quite different from the genes expressed in the native tissue state. Another method of obtaining a pure or enriched cell population involves manual extraction of the cells from the tissue sections. This method has been widely

used, but it requires a high level of manual dexterity and is often quite time consuming.

Laser capture microdissection (LCM) permits, under direct microscopic visualization, a quick and reliable means of procuring a selected cell population from a heterogeneous tissue, while maintaining the basic integrity of the RNA, DNA, and proteins within the cells (Emmert-Buck et al., 1996; Krizman et al., 1996; Simone et al., 1998, 2000; Banks et al., 1999).

Critical Parameters and Troubleshooting

Problems are rarely encountered during laser capture microdissection, but the most likely causes of difficulty are discussed below. The most probable cause for unsuccessful microdissection is improper handling of the tissue prior to microdissection. For best results, the use of plain uncharged and uncoated microscope slides is recommended. Although, some success has been achieved using positively charged slides, these are not recommended. Tissue sections should be flat and free of folds and wrinkles. The smallest of these imperfections in a prepared tissue section can prevent the proper seating of the polymer cap and limit effective cell capture.

Incomplete deparaffinization of embedded tissues also limits the amount of tissue transferred during microdissection. The times for deparaffinization listed in Table 2.5.2 are minimal and can be extended to allow for complete

removal of the paraffin from the sample, if necessary.

Incomplete dehydration of the tissue section prior to LCM will limit the operator's success in microdissection of the tissue. The xylene rinse, which is the final step of both staining protocols outlined in Table 2.5.2, is essential for the complete dehydration of the tissue on the slide. LCM is compatible with a wide range of immunohistochemical and fluorescent stains in addition to the outlined hematoxylin/eosin stain. It is necessary, however, for the final step of any staining protocol used to be a thorough dehydration step in xylene. If it is suspected that the slide is not completely dehydrated, allow it to sit in xylene for additional time.

Another cause for unsuccessful microdissection is improper focusing of the laser. To avoid this problem, the laser should be focused at the beginning of each LCM session, and whenever there is a change in the type of slide being used (see Basic Protocol).

Anticipated Results

Individual tissue cells can vary in their spacing density, shape, and volume. Therefore, the estimated cell yield is more precise when cells are collected using a greater number of laser pulses. Using the 30- μ m laser spot size, the operator can expect to collect, on the average, five to six cells per laser pulse (Fig. 2.5.3).

Time Considerations

The output of LCM is the molecular information derived from analysis of the procured cells. Great care should be taken to ensure the stability of DNA, RNA, or protein molecules analyzed. Delays incurred during staining or while performing LCM can adversely affect molecule stability. DNA is by far the most stable of cellular components. The cellular material collected on the cap for DNA analysis is stable when refrigerated or stored at -20°C until testing is resumed. In contrast, samples microdissected for RNA or proteomic analysis

are far less stable because of the endogenous RNases and proteinases. The amount of time that lapses between the start of the staining protocol and completion of microdissection should be closely monitored. Do not exceed 30 min from the time of thawing or deparaffinizing the tissue slide to refreezing of the final dissected sample at -80°C or immediate extraction.

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Preparation of Human Epidermal Keratinocyte Cultures

UNIT 2.6

**BASIC
PROTOCOL**

This unit describes a protocol for establishing keratinocyte cultures from human newborn foreskins. The foreskins are incubated with trypsin to dissociate the dermis and epidermis. Keratinocytes are obtained from the epidermis as described in the steps below, and fibroblasts can be obtained from the dermis as described in *UNIT 2.1*.

NOTE: All incubations are performed in a humidified 37°C, 5% CO₂ incubator.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique must be used.

Materials

- Human newborn foreskin (for sources, see Background Information)
- Betadine (Purdue Frederick) or 5% (v/v) Wescodyne (STERIS Corporation)
- HEPES-buffered saline (see recipe)
- 0.25% (w/v) trypsin in HEPES-buffered saline
- Keratinocyte primary culture medium (see recipe)
- Hanks' balanced salt solution (HBSS; *APPENDIX 2A*)
- Keratinocyte growth medium (either Keratinocyte-SFM from Invitrogen or KGM from Cambrex)
- Trypsin/EDTA solution (Invitrogen) diluted 1:1 with Hanks' balanced salt solution (final 0.025% w/v trypsin, 0.26 mM EDTA)
- 10 mg/ml soybean trypsin inhibitor (Sigma) in Hanks' balanced salt solution
- Freezing medium: 10% (v/v) DMSO/10% (v/v) heat-inactivated fetal bovine serum/80% (v/v) keratinocyte growth medium
- 100-mm sterile tissue culture dishes
- Small curved forceps (4 pairs), sterile
- Small sharp straight or curved scissors, sterile
- Coarse filter such as a tea strainer or several layers of cheesecloth taped over the top of a 50-ml centrifuge tube, sterile
- 15- and 50-ml sterile disposable centrifuge tubes
- 75-cm² sterile tissue culture flasks
- 1-ml sterile cryotubes
- Liquid nitrogen freezer
- Additional reagents and equipment for cell culture (*UNIT 1.1*)

CAUTION: When working with human tissue, appropriate biosafety practices must be followed.

Prepare foreskin

1. Incubate foreskin in Betadine or 5% Wescodyne for 1 min.
2. Wash foreskin four times, each time by incubating for 30 sec in HEPES-buffered saline at room temperature, to remove the disinfectant.
3. Place foreskin in a 100-mm tissue culture dish and trim away as much subcutaneous tissue as possible by snipping it away with a small pair of sharp scissors. Cut the foreskin into 4- to 6-mm pieces, and float them, epidermis-side-up, in 5 ml of 0.25% trypsin in a 100-mm tissue culture dish overnight at 4°C.

IMPORTANT NOTE: *The scissors used in this step must be sharp.*

**Preparation and
Isolation of Cells**

2.6.1

Contributed by Susan S. Yamada

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When the epidermis is ready to be removed (as described in step 4), there is a change from the shiny and transparent appearance of the fresh epidermis to a more dull and white/opaque surface. Alternatively, the trypsin incubation can be performed at 37°C for several hours. Exposure times must be carefully controlled to curtail trypsin damage to the cells (see *Critical Parameters and Troubleshooting*).

Remove epidermis

4. Using two pairs of forceps, peel the epidermis from the pieces of tissue and place the detached epidermis in a dish containing 5 ml keratinocyte primary culture medium.

If the epidermis does not detach from the tissue, incubate as in step 3 for another 8 to 24 hr in fresh trypsin at 4°C. The dermis can be used to isolate fibroblasts (see UNIT 2.1).

5. Release cells from the epidermis by pipetting up and down ten times with a 5-ml disposable plastic pipet.

The pieces of epidermis may have a tendency to stick to the inside of the pipet. Coating the inside of the pipet with serum proteins by rinsing it with keratinocyte primary culture medium may help prevent this problem.

6. Filter out the epidermal sheets and other debris by passing the cell suspension through a coarse filter such as a tea strainer or several layers of cheesecloth and collecting the filtrate in a 50-ml disposable centrifuge tube.
7. Centrifuge the filtered suspension 5 min at 200 × g, room temperature. Aspirate the supernatant and resuspend the cells in 10 ml keratinocyte primary culture medium. Plate the cells in a 75-cm² flask (see UNIT 1.1 for basic cell culture techniques).
8. Place the culture in a humidified 37°C, 5% CO₂ incubator. The next day, inspect for cells attached to the flask.

The cells may not spread overnight, but there should be some cells attached to the flask. If there are few or no cells attached after an overnight incubation, leave the culture in keratinocyte primary culture medium for up to 1 additional day before changing the medium. Many or most of the cells will never attach.

9. Wash the flask gently three times with Hanks' balanced salt solution and add 15 ml keratinocyte growth medium.
10. Incubate the flask, changing the medium every other day, until the keratinocytes have reached no more than 70% to 80% confluency.

IMPORTANT NOTE: *Do not permit the cells to become confluent. Confluent cells are very difficult to remove and can begin to differentiate and stop dividing (see *Critical Parameters and Troubleshooting*).*

Subculture cells

11. To subculture the cells, wash the flask twice with Hanks' balanced salt solution. Add 5 ml trypsin/EDTA warmed to 37°C and incubate at room temperature for about 5 min or until most of the cells are rounded up. Rap the side of the flask against the palm of the hand to release the cells from the flask. If most of the cells do not come off, wait 1 to 2 min and rap again.

*Not all of the cells will detach easily, and it is better to leave a few behind than to overtrypsinize the rest. In general, keratinocytes are more difficult to remove than fibroblasts, and they are also more sensitive to being overtrypsinized (see *Critical Parameters and Troubleshooting*). It is therefore important that the trypsin/EDTA solution be very fresh. One may wish to freeze aliquots and defrost only as much as needed.*

12. After the cells are released from the flask surface, add an equal volume of soybean trypsin inhibitor solution to the trypsinized cell suspension and transfer to a 15-ml disposable centrifuge tube. Centrifuge 5 min at $200 \times g$, at room temperature. Aspirate the supernatant and resuspend the pellet of cells in keratinocyte growth medium. Count cells (*UNIT 1.1*).

13. Plate at $\sim 6 \times 10^3$ cells per cm^2 in 75- cm^2 tissue culture flasks.

This represents about a 1:5 split ratio, and the cells will usually be ready to trypsinize again in about 4 or 5 days. At this split ratio, keratinocyte cultures will usually stop growing at around passage 10 or 11. However, other characteristics of the cultures may begin to change well before this time, and investigators should determine the maximum useful lifespan of these cells for their individual purposes.

14. Change the medium every other day.

Freeze cells and defrost at time of use

15. Resuspend the pellet of trypsinized cells in freezing medium. Dispense into 1-ml sterile cryotubes at 5×10^5 cells/ml, and place in a styrofoam box in a -80°C freezer overnight. Transfer the cryotubes to liquid nitrogen the next day.

16. To defrost cells, thaw a tube of cells in a 37°C water bath. As soon as the cell suspension is thawed, transfer to a 75- cm^2 tissue culture flask containing 15 ml keratinocyte growth medium and incubate at 37°C . As soon as most of the cells have attached, which should take several hours, wash gently several times with Hanks' balanced salt solution and add fresh keratinocyte growth medium.

The addition of serum to the freezing medium ensures better survival of the frozen cells, but results in high calcium levels once they have been defrosted. Therefore, it is important to wash the cells and change into fresh keratinocyte growth medium as soon as they have attached.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

HEPES-buffered saline

480 ml water
0.5 ml 1 M HEPES buffer solution (1 mM final)
0.9 g glucose (10 mM final)
1.5 ml 1 M KCl (3 mM final)
1 ml 0.5 M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (1 mM final)
3.8 g NaCl (130 mM final)
0.1 ml 0.5% (w/v) phenol red solution (final 1 $\mu\text{g}/\text{ml}$)
Adjust the volume to 500 ml
Filter sterilize through a 0.22- μm filter
Store up to 1 year at 4°C

Keratinocyte primary culture medium

500 ml DMEM (*APPENDIX 2B*)
12 ml 1 M HEPES (20 mM final)
100 ml fetal bovine serum (FBS; heat-inactivated 30 min at 55°C)
0.6 ml 100 $\mu\text{g}/\text{ml}$ EGF (Sigma-Aldrich; 10 ng/ml final)
0.6 ml 10^{-7} M cholera toxin (*Vibrio cholerae*, lyophilized powder; Sigma-Aldrich; 10^{-9} M final)

continued

**Preparation and
Isolation of Cells**

2.6.3

2.4 ml 100 µg/ml hydrocortisone (Sigma-Aldrich; 0.4 µg/ml final)
6 ml 100× penicillin/streptomycin (100 U/ml penicillin G and 100 µg/ml streptomycin sulfate final)
0.6 ml 250 µg/ml amphotericin B (0.25 µg/ml final)
0.6 ml 10 mg/ml gentamicin (10 µg/ml final)
Store up to 1 month at 4°C in the dark

As an alternative to the 100 ml of FBS, it is possible to use 50 ml calf serum and 50 ml FBS, both heat-inactivated.

CAUTION: *Cholera toxin is very toxic. Use appropriate precautions when handling.*

COMMENTARY

Background Information

Early attempts to grow keratinocytes used medium supplemented with serum and frequently required fibroblast feeder layers (Rheinwald and Green, 1975). The development of serum-free MCDB 153 (Boyce and Ham, 1983) permitted keratinocyte culture without feeder layers. Both of the commercially available serum-free media recommended in this unit (Keratinocyte-SFM from Invitrogen and KGM from Cambrex) are modifications of MCDB 153. They feature low Ca^{2+} concentrations to inhibit keratinocyte differentiation and suppress fibroblast growth and are supplemented with bovine pituitary extract as the primary mitogen. If the undefined composition of bovine pituitary extract is a problem in the experimental system, a medium that replaces it with several defined mitogens is available (Defined Keratinocyte-SFM from Invitrogen).

This protocol can also be adapted to use biopsies or samples of skin from surgical operations or cadavers. A similar protocol has been used to obtain murine keratinocytes (Dlugosz et al., 1995). However, murine keratinocytes are difficult to passage successfully, which can limit their usefulness.

Neonatal human foreskins from circumcisions may be available locally, or they can be obtained through the Cooperative Human Tissue Network (<http://www-chn.ims.nci.nih.gov/>). Foreskins can be stored immersed in HEPES-buffered saline or serum-free medium such as DMEM (APPENDIX 2B) with 100 U/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B, at 4°C for up to 3 days before use.

Critical Parameters and Troubleshooting

This protocol specifies two media: keratinocyte primary culture medium and keratinocyte growth medium. Keratinocyte primary culture

medium contains DMEM with 20% serum and is used initially to encourage the keratinocytes from the epidermis to attach. As soon as they attach, the medium is changed to keratinocyte growth medium, a commercially available low-calcium, serum-free medium, which is used in all subsequent cultures.

The concentration of calcium in the medium can regulate whether keratinocytes differentiate or continue to proliferate. The calcium levels in the recommended growth media are low: 0.09 mM in Keratinocyte-SFM from Invitrogen, and 0.15 mM in KGM from Cambrex. Raising the calcium concentration, either intentionally, e.g., by adding CaCl_2 to 1 mM, or unintentionally, e.g., by adding factors dissolved in solutions containing calcium, will cause keratinocytes to terminally differentiate. Their growth rate will greatly decrease and their morphology will change markedly (Fig. 2.6.1) from flattened cells with ruffled edges that associate only loosely with neighboring cells to more tightly packed and well-circumscribed colonies of less-spread cells. Differentiated cells are often more phase-dense by phase-contrast microscopy, with phase-bright borders where they contact neighboring cells because the cells are more closely packed and thicker. Ruffled cell margins, if present, are seen only in cells with free edges at the perimeter of colonies. Whereas cells in low calcium can be quite motile, particularly those with semicircular ruffles, cells in high calcium move much less and tend to associate strongly with other cells. Permitting the cells to become confluent will also signal them to slow their growth and differentiate, and cultures that have been allowed to grow to confluence should be replaced with new cells from a frozen stock.

Keratinocytes are relatively sensitive to overtrypsinization, so care should be exercised when passaging the cultures. Use fresh trypsin and expose the cultures for a minimal period.

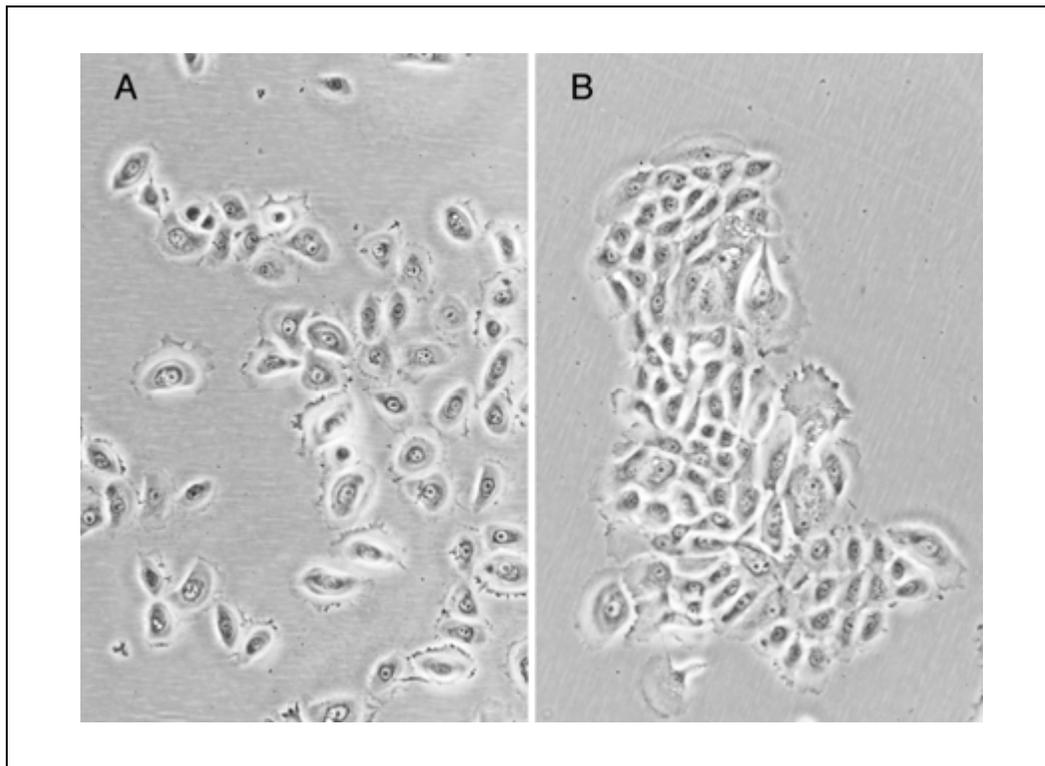


Figure 2.6.1 Human keratinocytes in culture (passage ~6) in: (A) low (0.09 mM) or (B) high (1 mM) calcium. Magnification: 130 \times .

Do not trypsinize until all the cells have been removed from the flask; there should always be some cells left attached. Immediately add soy-bean trypsin inhibitor solution to neutralize the trypsin.

Anticipated Results

After 1 to 2 days in keratinocyte primary culture medium, one should be able to see cells attached to the flask, although they may not be spread. After changing the medium to one of the low-calcium keratinocyte growth media, spread keratinocytes should be visible within 1 to 2 days. Cultures should be ready for passage within 1 or 2 weeks, depending on the initial yield. Early-passage cells should be frozen promptly. From a 75-cm² flask at about 80% confluence, it should be possible to harvest $\sim 2 \times 10^6$ cells.

Time Considerations

Trimming of the foreskin and placing it into trypsin may take 1 to 2 hr, with another hour or less the next day to peel off the epidermis and place the cells into culture. If the epidermis cannot be separated from the dermis after overnight trypsinization, another 8- to 24-hr incubation may be necessary.

Literature Cited

- Boyce, S.T. and Ham, R.G. 1983. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J. Invest. Dermatol.* 81:33s-40s.
- Dlugosz, A.A., Glick, A.B., Tennenbaum, T., Weinberg, W.C., and Yuspa, S.H. 1995. Isolation and utilization of epidermal keratinocytes for oncogene research. *Methods Enzymol.* 254:3-20.
- Rheinwald, J.G. and Green, H. 1975. Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. *Cell* 6:331-344.

Internet Resources

<http://www-chtn.ims.nci.nih.gov/>

Web site of the Cooperative Human Tissue Network, which is a program supported by the National Cancer Institute that can provide human tissue at nominal cost.

Contributed by Susan S. Yamada
National Institute of Dental and Craniofacial
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Bethesda, Maryland

Figure 2.6.1 Human keratinocytes in culture (passage ~6) in: (A) low (0.09 mM) or (B) high (1 mM) calcium. Magnification: 130 \times .

CHAPTER 3

Subcellular Fractionation and Isolation of Organelles

INTRODUCTION

In the early 1700s, Leeuwenhoek noted that avian and amphibian blood cells contained a “clear area,” almost certainly corresponding to the structure we now know as the nucleus. This represents probably the earliest recognition that eukaryotic cells are not simply sacs of protoplasm, but instead contain subcellular structures. Although the concept of the nucleated cell became firmly established in the 18th and 19th centuries, the real beginning of subcellular fractionation had to await the emergence in the mid-1940s of the art form known as electron microscopy. The electron microscope revealed much more complexity than Leeuwenhoek could have imagined. Concurrent with improvements in the techniques of electron microscopy was the development of methodologies for subcellular fractionation. Through the 1950s, these parallel approaches resulted in the discovery and isolation of the major organelles that comprise the eukaryotic cell. Finally, as biochemical functions were associated with specific subcellular compartments, a much clearer picture of the eukaryotic cell began to emerge and with it the field of modern cell biology.

Chapter 3 is dedicated to methods for subcellular fractionation. The chapter begins with an overview of cell fractionation (*UNIT 3.1*) which covers some basic principles as well as the instrumentation of centrifugation. The various centrifugation media, ranging from sucrose to the newer media (Ficoll, Percoll, metrizamide, Nycodenz, and Iodixanol), are described.

Much of the earliest work in subcellular fractionation utilized rat liver as starting material. The second unit of Chapter 3 provides protocols for the isolation of plasma membrane sheets and domains from rat liver (*UNIT 3.2*). This method is used to isolate plasma membrane sheets in sufficient yield and purity to be suitable for a variety of analytical and preparative purposes. For example, these preparations can be used as starting material for the isolation of hepatocyte plasma membrane proteins. The sheets also serve as the starting material for the protocols within *UNIT 3.2* that yield the apical and basolateral domains comprising the polarized hepatocyte plasma membrane.

The densities of mitochondria, peroxisomes, and lysosomes are very similar and as a result these organelles cosediment in density gradients as the so-called “light mitochondrial fraction.” The development of separation media has significantly improved the ability to separate these organelles from each other. *UNITS 3.3-3.6* deal with separation of these organelles.

UNITS 3.3 & 3.4 describe methods for isolating mitochondria from rat liver as well as other sources including other tissues, cultured mammalian cells, and yeast. *UNIT 3.3* focuses on methods involving differential centrifugation for rapid isolation of metabolically active mitochondria, whereas methods involving density-gradient centrifugation for further purification of mitochondria are described in *UNIT 3.4*.

UNIT 3.5 provides methods for the isolation of peroxisomes from mammalian tissues and cells in tissue culture. Also included in this unit are a method for preparation of peroxisomes from yeast and descriptions of assays for assessment of the success of subcellular fractionation of the various starting materials.

The yeast *Saccharomyces cerevisiae* contains all the membrane-bound subcellular organelles that are characteristic of higher eukaryotes, yet this organism has a haploid genome

that is less than 4 times the size of that of *E. coli*. The availability of the entire *S. cerevisiae* genome sequence together with the ease with which yeast are cultured (UNIT 1.6) and genetic manipulations performed have made this organism a popular model in eukaryotic cell biology. Indeed, it is yeast cell biology that may provide insights into the function of many yeast genes and their mammalian counterparts. While some attention to certain subcellular organelles from yeast is given in earlier units of this chapter, UNIT 3.7 provides a comprehensive overview of yeast subcellular fractionation.

UNIT 3.8 describes centrifugation-based methods for isolating most of the major organelles of the yeast *Saccharomyces cerevisiae*. Most of these procedures begin with the preparation of spheroplasts as described in the Support Protocol near the beginning of the unit. Lysates for subcellular fractionation can also be generated by glass-bead lysis to disrupt intact yeast cells (also described). Because of the fragility of most yeast organelles, lysates of spheroplasts are best used for most protocols; however, glass-bead lysates can be used for preparation of cytosol and plasma membranes. Several of the protocols are analytical for assessment of the distribution of proteins along exocytic, endocytic, and biosynthetic pathways in yeast cells. The unit also includes preparative methods for isolating yeast nuclei, vacuoles, mitochondria, peroxisomes, endoplasmic reticulum, plasma membranes, and cytosol.

The organelle named for the pioneering cell biologist, Camillo Golgi, was identified on the basis of a then-correct protocol developed by Golgi for fixing and staining tissues. Although observations of what he then referred to as the “internal reticular apparatus” probably date even earlier in the 19th century, the Golgi remains an intracellular site of intense investigation by today’s cell biologists. UNIT 3.9 describes the isolation of Golgi membranes on a preparative basis. Individual protocols describe isolation of dextrose-treated Golgi stacks from rat liver using a sucrose density barrier and by flotation from a light mitochondrial fraction. Isolation from cultured cells is also described as is a method to assay for the Golgi marker enzyme, UDP-galactose galactosyl transferase.

UNIT 3.10 focuses on the “clear area” of Leeuwenhoek—the nucleus. As noted above, rat liver has been the most frequently used source of material for subcellular fractionation. This unit contains two protocols for isolation of nuclei from rat liver. The first utilizes a sucrose density barrier, whereas the second employs Nycodenz as a density medium. Although the vast majority of methods for preparing nuclei uses soft tissue (e.g., liver), the protocols described in this unit can be applied to other source materials (e.g., cultured cells, plants) provided appropriate homogenization can be achieved. UNIT 3.10 also contains two protocols for purification of nuclear membranes from isolated nuclei and Support Protocols describing methods to assay DNA and RNA.

Free-flow electrophoresis (UNIT 3.11) provides the opportunities for both analytical and preparative applications for isolating subcellular organelles such as rat endosomes. Endosome-enriched fractions are prepared from rat liver using discontinuous sucrose gradients, and these are used to prepare endosomal subpopulations by free-flow electrophoresis. The analytical approach can be used to study the kinetics and cellular subpopulations involved in endocytic traffic. Three distinct subpopulations of endosomes are revealed in such studies. Support Protocols include methods for analyzing markers for endosomal subpopulations and for demonstrating retention of endosomal function (acidification) *in vitro*. Methods are also included for preparing and labeling ligands that traffic through endosomes.

Future supplements will provide protocols for isolation of additional organelles from cells of higher eukaryotes, including isolation of specialized organelles such as synaptic vesicles and chromaffin granules.

Joe B. Harford

Cell fractionation has enjoyed widespread use among cell biologists for half a century. It continues to be a fruitful, if not essential, approach in the reductionistic efforts to define the composition and functions of the multiple compartments in eukaryotic cells. It also provides the essential ingredients for the increasing number of cell-free assays now being used in test-tube reconstructions of complex cellular events involving intercompartmental interactions. Much of the knowledge regarding the composition and function of cell organelles has resulted from fractionation of mammalian tissues, where cells are both abundant and highly differentiated (and thus organelle-rich). However, with new techniques in molecular biology and widened interest in combining studies of intact cells and functional reconstitution, interest in fractionation has spread to cultured cell lines and genetically tractable lower eukaryotic cells.

The goals of cell fractionation often differ depending on the nature of the experiments being conducted. In preparative procedures, in which the intent is to isolate quantities of a particular cell organelle for further study or for subfractionation, the emphasis is on purity and (secondarily) on yield. In analytical experiments, in which the intent is not isolation of organelles but evaluation of associations of selected macromolecules with particular organelles, the emphasis is on using one-step procedures that result in different distributions of various organelles (as defined by marker activities) rather than on separating organelles outright. Finally, in preparing organelles for cell-free reconstitution, the goal is to maintain them in a functional state. The investigator generally has developed a specific assay for intercompartmental interaction that does not rely on organelle purity, so the extent of contamination by irrelevant organelles is less important.

The separation of distinct organelles during cell fractionation results from their differing physical properties—size (and shape), buoyant density, and surface charge density—which reflect their differing compositions. Particular fractionation techniques capitalize on one or more of these properties. For example, gel filtration separates on the basis of size, centrifugation separates on the basis of size and density,

and electrophoresis separates on the basis of surface charge density. As knowledge of the specific composition of particular organelles has developed, it has become possible to apply newer techniques such as affinity chromatography and selective density-shift perturbation. Whichever fractionation method is used, the procedures may have to be modified to adapt them to individual needs. Even isolation of a particular kind of organelle from different tissue or cell sources may necessitate adjustment of fractionation conditions. This also means that in addition to isolating the organelle of interest, one should also plan on confirming the identity of what has been isolated.

Centrifugation is the most widely used procedure in cell fractionation. It is the only approach commonly used to separate crude tissue homogenates (often having quite large volumes) into subfractions as starting material for more refined purification procedures. Further, the technology available, using rotors with a variety of geometries and diverse media that enable separation according to size, density, or both, now routinely permits refined separations on volumes ranging from submilliliter to several liters. No other technology is this versatile. Gel filtration is limited by the pore sizes of available resins, such that only regularly shaped vesicles with diameters <100 to 200 nm can be purified away from larger or irregularly shaped organelles. Use of electrophoresis for organelle purification, especially at the preparative level, is relatively recent, and, as with gel filtration, its success relies on generating starting material by centrifugation. Although it shows promise for purifying selected organelles (e.g., endosomes) that have been difficult to obtain otherwise, surface charge densities on most organelles may not be different enough (or able to be manipulated sufficiently) to make this a versatile procedure. For these reasons, the remaining comments in this overview focus primarily on fractionation of organelles by centrifugation. For more detailed coverage of each of the topics, the reader is referred to the excellent book edited by Rickwood (1984).

BASIC PRINCIPLES OF CENTRIFUGATION

One of the best ways to understand the rationale for various cell fractionation proce-

dures is to examine the Svedberg equation, which describes mathematically the sedimentation of a spherical particle in a viscous fluid:

$$\frac{dx/dt}{\omega^2 x} = \frac{2r^2 (\rho_p - \rho_m)}{9\eta}$$

where x is the distance from axis of rotation, t is time, ω is the angular velocity, r is the radius of the particle, ρ_p and ρ_m are the densities of the particle and medium, and η is the viscosity of the medium.

This equation states that the sedimentation velocity, dx/dt , per unit centrifugal field $\omega^2 x$ (which is set by the instrumentation) increases with the square of the particle radius and the difference in density between the particle and the medium, and decreases with increasing viscosity. In practical terms, when sedimentation is performed by differential velocity in a medium such as 0.25 M sucrose, which is less dense than all particles and has a low viscosity, the radius of the particle is the dominant factor that determines the sedimentation rate. In contrast, when isopycnic density gradient centrifugation is used, the density of the medium changes from top to bottom in the centrifugation tube, and the range of densities typically includes the buoyant densities of most particles (vesicles). Thus individual types of vesicles will sediment until $\rho_p = \rho_m$, and the vesicles will then stop at their isopycnic densities. Alternatively, in flotation procedures $\rho_p < \rho_m$ initially and dx/dt is negative, meaning that particles will move toward the axis of rotation (i.e., toward the top of the tube). Clearly, the equation also indicates that sedimentation or flotation, as the case may be, is slowed in media of higher viscosity. As discussed below, various media used in centrifugation are distinguished by their viscosities (and osmolarities) at high densities.

The Svedberg equation can also be used to discuss three other basic relationships encountered in using centrifugation. First, the velocity of a particle per unit centrifugal field (left side of the equation) is the sedimentation coefficient. Its dimensions are in seconds, and it is generally reported in units known as Svedbergs (S). $1\text{ S} = 10^{-13}\text{ sec}$, the same order of magnitude as the sedimentation coefficient of several biological macromolecules (e.g., 5S RNA). Second, the centrifugal field, $\omega^2 x$ is usually normalized to gravity and is expressed in the literature as a relative centrifugal force (RCF = $\omega^2 x/g$). Centrifugation conditions are best reported as $\times g$. RCF is usually expressed at the

midpoint of the tube (g_{av}). The centrifugal field term includes the angular velocity in units of radians per second, which is easily converted to revolutions per minute (rpm), the unit most often given in the literature, as follows: $\text{RCF} = 11.18 r(N/1000)^2$, where N is rpm. Third, integration of the Svedberg equation over time yields the following relationship:

$$t = \frac{1}{s\omega^2} \ln\left(\frac{X_{\max}}{X_{\min}}\right) = \frac{k}{s}$$

$$\text{where } k = \frac{1}{\omega^2} \ln\left(\frac{X_{\max}}{X_{\min}}\right)$$

This is a measure of the clearance time, the time required for a particle of given sedimentation coefficient to travel from the top to the bottom of the tube. Included in k are the geometry of the rotor (X_{\max} and X_{\min}) and the angular velocity. k -factors are reported in the printed information that accompanies most rotors. They are useful for estimating the length of centrifugation required to pellet a particular particle and for translating centrifugation conditions between two rotors. For a given particle or vesicle, k/t (rotor 1) = k/t (rotor 2).

INSTRUMENTATION

Several pieces of equipment must be available to carry out cell fractionation by centrifugation. These include homogenizers, centrifuges (generally one low-speed and one ultracentrifuge) with compatible rotors, a refractometer for measuring the refractive index of centrifugation media (before centrifugation) and gradient fractions (after centrifugation), a gradient-forming device for generating preformed density gradients, and a gradient-collecting device for unloading the gradients after the run. The refractometer, gradient-forming device, and gradient-collection device are very useful, if not essential, for density gradient and rate zonal sedimentation.

Centrifuges. Most organelle purification procedures require that samples be maintained at $\leq 4^\circ\text{C}$, so the centrifuges used need to be refrigerated. The low-speed centrifuge is used in the early steps of the typical purification procedure for sedimenting large particulates such as nuclei and unbroken cells, frequently from large volumes. The ultracentrifuge is used for subsequent spins at higher speeds and often with smaller volumes. These instruments have a vacuum system as the rotors are spun in an evacuated chamber to reduce friction (and thus heating).

Rotors. Commonly used rotors come in three configurations: fixed-angle, vertical (or near-vertical), and swinging-bucket (Fig. 3.1.1). Continuous-flow and zonal rotors, which are loaded and unloaded during the run, are less widely used. Fixed-angle rotors emphasize speed and capacity. Many of these rotors

generate RCFs in excess of $600,000 \times g$, meaning low k -factors, and can handle a few hundred milliliters of solution at one time. As a result, they are used frequently for pelleting fractions, particularly from large volumes. Most fixed-angle rotors hold tubes in a fixed incline substantially away from the vertical. When the

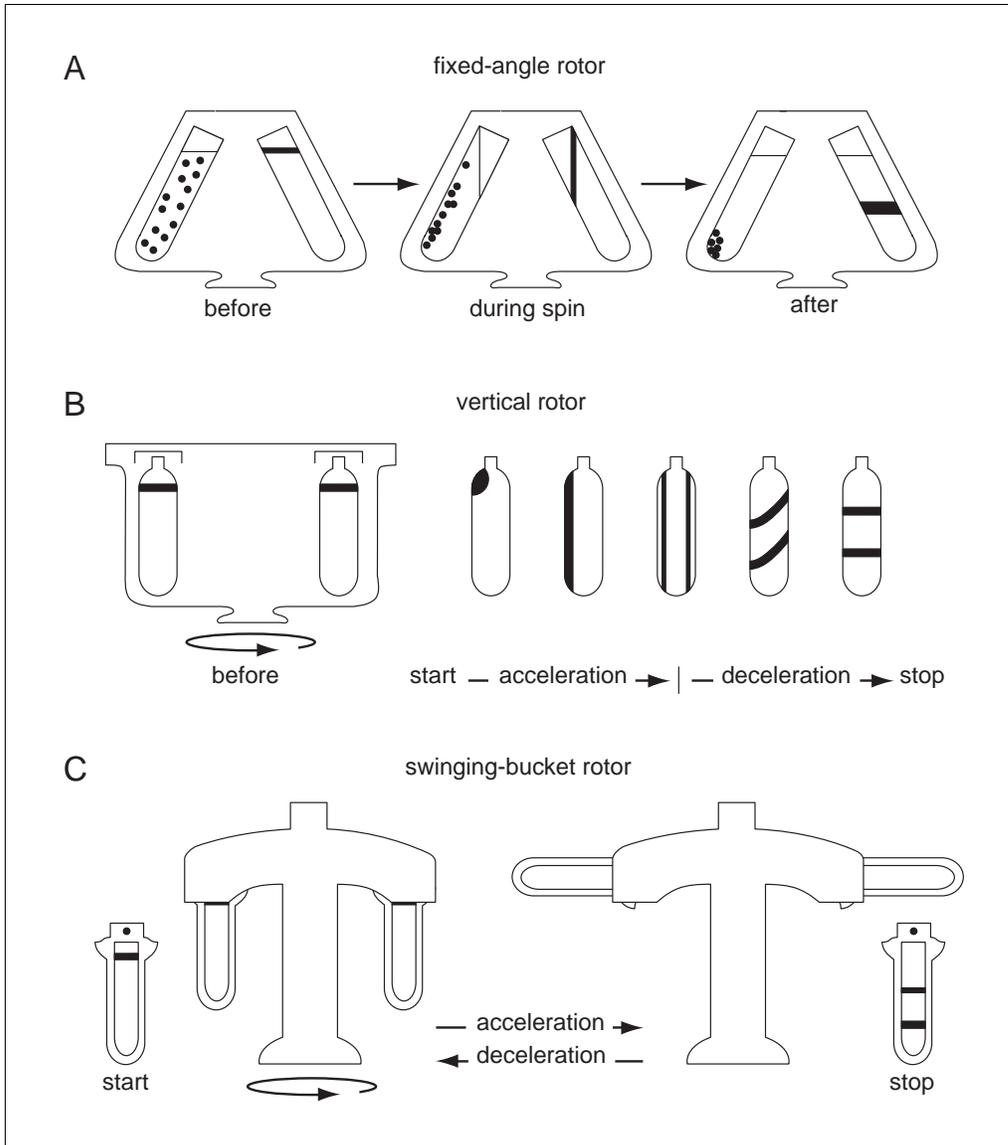


Figure 3.1.1 Rotors used in ultracentrifuges. **(A)** Fixed-angle rotor. In each profile, the tube on the left shows pelleting from a uniform suspension and the tube on the right shows sedimentation of a band layered at the top above a higher-density fluid. Sedimentation is radial (particles move toward the outer wall of the tube and then down this wall to the pellet). Note how the band in the right tube reorients during the spin and then again at the end of the run. **(B)** Vertical rotor. The rotor profile shows tubes with sample layered above a higher-density solution before a run. Note that during the run (successive tube profiles shown at right), the layer reorients along the inner wall of the tube and then the bands resolve vertically. During deceleration the bands reorient to a horizontal position. **(C)** Swinging-bucket rotor. Note the tube on the left in the upper profile has a sample layered at the top. The buckets are mounted vertically on the rotor. During the run, the buckets reorient to the horizontal position, and the bands separate along the length of the tube. Because sedimentation is radial, the particulates in the bands are more concentrated at the walls facing and away from the viewer than in the center of the tube. Toward the end of deceleration, the buckets reorient to the vertical position.

Table 3.1.1 Density and Refractive Index of Aqueous Sucrose Solutions at 20°C^a

Percent (w/w)	Molarity	Density	Refractive index	Percent (w/w)	Molarity	Density	Refractive index
2	0.06	1.006	1.3359	38	1.30	1.166	1.3958
4	0.12	1.014	1.3388	40	1.38	1.176	1.3997
6	0.18	1.022	1.3418	42	1.46	1.187	1.4036
8	0.24	1.030	1.3448	44	1.54	1.197	1.4076
10	0.30	1.038	1.3479	46	1.62	1.208	1.4117
12	0.37	1.046	1.3510	48	1.71	1.219	1.4158
14	0.43	1.055	1.3541	50	1.80	1.230	1.4200
16	0.50	1.064	1.3573	52	1.88	1.241	1.4242
18	0.56	1.072	1.3606	54	1.98	1.252	1.4285
20	0.63	1.081	1.3639	56	2.07	1.263	1.4329
22	0.70	1.090	1.3672	58	2.16	1.275	1.4373
24	0.77	1.099	1.3706	60	2.26	1.286	1.4418
26	0.84	1.108	1.3740	62	2.35	1.298	1.4464
28	0.91	1.118	1.3775	64	2.45	1.310	1.4509
30	0.99	1.127	1.3811	66	2.55	1.322	1.4558
32	1.06	1.137	1.3847	68	2.65	1.335	1.4605
34	1.14	1.146	1.3883	70	2.76	1.347	1.4651
36	1.22	1.156	1.3920				

^aModified from Hofmann (1977) by permission of ISCO, Inc.

centrifugal force is applied, it is radial rather than along the length of the tube. Thus the path length is across the tilted tube and is reasonably short; particles travel outward until they hit the outer wall and then “slide” down the wall to form a pellet (see Fig. 3.1.1). In vertical rotors, the path is only the width of the tube. As is evident from the figure, in both fixed-angle and vertical rotors the solution in the tubes reorients from the horizontal to the vertical during acceleration and reverses during deceleration. These rotors can be advantageous for density gradients because equilibrium is reached quite rapidly with the short path, and the separation of bands of different density increases as the contents of the tube reorient during deceleration. With swinging-bucket rotors, the tube and its contents reorient from vertical to horizontal during acceleration and reverse during the end of deceleration. The centrifugal force in this case is along the length of the tube. Note, however, that because the force is directed radially, sedimenting particles tend to concentrate peripherally near the tube walls. Also, the long path length and the generally lower centrifugal forces required for these rotors mean that *k*-factors are usually much higher than with fixed-angle rotors. Thus these rotors have been

used more for density gradient and rate zonal centrifugations and less for pelleting fractions.

Refractometers. A refractometer measures refractive indices of solutions used for organelle separations. Refractive index is linearly related to density, and tables relating these two parameters over a large range of concentrations have been prepared for sucrose (Table 3.1.1) and can readily be constructed for other gradient media by weighing aliquots of accurately prepared solutions of different concentrations. The measurement of refractive index/density is particularly useful for characterizing the position of organelles in gradients after centrifugation and for ensuring reproducibility of gradients from one experiment to another.

Gradient-forming devices. These devices consist of two chambers with an interconnecting channel along the bottom and an outlet from one chamber that connects to a peristaltic pump on its way to the centrifuge tube (see Fig. 3.1.2). The channel is gated by a stopcock. For linear gradients (the type most frequently used), the chambers have the same cross-sectional area. Solutions representing the extremes in density of the desired gradient are each placed in one chamber, and a stirring device is placed in the chamber with the outlet going to the tube. As

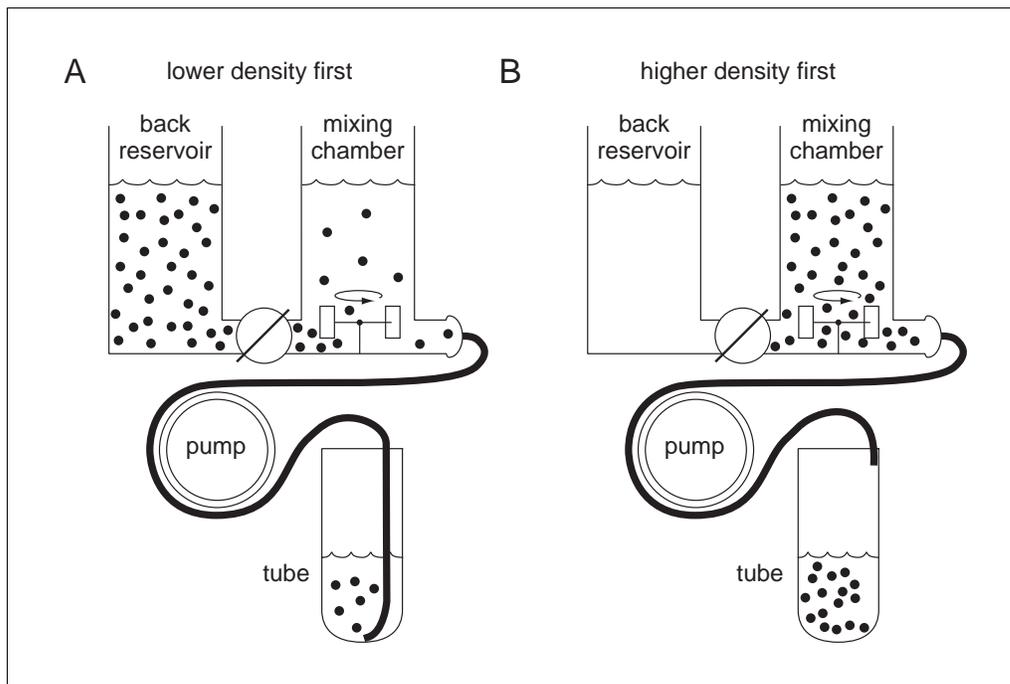


Figure 3.1.2 Linear gradient-forming device showing the back reservoir connected to the mixing chamber via a channel with a stopcock. A delivery tube leads from the mixing chamber to a peristaltic pump and then to the centrifuge tube. **(A)** Configuration used when the lower-density solution is delivered first; the lower-density solution is in the mixing chamber and the outlet is at the bottom of the centrifuge tube. **(B)** Configuration used when the higher-density solution is delivered first; the higher-density solution is in the mixing chamber and the outlet tube is always above the surface of the liquid entering the tube.

fluid is pumped out of the mixing chamber, fluid also flows through the connecting channel from the reservoir to maintain the same pressure head in the two chambers. Consequently, the density of the solution in the mixing chamber changes progressively. Gradients can be generated either highest density first, if the higher-density solution is placed in the mixing chamber, or lowest density first, if the lower-density solution is placed in the mixing chamber. Note that for highest density first, the delivery point must be located above the level of the solution filling the centrifuge tube, whereas for lowest density first, the delivery point must be at the bottom of the tube.

Gradient-collection devices. Gradient collection at the end of centrifugation requires a systematic and reproducible means of emptying the tube. Gradients can be collected from the top or from the bottom. With care, a pipettor (e.g., Pipetman, Rainin) of suitable volume can be used for manual collection from the top, taking care to withdraw equal-volume fractions from just below the meniscus. Alternatively, Buchler markets a pumping device (Autodensiflow) that continually adjusts to the height of the meniscus and withdraws gradients from the

top, and both MSE Scientific and Nyegaard market devices that pump high-density solution beneath the gradient and progressively displace the gradient, which is again collected at the top. For collection from the bottom, the centrifuge tube is punctured with a needle and the gradient is collected dropwise.

FRACTIONATION MEDIA

Early in the development of centrifugation as a tool for purification of cellular organelles, it was established that nonelectrolyte-based media are preferable to electrolyte-based media for reducing organelle aggregation (Hogeboom et al., 1948). Most media used today are nonelectrolyte-based, although some do have a substantial electrolyte content (e.g., STKM medium, which contains 50 mM Tris-Cl, 25 mM KCl, and 5 mM MgCl₂ in addition to 0.25 M sucrose; Adelman et al., 1973). Moreover, media are generally isoosmotic or hyperosmotic as compared to 0.15 M NaCl. Organelles are osmometers, with water moving across their limiting membranes to maintain osmotic equilibrium; thus lysis can occur in hypoosmotic media.

The nonelectrolyte solute most commonly used in subcellular fractionation is sucrose. High-quality sucrose (free of RNase) is reasonably inexpensive, very soluble, and can be used readily to prepare solutions that span the range of densities of most biological organelles (Table 3.1.1). In addition, procedures have been developed for purifying most biological organelles in sucrose. Sucrose, however, has limitations; at higher concentrations solutions are quite viscous and hyperosmotic. Isoosmotic sucrose (0.25 M; ~9% w/v) has a density of only 1.03 g/ml, which is at the lower-density end for biological organelles. Thus all isopycnic density gradient centrifugation performed in sucrose constitutes hyperosmotic conditions; intraorganellar water redistributes to the surrounding medium, and the organelles shrink and increase in density. These changes are reversible for some, but not all, organelles.

Various sugar alcohols have been used in place of sucrose in some procedures. Glycerol is one, but although its viscosity is lower than that of sucrose, so is its density at a given concentration, and it also permeates some organelles. Mannitol has been used in a few procedures where the use of sucrose is not feasible—e.g., for isolation of epithelial brush border membranes that contain sucrase (Malathi et al., 1979)—and sorbitol has been a favorite for fractionating yeast, which secrete invertase (a form of sucrase; e.g., Walworth and Novick, 1987).

Polysaccharides have also been employed as substitutes for sucrose. Ficoll 400 (Pharmacia Biotech), a chemical polymer of sucrose with epichlorohydrin that has an average molecular weight of 400,000, has been a popular choice (a 70,000-MW form is also available, as Ficoll 70). At low concentrations, it has very low osmolarity, but as can be seen in Figure 3.1.3, the osmolarity rises sharply above 30% (w/v; density ~1.09 g/ml), and it is quite viscous at all concentrations above 10% (w/v). Ficoll has been used widely for separating different cell populations and is frequently most useful as an additive to media containing other density-modifying agents.

Iodinated nonelectrolytes increase the density of fractionation media through most of the range of biological organelles while maintaining reasonably low viscosity and osmolarity as compared to sucrose. Three popular and closely related compounds are metrizamide, Nycodenz, and iodixanol (OptiPrep; marketed by Nyegaard; Accurate Chemical—listed in the SUPPLIERS APPENDIX—is the U.S. outlet). As seen

from Figure 3.1.3, 40% (w/v) solutions of both metrizamide and Nycodenz have a density >1.2 g/ml (above that of most organelles) and an osmolarity ≤400 mOsm with reasonable viscosity. 60% iodixanol has a density of 1.32 g/ml and an osmolarity of 260 mOsm with only moderate viscosity, making this medium especially useful for density gradient fractionation under isoosmotic conditions. With all these advantageous features, why aren't they used more? They are rather expensive, which certainly limits their use for large-scale work. In addition, they absorb in the UV and have been reported to inhibit selected enzyme activities.

A different alternative for achieving high densities at low viscosity and osmolarity involves the use of Percoll, colloidal silica coated with polyvinylpyrrolidone to reduce its adsorption to biological organelles. For fractionation of cellular organelles, Percoll is almost always used in combination with isoosmotic sucrose, because pure Percoll is only 10 mOsm. As can be seen from Figure 3.1.3, Percoll-containing media can achieve very high densities with reasonably low viscosities. Because Percoll particles have a significant size (range 30 to 150 Å) and density, they sediment during centrifugation. Thus, concentration gradients self-form fairly rapidly during a spin and the profile of density along the length of the tube continuously changes with time. The shallow gradients achieved with Percoll can be very advantageous in separating organelles whose densities differ only slightly; however, for reproducibility, the centrifugation conditions must be quite carefully set. Percoll also has its limitations: it absorbs in the UV, interferes with protein assays, precipitates in acid and organic solvents—do *not* attempt to precipitate Percoll-containing fractions with trichloroacetic acid (TCA) or acetone—is difficult to get rid of entirely.

EVALUATION OF FRACTIONATION

In setting up a procedure for separating or purifying cellular organelles, it is advisable to evaluate the steps of the protocol to confirm that the outcome is as desired and as stated. This means setting up assays for marker activities and protein and accounting for the totality of both throughout the procedure. This meticulousness enables the investigator to evaluate the homogenization, yield, fold purification, and extent of contamination by other undesirable organelles, and to account for 100% of the activity at each step. The extent of cell breakage

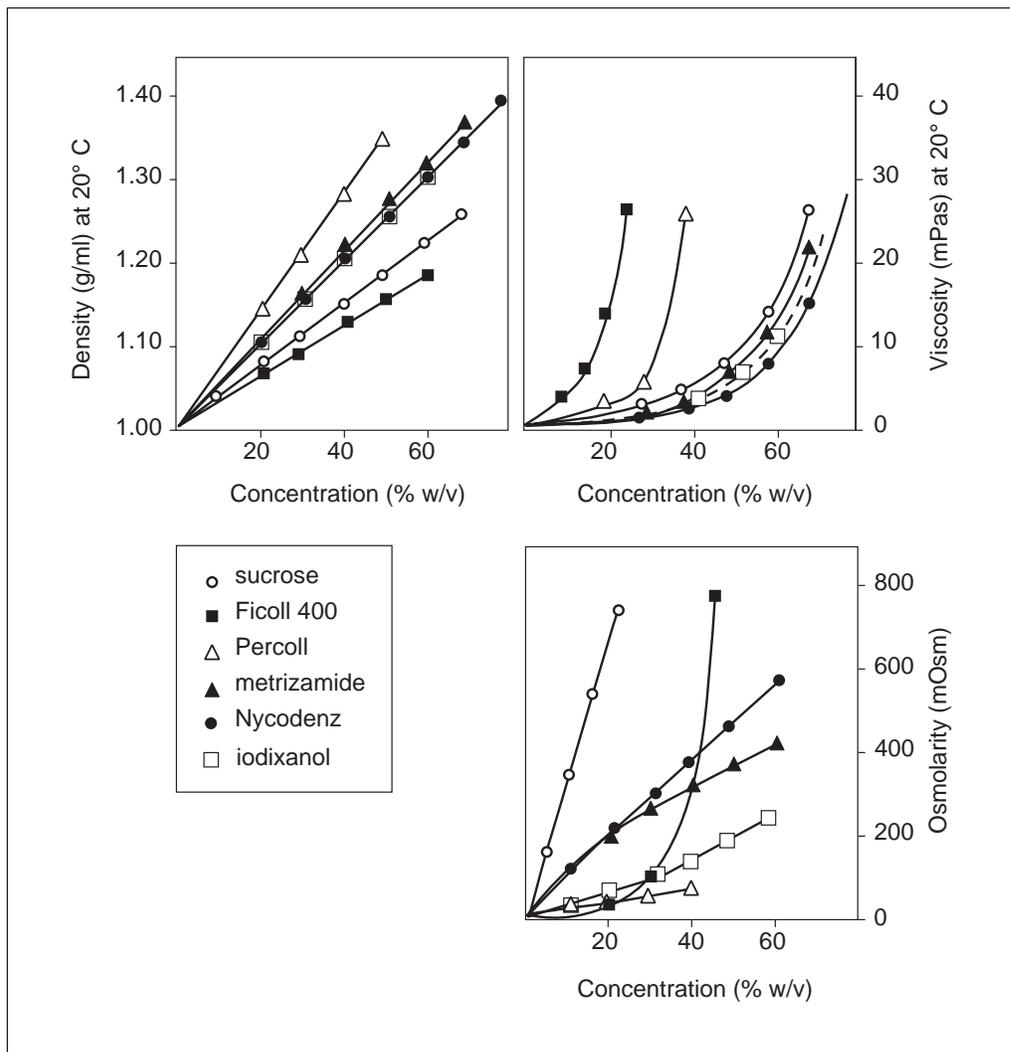


Figure 3.1.3 Plots of density, viscosity, and osmolarity of gradient media used to fractionate cellular organelles as a function of concentration. Data are shown for sucrose, Ficoll 400, Percoll, metrizamide, Nycodenz, and iodixanol. Modified from Rickwood (1984) by permission of IRL Press. Data for iodixanol by permission of Nycomed Pharma AS Diagnostics (Nycomed Pharma, 1996).

during homogenization can be estimated by determining the fraction of a marker activity of a particular organelle that pellets in the first low-speed centrifugation step (and is likely still to be associated with unbroken cells), while the extent of organelle breakage can be estimated by determining the fraction of the total amount of a soluble intraorganellar protein that is not sedimented by a high-speed spin that pellets all of the organelle. Following marker activity throughout purification enables the investigator to determine the yield of the desired organelle, and, if markers of other organelles are followed, the extent of residual contamination. Finally, if protein concentration is determined in parallel, calculation of the ratio of activity to protein (known as specific activity) at each step provides an estimate of fold purification or

enrichment. In the event that analytical rather than preparative procedures are used, determination of marker activity in every fraction is essential for specifying the distribution of a particular organelle. Finally, it is very important to note that subcellular fractionation is never perfect! The entirety of a particular organelle is never obtained in completely pure form. In fact, the term “fraction” signifies both incomplete yield and incomplete purity. The careful investigator will always be cognizant of the limitations of the procedures and the consequent limitations of the results.

DEFINITIVE PROCEDURES

A useful starting point in isolating a particular cellular organelle may be to examine a procedure that has been designed for the spe-

Table 3.1.2 Preparing Organelle Fractions from Mammalian Tissues and Cells

Organelle	Procedure	Reference
Nucleus	Centrifugation through high-density sucrose	Blobel and Potter (1966)
Endoplasmic reticulum	Discontinuous sucrose gradient	Adelman et al. (1973)
Golgi complex	Sucrose gradient, continuous and discontinuous	Bergeron et al. (1982)
Secretion granules		
Endocrine	Metrizamide gradient	Loh et al. (1984)
Exocrine	Discontinuous sucrose gradient	Cameron and Castle (1984)
Synaptic vesicles	Chromatography on controlled-pore glass	Carlson et al. (1978) Huttner et al. (1983)
Plasma membrane	Discontinuous sucrose gradient	Hubbard et al. (1983); <i>UNIT 3.2</i>
Endosomes	Free flow electrophoresis Density shift with sucrose gradient	Marsh et al. (1987) Beaumelle et al. (1990)
Lysosomes	Metrizamide gradient	Wattiaux et al. (1978)
Mitochondria	Velocity sedimentation in sucrose	Schnaitman and Greenawalt (1968)
Peroxisomes	Sucrose gradient, discontinuous and continuous	Leighton et al. (1968)

cific organelle of interest. Table 3.1.2 lists references to procedures for purifying most of the membranous organelles found in eukaryotic cells. Unfortunately, it is not possible to make this listing comprehensive, but the procedures selected have achieved unusually good purity and in many cases have documented their achievement by bookkeeping of marker activities. Although several of the procedures are reasonably old, they have been used either as starting points for subfractionation (e.g., nucleus, endoplasmic reticulum, secretion granules, plasma membranes, lysosomes, mitochondria, and peroxisomes) or for functional studies in cell-free assays (e.g., Golgi, endoplasmic reticulum, and mitochondria). The adventurous investigator may want to consider the newer-generation reagent iodixanol (OptiPrep). The ability to fractionate under isoosmotic conditions over a large density range seems quite appealing. Sample procedures for various fractionations in iodixanol can be obtained from Nycomed/Accurate Chemical Co.

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Isolation of Rat Hepatocyte Plasma Membrane Sheets and Plasma Membrane Domains

UNIT 3.2

The plasma membrane of polarized epithelial cells is separated into discrete domains (apical and basolateral) that are functionally distinct. In polarized hepatocytes, the basolateral surface includes the sinusoidal front, which is specialized for the exchange of metabolites with the blood, and the lateral surface, which is adjacent to neighboring hepatocytes. The apical or bile canicular plasma membrane domain is separated from the basolateral surfaces by tight junctions and is specialized for bile secretion. The functional and morphological differences between the two domains are matched by differing biochemical compositions. In order to examine the constituents of the hepatocyte plasma membrane, isolation of preparative amounts of this organelle is required.

This unit describes isolation of purified plasma membrane sheets from rat hepatocytes (see Basic Protocol 1). Since relatively high yields of plasma membrane sheets are obtained from this procedure, these preparations are suitable for a variety of analytical or preparative uses. For example, integral plasma membrane proteins can be extracted from preparative amounts of the sheets and further purified. The sheets also serve as the starting material for the separation of the two membrane domains (see Basic Protocol 2). The sheets are vesiculated by sonication, applied to continuous sucrose gradients, and centrifuged to equilibrium. The fractionation is generally an analytical procedure, and is useful for determining the distributions of plasma membrane-associated molecules between the two domains. The recovery and purity of plasma membrane sheets can be determined by measuring alkaline phosphodiesterase I activity (see Support Protocol 1); basolateral membrane recovery and purity can be assessed by measuring K^+ -stimulated *p*-nitrophenylphosphatase activity (see Support Protocol 2); and apical membrane recovery and purity can be assessed by measuring 5'-nucleotidase activity (see Support Protocol 3). Distributions of proteins at the hepatocyte plasma membrane can also be determined morphologically (see Support Protocol 4). The large plasma membrane sheets readily adhere to glass coverslips and are easily processed for indirect immunofluorescent detection of specifically labeled antigens. The biochemical and morphological procedures described in this unit provide a relatively simple, yet powerful approach to examining molecules associated with the plasma membrane in rat hepatocytes.

ISOLATION OF PLASMA MEMBRANE SHEETS

This protocol describes a rapid and effective method for the purification of plasma membrane sheets from rat hepatocytes. Rat livers are first gently homogenized in buffered sucrose, and the plasma membrane sheets are separated from other intracellular compartments by a series of four differential centrifugations followed by a single flotation through a one-step sucrose gradient.

NOTE: All solutions and glassware should be prechilled to 4°C before the procedure, and kept on ice throughout.

Materials

- 125- to 150-g male Sprague-Dawley rats
- Ether
- 0.9% (w/v) NaCl, ice cold (store 1 to 2 weeks at 4°C)
- 0.25 and 2.0 M STM solutions (see recipes), ice cold

continued

**BASIC
PROTOCOL 1**

**Subcellular
Fractionation and
Isolation of
Organelles**

Protease inhibitor solutions (see recipe)
0.25 M sucrose solution (see recipe), ice cold
40-ml Dounce homogenizer (Wheaton) with tight- and loose-fitting pestles (size A and B, respectively, although certain manufacturers reverse these designations)
Cheesecloth, grade 60
Abbe refractometer (Bausch and Lomb)
7-ml Dounce homogenizer (Wheaton) with loose-fitting pestle (size B)
Additional reagents and equipment for SDS-PAGE (UNIT 6.1), quantitative immunoblotting (UNIT 6.2), and densitometry (UNIT 6.3)

Subfractionate liver homogenate

1. Starve a 125- to 150-g male rat for 18 to 24 hr.
For analytical work, one 6- to 8-g rat liver provides sufficient material. For preparative work, use up to 40 g of liver at a time (five to six rats and scale up solution volumes proportionally to number of livers used). The protocol described here and the volumes of sucrose solutions given (see Reagents and Solutions) are for the isolation of plasma membrane sheets from one rat liver.
2. Anesthetize the rat with ether and sacrifice by decapitation. Drain the blood from the animal under cold running water and carefully excise the liver.
3. Rinse the liver with ice-cold 0.9% NaCl to remove excess blood and hair. Using a squirt bottle, perfuse the liver with 0.9% NaCl via the portal vein until the liver is blanched. Quickly weigh the perfused liver and place it in a prechilled beaker on ice.
This perfusion will remove any contaminating blood trapped within the liver. All subsequent steps are performed at 4°C.
4. Use scissors to mince the liver into ~0.5-cm³ pieces and add 4.5 vol (4.5 ml/g tissue) of 0.25 M STM solution with protease inhibitors. Pour the mixture into a 40-ml Dounce homogenizer.
If the liver weighs more than 8 g, divide it in half and homogenize separately.
5. Homogenize with 10 up-and-down strokes with the loose-fitting pestle (B).
Avoid producing bubbles during homogenization by moving the pestle slowly and steadily. Wipe off any connective tissue that sticks to the pestle.
6. Filter homogenate into a graduated cylinder through a funnel lined with four layers of grade-60 cheesecloth premoistened with 0.25 M STM solution. Add 0.25 M STM solution to adjust the filtered volume to five times the original wet weight (5 ml/g) of the liver, producing a 20% (w/v) homogenate. Mix by gently inverting 3 to 5 times.
7. Pour 25- to 30-ml aliquots of homogenate into 50-ml conical tubes. Centrifuge 5 min at $260 \times g$ (e.g., 1100 rpm in Beckman GS-6R), 4°C.
8. Carefully decant supernatant (S1) into a fresh tube, avoiding contamination from the soft pellet, and place on ice.
9. Resuspend pellet in one-half the original homogenate volume (step 6) of 0.25 M STM solution with protease inhibitors, using three strokes of the loose-fitting pestle (B) in a 40-ml Dounce homogenizer. Centrifuge as in step 7.
10. Decant supernatant and pool with supernatant from step 8 (S1). Centrifuge 25- to 30-ml aliquots of pooled supernatant per 50-ml conical tube, 10 min at $1500 \times g$ (e.g., 2600 rpm in Beckman GS-6R), 4°C.
11. Pour off supernatant (S2).
If assaying all fractions for recoveries, save S2 on ice and use on the same day.

Float plasma membrane sheets

12. Add two-thirds the original homogenate volume (step 6) of 0.25 M STM solution with protease inhibitors to the pellet (P2). Resuspend in a 40-ml Dounce homogenizer with three strokes of the loose-fitting pestle (B) followed by one stroke of the tight-fitting pestle (A).
13. Pour the suspension into a graduated cylinder and adjust to 2 times the original homogenate volume (step 6) with 2.0 M STM solution. Mix by gently inverting 3 to 5 times.
14. Check the density of the mixture with an Abbe refractometer, and adjust with either 0.25 M or 2.0 M STM solution until a density of 1.18 g/cm³ (refractive index = 1.4016) is achieved.
15. Fill ultracentrifuge tubes equally with the resuspended P2 to ~90% total volume. Carefully overlay each tube to within a few millimeters from the top with ice-cold 0.25 M sucrose solution.

For the Beckman SW28 rotor, 90% is 32 ml.

16. Centrifuge 60 min at 113,000 × g (e.g., 25,000 rpm in Beckman SW28 rotor), 4°C, in a swinging-bucket rotor with no brake.
17. Collect and pool the pellicules at the interface of each tube with a blunt-ended plastic transfer pipet.

If assaying all fractions for recoveries, pour off and save the load fraction and resuspend the pellet (P3) to the desired volume (e.g., 5 to 10 ml).

18. Resuspend pellicules in 0.25 M sucrose solution at 0.8 to 1.0 times the original homogenate volume (step 6) and gently homogenize with three strokes of the loose-fitting pestle in the 40-ml Dounce homogenizer.
19. Check density of the suspension and adjust to ≤1.05 g/cm³ (refractive index ≤1.3500) by diluting with 0.25 M sucrose solution, if necessary.

Recover and characterize plasma membrane sheets

20. Centrifuge 25- to 30-ml aliquots of resuspended pellicules per 50-ml conical tube, 10 min at 1500 × g (e.g., 2600 rpm in Beckman GS-6R), 4°C.
21. Carefully remove supernatant with a pipet and resuspend the pellet (plasma membrane sheets) in 1 to 2 ml of 0.25 M sucrose solution per liver in a 7.0-ml Dounce homogenizer with a loose-fitting pestle. Divide the purified plasma membrane sheets into 0.5- to 1.0-ml aliquots and save indefinitely at –80°C.

Use caution when removing the supernatant, as this pellet is loose.

22. Apply ~20 μl of resuspended membrane sheets to a glass slide and examine at 25× magnification by phase-contrast microscopy.

There should be relatively few small (≤1-μm) vesicles and membrane fragments, and an abundance of relatively large (20- to 40-μm), Y-shaped structures. If large (10-μm) spheres are also observed, these are nuclei. To avoid this form of contamination, pour less supernatant off of the pellet in step 8.

23. Check recovery and purity of an aliquot of the plasma membrane sheets by SDS-PAGE (UNIT 6.1) and immunoblotting (UNIT 6.2), assaying immunoreactivity with antibodies specific for various intracellular membrane marker proteins (see Table 3.2.1). Determine distributions by densitometric analysis (UNIT 6.3) of immunoreactive bands.

Alternatively, recovery and purity can be checked by using the relatively simple colorimetric assay for the plasma membrane marker alkaline phosphodiesterase I (see Support Protocol 1) and by assaying protein concentration (APPENDIX 3).

Table 3.2.1 Recovery and Enrichment of Various Organellar Markers During the Isolation of Plasma Membrane Sheets^a

Marker ^b	% Recovery	Fold-enrichment
Protein	0.4 ± 0.13	—
5'-nucleotidase (PM)	17.4 ± 6	39 ± 10
Alkaline phosphodiesterase I (PM)	17.0 ± 5.6	40 ± 9
Asialoglycoprotein binding activity (PM)	16.3 ± 6	47.8 ± 18
NADH-cytochrome <i>c</i> reductase (ER)	0.3 ± 0.2	1 ± 0.5
Glucose-6-phosphatase (ER)	0.5	0.8
β- <i>N</i> -acetylglucosaminidase (lysosomes)	0.22 ± 0.13	0.66 ± 0.3
Cytochrome oxidase (mitochondria)	0.12 ± 0.07	0.22 ± 0.1
DNA (nuclei)	0.26	0.3
Galactosyltransferase (Golgi)	1.0	1.9

^aValues are reported as the mean ± standard deviation ($n \geq 3$), when available. Reproduced from Hubbard et al. (1983) by copyright permission of the Rockefeller University Press.

^bPM, plasma membrane; ER, endoplasmic reticulum.

SUPPORT PROTOCOL 1

ASSAY FOR ALKALINE PHOSPHODIESTERASE I ACTIVITY

Alkaline phosphodiesterase I is a plasma membrane-associated enzyme that distributes equally between the two surface domains. Since its enzymatic activity is easily assayed (Touster et al., 1970), alkaline phosphodiesterase is an excellent marker for total plasma membranes in biochemical subcellular fractions. In general, the assay described below is used to determine the recovery and purity of plasma membrane sheets in the preparative fractions obtained during the isolation procedure (Basic Protocol 1).

Materials

Preparative fractions and isolated plasma membrane sheets (see Basic Protocol 1)
Alkaline phosphodiesterase I reaction buffer (see recipe)
5% and 10% (w/v) trichloroacetic acid (TCA), ice cold
2 N NaOH
0.25 mM *p*-nitrophenol in 5% (w/v) TCA
1-cm-pathlength cuvettes

Analyze samples

1. Determine the necessary volume of alkaline phosphodiesterase I reaction buffer by multiplying the number of assay points plus one substrate blank by 0.8 ml (0.2 ml/assay tube × 4 tubes/fraction) and prepare it fresh.

In general, all preparative fractions from the isolation of plasma membrane sheets, as well as the substrate blank, are assayed in duplicate for alkaline phosphodiesterase activity. Also, two incubation times are usually used.

Substrate blanks are processed along with the other fractions and account for the amount of substrate hydrolyzed without added enzyme.

2. Set up four disposable glass tubes per fraction and substrate blank, and distribute 0.2 ml reaction buffer to each tube at room temperature.
3. Add 50 μl of each fraction (kept on ice until ready to assay) to the appropriate sample tubes and 50 μl water to the substrate blank tubes. Vortex gently.
4. Incubate fractions and blanks at 37°C in a shaking water bath, half for 30 min and half for 60 min.

5. Stop the reaction by adding 0.25 ml ice-cold 10% TCA. Gently vortex and let stand on ice.
6. Add 1.5 ml of 2 N NaOH to each tube and gently vortex.
7. Read A_{400} using a 1-cm-pathlength cuvette.

Analyze standards

8. Distribute 0, 0.1, 0.25, 0.3, 0.4, and 0.5 ml of 0.25 mM *p*-nitrophenol in 5% TCA to duplicate tubes.

The corresponding amounts of p-nitrophenol are 0.0, 25.0, 62.5, 75, 100, and 125 nmoles/tube, respectively.

9. Adjust the volume of each tube to 0.5 ml with 5% TCA.
10. Add 1.5 ml of 2 N NaOH to each tube and read A_{400} as in step 7.

Perform calculations

11. Generate a standard curve by plotting A_{400} versus the amount of *p*-nitrophenol.
12. Subtract the substrate blank from each sample absorbance reading.
13. Determine the *p*-nitrophenol released in each sample tube from the standard curve.
If the unknown alkaline phosphodiesterase activity is too high, dilute the fraction, assay a smaller aliquot, or generate another standard curve in a higher concentration range. Do the opposite if the activity is too low.
14. Calculate enzyme activity (μ moles substrate hydrolyzed/hr/ml) by multiplying the number of micromoles of *p*-nitrophenol in the samples by 20 (to correct the sample volume to 1.0 ml), by 2 (for the 30-min sample only, to correct the time to 1 hr), and by 1/dilution (if sample was diluted before addition to tube in the 50- μ l aliquot).

ISOLATION OF PLASMA MEMBRANE DOMAINS

The isolated sheets prepared above (see Basic Protocol 1) are used as the starting material for the separation of the apical and basolateral domains, which constitute the intact rat hepatocyte plasma membrane. Gentle sonication of the plasma membrane sheets results in the formation of vesicles derived from either the apical or the basolateral domains. The two resultant vesicle populations are separated by equilibrium centrifugation on linear sucrose gradients and are identified by immunodetection of domain-specific antigens.

Materials

- Plasma membrane sheets (see Basic Protocol 1)
- 0.25, 0.46, and 1.42 M sucrose solutions (see recipes)
- Protease inhibitor solutions (see recipe)
- Antibodies specific for apical and basolateral domains (e.g., anti-dipeptidyl peptidase IV for apical domain and CE9 antibody for basolateral domain; Hubbard et al., 1985)
- Sonicating water bath (e.g., Laboratory Supplies)
- Peristaltic pump
- Gradient maker
- Abbe refractometer (Bausch and Lomb)
- Additional reagents and equipment for preparing and collecting sucrose gradients (UNIT 5.3), SDS-PAGE (UNIT 6.1), immunoblotting (UNIT 6.2), and densitometry (UNIT 6.3)

BASIC PROTOCOL 2

Subcellular Fractionation and Isolation of Organelles

3.2.5

1. Dilute purified plasma membrane sheets to 1.0 mg protein/ml with 0.25 M sucrose solution containing protease inhibitors.

If using a Beckman SW28 rotor, use 4 mg plasma membrane protein/32-ml gradient.

2. Sonicate 2.0-ml aliquots in 15-ml conical tubes using a sonicating water bath containing an ice/water slurry. Sonicate for 10-sec bursts with 1-min pauses on ice between bursts. Monitor vesiculation by phase-contrast microscopy (25× magnification; see Basic Protocol 1, step 22) every 1 to 5 bursts.

Vesiculation is complete when no, or very few, large Y-shaped structures are observed. In general, this takes 3 to 20 bursts, depending on the strength of sonication. If a sonicating water bath is not available, vesiculation can also be achieved using a Polytron homogenizer (Brinkmann) fitted with a 12-mm tip at a setting of 8.

3. Pour linear gradients from 0.46 to 1.42 M sucrose (UNIT 5.3).

If using a Beckman SW28 rotor, each gradient is 32.0 ml.

4. Layer the vesiculated plasma membrane sheets onto the gradients to within 2 to 5 mm from the top.

If using a Beckman SW28 rotor, this volume is ~3.9 to 4.0 ml. Scale sucrose sample volumes proportionally for other types of tubes.

5. Centrifuge 16 to 20 hr at $72,000 \times g$ (e.g., 38,000 rpm in Beckman SW28 rotor) with no brake at 4°C.

6. Collect fractions of desired volume (UNIT 5.3). Resuspend the pellet in 0.25 M sucrose to the same volume as the fractions. Invert each fraction to mix the sucrose solution and measure the refractive index of each with an Abbe refractometer. Store fractions indefinitely at -80°C.

For 36-ml gradients, 3.0-ml fractions are usually collected.

7. Determine the distributions of apical and basolateral plasma membrane proteins by SDS-PAGE (UNIT 6.1) and quantitative immunoblotting (UNIT 6.2) with specific antibodies (e.g., dipeptidyl peptidase IV antibodies for the apical plasma membrane marker and CE9 antibodies for the basolateral), followed by densitometric analysis (UNIT 6.3) of immunoreactive bands.

*Alternatively, domain distributions can be determined by assaying for marker enzyme activities. For the basolateral vesicles, assay K^+ -stimulated *p*-nitrophenyl phosphatase activity (see Support Protocol 2), and for the apical vesicles, assay 5'-nucleotidase activity (see Support Protocol 3).*

A typical plot of domain distribution is shown in Figure 3.2.1. For discussion, see Commentary.

SUPPORT PROTOCOL 2

ASSAY FOR K^+ -STIMULATED *p*-NITROPHENYLPHOSPHATASE ACTIVITY

K^+ -stimulated *p*-nitrophenylphosphatase activity is primarily associated with the basolateral plasma membrane in polarized epithelial cells. The assay outlined below (Stieger et al., 1986) is a straightforward method to determine the distribution of basolateral plasma membranes in sucrose gradient fractions containing vesiculated plasma membrane sheets (Basic Protocol 2). When used in combination with the assay for 5'-nucleotidase activity (Support Protocol 3), the distributions of both domains are easily determined for a single gradient.

Materials

Domain gradient fractions and resuspended pellet fraction (see Basic Protocol 2)
p-Nitrophenylphosphatase reaction buffer, with and without K^+ (see recipe)
Substrate mix (see recipe)

continued

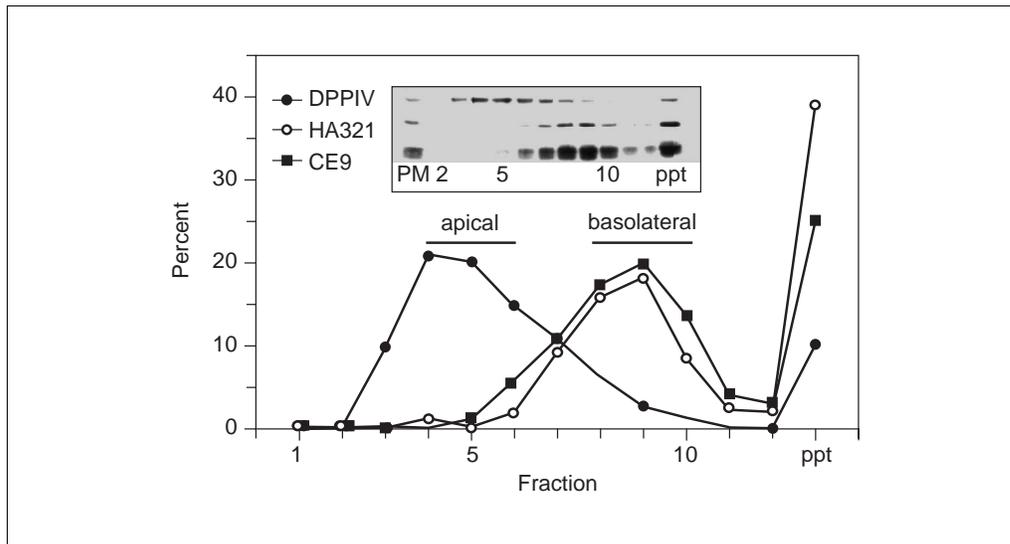


Figure 3.2.1 Distribution of one apical and two basolateral markers in the plasma membrane domain gradient. Isolated hepatocyte plasma membrane sheets were sonicated and the resulting vesicles separated on linear sucrose gradients. Collected fractions were analyzed by SDS-PAGE and quantitative immunoblotting with antibodies against known plasma membrane marker proteins. The data obtained from densitometry of the immunoreactive species detected in each fraction relative to the total recovered were plotted and indicated as percent distribution. The blotting data for each of the proteins are shown in the inset. The majority of the apical plasma membrane marker DPPIV was distributed to fractions 4 to 6, while the basolateral markers HA321 and CE9 were detected in fractions 8 to 10. The data shown are representative of three to ten experiments. Abbreviations: DPPIV, dipeptidyl peptidase IV; PM, plasma membrane; ppt, pellet. Reproduced from Fujita et al. (1998) by copyright permission of Portland Press.

1 and 10 N NaOH
 0.25 mM *p*-nitrophenol in 1 N NaOH
 1-cm-pathlength cuvette

Analyze samples

1. Determine the necessary volumes of K^+ and K^+ -free *p*-nitrophenylphosphatase reaction buffers by multiplying the number of assay points plus one substrate blank by 3.28 ml (0.82 ml/assay tube \times 4 tubes/fraction). Freshly prepare this volume of each reaction buffer.

In general, all gradient fractions (including the resuspended pellet fraction) and a substrate blank are assayed in duplicate for both K^+ -stimulated and K^+ -independent activity. Also, two incubation times are usually used.

Substrate blanks are processed along with the other fractions and account for the amount of substrate hydrolyzed without added enzyme.

2. Set up eight disposable glass tubes per fraction and substrate blank. Distribute 0.82 ml of each reaction buffer to four tubes/fraction. Distribute 0.87 ml of each reaction buffer to four substrate blank tubes.
3. Add 50 μ l of each gradient fraction and the pelleted fraction (kept on ice until ready to assay) to the appropriate sample tubes and vortex gently.
4. Add 30 μ l of substrate mix to all sample and blank tubes to begin the reaction. Incubate at 37°C in a shaking water bath, half for 30 min and half for 60 min.
5. Stop reaction by adding 100 μ l of 10 N NaOH and vortex.
6. Read A_{405} using a 1-cm-pathlength cuvette.

Analyze standards

7. Distribute 0, 0.1, 0.25, 0.3, 0.4, and 0.5 ml of 0.25 mM *p*-nitrophenol in 1 N NaOH to duplicate tubes.

The corresponding amounts of p-nitrophenol are 0, 25.0, 62.5, 75, 100, and 125 nmoles/tube, respectively.

8. Adjust the volume of each tube to 1.0 ml with 1 N NaOH and read A_{405} as in step 5.

Perform calculations

9. Generate a standard curve by plotting A_{405} versus the amount of *p*-nitrophenol.

10. Subtract the substrate blank from each sample absorbance reading.

11. Determine the *p*-nitrophenol released in each sample tube from the standard curve.

If the unknown phosphatase activities are too high, dilute the fraction, assay a smaller aliquot, or generate another standard curve in a higher concentration range. Do the opposite if the activity is too low.

12. Calculate enzyme activity (μ moles substrate hydrolyzed/hr/ml) by multiplying the number of micromoles of *p*-nitrophenol in the samples by 20 (to correct the sample volume to 1.0 ml), by 2 (for the 30 min sample only, to correct the time to 1 hr), and by 1/dilution (if sample was diluted before addition to tube in the 50- μ l aliquot).

13. To determine the K^+ -stimulated *p*-nitrophenylphosphatase activity (the basolateral domain marker enzyme), subtract the values obtained using the buffer containing K^+ from those obtained using the K^+ -free buffer.

ASSAY FOR 5'-NUCLEOTIDASE ACTIVITY

5'-nucleotidase is a plasma membrane-associated enzyme that distributes exclusively to the apical domain in many polarized epithelial cells. The assay described below (Widnell and Unkeless, 1968) serves as a straightforward means of determining the distributions of the apical plasma membranes in sucrose gradients containing vesiculated plasma membrane sheets (Basic Protocol 2).

Materials

Domain gradient fractions and resuspended pellet fraction (see Basic Protocol 2)
5'-Nucleotidase reaction buffer (see recipe)
5% and 30% (w/v) trichloroacetic acid (TCA), ice cold
10% (w/v) ascorbic acid, prepared fresh
0.42% (w/v) ammonium molybdate (see recipe)
0.1 mM KH_2PO_4 in 10% (w/v) TCA
1-cm-pathlength cuvette

Analyze samples

1. Determine the necessary volume of 5'-nucleotidase reaction buffer by multiplying the number of assay points plus one substrate blank by 1.8 ml (0.45 ml/assay tube \times 4 tubes/fraction) and prepare it fresh.

In general, all gradient fractions (including the resuspended pellet fraction) and the substrate blank are assayed in duplicate for 5'-nucleotidase activity. Also, two incubation times are usually used.

A substrate blank consists of a mixture of 0.45 ml reaction buffer and 50 μ l of H_2O also done in duplicate. These samples are processed along with the other fractions and account for the amount of substrate hydrolyzed without added enzyme. Two such blanks are required: one for the 30-min reaction and one for the 60-min reaction.

2. Set up four disposable glass tubes per fraction and substrate blank, and distribute 0.45 ml reaction buffer to each tube.
3. Add 50 μ l of each gradient fraction (kept on ice until ready to assay) to the appropriate sample tubes and 50 μ l water to the substrate blank tubes. Vortex gently.
4. Incubate at 37°C in a shaking water bath, half for 30 min and half for 60 min.
5. Stop reaction by adding 0.1 ml ice-cold 30% TCA and vortexing. Keep samples on ice.
6. Mix one part 10% ascorbic acid with six parts 0.42% ammonium molybdate (AA/AM).
7. To the tubes in step 4, add 1.5 ml AA/AM, cover with aluminum foil, and incubate an additional 20 min at 45°C.
8. Read A_{750} using a 1-cm-pathlength cuvette.

Analyze standards

9. Distribute 0, 0.05, 0.1, 0.2, 0.3, and 0.4 ml of 0.1 mM KH_2PO_4 in 10% TCA to duplicate tubes.
The corresponding amounts of PO_4 are 0, 5, 10, 20, 30, and 40 nmoles/tube, respectively.
10. Adjust the volume of each tube to 0.6 ml with 5% TCA.
11. Add 1.5 ml AA/AM to each tube and incubate at 45°C as in step 6.
12. Read A_{750} as in step 8.

Perform calculations

13. Generate a standard curve by plotting A_{750} versus the amount of PO_4 .
14. Subtract the substrate blank from each sample absorbance reading.
15. Determine the PO_4 released in each sample tube from the standard curve.
If the unknown 5'-nucleotidase activity is too high, dilute the fraction, assay a smaller aliquot, or generate another standard curve in a higher concentration range. Do the opposite if the activity is too low.
16. Calculate enzyme activity (μ moles substrate hydrolyzed/hr/ml) by multiplying the number of micromoles of PO_4 in the samples by 20 (to correct the sample volume to 1.0 ml), by 2 (for the 30 min sample only, to correct the time to 1 hr), and by 1/dilution (if sample was diluted before addition to tube in the 50- μ l aliquot).

INDIRECT IMMUNOFLUORESCENT DETECTION OF PROTEINS ASSOCIATED WITH PLASMA MEMBRANE SHEETS

The distribution of plasma membrane-associated molecules between the two domains can be easily determined by processing the purified sheets for indirect immunofluorescence. The sheets are settled onto glass coverslips by gravity and fixed with methanol at -20°C . The sheets are labeled with specific primary antibodies and visualized by indirect immunofluorescent detection of fluorophore-conjugated secondary antibodies. For additional discussion of immunofluorescent detection, see *UNIT 4.3*.

Materials

- Plasma membrane sheets (see Basic Protocol 1)
- 0.25 M sucrose solution (see recipe)
- PBS (*APPENDIX 2A*)

continued

SUPPORT PROTOCOL 4

Subcellular Fractionation and Isolation of Organelles

3.2.9

Methanol, prechilled to -20°C
PBS/1% (w/v) BSA, prepared fresh
Primary antibody for marker protein
PBS/0.2% (w/v) BSA, prepared fresh
Fluorochrome-conjugated secondary antibody specific for Ig of the species of the primary antibody
Phenylenediamine mounting medium (see recipe)
Nail polish
22 × 22–mm glass coverslips

Adhere plasma membrane sheets to glass coverslips

1. Dilute plasma membrane sheets to 1.0 mg protein/ml with 0.25 M sucrose solution. Apply 100 to 200 μl to a 22 × 22–mm glass coverslip and distribute evenly across the surface with the side of a pipet tip.
2. Allow the plasma membrane sheets to settle onto the coverslip by gravity for 20 to 30 min at room temperature.

The solution should not dry out during this time.

Prepare fixed samples

3. Wash off unattached plasma membrane sheets by rapidly dunking the coverslip 10 times in a small beaker of PBS. Remove excess liquid by touching an edge of the coverslip to a paper towel.
4. Quickly place the coverslip in prechilled (-20°C) methanol and incubate 5 min at -20°C .
5. Remove the coverslip from the methanol and rehydrate by washing three times in PBS for 5 min each.

This and all subsequent steps are carried out at room temperature.

Stain samples for immunofluorescence

6. Block the plasma membrane sheets by incubating 15 min with PBS/1% BSA.
7. Remove excess blocking reagent as in step 3 and replace with 100 μl fresh PBS/1% BSA containing the desired primary antibody. Incubate 30 min.
8. Wash the coverslip three times with PBS/0.2% BSA, 5 min each, and remove excess wash buffer as in step 3.
9. Incubate 15 min in 100 μl PBS/1% BSA containing the desired concentration of fluorochrome-conjugated secondary antibody.
10. Wash coverslip as in step 8.
11. Place a drop of the phenylenediamine mounting medium in the middle of a glass slide. Place the coverslip with plasma membrane sheets face down on top of the drop.
12. Wick excess liquid with filter paper placed adjacent to the coverslip. Seal the coverslip into place with nail polish.
13. Allow specimen to dry at least 15 min and view with a fluorescent microscope equipped with a 63× or 100× objective.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

Alkaline phosphodiesterase I reaction buffer

5.0 ml 100 mM Na₂CO₃/NaHCO₃, pH 10.5 (40 mM final)

1.25 ml 1% (v/v) Triton X-100 (0.1% final)

1.0 ml 20 mM TMP solution (see recipe; 2 mM final)

H₂O to 10.0 ml

Prepare fresh and use at room temperature

This volume is sufficient for assaying all the preparative fractions and substrate blanks in duplicate for two incubation times (enough for 50 assay tubes).

Ammonium molybdate, 0.42% (w/v)

27.8 ml concentrated H₂SO₄ (1 N final)

4.2 g ammonium molybdate

H₂O to 1 liter

Store indefinitely at room temperature

p-Nitrophenylphosphatase reaction buffer, with and without K⁺

5 ml 100 mM KCl (10 mM final)

1.5 ml 100 mM MgCl₂ (3 mM final)

25 ml 100 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*; 50 mM final)

45 mg theophylline (5 mM final)

H₂O to 50 ml

Adjust pH to 7.4

Prepare fresh and use at room temperature

For buffer without K⁺: Use 5 ml of 100 mM NaCl (10 mM final) in place of KCl.

This volume is sufficient for assaying all the gradient fractions and substrate blanks in duplicate for two incubation times (enough for 60 assay tubes).

5'-Nucleotidase reaction buffer

27.0 ml 100 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*; 90 mM final)

3.0 ml 100 mM MgCl₂ (10 mM final)

15 mg adenosine-5'-monophosphate

Prepare fresh and use at room temperature

This volume is sufficient for assaying all the gradient fractions and substrate blanks in duplicate for two incubation times (enough for 66 assay tubes).

Phenylenediamine mounting medium

2.5 ml 2× TBS (see recipe)

2.5 ml glycerol

10 mg phenylenediamine (2 mg/ml final)

Adjust pH to between 9.5 and 10.5. Place medium in a 5-ml syringe, attach a 0.22- μ m syringe-tip filter, and wrap in aluminum foil. Prepare fresh and keep on ice. Pass solution directly onto slide through the filter when needed.

This medium is very light sensitive.

Protease inhibitor solutions

Antipain: Prepare a 5-ml aqueous solution containing 5 mg antipain (1 mg/ml final) and 10% (v/v) dimethyl sulfoxide (DMSO). Divide into aliquots and store up to 1 year at -20°C . Add immediately before use at a 1:1000 dilution (1 $\mu\text{g/ml}$ final).

continued

Aprotinin: Prepare a 10-ml aqueous solution containing 13.6 mg (1.36 mg/ml final) aprotinin. Store up to 1 year at 4°C. Add immediately before use at a 1:200 dilution (6.8 mg/ml final).

Benzamidine: Prepare a 10-ml aqueous solution containing 0.313 g benzamidine (200 mM final). Divide into aliquots and store up to 1 year at -20°C. Add immediately before use at a 1:200 dilution (1 mM final).

Leupeptin: Prepare a 5-ml aqueous solution containing 5 mg leupeptin (1 mg/ml final) and 10% (v/v) DMSO. Divide into aliquots and store up to 1 year at -20°C. Add immediately before use at a 1:1000 dilution (1 µg/ml final).

Phenylmethylsulfonyl fluoride (PMSF): Prepare a 10-ml solution of 100% ethanol containing 0.348 g PMSF (200 mM final). Store indefinitely at 4°C. Add immediately before use at a 1:200 dilution (1 mM final).

STM (sucrose/Tris/MgCl₂) solution, 0.25 M

17.12 g sucrose (0.25 M final)

2.0 ml 1.0 M Tris·Cl (APPENDIX 2A), pH 7.4 (10 mM final)

0.2 ml 1.0 M MgCl₂ (APPENDIX 2A; 1.0 mM final)

H₂O to 200 ml

Adjust to pH 7.4. Determine density (refractive index) at room temperature. Filter through a 0.2-µm nitrocellulose filter and store up to 48 hr at 4°C.

Refractive index = 1.3453 ± 0.0005. Solution can also be stored up to 1 year at -20°C, but density and pH should be checked before use.

STM solution, 2.0 M

Prepare as for 0.25 M STM solution (see recipe), but use 68.4 g sucrose per 100 ml (2.0 M final), and filter with a 1.2-µm nitrocellulose filter.

Refractive index = 1.4295 ± 0.0005.

Substrate mix

1.0 ml 10% (w/v) saponin (0.5% final)

0.92 g *p*-nitrophenylphosphate ditris salt (100 mM final)

H₂O to 20 ml

Store for up to 1 year at -20°C

Sucrose solution, 0.25 M

12.84 g sucrose

H₂O to 150 ml

Adjust to pH 7.4. Determine density (refractive index) at room temperature. Filter through a 0.2-µm nitrocellulose filter and store up to 48 hr at 4°C.

Refractive index = 1.3453 ± 0.0005. Solution can also be stored up to 1 year at -20°C, but density and pH should be checked before use.

Sucrose solution, 0.46 M

7.87 g sucrose

0.5 ml 1.0 M Tris·Cl (APPENDIX 2A), pH 7.5 (10 mM final)

H₂O to 50 ml

Adjust to pH 7.5. Determine density (refractive index) at room temperature. Filter through a 0.2-µm nitrocellulose filter and store up to 48 hr at 4°C.

Refractive index = 1.3557 ± 0.0005. Solution can also be stored up to 1 year at -20°C, but density and pH should be checked before use.

Sucrose solution, 1.42 M

Prepare as for 0.46 M sucrose solution (see recipe) but use 24.3 g sucrose.

Refractive index = 1.4016 ± 0.0005.

Thymidine-5'-monophospho-p-nitrophenyl ester (TMP), 20 mM

92 mg TMP

H₂O to 10.0 ml

Store up to 1 year at –20°C

Tris-buffered saline (TBS), 2×

2.42 g Tris base (100 mM final)

3.51 g NaCl (300 mM final)

H₂O to 200 ml

Adjust pH to 10.5 with concentrated HCl

Store indefinitely at 4°C

COMMENTARY

Background Information

The plasma membrane of polarized epithelial cells is divided into two functionally and compositionally distinct plasma membrane domains: the apical domain and the basolateral domain. In order to examine the biochemical constituents of each domain, isolation of highly purified plasma membranes is required. Not only must the preparations be pure, they must also contain each of the domains in similar amounts to that found in intact hepatocytes.

Early stereological studies revealed that the basolateral surface accounts for ~87% of the total plasma membrane surface area in hepatocytes; the apical domain for only ~13% (Weibel, 1976; Weibel et al., 1969; Blouin et al., 1977). However, early purification methods were deficient in substantial amounts of the basolateral surface, as discussed in Hubbard et al. (1983). This is largely due to the fact that the plasma membranes were vesiculated in the initial homogenization step, rendering the basolateral-derived vesicles both physically and morphologically indistinguishable from other intracellular vesicular structures. To circumvent this problem, the method presented here (first presented in Hubbard et al., 1983) avoids vesiculation of plasma membranes in the initial purification. Rather, using gentle homogenization, entire plasma membrane sheets are prepared and purified. These purified sheets retain both the apical and basolateral domains in near-normal surface area ratios (Hubbard et al., 1983), as well as a full complement of intercellular junctions and the subplasmalemmal cytoskeletal network (Hubbard and Ma, 1983).

This isolation procedure offers several other advantages over previously published methods. For one, this procedure is fast, taking a total of only 4 to 5 hr. It is also simple, requiring only four low-speed centrifugation steps and a single flotation through a one-step sucrose gradient. Perhaps most importantly, this method is effective, with

routinely high yields and substantial enrichment of plasma membrane marker proteins (see Anticipated Results). This preparation also has substantially lower recoveries of membranes derived from other organelles, including the plasma membranes of other liver cell types (e.g., Kupffer and endothelial cells; see Table 3.2.1).

Once isolated, the plasma membrane sheets are useful for a variety of preparative and analytical procedures ranging from the identification of plasma membrane-associated molecules to their purification. Because domain-specific integral membrane proteins of the rat hepatocyte maintain their membrane localizations during isolation (Hubbard et al., 1983), the sheets are also useful as the starting material for purification of plasma membrane domains and determination of the distribution of molecules between domains. For this procedure, the plasma membrane sheets are vesiculated by sonication, applied to linear sucrose gradients, and centrifuged to equilibrium (Bartles et al., 1985). The basolateral-derived vesicles are more dense, probably due to associated cytoskeletal elements, and are readily separated from apical membrane vesicles. However, since the apical and basolateral vesicles partially overlap in the gradients (see Fig. 3.2.1), this method is only appropriate for analytical purposes.

The distributions of plasma membrane-associated molecules between domains can also be determined by processing isolated sheets for indirect immunofluorescence (Fujita et al., 1998). The large sheets readily adhere to uncoated glass coverslips and can be fixed in place with methanol. Conventional methods are then used to specifically label antigens with primary antibodies and visualize them with fluorophore-conjugated secondary antibodies. This method provides a much less time-consuming and labor-intensive alternative to density centrifugation followed

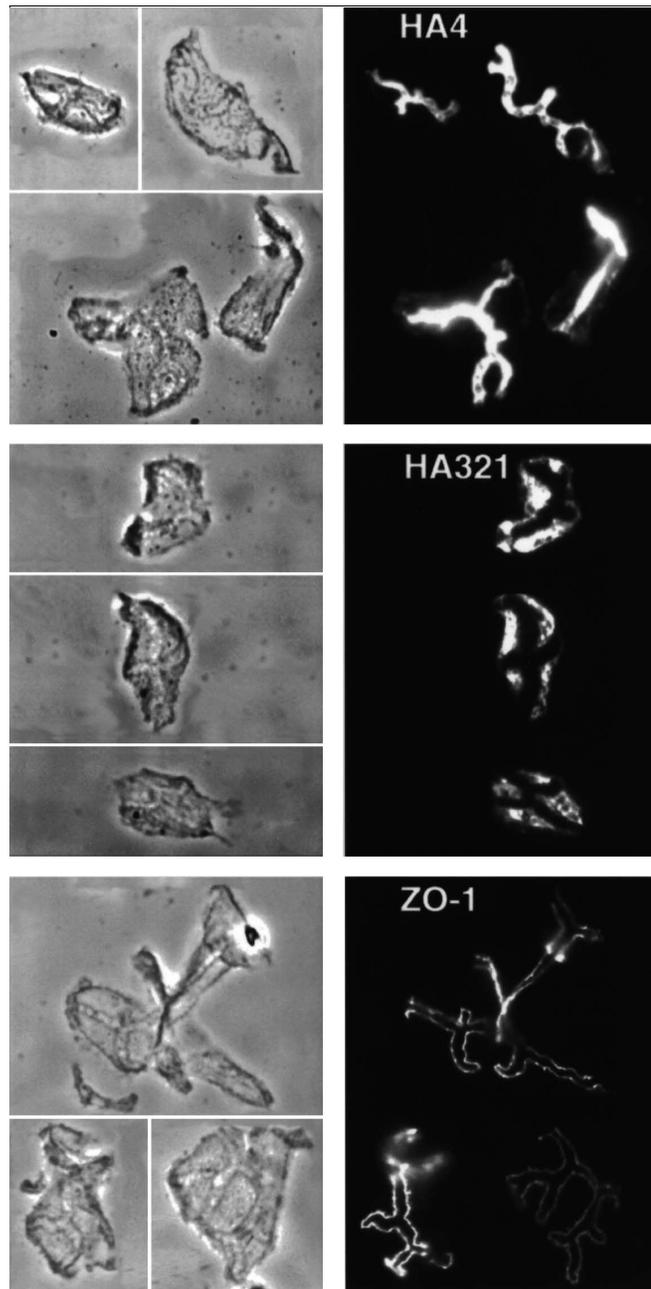


Figure 3.2.2 Immunofluorescent detection of domain-specific proteins and a tight junction-associated molecule in isolated plasma membrane sheets. Isolated plasma membrane sheets (~0.1 mg) were absorbed to glass coverslips, fixed with methanol for 5 min at -20°C , and processed for indirect immunofluorescence. (A), (B), and (C) are phase images of the plasma membrane sheets labeled for HA4 (D), HA321 (E), and ZO-1 (F), respectively. HA4 (an apical plasma membrane protein) was localized strictly to the bile canalicular area, whereas HA321 (a basolateral plasma membrane marker) was excluded from these membranes. The tight junctions, as indicated by ZO-1 staining, were detected in narrow regions immediately adjacent to the canalicular membranes. Reproduced from Fujita et al. (1998) by copyright permission of Portland Press.

by immunoblotting. In addition, the fluorescent staining patterns for domain-specific markers and tight junction components are dramatic and distinct (see Anticipated Results and Fig. 3.2.2). The authors have also found that plasma membrane proteins that were not observed in intact cells can often be detected in isolated sheets (Fujita et al., 1998). The reasons for this are not clear, but may be linked to increased accessibility of antigens for antibody binding. The sheets can also be processed for immunostaining by electron microscopy (Hubbard et al., 1983). However, the time and expertise required for ultrastructural analysis far exceed that required for light-level evaluation, making the latter approach much more accessible to the average experimenter.

Critical Parameters and Troubleshooting

Several features of the isolation procedure are essential to the successful preparation of purified plasma membrane sheets. To reduce aggregation of subcellular organelles by glycogen, rats must be starved at least 18 hr prior to sacrifice. Rat livers from younger rats (125 to 150 g) are preferable for isolation and should be used to avoid the increased amounts of connective tissue and plasma membrane-associated filaments found in preparations from larger rats. Homogenization of the perfused livers should be performed within 5 min of excision to avoid autolysis. To reduce both the vesiculation of the plasma membrane sheets and/or the production of membrane fragments, homogenization should be gentle, with minimal generation of bubbles or vacuum during the up and down strokes with the pestle. To reduce contamination by endoplasmic reticulum, it is recommended to resuspend the second pellet (P2, after centrifugation at $1500\times g$) in twice the initial homogenate volume in preparation for flotation (see Basic Protocol 1, steps 12 and 13).

The most commonly encountered problem in the domain separation procedure is incomplete vesiculation of the membrane sheets resulting from under-sonication. Incomplete vesiculation is characterized by $>25\%$ of the plasma membrane markers being found in the pelleted fraction and by the apical plasma membrane markers having more basolateral-like distributions. This problem can be corrected by increasing the number of sonication bursts until no Y-shaped membrane structures are visible by phase-contrast microscopy. Over-sonication is a less prevalent problem, but results in basolateral markers having a more apical-like distribution in the gradient. This problem can be avoided by more closely monitoring

vesiculation (e.g., after every sonication burst) by phase-contrast microscopy.

The assays to determine the recovery and purity of the plasma membrane sheets and of the two domains are relatively straightforward and easily interpreted. The most common problem is determining the appropriate dilution of the preparative fractions such that their activity levels fall within the values of the standard curve. In Table 3.2.2, suggested dilutions for preparative fractions are listed for both the enzyme assays and protein concentration determination using BCA reagent (Pierce). Alternatively, different standard curves can be prepared shifting the concentrations either higher or lower, or different incubation times can be used. In general, dilution of the domain gradient fractions is not suggested when assaying enzyme activities. Thus, altering the standard curve concentrations and incubation times are advised if problems are encountered.

Anticipated Results

The plasma membrane sheets purified according to Basic Protocol 1 are enriched 20- to 40-fold in plasma membrane markers. This preparation contains substantial amounts of both domains in continuity with each other in ratios approaching those of intact hepatocytes. The yield is 10% to 20% of total plasma membranes. The protein concentration of the purified sheets generally ranges from 1 to 2 mg/ml, corresponding to ~ 1 mg of plasma membrane protein/g of starting liver wet weight. The major contaminant of the isolated sheets is endoplasmic reticulum, which is enriched in these fractions 1-fold (see Table 3.2.1).

The vesicles derived from the apical and basolateral domains are partially resolved on the basis of differences in equilibrium density (see Fig. 3.2.1). The density profile for apical vesicles is characterized by a single peak with its center at a density of 1.10 g/cm^3 (refractive index = 1.3713). Basolateral vesicles have a bimodal distribution, with a peak centered at 1.14 g/cm^3 (refractive index = 1.3859) and a smaller (and variable) amount found in the pelleted fraction.

The apical and basolateral plasma membrane antigens, as well as tight junction components, exhibit distinct staining patterns in plasma membrane sheets. As shown in Fig. 3.2.2, the relatively intense staining for HA4 (an apical protein) is restricted to the bile canalicular membranes in an evenly distributed pattern. In contrast, staining for HA321 (a basolateral protein) is excluded from the canalicular structures and is detected in the surrounding membranes as a more diffuse and less intense signal. The tight junction protein ZO-1 is detected in regions immediately adjacent

Table 3.2.2 Suggested Dilutions for Analysis of Plasma Membrane Preparative Fractions

Fraction	Dilutions for enzyme assays ^a	Dilutions for BCA protein assay
H	1:50; 1:100	1:50; 1:100
S1	1:25; 1:50	1:25; 1:50
P1	1:50; 1:100	1:20; 1:40
S2	1:20; 1:40	1:25; 1:50
P2	1:10; 1:20	1:5; 1:10
I	1:10; 1:20	No dilution; 1:2
1.18 g/m ³	1:2; 1:4	1:2; 1:4
P3	1:5; 1:10	1:50; 1:100
S3	1:2; 1:4	No dilution
PM	1:50; 1:100	1:5; 1:10

^aIn these listings, "1:50" indicates 1 part enzyme in a total of 50 parts (i.e., 1 part enzyme plus 49 parts diluent).

to (outside) the apical plasma membrane in a ring-like pattern, indicating the location of the junctional complexes that form the barrier between plasma membrane domains.

Time Considerations

The isolation of plasma membrane sheets starting from the excision of the rat liver to the final plasma membrane pellet takes only 4 to 5 hr. Preparation of the sheets for density centrifugation by sonication generally takes 15 to 30 min. Each of the enzyme assays takes approximately 3 to 4 hr to perform, including setting up the assay, incubating the samples, reading the absorbances, and performing the calculations. Processing the sheets for indirect immunofluorescence is accomplished within 2 to 3 hr. SDS-PAGE also takes 2 to 3 hr and immunoblotting takes 18 to 24 hr.

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Isolation of Mitochondria from Tissues and Cells by Differential Centrifugation

UNIT 3.3

The protocols in this unit are simple and rapid methods for the isolation of a mitochondrial fraction from three different mammalian tissues (liver, heart, and skeletal muscle), from cultured cells, and from yeast. Unlike the protocols in *UNIT 3.4*, they only require routine differential centrifugation in low- and high-speed centrifuges and should be accessible to any laboratory. These mitochondrial fractions will be contaminated to varying degrees by smaller particles (lysosomes and peroxisomes), although the heavy mitochondrial fraction from rat liver is relatively pure (~90%). These preparations can be used as starting material for the density-gradient separations described in *UNIT 3.4*.

Basic Protocol 1 describes the isolation of the heavy mitochondrial fraction from rat liver; this fraction has high respiratory control and can be used in oxygen electrode studies for ≥ 4 hr after preparation. Centrifuging the postnuclear supernatant at only $3000 \times g$ avoids significant contamination of the pellet by other more slowly sedimenting organelles (e.g., lysosomes, peroxisomes, endoplasmic reticulum). Contamination is reduced further by gently washing the $3000 \times g$ pellet. A mannitol-containing buffer is the medium of choice for this preparation.

Other protocols describe methods for obtaining crude total mitochondrial fractions from bovine heart (see Basic Protocol 2), rat skeletal muscle (see Basic Protocol 3), cultured cells (see Basic Protocol 4), and yeast (see Basic Protocol 5). The major differences between these protocols is their mode of homogenization. Although the easy availability of rat liver in most laboratories makes it a popular choice as a source of mitochondria, those from bovine heart are also often used for respiratory studies. Indeed, they may even be more tightly coupled than liver mitochondria, and they can generally be stored for longer periods while maintaining good functional integrity. This may be allied to the lower levels of proteases and slower release of fatty acids in this tissue. Heart mitochondria also tend to provide better yields of the various structural components of electron transport and ATP synthesis. Basic Protocol 2 also provides a strategy for large-scale preparation. The increasing use of yeast as a model for mammalian membrane and organelle synthesis points to the importance of Basic Protocol 5. Although rat brain is another widely used source of mitochondria, they are rarely purified by differential centrifugation alone (see *UNIT 3.4*).

Methods for measuring succinate dehydrogenase, catalase, and β -galactosidase (as mitochondrial, peroxisomal, and lysosomal markers, respectively) in density-gradient fractions are given in *UNIT 3.4*, but they can also be applied to assessing the purity of mitochondria prepared by differential centrifugation.

Protease inhibitors (see *UNIT 3.4*, Reagents and Solutions) can be included in any or all of the media at the discretion of the investigator, except in the protease-containing solutions used in Basic Protocols 3 and 5.

NOTE: For all protocols, all g values are given as g .

PREPARATION OF THE HEAVY MITOCHONDRIAL FRACTION FROM RAT LIVER

Young adult male animals (150 to 200 g) are routinely used, providing livers of ~10 g wet weight. This protocol is designed for one such liver, but can be scaled up or down proportionally for different amounts of liver. The animals are normally deprived of food overnight to reduce the glycogen content of the liver; this facilitates the separation process.

BASIC PROTOCOL 1

Subcellular Fractionation and Isolation of Organelles

3.3.1

Contributed by John M. Graham

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Supplement 4

A homogenization medium containing mannitol and sucrose, a chelating agent (either EGTA or EDTA), and a buffer (normally HEPES or MOPS) is best suited to respiratory studies.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. When handling the glass vessel of the Potter-Elvehjem homogenizer, a thermally insulated glove or silicone rubber hand protector should be used, not only to avoid heat transfer from the skin, but also to protect the hand in the unlikely event that the vessel breaks.

Materials

- 150- to 200-g male Sprague-Dawley rat
- Liver homogenization medium (LHM; see recipe), ice cold
- Dissecting tools
- Potter-Elvehjem homogenizer (~0.09-mm clearance; 25-ml working volume)
- Overhead high-torque electric motor (thyristor-controlled)
- Low-speed centrifuge with swinging bucket rotor and appropriate tubes
- High-speed centrifuge with fixed-angle rotor and 40- to 50-ml polycarbonate tubes
- Vacuum pump
- Dounce homogenizer (30- to 40-ml volume) with loose-fitting pestle (Wheaton type B)

Isolate liver

1. Deprive a 150- to 200-g male Sprague-Dawley rat of food overnight.
2. Sacrifice the animal by cervical dislocation or decapitation.
This must be supervised or carried out by an experienced animal technician.
3. Open the abdominal cavity and transfer the liver to a chilled beaker containing ~20 ml LHM.
4. Decant the medium and finely mince the liver using scissors.
The pieces of liver should be no more than ~25 mm³.
5. Agitate the minced tissue in ~30 ml ice-cold LHM and allow the pieces to settle out.
6. Decant the medium and replace with ~40 ml fresh medium.

Homogenize liver

7. Transfer half the suspension to the chilled glass vessel of a Potter-Elvehjem homogenizer.
8. Attach the cold pestle to an overhead high-torque electric motor and homogenize the minced liver using five to six up-and-down strokes of the pestle, rotating at ~500 rpm. Decant the homogenate into a beaker on ice.
The motor should be securely mounted either to a wall, to a bench via a G clamp, or in a floor-standing cradle. Attachment to a free-standing retort stand is not adequate.
9. Rinse the homogenizer with medium and wipe the pestle to remove any adhering connective tissue. Repeat the procedure with the other half of the suspension.

Isolate mitochondria

10. Centrifuge the homogenate 10 min at $1000 \times g$, 4°C , in a swinging-bucket rotor using a low-speed centrifuge.
11. Aspirate the supernatant and transfer to 40- to 50-ml polycarbonate tubes.

It is convenient to use a 20- to 30-ml syringe attached to a metal filling cannula (i.d. 0.8 to 1.0 mm) to aspirate supernatants that are to be recentrifuged.
12. Centrifuge the supernatant 10 min at $3000 \times g$, 4°C , in a fixed-angle rotor using a high-speed centrifuge.
13. Using a glass Pasteur pipet attached to some form of vacuum pump, aspirate the supernatant from each tube, keeping the tip of the pipet at the meniscus to remove as much of the floating lipid layer as possible. Also remove as much as possible of the loose-packed pinkish layer that overlies the brown mitochondria.
14. Wipe away any remaining lipid adhering to the wall of the tube with a paper tissue.

Removal of this lipid is essential, as free fatty acids are potent uncouplers of phosphorylation from electron transport.
15. Add a small amount of LHM (~8 ml) to each pellet and crudely resuspend the pellet with a glass rod. Then resuspend fully using 3 to 4 very gentle strokes in a Dounce homogenizer.
16. Make up to the original volume with LHM, transfer to new tubes, and recentrifuge 10 min at $3000 \times g$ in the high-speed centrifuge.
17. Repeat steps 13 to 16 twice more.

For large-scale preparations, the total volume of LHM used to resuspend the pellet can be reduced by ~50% for the second and third washes.
18. Resuspend the purified mitochondria in a buffer whose composition is compatible with any subsequent analysis or processing; in many instances, LHM will be satisfactory.

See Time Considerations for information about storage of mitochondria prior to further processing.

LARGE-SCALE PREPARATION OF MITOCHONDRIA FROM BOVINE HEART

It is important that fresh slaughterhouse material be used for this preparation, and that any adhering connective and adipose tissue be carefully removed. Because of the scale of the preparation and the size of the homogenization equipment, the procedure must be carried out in a cold room. The procedure is suitable for 500 to 600 g of material, and is adapted from Smith (1967) and Rice and Lindsay (1997).

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice or in a cold room throughout.

Materials

- Bovine heart, freshly isolated
- Heart wash buffer (see recipe)
- 2.0 M Tris base
- Sucrose/succinate solution (SS; see recipe)

BASIC PROTOCOL 2

Subcellular Fractionation and Isolation of Organelles

3.3.3

Commercial mincer with a total capacity of 2 to 3 liters
Waring blender or other large-capacity rotating blades homogenizer
Cotton muslin
Low-speed centrifuge with swinging-bucket rotor and 250- to 750-ml bottles
High-speed centrifuge with fixed-angle rotor
Glass rod
Dounce homogenizer (50-ml volume) with loose-fitting pestle (Wheaton type B)

Prepare the heart

1. Cut freshly isolated bovine heart tissue into small cubes (~4 cm³) and pass once through a commercial mincer.
2. Suspend in 800 ml ice-cold heart wash buffer. While stirring, adjust the pH to 7.8 by adding 2.0 M Tris base.
3. Pour through two layers of cotton muslin and then squeeze to remove as much of the liquid as possible.
4. Transfer minced tissue to a clean beaker and suspend in 800 ml ice-cold SS.

Homogenize the heart

5. Transfer half the suspension to a Waring blender and homogenize at high speed for 20 sec.
6. Readjust the pH to 7.8 using 2.0 M Tris base and then reblend for 60 sec.
7. Repeat the procedure with the other half of the suspension. Combine the two homogenates and dilute with ice-cold SS to ~2.2 liters.

Isolate mitochondria

8. Transfer homogenate to 250- to 750-ml centrifuge bottles and centrifuge 20 min at $800 \times g$, 4°C, in a swinging-bucket rotor using a low-speed centrifuge.
9. Carefully decant the supernatants and recentrifuge 20 min at $26,000 \times g$, 4°C, in a fixed-angle rotor using a high-speed centrifuge.

A rotor such as the Sorvall SLA-1500 will allow this to be carried out in two centrifugations.

10. Decant and discard the supernatant.
The pellet is clearly tripartite.
11. Tilting the bottles, gently pour ~10 ml SS on top of each pellet and gently swirl the contents to resuspend the top light-brown layer of partially disrupted mitochondria. Discard this material.
12. Crudely resuspend the remaining dark-brown mitochondria in ~20 ml SS using a glass rod, avoiding the almost-black hard-packed button at the bottom.
13. Completely resuspend mitochondria using 2 to 3 gentle strokes in a Dounce homogenizer.
14. Dilute the suspension to ~300 ml with SS and recentrifuge 20 min at $26,000 \times g$, 4°C.
15. Collect and resuspend the middle layer of the pellet as in steps 10 to 13.

The upper and lower layers of this second pellet should be relatively minor components.

16. If the composition of the SS medium is incompatible with any subsequent analysis, centrifuge the suspension 20 min at $26,000 \times g$ and resuspend the pellet in a suitable medium.

See Time Considerations for information about storage of mitochondria prior to further processing.

PREPARATION OF MITOCHONDRIA FROM SKELETAL MUSCLE

This protocol uses a commercially available protease to facilitate the homogenization of the muscle tissue. Using this approach, the severity of the shear forces used to disrupt the tissue can be reduced, thus minimizing any damage to the mitochondria. The protocol is adapted from Bhattacharya et al. (1991). The protocol is designed for 4 to 5 g muscle tissue, but can be scaled up or down proportionally.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice or in a cold room throughout. When handling the glass vessel of the Potter-Elvehjem homogenizer, a thermally insulated glove or silicone rubber hand protector should be used, not only to avoid heat transfer from the skin, but also to protect the hand in the unlikely event that the vessel breaks.

Materials

- 150- to 200-g male Sprague-Dawley rat
- Muscle wash buffer (see recipe)
- Muscle homogenization medium I (see recipe)
- Muscle homogenization medium II (see recipe)

- Dissecting tools
- Potter-Elvehjem homogenizer (~0.3-mm clearance, 40-ml working volume)
- Overhead high-torque electric motor (thyristor-controlled)
- Fine nylon mesh (200- μ m pore size)
- Low-speed centrifuge with swinging-bucket rotor and appropriate tubes
- High-speed centrifuge with fixed-angle rotor
- Dounce homogenizer (30-ml volume) with loose-fitting pestle (Wheaton type B)

Isolate muscle tissue

1. Sacrifice a 150- to 200-g male Sprague-Dawley rat by cervical dislocation or decapitation.

This must be supervised or carried out by an experienced animal technician.

2. Rapidly dissect out 4 to 5 g of striated leg muscle and wash it twice in ~50 ml muscle wash buffer.
3. Mince the muscle finely on a cold surface (e.g., a glass plate on crushed ice) using two scalpels.

The muscle pieces should be <math><30\text{ mm}^3</math>. The smaller the muscle pieces, the more efficient is the enzyme softening.

Homogenize tissue

4. Add 40 ml muscle homogenization medium I to the tissue and incubate for 5 min at 0° to 4°C.
5. Transfer to an ice-cold glass vessel of a Potter-Elvehjem homogenizer.
6. Attach the cold pestle to an overhead high-torque electric motor and homogenize using eight up-and-down strokes of the pestle, rotating at ~700 rpm.

The motor should be securely mounted either to a wall, to a bench via a G clamp, or in a floor-standing cradle. Attachment to a free-standing retort stand is not adequate.

7. Incubate the homogenate with stirring for 5 min at 0° to 4°C.
8. Dilute with an equal volume of muscle homogenization medium II and rehomogenize as in step 6.

Isolate mitochondria

9. Filter the homogenate through fine nylon mesh and then centrifuge the filtrate 10 min at $2000 \times g$, 4°C , in a swinging-bucket rotor using a low-speed centrifuge.
10. Decant the supernatant and keep on ice.
11. Rehomogenize the pellet in 20 ml muscle homogenization medium II using three up-and-down strokes of the pestle, recentrifuge, and combine the supernatant with the first supernatant.
12. Centrifuge mitochondria 10 min at $10,000 \times g$, 4°C , in a fixed-angle rotor using a high-speed centrifuge.
13. Wash the pellet by resuspending in ~ 20 ml muscle homogenization medium II using 2 to 3 gentle strokes of the pestle in a Dounce homogenizer.
14. Repeat centrifugation as in step 12 and resuspend the pellet in a medium whose composition is compatible with any subsequent analysis or processing; in many instances muscle homogenization medium II or LHM (see recipe) will be quite satisfactory.

See Time Considerations for information about storage of mitochondria prior to further processing.

BASIC PROTOCOL 4

PREPARATION OF MITOCHONDRIA FROM CULTURED CELLS

This protocol, which is adapted from Attardi and Ching (1979) and Rice and Lindsay (1997), is designed for cultured cells ($\sim 2 \times 10^8$) grown as a monolayer. The method may also be satisfactory for suspension culture cells, as the homogenization medium is hypoosmotic. Inclusion of Mg^{2+} and K^{+} in this medium is crucial; not only do these cations protect the nuclei from breakage, the KCl helps prevent some of the cytoplasmic proteins from forming a gel. To protect the released organelles from the hypoosmotic environment, the volume ratio of cells to medium should be as high as possible.

NOTE: All solutions (except PBS), glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice or in a cold room throughout. When handling the glass vessel of the Potter-Elvehjem homogenizer, a thermally insulated glove or silicone rubber hand protector should be used, not only to avoid heat transfer from the skin, but also to protect the hand in the unlikely event that the vessel breaks.

Materials

- Confluent monolayer cultured cells (total 2×10^8)
- PBS (*APPENDIX 2A*)
- Cell homogenization medium (CHM; see recipe)
- CHM containing 1 M sucrose
- Sucrose/ Mg^{2+} medium (see recipe)
- Mitochondrial suspension medium I (see recipe)
- Rubber policeman
- Potter-Elvehjem homogenizer (~ 0.09 -mm clearance, 10- to 15-ml working volume)
- Overhead high-torque electric motor (thyristor-controlled)
- Low-speed centrifuge with swinging-bucket rotor and appropriate tubes
- High-speed centrifuge with fixed-angle rotor and appropriate tubes
- Dounce homogenizer (5- to 10-ml volume) with loose-fitting pestle (Wheaton type B)

Prepare cells

1. Wash the monolayer cultures twice with ~60 ml PBS per wash at room temperature.
2. Using a rubber policeman, scrape the cells into 60 to 80 ml PBS and pellet the cells at $1000 \times g$, room temperature for 15 min.

It is important to form a compact pellet so that all of the PBS can be removed in step 3.

3. Aspirate or decant all of the supernatant and resuspend the cells in ice-cold cell homogenization medium. Use a volume of medium equal to six times the volume of the pellet.
4. Leave on ice for 2 min.

Homogenize cells

5. Attach the cold pestle of a Potter-Elvehjem homogenizer to an overhead high-torque electric motor and homogenize the cells using five up-and-down strokes at 500 rpm. Confirm that $\geq 90\%$ cell breakage has occurred by examining the homogenate under a phase-contrast microscope.

The motor should be securely mounted either to a wall, to a bench via a G clamp, or in a floor-standing cradle. Attachment to a free-standing retort stand is not adequate.

Some cells may require up to ten strokes of the pestle. If adequate cell breakage has not occurred at this stage, a tight-fitting Dounce homogenizer (Wheaton type A) should be used.

6. Add $\frac{1}{3}$ vol ice-cold CHM containing 1 M sucrose (final 0.25 M) and mix gently by repeated inversion.

Do not create foaming by rapid agitation.

Isolate mitochondria

7. Pellet nuclei by centrifuging 5 min at $1000 \times g$, 4°C , in a swinging-bucket rotor using a low-speed centrifuge.

Washing the nuclear pellet to recover more mitochondria cannot be uniformly recommended for all cultured cells, as the nuclei of many cell lines tend to be rather fragile and may release DNA upon being resuspended.

8. Decant or aspirate the supernatant and centrifuge 10 min at $5000 \times g$, 4°C , in a fixed-angle rotor using a high-speed centrifuge.
9. Resuspend the pellet in ~10 ml ice-cold sucrose/ Mg^{2+} medium using two to three gentle strokes of the pestle in a Dounce homogenizer.
10. Recentrifuge at $5000 \times g$, 10 min, 4°C . Resuspend the pellet in 2 to 3 ml ice-cold mitochondrial suspension medium I or other suitable medium for further processing.

See Time Considerations for information about storage of mitochondria prior to further processing.

PREPARATION OF MITOCHONDRIA FROM YEAST (*SACCHAROMYCES CEREVISIAE*)

This protocol uses Zymolase to digest the tough outer coat of yeast to produce spheroplasts. It is probably best suited to the relatively small-scale cultures used in cell and molecular biology research. The rather harsh mechanical shear forces imposed by commercial homogenizers and ball mills that are often employed for large-scale cultures are not very well suited to the preparation of mitochondria. Zymolase works well with most wild-type yeast strains (e.g., D273-10B or KL14-4A). The yeast should be harvested in early- or mid-log growth phase, as the tougher coat that develops in late-log or stationary phase renders the enzyme digestion less satisfactory.

BASIC PROTOCOL 5

Subcellular Fractionation and Isolation of Organelles

3.3.7

The yeast should be grown in YPD medium (UNIT 1.6) and washed in distilled water and a dithiothreitol-containing buffer. This common strategy seems to facilitate subsequent spheroplast formation (Goud et al., 1988). The protocol is adapted from Rice and Lindsay (1997).

Materials

- Yeast cultures in early- to mid-log phase, grown in YPD medium (1% yeast extract/2% Bacto Peptone/2% glucose; see UNIT 1.6)
- DTT buffer (see recipe)
- Yeast sorbitol buffer (see recipe)
- Zymolase-100T (from *Arthrobacter luteus*, 100,000 U/g; e.g., ICN Biomedicals, Sigma)
- Spheroplast homogenization medium (see recipe), ice cold
- Mitochondrial suspension medium II (see recipe), ice cold
- Low-speed centrifuge with swinging-bucket rotor and appropriate tubes
- High-speed centrifuge with fixed-angle rotor and appropriate tubes
- 30°C incubator
- Dounce homogenizer (5- to 10-ml volume) with loose-fitting pestle (Wheaton type B)

Harvest yeast

1. Harvest yeast by centrifuging the culture (~1 liter) 10 min at $1000 \times g$, room temperature, in a swinging-bucket rotor using a low-speed centrifuge. Use a preweighed centrifuge tube, and calculate the wet weight of yeast by reweighing the tube after removing the supernatant.

An early- to mid-log phase yeast culture is equivalent to an OD_{600} of ~0.6. Such a culture contains $\sim 10^7$ cells/ml.

2. Wash cells once in 3 to 4 vol distilled water, centrifuge again, and resuspend in 2 vol DTT buffer.
3. Incubate 30 min at 30°C and then centrifuge again.
4. Wash the cells once in 3 to 4 vol yeast sorbitol buffer and then resuspend in this medium at 0.15 g wet weight/ml.

Make spheroplasts

5. Stir in Zymolase-100T at 0.25 mg/100 g wet weight and incubate for ~1 hr at 30°C.

Spheroplast formation can be checked by adjusting a small sample of the suspension to 0.1% (w/v) sodium dodecyl sulfate (SDS) to solubilize the spheroplasts and observing the remaining intact yeast cells under a microscope.

Zymolase is sold by Sigma under the trade name Lyticase.

6. Harvest spheroplasts by centrifuging 10 min at $1000 \times g$, 4°C, and then wash the pellet once in 3 to 4 vol yeast sorbitol buffer.

Homogenize spheroplasts

7. Resuspend spheroplasts in 2 vol ice-cold spheroplast homogenization medium and carry out all subsequent operations at 0° to 4°C.
8. Homogenize in a Dounce homogenizer using no more than ten up-and-down strokes of the pestle.

Isolate mitochondria

9. Dilute homogenate with an equal volume of spheroplast homogenization medium and remove the nuclei and unbroken spheroplasts by centrifuging 10 min at $1000 \times g$, 4°C .
10. Decant or aspirate the supernatant and centrifuge 10 min at $6500 \times g$, 4°C , in a fixed-angle rotor using a high-speed centrifuge.
11. Wash the pellet once in ~ 40 ml ice-cold mitochondrial suspension medium II, recentrifuge, and resuspend in ~ 10 ml of the same buffer.

See Time Considerations for information about storage of mitochondria prior to further processing.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocols (except where indicated). For common stock solutions see APPENDIX 2A; for suppliers see SUPPLIERS APPENDIX.

Cell homogenization medium (CHM)

To 100 ml H₂O add:

30 μl 1 M MgCl_2 (150 mM final)

0.15 g KCl (10 mM final)

2.0 ml 1 M Tris·Cl (APPENDIX 2A; 10 mM final)

Adjust pH to 6.7

Add H₂O to 200 ml

Store up to 1 to 2 days at 4°C

For CHM containing 1 M sucrose, add 68.4 g sucrose to 200 ml CHM.

Dithiothreitol (DTT) buffer

To 100 ml H₂O add:

0.31 g dithiothreitol (10 mM final)

20 ml 1 M Tris base (0.1 M final)

Adjust pH to 9.3 with HCl

Add H₂O to 200 ml

Store up to 1 to 2 days at 4°C

Heart wash buffer

To 500 ml H₂O add:

85.5 g sucrose (0.25 M final)

10 ml 1 M Tris·Cl (APPENDIX 2A; 10 mM final)

Adjust pH to pH 7.8

Add H₂O to 1 liter

Store up to 1 to 2 days at 4°C

Liver homogenization medium (LHM)

To 250 ml H₂O add:

18.2 g mannitol (0.2 M final)

8.55 g sucrose (50 mM final)

0.37 g KCl (10 mM final)

5.0 ml 100 mM Na_2EDTA (1 mM final)

50 ml 100 mM HEPES (10 mM final)

Adjust pH to 7.4 with KOH

Add H₂O to 500 ml

Store up to 1 to 2 days at 4°C

Mitochondrial suspension medium I

To 50 ml H₂O add:

8.5 g sucrose (0.25 M final)
1.0 ml 1 M Tris base (10 mM final)
Adjust pH to 7.0 with acetic acid
Add H₂O to 100 ml
Store up to 1 to 2 days at 4°C

Mitochondrial suspension medium II

To 100 ml H₂O add:

21.9 g mannitol (0.6 M final)
40 ml 100 mM HEPES (20 mM final)
Adjust pH to 7.4 with KOH
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

Muscle homogenization medium I

To 100 to <200 ml H₂O add:

6.84 g sucrose (0.1 M final)
0.686 g KCl (46 mM final)
20 ml 100 mM Na₂EDTA (10 mM final)
2.0 ml 1 M Tris·Cl (APPENDIX 2A; 10 mM final)
Adjust pH to 7.4
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C
Immediately before use, add 1.0 g bovine serum albumin (BSA; 0.5% w/v final)
and 40 mg Nagarse (0.2 mg/ml final)

Sigma now markets Nagarse under the name Protease Type VII.

Muscle homogenization medium II

To 100 ml H₂O add:

6.84 g sucrose (0.1 M final)
0.686 g KCl (46 mM final)
20 ml 100 mM Na₂EDTA (10 mM final)
2.0 ml 1 M Tris·Cl (APPENDIX 2A; 10 mM final)
Adjust pH to 7.4
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

Muscle wash buffer

To 100 ml H₂O add:

7.64 g mannitol (0.2 M final)
4.78 g sucrose (70 mM final)
0.2 ml 100 mM Na₂EDTA (0.1 mM final)
2.0 ml 1 M Tris·Cl (APPENDIX 2A; 10 mM final)
Adjust pH to 7.4
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

Spheroplast homogenization medium

To 100 ml H₂O add:

21.9 g mannitol (0.6 M final)
40 ml 100 mM HEPES (20 mM final)
Adjust pH to 7.4 with KOH

Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C
Immediately before use add 1 ml 200 mM phenylmethylsulfonyl fluoride
(PMSF; 1 mM final)

Sucrose/Mg²⁺ medium

To 100 ml H₂O add:
30 μl 1 M MgCl₂ (0.15 M final)
17.1 g sucrose (0.25 M final)
2 ml 1 M Tris·Cl (APPENDIX 2A; 10 mM final)
Adjust pH to 6.7
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

Sucrose/succinate solution (SS)

To 1.5 liter H₂O add:
256.5 g sucrose (0.25 M final)
0.81 g disodium succinic acid hexahydrate (1 mM final)
6.0 ml 100 mM Na₂EDTA (0.2 mM final)
30 ml 1 M Tris·Cl (APPENDIX 2A; 10 mM final)
Adjust to pH 7.8
Add H₂O to 3 liters
Store up to 1 to 2 days at 4°C

Yeast sorbitol buffer

To 100 ml H₂O add:
43.7 g sorbitol (1.2 M final)
40 ml 100 mM KH₂PO₄ (20 mM final)
Adjust pH to 7.4 with KOH
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

COMMENTARY

Background Information

Differential centrifugation is often used merely as a preliminary procedure to prepare a crude fraction of membranes or subcellular organelles prior to further purification in a density gradient. This technique separates particles according to their velocity of sedimentation (v), which depends principally on the diameter of the particle (d), although particle density (ρ_p) can also be important in some cases (e.g., nuclei). For the other parameters, ρ_l (the density of the liquid) and μ (its viscosity), are constant, and g is the applied centrifugal force.

$$v = \frac{d^2(\rho_p - \rho_l)}{18\mu}g$$

Table 3.3.1 gives the dimensions of some of the major particles in mammalian liver. Since v is proportional to d^2 , it is clear from these values that the sedimentation rate of the majority of nuclei is much greater than that of the

other particles, while only the largest mitochondria are likely to be separable from organelles such as lysosomes and peroxisomes. This is enhanced by the tendency of the larger mitochondria to be slightly more dense than lysosomes and peroxisomes. Thus centrifugation of a homogenate at 500 to 1000 $\times g$ for 5 to 10 min will pellet essentially all of the nuclei, a slightly higher relative centrifugal force (RCF) of 3000 $\times g$ for 10 min will pellet the “heavy” fraction of the mitochondria, and the considerably higher RCFs (e.g., 10,000 to 15,000 $\times g$) required to pellet all of the smallest mitochondria will also pellet all lysosomes and peroxisomes.

The heterogeneity in the size of different organelles is bound to compromise separations of particles on the basis of sedimentation rate, but differential centrifugation (as opposed to rate-zonal centrifugation) imposes a further problem that arises from the sedimentation path length of the rotor and the radial increase in g

Table 3.3.1 Size (d) and d^2 of Major Organelles from Rat Liver

Particle	Size (μm)	d^2
Nucleus	4-12	16-144
Plasma membrane sheet ^a	3-20	9-400
Golgi membranes	1-2	1-4
Mitochondria	0.4-2.5	0.16-6.25
Lysosomes	0.4-0.8	0.16-0.64
Peroxisomes	0.4-0.8	0.16-0.64
Most vesicles	0.05-0.3	0.0025-0.09

^aOnly organized tissues such as liver produce plasma membrane sheets under certain homogenization conditions.

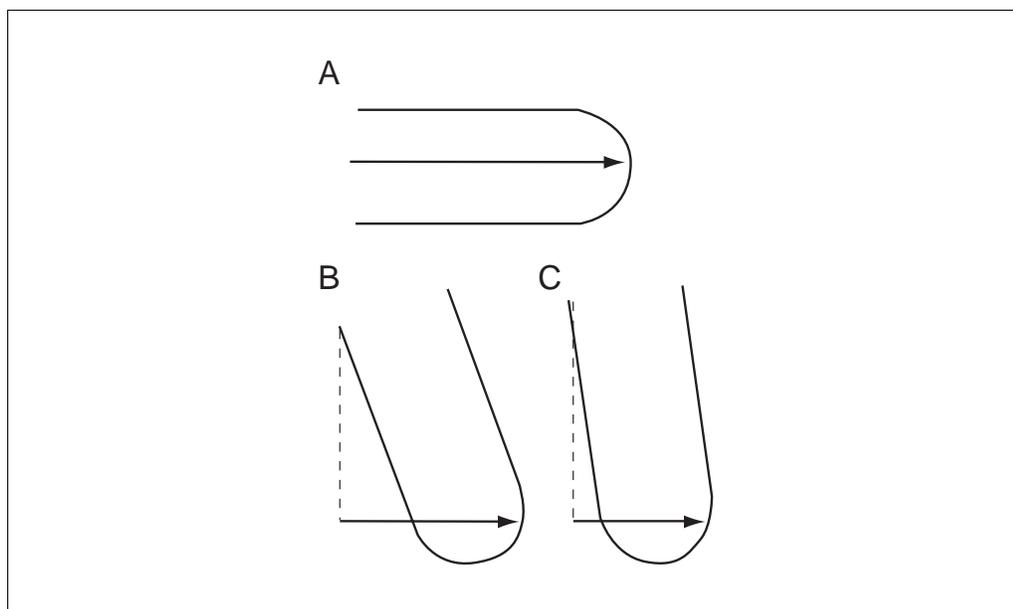


Figure 3.3.1 Sedimentation path length of rotors: (A) swinging-bucket; (B) high-angle fixed-angle; (C) low-angle fixed-angle. The sedimentation path length is indicated by the arrowed line.

force in the spinning rotor. If the homogenate fills the centrifuge tube, then the RCF at the top of the sample is considerably less than that at the bottom of the sample. On the other hand, particles at the top of the sample have the furthest distance to travel to form a stable pellet. The above equation shows that a particle with a diameter of 5 μm will sediment four times faster than one with a diameter of 2.5 μm and equal density. In such cases, all the smaller particles in the lower quarter of the centrifuge tube will sediment in the time taken for the larger particles, initially at the top of the tube, to pellet. Thus, in differential centrifugation, if a rotational speed and centrifugation time are chosen to allow all of the nuclei to pellet, then some the mitochondria towards the bottom of the tube—which are exposed to the highest RCF and have the shortest distance to travel—

will also pellet. It is for this reason that “washing” of a differential centrifugation pellet is a common practice; resuspending any pellet in medium and repeating the centrifugation will reduce contamination by the smaller particles. However, it will also reduce the yield of the particle of interest.

The sedimentation path length and the difference in RCF between the top and bottom of the sample are both less in a fixed-angle rotor than in a swinging-bucket rotor of the same tube volume (Fig. 3.3.1). Thus, differential centrifugation should always be carried out in a fixed-angle rotor for maximum resolution, and the lower the tube angle the better. However, as the tube angle decreases, the pellet tends to spread up the wall of the tube and become less compact and sometimes less stable, making removal of the supernatant more difficult. In practice,

therefore, a tube angle of $\sim 30^\circ$ is probably a good compromise. It might, therefore, be surprising that in all of the protocols presented here, the first low-speed centrifugation to remove the nuclei is often executed in a swinging-bucket rotor. In this case, the difference in the rate of sedimentation between the bulk of the nuclei and the mitochondria is so great that a fixed-angle rotor offers no significant advantage. More information on the use of differential centrifugation for the isolation of subcellular organelles can be obtained from Evans (1992), Graham (1997), and Hinton and Mullock (1997).

For studies on oxidative phosphorylation, mitochondria prepared by simple differential centrifugation are often preferred to those purified by gradient centrifugation. Here the aim is not to achieve high recoveries, but to prepare the organelles as rapidly and as gently as possible in a medium known to promote the retention of a highly coupled state. Hence, it is best to avoid the use of time-consuming density gradients, which may also expose the mitochondria to potentially damaging high g forces and unsuitable media. Bovine heart (see Basic Protocol 2) and the heavy fraction from rat liver (see Basic Protocol 1) are widely used for this purpose. See Rice and Lindsay (1997) for a review of methods for the measurement of functions associated with electron transport and oxidative phosphorylation and the preparation of submitochondrial particles.

Purification of subcellular organelles such as mitochondria has been given fresh impetus recently from the increasing number of projects aimed at mapping all proteins within a tissue (proteomics).

Critical Parameters and Troubleshooting

The composition of solutions used for isolating mitochondria is very much dependent on the source material. While a mannitol/sucrose-containing medium (see Basic Protocol 1) appears to offer good retention of functional integrity for liver mitochondria, it is not apparently a universal requirement. Although the inclusion of divalent cations (e.g., Mg^{2+}) is usually avoided (indeed, addition of EDTA or EGTA is often beneficial), the selection of a suitable homogenization medium may be dictated by the choice of material. This is most clearly the case with cultured cells, which may require a hypoosmotic medium to effect cell breakage; in these instances, Mg^{2+} and K^+ are

present to protect the nuclei from disruption. The medium used in Basic Protocol 4 is a good general-purpose medium for adherent cultured cells (Attardi and Ching, 1979) that may also be suitable for suspension cultured cells, its composition being not unlike that used by Goldberg and Kornfeld (1983) for isolating membrane compartments of the secretory system. The presence of KCl is quite compatible with the isolation of functionally intact mitochondria, and it is often included to solubilize proteins that tend to form gels in homogenates of cultured cells and skeletal muscle (Rice and Lindsay, 1997). However, some monolayer cells can be homogenized in an isoosmotic sucrose medium containing 0.25 M sucrose, 1 mM EDTA, 10 mM triethanolamine/acetic acid, pH 7.4 (Marsh et al., 1987; Graham, 1997). The efficacy of the medium is related to the particular buffer, for which there is a strict requirement. If a hypoosmotic Mg^{2+} -containing medium is used, the suspending medium must be made isoosmotic as soon as possible and the mitochondria suspended in a Mg^{2+} -free medium. Once the nuclei have been removed, it is acceptable to add EDTA to the postnuclear supernatant to chelate the Mg^{2+} .

Scaling up of procedures should always be accompanied by a proportional scaling up of the homogenization volume, but the volumes of buffer used for washing centrifugation pellets may be reduced. For example, in Basic Protocol 1, the ratio of liver to homogenization medium must be maintained at ~ 40 ml per 10 g of liver; for washing the $3000 \times g$ pellets, the volume of LHM can be reduced by about one third.

The centrifugation conditions used to isolate mitochondria from the postnuclear supernatant are tailored to individual tissues or cells; however, any RCF between 5,000 and $10,000 \times g$ is generally adequate, and the reason for using $26,000 \times g$ for bovine heart mitochondria (Smith, 1967) is not entirely clear. Lysosome and peroxisome contamination will tend to increase as the RCF increases, although the yields of mitochondria will also increase. The RCFs required for the pelleting of mitochondria from cultured cells may well depend on the type of cell and the homogenization schedule used. With this type of material, therefore, it may be prudent to determine the RCFs empirically by investigating the composition of pellets produced at a series of RCFs such as 500, 1000, 3000, 6000, 10,000, and $15,000 \times g$ (each for 10 min).

Respiratory studies on mitochondria are particularly demanding on the integrity of the product. Thus, at all stages in their preparation, great attention must be paid to the use of the mildest and gentlest of manipulations. It cannot be stressed too strongly that excessive shear forces during homogenization can be highly deleterious to these organelles, and the resuspension of mitochondrial pellets must be carried out with the very minimum of liquid shear forces.

Anticipated Results

The yield of succinate dehydrogenase in the heavy mitochondrial pellet from rat liver may be as much as 80% of the total, with relatively little contamination from lysosomes and peroxisomes. The contamination levels vary with the efficacy of the washing procedure, but 5% to 10% of the total is not uncommon. Levels are much higher in the unwashed material (20% to 25% of the total). Oxygen electrode studies on this fraction reveal that oxygen uptake in the presence of either succinate or glutamate plus malate is very low upon depletion of endogenous ADP, and that addition of exogenous ADP increases the rate of oxygen consumption by as much as ten to fifteen fold, indicating that they are highly coupled. Recoveries of mitochondria in the other protocols are routinely 90% to 95%. However, because the RCFs used to produce the mitochondrial pellet are higher, the contamination by peroxisomes and lysosomes is correspondingly higher (25% to 30%), and these levels are less easily reduced by washing.

Time Considerations

There are no points at which these relatively short protocols may be suspended. Indeed, rapid preparation is the key to the recovery of functionally intact mitochondria. All solutions can be made ahead of time and stored at 4°C for 1 to 2 days or frozen at -20°C for longer period (e.g., 2 to 3 months). Note that any enzymes, BSA, PMSF, or other protease inhibitors should be added immediately prior to use.

Basic Protocols 1, 3, and 4 should be accomplished in 2 to 3 hr, while Basic Protocol 5 will require ≥ 4 hr because of the two incubation steps. The time required for Basic Protocol 2 will depend on the availability of suitable large-capacity rotors; it may be necessary to split the material between two centrifugations, if the 2.2 liters cannot be accommodated in the rotor. Large-capacity rotors also decelerate slowly

(up to 15 min) so the whole procedure may take ~3.5 hr.

Functional assays should be carried out on the mitochondria as soon as possible after their preparation: this applies particularly to studies on oxidative phosphorylation and other measurements which rely on the permeability properties of the mitochondrial membranes. Storage for up to 4 hr at 0° to 4°C (<8 hr for heart mitochondria) is permissible but not recommended. Other functional assays (e.g., the succinate dehydrogenase assay described in *UNIT 3.4*) which are less demanding on structural integrity may be performed on material stored at 0° to 4°C for <16 h. Such assays and compositional measurements may also be carried out on material frozen to -20°C or -80°C. There are, however, no general rules regarding the suitability of a particular storage regime, and it is strongly recommended that the validity of results on stored material be checked by comparison with those made on the fresh organelles.

NOTE: If the mitochondria are to be further purified using one of the Basic Protocols described in *UNIT 3.4*, this must be carried out without delay.

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Purification of a Crude Mitochondrial Fraction by Density-Gradient Centrifugation

Metabolic studies of mitochondria are often carried out on organelles prepared by differential centrifugation (UNIT 3.3), avoiding the use of gradients, which extend the preparation time. However, to obtain high yields of mitochondria in a relatively pure form, or to determine the specificity of the localization of a particular component or function to mitochondria (as compared to other organelles), density-gradient centrifugation is the most obvious strategy. Most mitochondrial fractions produced by differential centrifugation, with the exception of the heavy fraction from some mammalian tissues (UNIT 3.3), are contaminated to a significant degree by other organelles—lysosomes, peroxisomes, tubular Golgi membranes, and small amounts of endoplasmic reticulum. The Basic Protocols in this unit are therefore aimed at the optimal resolution of mitochondria from these other membrane particles in density gradients that can be used either preparatively or analytically.

A continuous sucrose gradient system for mammalian liver mitochondria is described in Basic Protocol 1. Although discontinuous sucrose gradients seem to lack the resolution necessary for mammalian systems, they can be used for yeast (also see Basic Protocol 1). Because of the hyperosmotic nature of all sucrose gradient systems, they lack the resolution possible with media such as Nycodenz, iodixanol, and Percoll, which can form isoosmotic (or nearly isoosmotic) gradients. Basic Protocol 2 describes a discontinuous Percoll gradient as applied to the isolation of rat brain mitochondria. Although the Percoll gradient in this protocol is probably satisfactory for mitochondria from sources other than rat brain, an alternative discontinuous gradient designed for rat liver is also included (see Alternate Protocol). Basic Protocol 3 describes a self-generated Percoll gradient for rat liver mitochondria. Some of the variations of these Percoll-based methods are discussed later (see Commentary). Although iodixanol can also be used in self-generated mode, the g forces required are rather high ($\geq 180,000 \times g$); therefore, Basic Protocol 4 describes the use of a preformed continuous iodixanol gradient. All of the gradients covered in these protocols should be broadly applicable to most material sources; however, because the sedimenting properties and density of organelles from different tissues and cells can vary, it may be necessary to modulate either the density profile of the gradient or the centrifugation conditions.

With the exception of Basic Protocol 2 (for rat brain), the methods do not include homogenization schedules, and any differential centrifugation is designed for mammalian liver. Thus, the homogenization medium is also used for all stages of the differential centrifugation and for suspending the final crude mitochondrial fraction. This is often not the case with other material such as cultured cells, skeletal muscle, or yeast (UNIT 3.3). Therefore, the strategy and media recommended in UNIT 3.3 should be used to prepare crude mitochondria from sources other than mammalian liver.

It should be pointed out that the precise composition of the homogenization medium, the medium used to suspend the crude mitochondrial pellet, and the medium used to prepare the gradients from Percoll and OptiPrep (Basic Protocols 2-4 and Alternate Protocol) can be adapted to subsequent operational requirements. For example, because Percoll itself has no significant osmotic activity, Reinhart et al. (1982) were able to use medium containing 0.21 M mannitol, 60 mM sucrose, 10 mM succinate, 10 mM KCl, 1 mM ADP, 0.25 mM DTT, 10 mM HEPES-KOH, pH 7.4, throughout the entire procedure. As OptiPrep has an osmolality of approx 170 mOsm, it could also be diluted with a similar medium. Such media doubtless have many beneficial effects on the retention of respiratory competence by the organelles. Because of the high concentrations of sucrose used in the gradients of Basic Protocol 1, however, the use of mannitol in the homogenization and differential centrifugation stages is

probably of little benefit, although the inclusion of KCl, ADP, DTT in all the solutions might be worthwhile. If the aim of the gradient purification is to identify the localization of some functional or compositional parameter or it is a preliminary process to some subsequent extraction, then the use of a simple general-purpose medium throughout the preparation is probably satisfactory. In the following protocols therefore basic general-purpose media containing either sucrose or mannitol, a chelating agent, and an organic buffer have generally been recommended, but these may be adjusted by the investigator to include low concentrations ≤ 10 mM) of substrates, co-factors, and sulphhydryl reagents. Note, also, that the relative centrifugal force (RCF) used to pellet this mitochondrial fraction can be between 6,000 and 20,000 $\times g$, as the purpose of the gradient is to resolve mitochondria from contaminating lysosomes, peroxisomes, and Golgi, if present.

Support Protocols describe simple assays for succinate dehydrogenase (see Support Protocol 1), β -galactosidase (see Support Protocol 2), and catalase (see Support Protocol 3), which have been adapted specifically for the detection of mitochondria, lysosomes, and peroxisomes, respectively, in gradient fractions, but they can be used for any mitochondrial fraction.

NOTE: For all protocols, all g values are given as g_{au} .

BASIC PROTOCOL 1

RESOLUTION OF A RAT LIVER MITOCHONDRIAL FRACTION IN A CONTINUOUS SUCROSE GRADIENT

This protocol, adapted from Diczfalusy and Alexson (1988) and Vamecq and Van Hoof (1984), was designed for mitochondria from mammalian liver. A crude light mitochondrial fraction should be suspended in a medium that is suitable to mitochondria, but there is little merit in using a mannitol-containing medium, as the mitochondria will be exposed to high hyperosmotic concentrations of sucrose in the gradient. The protocol is best carried out on Triton WR1339-treated rats (see Background Information) in order to reduce the density of the lysosomes. Optimal resolution in these sucrose gradients is carried out in a large-volume vertical rotor. Although this protocol is designed for mitochondria from rat liver, it can be modified for other mammalian tissues and for yeast. In the latter case, discontinuous sucrose gradients are used in a swinging-bucket rotor according to the method of Beauvoit et al. (1989).

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: Supplements (see recipe) to the media used in this protocol are optional and can be added to any or all of the solutions.

Materials

- General-purpose homogenization medium (GHM; see recipe), ice cold
- Sucrose gradient solutions (see recipe)
- Sucrose cushion (see recipe)
- Protease inhibitors (optional; see recipe)

- Dounce homogenizer (5- to 10-ml with loose-fitting, Wheaton type B pestle)
- Low-speed centrifuge with swinging-bucket rotor and appropriate tubes
- High-speed centrifuge with fixed-angle rotor and appropriate tubes
- Gradient maker (two-chamber or Gradient Master)
- Ultracentrifuge (e.g., Beckman VTi 50 with vertical rotor) and 39-ml tubes
- Gradient unloader (optional)

Additional reagents and equipment for preparing homogenates (*UNIT 3.3*)

1. Prepare rat liver homogenate as described in *UNIT 3.3* (Basic Protocol 1, steps 1 to 9), but use ice-cold GHM instead of LHM.

For sources other than mammalian liver it may be necessary to use a different homogenization medium (see UNIT 3.3), in which case GHM should be used only for resuspending the final crude mitochondrial pellet (step 4 below).

2. Pellet nuclei by centrifuging 5 min at $1000 \times g$, 4°C , in a swinging-bucket rotor using a low-speed centrifuge. If desired, wash the nuclei once to maximize the recovery of mitochondria.
3. Pellet mitochondria from the postnuclear supernatant by centrifuging 10 min at $15,000 \times g$, 4°C in a fixed-angle rotor using a high-speed centrifuge.
4. Resuspend the mitochondrial pellet in ~ 8 ml ice-cold GHM using 3 to 4 gentle strokes of the Dounce homogenizer.
5. Using a two-chamber gradient maker, prepare linear 28-ml sucrose gradients from 34% and 64% (w/v) sucrose gradient solutions in 39-ml tubes for a Beckman VTi 50 ultracentrifuge. Underlay 4 ml sucrose cushion in each tube.

Alternatively, a Gradient Master can be used to prepare the gradients.

6. Layer 7 to 8 ml sample on top of the gradients and centrifuge 65 min at $170,000 \times g$, 4°C .

For a discontinuous gradient (yeast), layer 11 ml each of 30%, 40%, and 55% (w/v) sucrose gradient solutions (see recipe) in 38-ml centrifuge tubes for an ultracentrifuge swinging-bucket rotor (e.g., Beckman SW28 or 28.1, or Sorvall AH 629). Then layer 5 ml sample on top of the gradients and centrifuge 20 to 30 min at $150,000 \times g$, 4°C . For 17-ml tubes, use 5 ml of each sucrose solution and 2 ml mitochondrial fraction.

7. Collect the gradient in 1- to 2-ml fractions using a gradient unloader or harvest the brownish mitochondrial band just above the middle of the tube (continuous gradient).

For discontinuous gradients, harvest mitochondria from the 40%/55% interface.

8. Dilute sample(s) with ≥ 2 vol ice-cold GHM.
9. Centrifuge 20 min at $20,000 \times g$, 4°C .
10. Resuspend pellet(s) in ice-cold GHM or another suitable medium at 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some assays, such as those in Support Protocols 1 to 3, can be carried out after storage at 4°C for < 16 hr. Many assays can be carried out on material frozen to -20°C or -80°C ; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with measurements made on fresh material.

ISOLATION OF MITOCHONDRIA FROM RAT BRAIN USING A DISCONTINUOUS PERCOLL GRADIENT

Methods for isolating rat brain mitochondria rarely rely solely on differential centrifugation as a purification process, probably because of contamination by synaptosomes and myelin. This particular purification is thus aimed at the isolation of nonsynaptosomal mitochondria. The method, derived from Sims (1990), appears to work optimally when ≥ 500 mg of tissue is processed. A discontinuous Percoll gradient for liver is described in the Alternate Protocol.

BASIC PROTOCOL 2

Subcellular Fractionation and Isolation of Organelles

3.4.3

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: Supplements (see recipe) to the media used in this protocol are optional and can be added to any or all solutions.

Materials

150- to 200-g Sprague-Dawley rats
Brain homogenization medium (BHM; see recipe), ice cold
Percoll solutions in BHM: 15% (v/v), 23% (v/v), and 40% (v/v) (see recipe)
Protease inhibitors (optional; see recipe)
10-ml Dounce homogenizer with tight-fitting and loose-fitting pestles (Wheaton type A and B, respectively)
Low-speed centrifuge with swinging-bucket rotor and appropriate tubes
High-speed centrifuge with fixed-angle rotor and appropriate tubes
Ultracentrifuge with swinging-bucket rotor for 12-ml tubes

Prepare homogenate

1. Starve 150- to 200-g Sprague-Dawley rats overnight and sacrifice by decapitation.
This must be supervised or carried out by an experienced animal technician.
2. Dissect out brain tissue, place ~500 mg in ice-cold BHM, and wash in three changes of this medium.
3. After mincing the tissue finely with scissors, suspend it in ~5 ml BHM and homogenize using five up-and-down strokes with the loose-fitting pestle of a 10-ml Dounce homogenizer, followed by ten more up-and-down strokes with the tight-fitting pestle.
4. Centrifuge 3 min at $1300 \times g$, 4°C, in a swinging-bucket rotor using a low-speed centrifuge. Decant and retain the supernatant.
5. Resuspend the pellet up to the original volume with BHM and rehomogenize using 10 strokes with the tight-fitting pestle.
6. Repeat centrifugation, decant the supernatant, and combine it with the first (step 4).

Isolate mitochondria

7. Centrifuge 10 min at $21,000 \times g$, 4°C, in a fixed-angle rotor using a high-speed centrifuge.
8. Discard the supernatant and resuspend the pellet in 5 ml of 15% Percoll solution.
9. In a suitable tube for a swinging-bucket rotor, layer 4.0 ml each of 23% and 40% Percoll solutions in BHM. Layer 3 ml mitochondrial suspension on top and centrifuge 5 min at $31,000 \times g$, 4°C.
Add additional BHM on top if necessary to fill the tube.
10. Harvest the mitochondria that band at the lowest interface, and dilute with 4 vol ice-cold BHM.
11. Centrifuge 10 min at $17,000 \times g$, 4°C in a fixed-angle rotor.

12. Remove the supernatant and resuspend the mitochondrial pellet as appropriate at 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some assays, such as those in Support Protocols 1 to 3, can be carried out after storage at 4°C for <16 hr. Many assays can be carried out on material frozen to -20°C or -80°C; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with measurements made on fresh material.

ISOLATION OF MITOCHONDRIA FROM RAT LIVER USING A DISCONTINUOUS PERCOLL GRADIENT

ALTERNATE PROTOCOL

A similar discontinuous Percoll gradient, covering a slightly higher density range, has been used by Reinhart et al. (1982) to isolate a mitochondrial fraction from a total rat liver homogenate; this approach eliminates the resuspension of a crude mitochondrial pellet which may damage the organelles. Presumably to avoid contamination of the gradient by erythrocytes, the authors perfused the liver with a standard balanced salt medium containing 1.3 mM CaCl₂. Alternatively a post-nuclear supernatant could be used from an unperfused liver (as described in this protocol). The method is restrictive inasmuch as only 2 ml of homogenate can be processed on a single gradient, and the authors used only the median lobe of the liver in a total homogenate volume of ~10 ml. The method is adapted from Reinhart et al. (1982).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care of laboratory animals.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: Supplements (see recipe) to the media used in this protocol are optional and can be added to any or all solutions.

Materials

150- to 200-g Sprague-Dawley rats

Mannitol-sucrose homogenization medium (MSHM; see recipe), ice-cold

Percoll solutions in MSHM: 19%, 31%, 42%, and 52% (v/v) (see recipe)

Protease inhibitors (optional; see recipe) can be added to any or all of the solutions

Low-speed centrifuge with swinging-bucket rotor and appropriate tubes

High-speed centrifuge with fixed-angle rotor (e.g., Sorvall SS34) with ~14-ml tubes

Additional reagents and equipment for preparing liver homogenate (*UNIT 3.3*)

1. Prepare rat liver homogenate from the median liver lobe as described in *UNIT 3.3* (Basic Protocol 1, steps 1 to 9), but use ice-cold MSHM (10 ml) instead of LHM.

For sources other than mammalian liver it may be necessary to use a different homogenization medium (see UNIT 3.3), in which case only use MSHM to prepare the Percoll solutions.

2. Pellet the nuclei by centrifuging 5 min at 1000 × g, 4°C, in a swinging-bucket rotor using a low-speed centrifuge.
3. Remove the postnuclear supernatant into a beaker and keep on ice
4. In a tubes for the ultracentrifuge swinging-bucket rotor layer 3 ml of each of the 31%, 42% and 52% Percoll solutions and 2 ml each of the 19% Percoll and post nuclear supernatant.

Subcellular Fractionation and Isolation of Organelles

3.4.5

5. Centrifuge at $36,000 \times g$ for 30 sec at speed.

The acceleration time should be ~1.5 min and the deceleration time ~3 min.

6. Collect the mitochondria that band at the lowest interface and dilute with 4 vol of MSHM. Centrifuge 10 min at $17,000 \times g$, 4°C , in a fixed-angle rotor.
7. Remove the supernatant and resuspend the mitochondria as appropriate at 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some assays, such as those in Support Protocols 1 to 3, can be carried out after storage at 4°C for <16 hr. Many assays can be carried out on material frozen to -20°C or -80°C ; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with measurements made on fresh material.

BASIC PROTOCOL 3

RESOLUTION OF A MITOCHONDRIAL FRACTION IN A SELF-GENERATED PERCOLL GRADIENT

A mitochondrial fraction prepared by differential centrifugation is loaded on to a 30% (v/v) solution of Percoll containing 225 mM mannitol and any other additives that may be required for the retention of respiratory activity. Gradient formation is normally carried out in a fixed-angle rotor at $95,000 \times g$. The protocol for rat liver mitochondria is adapted from Hovius et al. (1990).

Mitochondria band at ~ 1.09 g/ml in Percoll. It is important that the differential centrifugation fraction that is used contain as little endoplasmic reticulum (ER) as possible, as the density of mitochondria and ER tend to overlap quite significantly in these gradients. It is therefore recommended that the centrifugation speed be no more than $10,000 \times g$ to produce the crude mitochondrial fraction.

Compared to the discontinuous Percoll gradients described in Basic Protocol 2 and Alternate Protocol, the centrifugation time for self-generated gradients is considerably longer, but the time taken in the preparation of the gradients is much reduced and considerably more simple to execute. Self-generated gradients may therefore be the method of choice for large numbers of samples. The gradients that are formed by self-generation are also highly reproducible. Alternate Protocol allows the use of a whole homogenate but this necessarily restricts the amount of mitochondria which can be purified on one gradient.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: Supplements (see recipe) to the media used in this protocol are optional and can be added to any or all solutions.

Materials

Mannitol buffer A (see recipe), ice cold
30% (v/v) Percoll solution (see recipe), ice cold
Protease inhibitors (optional; see recipe)

Low-speed centrifuge with swinging-bucket rotor and appropriate tubes (~ 30 ml)
5- and 30-ml Dounce homogenizers with loose-fitting pestles (Wheaton type B)
High-speed centrifuge with fixed-angle rotor and appropriate tubes (~ 30 ml)
Ultracentrifuge with fixed-angle rotor (e.g., Beckman 60 Ti or Sorvall T-1250) and appropriate tubes

Additional reagents and equipment for preparing homogenates (UNIT 3.3)

Isolate light mitochondrial fraction

1. Prepare rat liver homogenate as described in *UNIT 3.3* (Basic Protocol 1, steps 1 to 9), but use ice-cold mannitol buffer A instead of LHM.

For sources other than mammalian liver it may be necessary to use a different homogenization medium (see UNIT 3.3), in which case mannitol buffer A should be used only for resuspending the final crude mitochondrial pellet (step 9 below).

2. Centrifuge homogenate 10 min at $1000 \times g$, 4°C , in a swinging-bucket rotor using a low-speed centrifuge.
3. Aspirate supernatant into new centrifuge tubes and set aside.
4. Resuspend pellets in ~ 20 ml ice-cold mannitol buffer A using two to three gentle strokes in a 30-ml Dounce homogenizer.
5. Centrifuge 10 min at $1000 \times g$, aspirate supernatant, and combine with the supernatant in step 3.
6. Centrifuge supernatants 15 min at $10,000 \times g$, 4°C , in a fixed-angle rotor using a high-speed centrifuge.
7. Discard supernatant and resuspend pellet in ~ 20 ml ice-cold mannitol buffer A as in step 4.
8. Centrifuge 15 min at $10,000 \times g$, 4°C , and discard the supernatant.
9. Resuspend the light mitochondrial pellet in 5 ml mannitol buffer A using 2 to 3 gentle strokes in a 5-ml Dounce homogenizer.

Purify mitochondria

10. Transfer 20 ml of 30% Percoll solution into each of four tubes for a suitable fixed-angle rotor (e.g., a Beckman 60Ti or Sorvall T-1250) and layer the resuspended mitochondria on top.
11. Centrifuge 30 min at $95,000 \times g$, 4°C . During deceleration below 1000 rpm, either switch off the brake or use a controlled deceleration program (if available on the ultracentrifuge).
12. Collect the gradient in 1- to 2-ml fractions.

The lower part of the dense brown/yellow band contains the bulk of the purified mitochondria; once their position has been confirmed, they can, in future separations, be recovered simply by aspiration. A syringe and long metal cannula is the best tool for this task.

13. Dilute the gradient fraction(s) with ≥ 2 vol ice-cold mannitol buffer A.
14. Pellet organelles by centrifuging 10 min at $6300 \times g$, 4°C , in a fixed-angle rotor using a high-speed centrifuge.
15. Wash the mitochondrial pellet at least twice with ~ 10 ml of this buffer and resuspend in a suitable medium at 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some assays, such as those in Support Protocols 1 to 3, can be carried out after storage at 4°C for <16 hr. Many assays can be carried out on material frozen to -20°C or -80°C ; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with measurements made on fresh material.

**RESOLUTION OF A RAT LIVER MITOCHONDRIAL FRACTION IN AN
IODIXANOL GRADIENT**

Although this protocol is carried out with a light mitochondrial fraction, any appropriate differential centrifugation fraction can be used. The fraction is adjusted to $\rho = 1.177$ g/ml, layered beneath a shallow linear gradient of iodixanol $\rho = 1.124$ to 1.162 g/ml, and centrifuged 2 hr at $110,000 \times g$ in a swinging-bucket rotor. As an alternative (if preferred), the mitochondrial fraction can be layered on top in the homogenization medium.

This protocol has been devised for mammalian liver mitochondria in a general-purpose medium. Any of the mitochondrial fractions from other sources covered in *UNIT 3.3* should behave in a similar manner, although the detail of the organelle banding may vary slightly and require modulation for optimal results. See Commentary for variations of this separation strategy.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout.

NOTE: Supplements (see recipe) to media used in this protocol are optional and can be added to any or all solutions.

Materials

- Iodixanol buffer A (see recipe), ice cold
- 50% (w/v) iodixanol solution: 1 vol iodixanol buffer B (see recipe) in 5 vol OptiPrep (60% iodixanol; Nycomed Pharm, Life Technologies, Accurate Chemicals, Mediatech)
- 19% and 27% iodixanol solutions: dilute 50% iodixanol with iodixanol buffer A
- Protease inhibitors (optional; see recipe)
- Low-speed centrifuge with swinging-bucket rotor and appropriate tubes
- 5- and 20-ml syringes and metal cannulae (i.d. ~ 0.7 mm)
- 5- and 30-ml Dounce homogenizers with loose-fitting pestles (Wheaton type B)
- High-speed centrifuge with fixed-angle rotor and appropriate tubes
- Ultracentrifuge with swinging-bucket rotor (e.g., Beckman SW 28.1 or Sorvall AH-629) and appropriate tubes
- Refractometer (optional)
- Gradient maker: two-chamber or Gradient Master
- Additional reagents and equipment for preparing homogenates (*UNIT 3.3*)

Isolate mitochondrial fraction

1. Prepare rat liver homogenate as described in *UNIT 3.3* (Basic Protocol 1, steps 1 to 9), but use ice-cold iodixanol buffer A instead of LHM.

For sources other than mammalian liver it may be necessary to use a different homogenization medium (see UNIT 3.3), in which case iodixanol buffer A should be used only for resuspending the final crude mitochondrial pellet (step 10 below).

2. Centrifuge homogenate 10 min at $1000 \times g$, 4°C , in a swinging-bucket rotor using a low-speed centrifuge.
3. Transfer the supernatant to new centrifuge tubes using a 20-ml syringe and metal cannula, and set aside.
4. Resuspend pellets in ~ 20 ml ice-cold iodixanol buffer A using two to three gentle strokes in a 30-ml Dounce homogenizer.

5. Centrifuge 10 min at $1000 \times g$, remove supernatant, and combine with the supernatant in step 3.
6. Centrifuge supernatants 10 min at $3000 \times g$, 4°C , in a fixed-angle rotor using a high-speed centrifuge.

This step can be omitted if the heavy mitochondrial fraction is not required as a separate fraction or if the source material does not exhibit such a fraction.
7. Remove supernatants and centrifuge 15 min at $15,000 \times g$, 4°C .
8. Discard supernatant and resuspend pellet in ~ 20 ml ice-cold iodixanol buffer A using three to four gentle strokes in the 30-ml Dounce homogenizer.
9. Centrifuge 15 min at $15,000 \times g$, 4°C , and discard the supernatant.
10. Resuspend the light mitochondrial pellet in 3 ml iodixanol buffer A using 2 to 3 gentle strokes in a 5-ml Dounce homogenizer.

Purify by Iodixanol gradient

11. Mix the suspension with 4.5 ml of 50% iodixanol solution by repeated inversion.

Do not shake the tube vigorously to create foaming. If the sample is going to be loaded on top of the gradient rather than beneath it, then omit this step and the next.
12. Check that the refractive index is $1.3864 (\pm 0.0004)$ and adjust with 50% iodixanol solution or iodixanol buffer A, if necessary.
13. Prepare a linear iodixanol gradient in a ~ 17 -ml centrifuge tube for a swinging-bucket rotor (e.g., a Beckman SW28.1 or a Sorvall AH-629) using 6 ml each of 19% and 27% iodixanol solution.

If the gradient is prepared with a Gradient Master, use an 80° angle at 20 rpm and a time of 1 min 50 sec.
14. Underlayer the gradient with 3 ml sample (step 12), using a 5-ml syringe and a metal cannula.
15. Overlay the gradient with 1 ml iodixanol buffer A.

Alternatively, underlayer the gradient with 1 ml of 30% iodixanol (3 vol iodixanol stock solution plus 2 vol iodixanol buffer A), and then layer 3 ml sample on top of the gradient.
16. Centrifuge 1.5 hr at $110,000 \times g$, 4°C .
17. Collect the gradient in 1-ml fractions starting at the low-density end, using upward displacement with a dense liquid. Alternatively, collect the mitochondria that band just above the middle of the gradient using a syringe and cannula.
18. Dilute the sample with ≥ 2 vol iodixanol buffer A.
19. Centrifuge 20 min at $20,000 \times g$, 4°C , and resuspend the pellet in this buffer or another suitable medium at 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some assays, such as those in Support Protocols 1 to 3, can be carried out after storage at 4°C for <16 hr. Many assays can be carried out on material frozen to -20°C or -80°C ; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with measurements made on fresh material.

SUCCINATE DEHYDROGENASE ASSAY FOR MITOCHONDRIA

Although any component of the tricarboxylic acid cycle can be used as a functional marker for mitochondria, the most frequently used marker is succinate dehydrogenase with either the native electron acceptor (cytochrome *c*) or the artificial acceptor *p*-iodonitrotetrazolium violet (INT). Measuring the reduction of cytochrome *c* ideally requires a recording spectrophotometer, while the INT variant can be performed with a nonrecording instrument. INT is also far more stable than cytochrome *c* and avoids the use of cyanide, which is used to prevent reoxidation of cytochrome *c*. The INT method presented here is from Graham (1993).

Sample handling depends on the type of gradient and the concentration of mitochondria (and other organelles) in the fractions (see Commentary). Ideally, the protocol should be carried out on gradient fractions from which the gradient solute or Percoll has been removed by centrifugating as described in the final steps of each Basic Protocol. The gradient fractions should be resuspended in 0.25 M sucrose/10 mM HEPES-NaOH, pH 7.4, or an equivalent buffer.

Materials

Mitochondrial gradient fraction (see Basic Protocols 1 to 4)
Succinate solution (see recipe)
INT solution (see recipe)
Stop solution I (see recipe)
Spectrophotometer and glass cuvettes

1. Place 0.3 ml succinate solution in 1.5- to 2.0-ml microcentrifuge tubes, including a set of tubes to provide blanks for each fraction. Perform the assay in duplicate.

As long as the protein content of each assay is approximately the same, a blank for each fraction may not be necessary. A single blank containing buffer instead of sample may suffice for many gradients. Alternatively, blanks may be prepared by adding stop solution to the gradient fraction before adding INT solution in step 3.

2. Add 10 to 20 μ l of each gradient fraction to separate sample tubes. Incubate samples and blanks 10 min at 37°C.

The sample should contain ~20 μ g protein.

3. Add 0.1 ml INT solution and incubate for another 10 to 20 min at 37°C.
4. Terminate the reaction by adding 1 ml stop solution I.
5. Remove any precipitate by microcentrifuging for 2 min at maximum speed.
6. Using glass cuvettes, measure the absorbance at 490 nm against a suitable blank.
7. Calculate the enzyme activity as μ moles INT reduced per milligram protein.

The molar extinction coefficient of reduced INT is 19,300 $\text{cm}^{-1}\text{M}^{-1}$.

β -GALACTOSIDASE ASSAY FOR LYSOSOMES

β -Galactosidase is one of several glycosidases that can be used to detect lysosomes. The assay is facilitated by the availability of *p*-nitrophenol derivatives of a number of monosaccharides, which mimic the native oligosaccharide substrates. It is linked to a simple spectrophotometric measurement of the released nitrophenol. The method is as described by Graham (1993).

Sample handling depends on the type of gradient and the concentration of lysosomes (and other organelles) in the fractions (see Commentary). Ideally, the protocol should be carried out on gradient fractions from which the gradient solute or Percoll has been removed by

centrifugation as described in the final steps of each Basic Protocol. The gradient fractions should be resuspended in 0.25 M sucrose/10 mM HEPES-NaOH, pH 7.4, or an equivalent buffer.

Materials

Mitochondrial gradient fraction (see Basic Protocols 1 to 4)
Substrate solution (see recipe)
Stop solution II (see recipe)
Spectrophotometer and plastic cuvettes

1. Add 20 to 40 μ l of each gradient fraction (containing \sim 30 μ g protein) to separate microcentrifuge tubes containing 0.5 ml substrate solution. Perform the assay in duplicate.
2. Prepare a blank for each fraction by adding 1 ml stop solution II before adding the gradient fraction.

As long as the protein content of each assay is approximately the same, a blank for each fraction may not be necessary. A single blank containing buffer instead of sample may suffice for many gradients.

3. Incubate test samples 30 min at 37°C.
4. Add 1 ml stop solution II to test samples.
5. Remove any precipitate by microcentrifuging 1 to 2 min at maximum speed.
6. Using plastic cuvettes, measure the absorbance of tests at 410 nm against the chosen blank(s).

The molar extinction coefficient of nitrophenol is 9620 $\text{cm}^{-1}\text{M}^{-1}$.

CATALASE ASSAY FOR PEROXISOMES

The titanium oxysulfate method, adapted from Baudhuin (1974), is the method of choice for peroxisomes and provides very reliable data. It is more sensitive than the permanganate-based method and is certainly easier to execute.

In peroxisomes from rat liver, catalase is an extremely active enzyme, and purified fractions from gradients may require dilution of up to 100 fold. Assays are usually carried out at 0°C. Sample handling depends on the type of gradient and the concentration of peroxisomes (and other organelles) in the fractions (see Commentary). Ideally, the protocol should be carried out on gradient fractions from which the gradient solute or Percoll has been removed by centrifuging as described in the final steps of each Basic Protocol. The gradient fractions should be resuspended in 0.25 M sucrose/10 mM HEPES-NaOH, pH 7.4, or an equivalent buffer.

CAUTION: Exercise care when handling titanium oxysulfate, as this reagent is extremely corrosive.

Materials

Mitochondrial gradient fraction (see Basic Protocols 1 to 4)
Stock peroxide solution (see recipe)
Tris/BSA solution (see recipe)
Titanium oxysulfate reagent (see recipe)
Sample buffer (see recipe)
Spectrophotometer

NOTE: Carry out all operations on ice in 2-ml microcentrifuge tubes.

SUPPORT PROTOCOL 3

Subcellular Fractionation and Isolation of Organelles

3.4.11

1. Prepare substrate mixture by diluting 8.5 ml stock peroxide solution to 100 ml with Tris/BSA solution and keep at 0°C.
2. Add 1.0 ml titanium oxysulfate reagent to 0.5 ml substrate mixture and measure the absorbance at 405 nm.

A₄₀₅ should be ~1.5; if it is not, adjust the concentration of hydrogen peroxide accordingly.

3. Mix 10 µl gradient fraction with 30 µl sample buffer. Also set up a reagent control using 40 µl sample buffer. Perform assay in duplicate.

With mammalian liver, because it is necessary to dilute the gradient fractions 10 to 100 fold, it is never necessary to set up blanks for each fraction. A single reagent blank is adequate. If a sample blank is necessary, replace 0.5 ml substrate mixture with buffer.

Catalase activities in fractions from other sources may be much lower; test the pregradient mitochondrial fraction first.

4. Add 0.5 ml substrate mixture to all tubes. Do this in batches of 6 tubes, adding the assay mixture to successive tubes at timed 10-sec intervals.
5. After exactly 1 min, add 1.0 ml titanium oxysulfate reagent (also at 10-sec intervals).
6. Remove any precipitate by microcentrifuging 2 min at maximum speed, 4°C.
7. Transfer the tubes to room temperature and measure the absorbance at 405 nm against a blank containing 0.5 ml Tris/BSA solution and 1.0 ml titanium oxysulfate reagent.
8. Calculate the activity of the enzyme by deducting the test absorbance of each gradient fraction from that of the reagent control (see Commentary).

Note that catalase is estimated by "back-titration"; i.e., the residual H₂O₂ is measured in the assay mixture after being acted on by the enzyme.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocols (except where indicated). For common stock solutions see APPENDIX 2A; for suppliers see SUPPLIERS APPENDIX.

Brain homogenization medium (BHM)

To 100 ml H₂O add:

- 21.9 g sucrose (0.32 M final)
- 2.0 ml 100 mM K₂EDTA (1 mM final)
- 2.0 ml 1 M Tris·Cl (APPENDIX 2A; 10 mM final)
- Adjust pH to 7.4
- Add H₂O to 200 ml
- Store up to 1 to 2 days at 4°C

BHM, 2×

To 50 ml H₂O add:

- 21.9 g sucrose (0.64 M final)
- 2.0 ml 100 mM K₂EDTA (2 mM final)
- 2.0 ml 1 M Tris·Cl (APPENDIX 2A; 20 mM final)
- Adjust to pH 7.4
- Add H₂O to 100 ml
- Store up to 1 to 2 days at 4°C

Citrate/phosphate buffer

Dissolve 0.71 g Na₂HPO₄ in 100 ml H₂O (50 mM final). Dissolve 0.96 g citric acid in another 100 ml H₂O (50 mM final). Add Na₂HPO₄ to citric acid to reach pH 4.3. Store up to 1 to 2 days at 4°C

General-purpose homogenization medium (GHM)

To 100 ml H₂O add:

- 17.1 g sucrose (0.25 M final)
- 2.0 ml 100 mM Na₂EDTA (1 mM final)
- 2.0 ml 1 M Tris-Cl (*APPENDIX 2A*; 10 mM final)
- Adjust pH to 7.4
- Add H₂O to 200 ml
- Store up to 1 to 2 days at 4°C

Iodixanol buffer A

To 100 ml H₂O add:

- 17.1 g sucrose (0.25 M final)
- 2.0 ml 100 mM Na₂EDTA (1 mM final)
- 20 ml 100 mM HEPES (10 mM final)
- Adjust pH to 7.4
- Add H₂O to 200 ml
- Store up to 1 to 2 days at 4°C

Iodixanol buffer B

To 50 ml H₂O add:

- 8.55 g sucrose (0.25 M final)
- 6.0 ml 100 mM Na₂EDTA (6 mM final)
- 60 ml 100 mM HEPES (60 mM final)
- Adjust pH to 7.4
- Add H₂O to 100 ml
- Store up to 1 to 2 days at 4°C

p-Iodonitrotetrazolium violet (INT) solution

- 25 mg INT (2.5 mg/ml final)
- Phosphate buffer, pH 7.4 (see recipe), to 10 ml
- Store up to 2 to 3 months at -20°C

Mannitol buffer A

To 100 ml H₂O add:

- 9.13 g mannitol (0.25 M final)
- 38 mg EGTA (0.5 mM final)
- 0.2 g bovine serum albumin (BSA; 0.1% w/v final)
- 10 ml 100 mM HEPES (5 mM final)
- Adjust pH to 7.4
- Add H₂O to 200 ml
- Store 1 to 2 days at 4°C

Mannitol buffer B

To 100 ml H₂O add:

- 16.4 g mannitol (0.45 M final)
- 152 mg EGTA (2.0 mM final)
- 0.4 g BSA (0.2% w/v final)
- 100 ml 100 mM HEPES (50 mM final)
- Adjust pH to 7.4
- Add H₂O to 200 ml
- Store up to 1 to 2 days at 4°C

Mannitol-sucrose homogenization medium (MSHM)

To 100 ml H₂O add:
7.64 g mannitol (0.21 M final)
4.10 g sucrose (60 mM final)
0.15 g KCl (10 mM final)
2.0 ml 1 M HEPES-KOH (10 mM final)
Adjust to pH 7.4
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

MSHM, 4×

To 50 ml H₂O add:
15.3 g mannitol (0.21 M final)
8.2 g sucrose (60 mM final)
0.3 g KCl (10 mM final)
4.0 ml 1 M HEPES-KOH (10 mM final)
Adjust to pH 7.4
Add H₂O to 100 ml
Store up to 1 to 2 days at 4°C

Percoll solution, 30% (v/v)

3 vol Percoll
2 vol water
5 vol mannitol buffer B (see recipe)
Store up to 1 to 2 days at 4°C

Percoll solutions in BHM

Prepare a 50% (v/v) Percoll stock solution by adding 20 ml Percoll to 20 ml 2× BHM (see recipe). Store up to 1 to 2 days at 4°C
To prepare gradient solutions, dilute 50% Percoll stock with BHM to provide 15%, 23%, and 40% (v/v) Percoll. Store up to 1 to 2 days at 4°C

Percoll solution in MSHM

Prepare a 75% (v/v) Percoll solution by adding 30 ml Percoll to 10 ml of 4× MSHM (see recipe). Store up to 1 to 2 days at 4°C
To prepare gradient solutions, dilute Percoll (75% v/v) stock with MSHM to provide 19%, 31%, 42%, and 52% (v/v) Percoll. Store up to 1 to 2 days at 4°C

Phosphate buffer, pH 7.4

Dissolve 0.71 g Na₂HPO₄ in 100 ml water (50 mM final). Dissolve 0.68 g KH₂PO₄ in another 100 ml water (50 mM final). Add KH₂PO₄ to Na₂HPO₄ to reach pH 7.4.
Store up to 1 to 2 days at 4°C

Protease inhibitors

Add the following to solutions as required so that the final concentrations are 1 mM for PMSF and 2.0 μg/ml for each of the other inhibitors.

PMSF:

0.348 g phenylmethylsulfonyl fluoride (200 mM final)
10 ml dried propan-2-ol or ethanol
Store up to 2 to 3 months at 4°C

Antipain:

10 mg antipain (1 mg/ml final)
10 ml 10% (v/v) dimethyl sulfoxide (DMSO)
Store in aliquots up to 2 to 3 months at -20°C

Aprotinin:

10 mg aprotinin (1 mg/ml final)

10 ml H₂O

Store in aliquots up to 2 to 3 months at -20°C

Leupeptin:

10 mg leupeptin (1 mg/ml final)

10 ml of 10% (v/v) dimethyl sulfoxide (DMSO)

Store in aliquots up to 2 to 3 months at -20°C

Sample buffer

2 g Triton X-100 (2% w/v final)

H₂O to 100 ml

Mix 1 vol of this solution with 2 vol Tris/BSA solution (see recipe)

Store up to 1 to 2 days at 4°C

Stock peroxide solution

0.67 g 30% (w/w) H₂O₂ (30 mM final)

Tris/BSA solution (see recipe) to 200 ml

Prepare fresh

Stop solution I

10 g trichloroacetic acid

50 ml ethyl acetate

50 ml ethanol

Store up to 1 month at room temperature

Stop solution II

To 50 ml H₂O add:

1.87 g glycine (0.25 M final)

25 ml 1.0 M NaOH (0.25 M final)

Adjust pH to 10 (if necessary)

Add H₂O to 100 ml

Store up to 1 to 2 days at 4°C

Substrate solution

90 mg *o*-nitrophenyl β-D-galactopyranoside (6 mM final)

0.25 g Triton X-100 (0.5% w/v final)

Citrate/phosphate buffer (see recipe) to 50 ml

Store up to 1 to 2 days at 4°C

Succinate solution

0.27 g sodium succinic acid hexahydrate (10 mM final)

Phosphate buffer, pH 7.4 (see recipe), to 100 ml

Adjust to pH 7.4 if necessary

Store up to 1 to 2 days at 4°C

Sucrose cushion (continuous gradient only)

67 g sucrose (67% w/w final)

Dissolve the sucrose in 33 ml of 10 mM Tris·Cl, pH 7.4

Store up to 1 to 2 days at 4°C

Sucrose gradient solutions

Dilute 70% (w/v) sucrose stock (see recipe) with sucrose stock diluent (see recipe) to provide 34% and 64% (w/v) sucrose (continuous gradient); or 30%, 40%, and 54% (w/v) sucrose (discontinuous gradient)

Sucrose stock diluent

1.0 ml 100 mM Na₂EDTA (1 mM final)
1.0 ml 1 M Tris-Cl stock (10 mM final)
H₂O to 100 ml
Adjust pH to 7.4 after adding half the water
Store 1 to 2 days at 4°C

Sucrose stock solution, 70% (w/v)

70 g sucrose (70% w/v final)
1.0 ml 100 mM Na₂EDTA (1 mM final)
1.0 ml 1 M Tris-Cl stock (10 mM final)
H₂O to 100 ml
Adjust pH to 7.4 after adding most of the water
Store 1 to 2 days at 4°C

Supplements

0.27 g sodium succinic acid hexahydrate (10 mM final)
50 mg ADP (potassium salt) (1 mM final)
3.8 mg DTT (0.25 mM final)

Any or all of the above may be added to 100 ml of the homogenization, suspension, and gradient media, before adjusting the pH.

Titanium oxysulfate reagent

Dissolve 5.56 ml of 18 M H₂SO₄ in ~90 ml H₂O (1 M final). Add 0.225 g titanium oxysulfate (2.25 g/liter final). Bring to 100 ml with H₂O. Prepare fresh.

CAUTION: Both the acid and the titanium oxysulfate are highly corrosive. Exercise great care when adding pure H₂SO₄ to water. Use a face mask and protective gloves (not disposable plastic gloves, as any splashes will cause them to "melt" and adhere to the skin).

Tris/BSA solution

To 100 ml H₂O add:
0.2 g bovine serum albumin (1 g/liter final)
4.0 ml 1.0 M Tris-Cl (APPENDIX 2A; 20 mM final)
Adjust pH to 7.0
Add H₂O to 200 ml
Prepare fresh

COMMENTARY

Background Information

As a general rule, metabolic studies on liver, heart, or muscle mitochondria are carried out on organelles prepared by differential centrifugation (see UNIT 3.3). Density-gradient separations, although undoubtedly able to provide a more pure preparation, are frequently avoided simply because the speed of preparation is the prime consideration. With the exception of the heavy mitochondrial fraction (UNIT 3.3), however, these preparations contain significant contamination by other organelles (lysosomes, per-

oxisomes), sometimes by tubular Golgi elements (if present in the homogenate), and also by some of the more rapidly sedimenting endoplasmic reticulum vesicles. Such contamination can only be reduced by gradient centrifugation. The use of higher relative centrifugal forces (RCFs) to sediment the mitochondria from the postnuclear supernatant results in higher contamination. Gradients are also very useful if analytical work is to be carried out to determine the localization of a particular component or function. In this case, the distribution

Table 3.4.1 Density (g/ml) of Major Organelles from Mammalian Liver in Iodixanol, Sucrose, and Percoll

Particle	Density in iodixanol	Density in sucrose	Density in Percoll
Nuclei	1.23-1.25	>1.32	NA ^a
Peroxisomes	1.18-1.21	1.19-1.23	1.075-1.085
Mitochondria	1.14-1.16	1.17-1.21	1.085-1.100
Lysosomes	1.11-1.13	1.19-1.21	1.105-1.111
Golgi	1.03-1.08	1.05-1.12	1.03-1.105

^aNot available.

of the component or function in the gradient is compared with known markers for the different organelles.

Table 3.4.1 compares the density of mammalian liver mitochondria and other organelles in sucrose, iodixanol, and Percoll. In sucrose, the density of mitochondria overlaps that of lysosomes and peroxisomes. As mitochondria (and lysosomes) sediment through a sucrose gradient, their densities rise to a limiting value as water is removed from their enclosed spaces by the high and rising osmolarity of the gradient. In other media that are true solutes (e.g., Nycodenz and iodixanol) and that can provide gradients of much lower osmolarity, the density of these organelles are lower and more distinctive (Ford et al., 1983; Graham et al., 1990, 1994). Peroxisomes present an exception to this rule; because they do not have an osmotic space in these media, their density is almost identical in sucrose, Nycodenz, and iodixanol (Graham et al., 1994). Although both Percoll and iodixanol can provide isoosmotic gradients over the entire density range of these organelles, the densities of organelles in Percoll are lower. In iodinated density-gradient media, the density of the major organelles always increases in the order lysosomes, mitochondria, peroxisomes. In Percoll the order is sometimes completely reversed (Symons and Jonas, 1987), and in other cases the mitochondria are denser than the lysosomes (Singh et al., 1987). The reason for these differences is not clear. Hence, the popularity of Percoll methods whose efficiency relies as much on the higher sedimentation rate of the mitochondria as on their banding density.

The continuous sucrose gradient system in a vertical rotor used by Vamecq and Van Hoof (1984) can resolve mitochondria and peroxisomes reasonably well. Lysosomes, however, band broadly between these two organelles unless their density can be reduced by prior injection of the animals with Triton WR1339 (85 mg/100 g body weight) 3 to 5 days before

sacrifice (Leighton et al., 1968). Diczfalusy and Alexson (1988) used this approach, which makes the lysosomes significantly less dense than the mitochondria. A discontinuous sucrose gradient system was developed by Beauvoit et al. (1989) for yeast and might be applicable to other source materials (see Basic Protocol 1).

A major advantage of using Percoll for the density purification of mitochondria is that the medium forms self-generated gradients at relatively low *g* forces. Thus, the centrifugation conditions that are required are not unlike those used for purification through preformed gradients of other gradient media. One of the problems of using Percoll, however, is the significant overlap of mitochondria and endoplasmic reticulum (ER; Patel et al., 1991). The method of Hovius et al. (1990) uses a relatively low RCF (10,000 × *g* for 10 min) to produce the crude mitochondrial fraction in order to reduce the ER content (see Basic Protocol 3). In an alternative approach, Reinhart et al. (1982) separated the mitochondria principally on the basis of sedimentation rate in a preformed four-step discontinuous gradient of 19%, 31%, 42%, and 52% (v/v) Percoll (Alternate Protocol). Centrifugation was carried out at 37,000 × *g* for just 30 sec, so that the ER vesicles did not have time to reach their banding density. Although this method permits the use of a whole homogenate, only 2 ml can be loaded onto a single 11-ml gradient. A similar sedimentation rate approach was used by Sims (1990) for rat brain mitochondria (see Basic Protocol 2), and the slightly longer centrifugation time of 5 min is probably easier to standardize. Because of the speed of these Percoll systems, the mitochondria tend to display good respiratory control.

Generally speaking, although Percoll gradients can be tailored to the isolation of a particular organelle (e.g., mitochondria), they are less well suited to producing relatively pure fractions of other organelles simultaneously. Both Nycodenz and iodixanol are better suited to this

purpose. A linear gradient of iodixanol from 10% to 30% (w/v) was first developed by Graham et al. (1994), in which the crude mitochondrial fraction was loaded in 35% iodixanol beneath the gradient and centrifuged at $\sim 50,000 \times g$ for 1.5 hr. Although this may be a good starting point, it is now recognized that a more shallow gradient (19% to 27%) is probably better for resolving the mitochondria and lysosomes, and indeed this range of gradient concentration is not unlike the discontinuous metrizamide system used by Wattiaux et al. (1983). Note that the density of liver mitochondria in iodixanol is slightly lower than in Nycodenz (Graham et al., 1994).

A significant advantage of iodixanol (and Nycodenz) over Percoll is that, as long as the organelles are sufficiently concentrated in the gradient fractions, the gradient solute does not have to be removed prior to enzyme analysis using visible wavelength spectrophotometry. Because of the light-scattering properties of Percoll, this medium must be removed prior to such analysis, and since the RCFs required to pellet both Percoll particles and organelles are similar, significant loss of organelles can occur (Osmundsen, 1982).

Critical Parameters and Troubleshooting

Density-gradient centrifugation

See *UNIT 3.3* for information regarding the preparation of crude mitochondrial fractions by differential centrifugation.

The preparation of a preformed continuous gradient (see Basic Protocols 1 and 4) using a two-chamber gradient maker requires considerable practice to achieve highly reproducible gradients; some of the important points in operating such devices are covered by Dobrota and Hinton (1992). A Gradient Master, which involves simply layering the most dense and lightest solutions in the centrifuge tube, provides a more expensive but reproducible alternative. If neither of these are available, a discontinuous gradient constructed from three or four solutions spanning the required density range can be prepared and allowed to diffuse. Note that the time required for complete linearity to be achieved will depend on the tube diameter and the volume of each layer. Often this carried out at 4°C overnight without any consideration as to whether the timing is optimal. Always collect a blank gradient and check

(by refractive index) that the gradient is indeed linear and covers the appropriate density range.

Although the sample is layered under the iodixanol gradient (see Basic Protocol 4), overlaying can be used as an alternative and may even be beneficial if the separation of the organelles needs to be carried out partly on the basis of sedimentation rate (as with Basic Protocol 2). However, with overlaying, care may be required to avoid overloading the gradient, especially if the top of the gradient is adjusted to a lower density in order to customize the method to a particular source material. Overloading occurs when the total density of the sample layer (liquid plus particles) is greater than that of the top of the gradient; as a consequence, the sample falls through the gradient as a droplet. This is very unlikely to occur in Basic Protocol 1, as the top of the gradient is quite dense.

Self-generated gradients (see Basic Protocol 3) form optimally in tubes of relatively short sedimentation path length. Use of a rotor and gradient volume other than those recommended will mean that the density profile of the gradient, and consequently the separation of the organelles, will be altered. For self-generated Percoll gradients, fixed-angle rotors with an angle of $\sim 24^\circ$ appear to be the best suited.

It is possible that the density of organelles from other sources (e.g., cultured cells) may be sufficiently different for sucrose gradients to provide some degree of resolution. Poor resolution of organelles may occur as a result of an inappropriate density gradient, but it is just as likely that the basic problem lies with the earlier homogenization and differential centrifugation. A homogenate containing 0.25 M sucrose/10 mM HEPES-NaOH, pH 7.4/1 mM EDTA is suitable; the presence of KCl is permissible, but buffers containing Mg^{2+} should be avoided. If Mg^{2+} is essential for the homogenization of a cultured cell, then the mitochondria should be treated as described in *UNIT 3.3*. It is particularly difficult to predict the precise density of organelles separated from cells that have been homogenized in hypoosmotic conditions. In such circumstances, the Basic Protocols should only be regarded as a suitable starting point.

Enzyme assays

Prior to the execution of an enzyme assay (or other measurement), it is common practice

to remove the gradient medium from each gradient fraction by dilution with 2 to 4 volumes of a low-density medium, centrifugation to pellet the organelles, and resuspension in the same low-density medium. The latter is often the homogenization medium or, if it is isoosmotic, a medium used for the subsequent assay. This practice eliminates any possible interference of the gradient medium on the subsequent procedure(s), and it is described in the final steps of each Basic Protocol. In many instances, however, the sample volumes used in the assay are sufficiently small (as in the Support Protocols), that the gradient solute is sufficiently diluted not to cause any interference in the assay, even at the highest density. Thus, so long as the concentration of organelles in the gradient fraction is high enough for the enzyme activity to be detectable, the assay may be performed directly without washing. If, however, either of these requirements are not met, then dilution, pelleting, and resuspension of the pellet in a smaller volume are unavoidable. In the case of Percoll gradients, it is essential to remove the gradient medium prior to any spectrophotometric analysis to avoid variable interference from light scattering. It should also be pointed out that during these postgradient manipulations losses of functional activity can occur. If the assays are carried out in microcentrifuge tubes (as in the Support Protocols) a convenient strategy, which minimizes these losses, is to dilute and centrifuge (maximum speed) the organelles from an appropriate volume of gradient fraction and to use the resuspended pellet directly in the assay.

The only notable source of error in the enzyme assays themselves concerns the β -galactosidase assay. The stop solution for this assay should not have a pH >10; higher pH causes hydrolysis of the substrate.

In order to determine specific activities of measured enzymes, it is also necessary to estimate the protein content of fractions. Most gradient media interfere to varying extents with methods using the Folin-Ciocalteu reagent, although as with the enzyme assays, if by using small volumes ($\leq 100 \mu\text{l}$) of fraction in a 1 to 2 ml assay volume the concentration of gradient solute in the assay solution is <10% (w/v), the interference is marginal. Methods based on Coomassie blue are generally unaffected by any concentration of gradient solute, although because of light-scattering

problems, it is always advantageous to remove any Percoll.

With the sample volumes used in Support Protocols 1 to 3, the gradient solute is sufficiently diluted to not cause any inhibition of enzyme activity, even at the highest density. If the gradient fraction must be concentrated, the organelles must be pelleted after dilution of the fraction with 2 volumes of an isoosmotic buffer (buffered 0.25 M sucrose is normally adequate). As the assays are carried out in microcentrifuge tubes, a good and convenient strategy is to pellet the particles in a microcentrifuge for 15 to 20 min at 4°C, then suspend the pellet directly in the enzyme assay buffer.

Anticipated Results

Basic Protocol 1

With rat liver, Triton WR1339 shifts the density of lysosomes in sucrose to ~1.15 to 1.17 g/ml (~0.4 g/ml lower than in the normal animal; Table 3.4.1), while mitochondria and peroxisomes are unchanged at 1.17 to 1.21 g/ml and 1.19 to 1.23 g/ml, respectively. Typical separations that might be achieved from normal and Triton-treated animals are shown in Figure 3.4.1 and Figure 3.4.2, respectively. The lysosomes in the Triton WR1339-treated animals are clearly and significantly shifted to lower densities (Diczfalusy and Alexson, 1988) compared to those from the untreated animal (Vamecq and Van Hoof, 1984).

Basic Protocol 2 and Alternate Protocol

In the case of liver mitochondria (Reinhart et al., 1982), ~60% of total mitochondria band at the lowest interface. The major contamination is from peroxisomes and lysosomes (~10% of total; see Table 3.4.2), whereas there is essentially no contamination from either endoplasmic reticulum or plasma membrane. All the enzyme markers for organelles other than mitochondria are broadly distributed across the other interfaces. Although the corresponding band from rat brain (Sims, 1990) only contains ~12% of the total mitochondria, many of these are present in synaptosomes, which band at a much lower density. The author found only 0.3% of the total lactate dehydrogenase and 0.1% of the total 2',3'-cyclic nucleotide-3'-phosphodiesterase in the bottom interfacial material, attesting to very low contamination by synaptosomes or myelin, respectively.

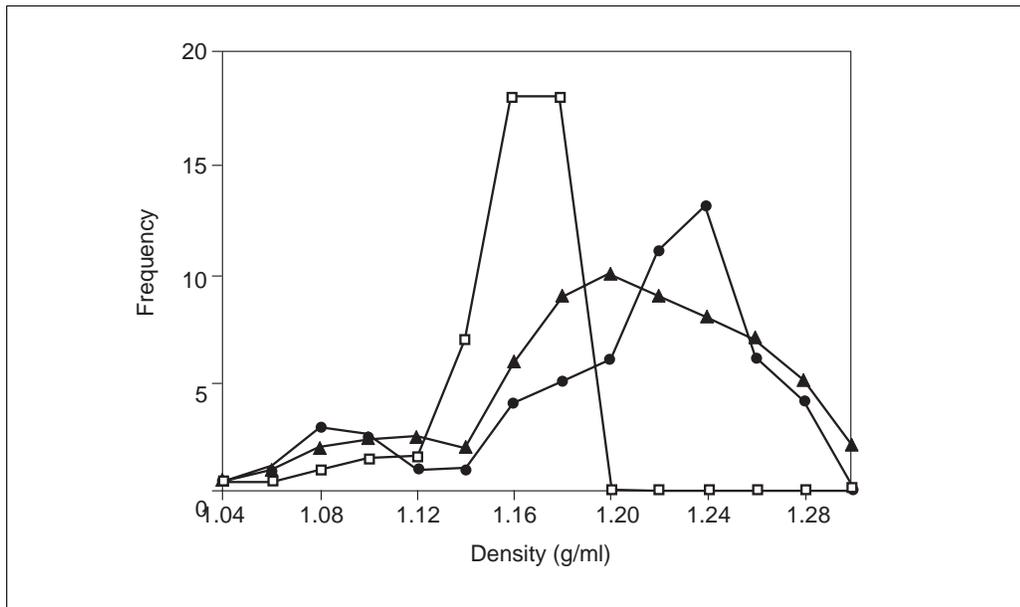


Figure 3.4.1 Frequency distribution of enzyme markers after separation of a crude mitochondrial fraction in a sucrose gradient. Cytochrome *c* oxidase (mitochondria; open squares); *N*-acetyl- β -glucosaminidase (lysosomes; filled triangles); catalase (peroxisomes; filled circles). Data from Vamecq and Van Hoof (1984). Frequency is the amount of enzyme in a fraction divided by the density interval covered by the fraction.

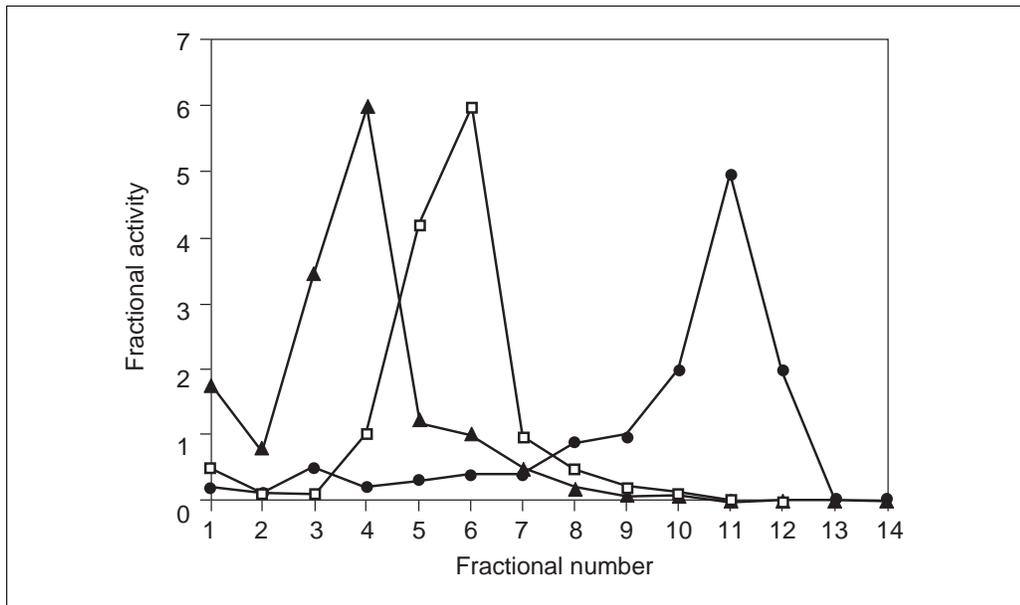


Figure 3.4.2 Fractional activity of enzyme markers after separation of a crude mitochondrial fraction from Triton WR1339-treated rats. Cytochrome *c* oxidase (mitochondria; open squares); *N*-acetyl- β -glucosaminidase (lysosomes; filled triangles); catalase (peroxisomes; filled circles). Fraction 1 is the top of the gradient; data from Diczfalusy and Alexson (1988).

Basic Protocol 3

An ~5-fold enrichment of succinate dehydrogenase is expected in the mitochondrial band over the homogenate, while marker enzymes characteristic of contaminating organelles are significantly depleted (Hovius et al., 1990).

Basic Protocol 4

Figure 3.4.3 describes a typical separation with rat liver on a preformed iodixanol gradient. Fractions 6 to 8 in the middle of the gradient contain >50% of the total mitochondria recovered from the gradient. This gradient also provides an excellent enrichment of lysosomes in fraction 2, and although the peroxisomes are rather broadly banded in the bottom of the gradient, the bottom five fractions contain very little contaminating enzyme markers. This gra-

dient would therefore be very useful analytically as well as preparatively.

Time Considerations

There are no obvious points at which any of the Basic Protocols can be discontinued. Preformed gradients should be made up during the initial differential centrifugation of the homogenate and kept on ice until required. If they are prepared at room temperature, they must be given time to cool down to 4°C. The enzyme marker assays need not be carried out immediately; overnight storage of mitochondria at 4°C is permissible unless metabolic studies are also to be carried out. Enzyme markers can be measured on previously frozen material, but any concentration of the organelle fractions and/or removal of gradient medium must be carried out prior to freezing.

Table 3.4.2 Marker Enzyme Content of a Rat Liver Mitochondrial Fraction after Discontinuous Percoll Gradient Separation^a

Enzyme marker	Percent of total recovered
Cytochrome <i>c</i> oxidase	55
Glucose-6-phosphatase	2
5'-Nucleotidase	4
Acid phosphatase	8
Catalase	11

^aData from Reinhart et al. (1982).

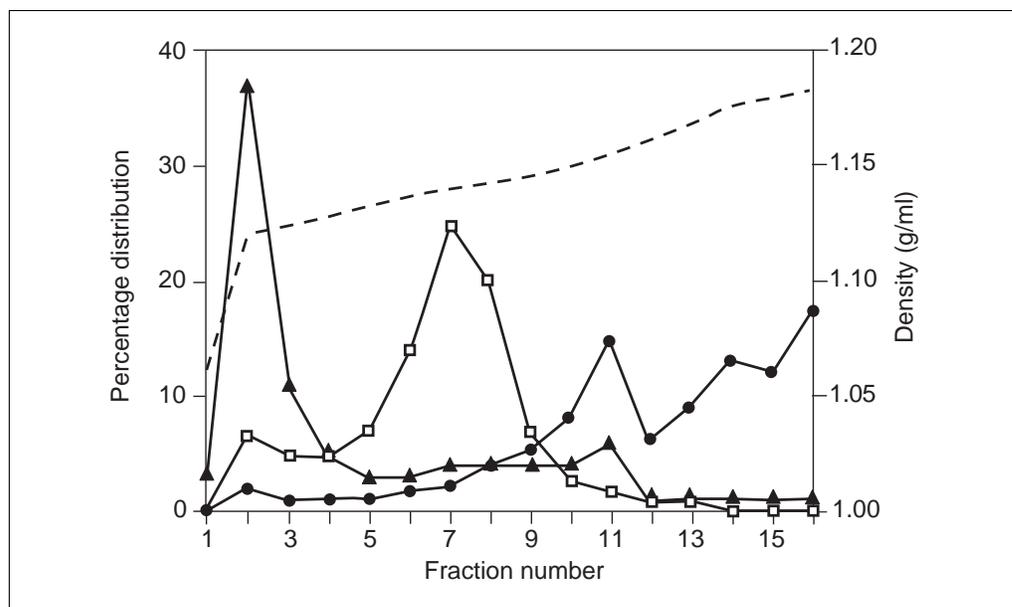


Figure 3.4.3 Percent distribution of enzyme markers after separation of a crude mitochondrial fraction in a preformed continuous iodixanol gradient. Succinate dehydrogenase (mitochondria; open squares); β -galactosidase (lysosomes; filled triangles); catalase (peroxisomes; filled circles).

The figures for the total time (given below) required to complete the gradient separation of mitochondria, include preparation of the homogenate (but omit the optional step of removing the gradient medium) and are calculated for a single rat liver. It is assumed that any preparation of either preformed or continuous gradients is carried out during the periods of differential centrifugation. With the exception of Basic Protocol 2 and Alternate Protocol, total preparation time is probably 3.5 to 4.5 hr. The very short gradient centrifugation times in Basic Protocol 2 and Alternate Protocol mean that overall times are reduced to 2 to 3 hr and 1 to 2 hr respectively. Although the number of pre-gradient operations in Alternate Protocol are also reduced, time has to be taken for preparing a larger number of multi-step discontinuous gradients.

Harvesting the banded organelles from the gradients with a syringe and metal cannula only takes a few minutes, while complete unloading of a single tube into a series of equivolume fractions will take 15 min per tube. Removal of the medium, by washing the pellet once, will add a further 0.5 to 1.0 hr depending on the number of fractions being handled.

All solutions can be made up ahead of time and stored at 4°C for 1 to 2 days or frozen to -20°C for longer periods (2 to 3 months). However, note that any enzymes, BSA, PMSF, or other protease inhibitors should be added immediately prior to use.

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Isolation of Peroxisomes from Tissues and Cells by Differential and Density Gradient Centrifugation

Almost without exception, the purification of peroxisomes from a mammalian tissue homogenate is carried out in two stages: (1) differential centrifugation to produce a light mitochondrial fraction and (2) resolution of the light mitochondrial fraction in some form of density gradient. Iodinated density gradient media (e.g., iodixanol) are preferred for the second phase of the purification (see Commentary). Moreover, because the difference in density between mitochondria and peroxisomes is greater in iodixanol than in Nycodenz, and because the osmolarity of the gradients can be more easily controlled with iodixanol, it is the medium of choice. The much wider use of Nycodenz merely reflects the fact that it has been commercially available for more than 15 years, while iodixanol (OptiPrep) has only been available since 1994.

The unit first describes the homogenization and differential centrifugation process for the most commonly used source material—rat liver (see Basic Protocol 1). This protocol serves as a preliminary methodology for all of the density gradient separations subsequently outlined. Next described is a preformed, continuous iodixanol gradient system for isolation of peroxisomes (see Basic Protocol 2). Iodixanol can also be used as a self-generated gradient (see Alternate Protocol 1). Methods are also presented for the use of Nycodenz: a protocol for a continuous Nycodenz gradient (see Basic Protocol 3) and for a simple Nycodenz barrier (see Alternate Protocol 2) are included. The relative merits of these protocols are discussed in the Commentary. There are a large number of published methods based on Nycodenz and there is variation in both the precise density range covered by the gradients and the centrifugation conditions; some of these are described in the italicized annotations to the appropriate steps of the Nycodenz protocols.

Lastly, the unit describes the isolation of peroxisomes from yeast (see Basic Protocol 4) and cultured monolayer cells (see Basic Protocol 5). In the case of mammalian cultured cells, the differential centrifugation system is truncated and a post-nuclear supernatant is used as the gradient input.

After collecting the banded material in the gradients, the success of the fractionation is routinely measured by assaying for various marker enzymes: catalase (peroxisomes), succinate dehydrogenase (mitochondria), and acid phosphatase or a glycosidase such as β -galactosidase (lysosomes). These assays are provided in *UNIT 3.4*. Although the endoplasmic reticulum (ER) is not a major contaminant of the light mitochondrial fraction, some ER is always present, and therefore an assay for a suitable marker such as NADPH-cytochrome *c* reductase or glucose-6-phosphatase is recommended. This problem becomes more significant if a post-nuclear fraction is loaded on to the gradient.

NADPH-cytochrome *c* reductase is ubiquitously found in the ER, while glucose-6-phosphatase is only present in significant amounts in the ER from liver and kidney. NADPH-cytochrome *c* reductase is also considerably easier to measure than is glucose-6-phosphatase; a method is included for assay of this oxidoreductase (see Support Protocol).

**ISOLATION OF A LIGHT MITOCHONDRIAL FRACTION FROM RAT
LIVER**

A standard homogenization medium such as 0.25 M sucrose/1 mM EDTA/10 mM HEPES-NaOH, pH 7.4, is satisfactory for a tissue such as liver, and may be chosen particularly if other organelles are to be isolated from the same homogenate. However if the aim is solely to isolate peroxisomes, the medium is supplemented with 0.1% ethanol to aid the recovery of functionally intact organelles, and the preferred buffer is often MOPS rather than HEPES (or Tris). Sometimes 1 to 5 mM KCl is also included, and the supplementation of any medium with protease inhibitors is at the discretion of the investigator. A standard three-step differential centrifugation system is described, although some workers use a two-step system (see Commentary for more information).

The following protocol is suitable for a single 10- to 12-g liver.

NOTE: All protocols using live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to government regulations regarding the care and use of laboratory animals.

Materials

- 150- to 200-g Sprague-Dawley rats
- Homogenization medium (HM; see recipe)
- Protease inhibitors (optional; see recipe) added to any or all solutions at concentrations indicated in recipe
- Dissecting equipment
- Overhead high-torque electric motor (thyristor-controlled) for homogenizers
- Potter-Elvehjem homogenizer (clearance ~0.09 mm), 25-ml working volume, prechilled
- Dounce homogenizer (loose-fitting Wheaton type B pestle, ~30 ml), prechilled
- Dounce homogenizer (loose-fitting Wheaton type B pestle, ~5 ml), prechilled
- Low-speed refrigerated centrifuge with swinging-bucket rotor accommodating 50-ml tubes
- 40- to 50-ml polycarbonate centrifuge tubes
- High-speed refrigerated centrifuge with fixed-angle rotors accommodating 50-ml tubes (e.g., Sorvall SS-34)

NOTE: All solutions, glassware, centrifuge tubes and equipment should be pre-cooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature. When handling the glass vessel of the Potter-Elvehjem homogenizer, a thermally insulated glove or silicone rubber hand protector should be used, not only to avoid heat transfer from the skin but also to protect the hand in the unlikely event of breakage of the vessel.

Isolate liver

1. Starve the animal overnight.
2. Sacrifice the animal by cervical dislocation or decapitation.
This must be supervised or carried out by an experienced animal technician.
3. Open the abdominal cavity and remove the liver to a chilled beaker containing ~20 ml of ice-cold HM.
4. Decant the medium and finely mince the liver using scissors.

The pieces of liver should be no more than ~25 mm³ each.

Homogenize liver

5. Suspend the mince in ~40 ml of HM and transfer half of this suspension to the glass vessel of the Potter-Elvehjem homogenizer.

The motor should be mounted either to a wall, via a G-clamp to the bench, or in a floor-standing cradle. Attachment to a free-standing retort stand is not adequate.

6. Attach the cold pestle to the electric motor and homogenize the liver mince using 4 to 5 up-and-down strokes of the pestle, rotating at ~500 rpm.
7. Decant the homogenate into a beaker on ice, and after rinsing the homogenizer with medium and wiping the pestle to remove any connective tissue that may be adhering, repeat steps 5 and 6 with the other half of the mince and combine the homogenates.

Isolate light mitochondrial fraction

8. Centrifuge the homogenate 10 min at $750 \times g$, 0° to 4°C , in the low-speed centrifuge.

Steps 8 to 12 are omitted in the two-step protocol (see Commentary). Proceed directly to step 13, centrifuging the homogenate from step 7 in the high-speed centrifuge at $3500 \times g$.

9. Decant and retain the supernatant.
10. Resuspend the pellet in 20 ml of HM, first using a glass rod and then more completely using 3 to 4 gentle strokes of the pestle of the 30-ml Dounce homogenizer.
11. Centrifuge, decant, and retain the supernatant (steps 8 and 9).
12. Combine the supernatants from steps 9 and 11 and transfer to 40- to 50-ml polycarbonate tubes.
13. Centrifuge 10 min at $3500 \times g$, 0° to 4°C , in the high-speed centrifuge.
14. Decant and retain the supernatant.

In the two-step method the pellet from this step should be resuspended in HM as in step 10, centrifuged at $3500 \times g$ as in step 13, the supernatant decanted as in step 14, and the two supernatants combined.

Fractionate liver homogenate

15. Centrifuge the $3500 \times g$ supernatant(s) from step 14 in the high-speed centrifuge, 20 min at $23,000 \times g$, 0° to 4°C .
16. Decant and discard the supernatant.

If a microsomal fraction is required for complete bookkeeping, centrifuge this supernatant plus the supernatant from step 18 for 45 min at $100,000 \times g$.

17. Resuspend the light mitochondrial pellet (LMP) in HM (~20 ml) using a glass rod followed by 3 to 4 gentle strokes of the pestle of the 30-ml Dounce homogenizer.
18. Recentrifuge 20 min at $23,000 \times g$, 0° to 4°C , and discard the supernatant.
19. Resuspend the LMP in HM (~6 ml) using a glass rod and then 3 to 4 gentle strokes of the pestle of the 5-ml Dounce homogenizer (do this in two batches).

This is equivalent to ~0.5 ml per gram of liver tissue. Use this for all subsequent density-gradient separations of rat liver peroxisomes.

ISOLATION OF PEROXISOMES FROM A RAT LIVER LIGHT MITOCHONDRIAL FRACTION USING A PREFORMED CONTINUOUS IODIXANOL GRADIENT

In the past, both metrizamide (e.g., Völkl and Fahimi, 1985; Wanders et al., 1986) and Nycodenz gradients (see Basic Protocol 3) have been used to purify peroxisomes from mammalian liver, but improved resolution can be achieved with iodixanol gradients (Graham et al., 1994; Van Veldhoven et al., 1996). Peroxisomes can be purified from a light mitochondrial fraction in preformed continuous 20% to 40% (w/v) iodixanol gradients at high yield (80% to 90%), with no detectable contamination from any other organelle. In this protocol, which is adapted from the method of Van Veldhoven et al. (1996), the gradient has been designed to be accurately isoosmotic over its whole range, by inclusion of an inverse gradient of sucrose. Because of the large difference in density between the peroxisomes and the other organelles, the separation can be carried out easily in open-topped tubes for a fixed-angle rotor. This considerably simplifies the loading procedures and also increases the capacity of the system. Since the peroxisomes are the densest organelle in the light mitochondrial fraction, it is normal practice to unload the gradient dense-end-first.

Materials

- Iodixanol gradient solutions A, B, and C (see recipe)
- Light mitochondrial pellet (LMP) suspension (see Basic Protocol 1)
- Homogenization medium (HM; see recipe)
- Protease inhibitors (optional; see recipe) added to any or all solutions at concentrations indicated in recipe
- Gradient maker: two-chamber or Gradient Master (Accurate Chemical)
- 5-ml syringe with metal cannula (1-mm i.d.).
- Ultracentrifuge and thick-walled polycarbonate tubes (30-ml) for a fixed-angle rotor (e.g., Beckman 60 Ti or Sorvall T-865)
- Gradient unloader for dense-end-first collection (thin metal cannula connected to a peristaltic pump)
- Additional reagents and equipment for protein assay (APPENDIX 3B)

NOTE: All solutions, glassware, centrifuge tubes and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

1. Using the two-chamber gradient maker (or Gradient Master) prepare two linear gradients from 9 ml each of iodixanol gradient solutions B and C in the thick-walled polycarbonate tubes for the ultracentrifuge rotor.

Alternatively, a continuous gradient can be generated from a discontinuous one by diffusion. Layer equal volumes of 20%, 25%, 30%, 35%, and 40% (w/v) iodixanol and let stand 16 hr at 4°C.

2. Using a syringe and metal cannula, underlayer each gradient with 2 ml of gradient solution A.

If thin-walled tubes, which require complete filling, are used the volume of solution A may need to be increased.

3. Layer 3 ml of the LMP suspension over each gradient and ultracentrifuge 1 hr at 105,000 × g, 0° to 4°C.

If the centrifuge has a slow acceleration facility use a program that allows the rotor speed to increase from 0 to 2000 rpm over a period of ~4 min.

4. Allow the rotor to decelerate from 2000 rpm without the brake (or on a controlled deceleration program) and collect the gradient in 1-ml fractions, dense-end-first, by aspiration using the gradient unloader.

Thin-walled tubes may be unloaded by tube puncture.

Peroxisomes have a median density of $\rho = 1.17$ g/ml in iodixanol; the peak fraction is 7 to 8 ml from the bottom.

5. Dilute the fraction(s) with ≥ 2 vol of HM and centrifuge 20 min at $30,000 \times g$, 0° to 4°C .
6. Perform a protein assay (*APPENDIX 3B*) on an aliquot of the pellet(s) resuspended in 1 to 2 ml of HM or other suitable medium, then adjust the volume to 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20° or -80°C ; always check that any measurements are unaffected by the storage conditions by comparing with those made on fresh material.

ISOLATION OF PEROXISOMES FROM A RAT LIVER LIGHT MITOCHONDRIAL FRACTION IN A SELF-GENERATED IODIXANOL GRADIENT

ALTERNATE PROTOCOL 1

The LMP suspension is mixed with OptiPrep to a starting iodixanol concentration of 25% (w/v) and centrifuged in a vertical, near-vertical, or low-angle fixed-angle rotor in tubes of ~12 ml volume at $\sim 180,000 \times g$ for 2 to 3 hr (depending on rotor type). The protocol is adapted from Graham et al. (1994).

Additional Materials (also see *Basic Protocol 2*)

OptiPrep (60%, w/v iodixanol; Life Technologies, Accurate Chemical, Sigma, or Aldrich)

Diluent medium (DM; see recipe)

Vertical (e.g., Beckman VTi 65.1 or Sorvall 65V13), near-vertical (e.g., Beckman NVT 65), or low-angle (20° to 26°) fixed-angle rotor for an ultracentrifuge with 11- to 12-ml sealed tubes

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

1. Make a 50% (w/v) iodixanol working solution by diluting 5 vol of OptiPrep with 1 vol of DM.
2. Mix equal volumes of 50% (w/v) iodixanol stock and LMP suspension by repeated gentle inversion.
3. Transfer to a suitable sealed tube.
4. Centrifuge 1.5 to 3 hr at $180,000 \times g$, 0° to 4°C , in vertical, near-vertical, or low-angle fixed angle rotor.

The time will depend on the sedimentation path length of the rotor. For a vertical or near-vertical rotor with a path length of 17 to 25 mm, 1.5 hr will be sufficient; for longer path-length fixed-angle rotors the time will be up to 3 hr. The aim is to generate a gradient that is shallow in the middle to allow satisfactory separation of peroxisomes and mitochondria (see Commentary).

Subcellular Fractionation and Isolation of Organelles

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5. Decelerate the rotor from 2000 rpm to rest either using a controlled deceleration program or without the brake to allow a smooth reorientation of the gradient.
6. Collect the gradient, dense-end-first, in ~1-ml fractions by tube puncture or aspiration.

The peroxisomes band in the bottom third of the gradient.

7. Dilute the fraction(s) with ≥ 2 vol of HM and centrifuge 20 min at $30,000 \times g$, 0° to 4°C .
8. Perform a protein assay (*APPENDIX 3B*) on an aliquot of the pellet(s) resuspended in 1 to 2 ml of HM or other suitable medium, then adjust the volume to 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20° or -80°C ; always check that any measurements are unaffected by the storage conditions, by comparing with those made on fresh material.

**BASIC
PROTOCOL 3**

**ISOLATION OF PEROXISOMES FROM A RAT LIVER LIGHT
MITOCHONDRIAL FRACTION USING A PREFORMED CONTINUOUS
NYCODENZ GRADIENT**

Because the difference in density between peroxisomes and mitochondria in Nycodenz is less than that in iodixanol, it is common practice to use the high resolving power of a vertical rotor. The short sedimentation path length of such rotors permits the use of relatively short centrifugation times at low relative centrifugal forces of $35,000$ to $75,000 \times g$. This approach also reduces potential contamination of the peroxisomes by vesicles of the endoplasmic reticulum. Published methods use a variety of gradients and centrifugation conditions, and the following protocol is adapted from a number of these (Appelqvist et al., 1990; Kase and Björkhem, 1989; Prydz et al., 1988; Wilcke and Alexson, 1994).

Materials

- 20% (w/v) Nycodenz in homogenization medium (HM; see recipe)
- 50% and 60% (w/v) Nycodenz in high-density diluent (HD; see recipe)
- Light mitochondrial pellet (LMP) suspension (see Basic Protocol 1)
- Homogenization medium (HM; see recipe)
- Protease inhibitors (optional; see recipe) added to any or all of the solutions at concentrations indicated in recipe
- Gradient maker: two-chamber or Gradient Master (Accurate Chemical)
- 5-ml syringe with metal cannula (1-mm i.d.)
- Ultracentrifuge with vertical rotor (e.g., Beckman VTi50 or Sorvall 50V39) with appropriate sealed tubes (~39 ml)
- Gradient unloader for dense-end-first collection (tube-puncturing device or thin metal cannula connected to a peristaltic pump)
- Additional reagents and equipment for protein assay (*APPENDIX 3B*)

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

1. Using the two-chamber gradient maker (or Gradient Master) prepare linear gradients from 17 ml each of the 20% and 50% Nycodenz solutions in tubes appropriate for the vertical rotor of the ultracentrifuge.

Alternatively, a continuous gradient can be generated from a discontinuous one by diffusion. Layer equal volumes of 20%, 30%, 40% and 50% (w/v) Nycodenz and let stand for 16 hr at 4°C.

Variants of this gradient include 18% to 50% (Hartl et al., 1985); 13% to 48% (Kase and Björkhem, 1989); 15% to 48% (Prydz et al., 1988); and 25% to 50% (Wilcke and Alexson, 1994).

2. Using the syringe and metal cannula, underlayer the gradient with 2 ml of the 60% Nycodenz as a density cushion.

A number of published methods used the nonaqueous, low-viscosity Maxidens as a density cushion, but this is no longer readily available commercially.

3. Layer 2 to 3 ml of the LMP suspension on top of the gradient.
4. Centrifuge 75 min at $60,000 \times g$, 0° to 4°C, using programmed acceleration and deceleration to ensure a smooth reorientation of the gradient in the tubes.

If programmed deceleration is not available then turn off the brake below 2000 rpm.

Variants of the centrifugation parameters include 20 min at $74,000 \times g$ (Prydz et al., 1988) and 75 min at $35,000 \times g$ (Appelqvist et al., 1990).

If a vertical rotor is not available, use a swinging-bucket rotor for 2 hr at $70,000 \times g$ (Hartl et al., 1985).

5. Collect the gradient, dense-end-first, in 1- to 2-ml fractions, using a gradient unloader.

The peroxisomes band close to the bottom of the gradient.

6. Dilute the fraction(s) with ≥ 2 vol of HM and centrifuge 20 min at $30,000 \times g$, 0° to 4°C.

7. Perform a protein assay (APPENDIX 3B) on an aliquot of the pellet(s) resuspended in 1 to 2 ml of HM or other suitable medium, then adjust the volume to 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20° or -80°C; always check that any measurements are unaffected by the storage conditions by comparing with those made on fresh material.

ISOLATION OF PEROXISOMES FROM A RAT LIVER LIGHT MITOCHONDRIAL FRACTION USING A NYCODENZ BARRIER

ALTERNATE PROTOCOL 2

This is the simplest of all the systems. There is a tendency for the contamination from mitochondria to be slightly higher than with other systems, and the formation of a pellet rather than a band in a gradient may be detrimental to the integrity of the rather fragile organelles. The method is adapted from Ghosh and Hajra (1986).

Additional Materials (also see Basic Protocol 3)

30% (w/v) Nycodenz in high-density diluent (HD; see recipe)

Fixed-angle rotor with ~25-ml thick-walled polycarbonate tubes (e.g., Beckman 55.2 Ti or Sorvall T-865)

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

1. Transfer 15 ml of 30% Nycodenz to a 25-ml polycarbonate centrifuge tube.
2. Layer 2 ml of the LMP suspension over the Nycodenz barrier.
3. Centrifuge 1 hr at $130,000 \times g$, 0° to 4°C.

Subcellular Fractionation and Isolation of Organelles

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4. Remove all of the supernatant carefully by aspiration and resuspend the pellet in 1 to 2 ml of HM.

Do not decant the supernatant, since the pellet is not firmly packed.

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20° or -80°C; always check that any measurements made are unaffected by the storage conditions by comparing with those made on fresh material.

ISOLATION OF PEROXISOMES FROM YEAST SPHEROPLASTS USING A PREFORMED CONTINUOUS NYCODENZ GRADIENT

Although wild-type yeast appears to contain rather few peroxisomes, they can be induced to proliferate, and there are a number of peroxisome gene-deletion strains of the organism (Thieringer et al., 1991; Watkins et al., 1998). Yeast has therefore become an increasingly popular means of investigating peroxisome development. In the following protocol, spheroplasts are homogenized in a sorbitol-containing medium using a Dounce homogenizer. Some variation exists in the sorbitol concentration in this medium; Watkins et al. (1998) used 0.6 M, while Crane et al. (1994) used 1.0 M. As with mammalian liver, a continuous Nycodenz gradient is used to fractionate a light mitochondrial fraction prepared from the homogenate. This method is adapted from Crane et al. (1994) and Watkins et al. (1998).

Materials

- Yeast homogenization medium (YHM; see recipe)
- 15% (w/v) Nycodenz in yeast low-density diluent (YLD; see recipe)
- 42.5% and 50% (w/v) Nycodenz in yeast high-density diluent (YHD; see recipe)
- Yeast low-density diluent (YLD; see recipe)
- Yeast high-density diluent (YHD; see recipe)
- Protease inhibitors (optional; see recipe) added to any or all solutions at concentrations indicated in recipe
- Dounce homogenizer (Wheaton Type B, 40-ml)
- Dounce homogenizer (Wheaton Type B, 5- to 10-ml)
- Gradient maker: two-chamber or Gradient Master (Accurate Chemical)
- 5-ml syringe with metal cannula (1-mm i.d.)
- Ultracentrifuge with vertical rotor (e.g., Beckman VTi65.1 or Sorvall 65V13) with appropriate sealable tubes (~13 ml)
- Gradient unloader for dense-end-first collection (tube-puncturing device or thin metal cannula connected to a peristaltic pump)
- High-speed refrigerated centrifuge with fixed-angle rotor to accommodate 50-ml tubes (e.g., Sorvall SS34)
- Additional reagents and equipment for preparation of yeast spheroplasts (*UNIT 3.3*) and protein assay (*APPENDIX 3B*)

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Prepare light mitochondrial fraction from spheroplasts

1. Prepare spheroplasts from 1 liter of yeast culture, grown in YPD medium to an OD₆₀₀ of 0.5 to 1.0 (*UNIT 3.3*).
2. Suspend the spheroplasts in 35 ml of yeast homogenization medium (YHM) and homogenize in the 40-ml Dounce homogenizer, using 10 up-and-down strokes of the pestle.

3. Centrifuge the homogenate 10 min at $1500 \times g$, 0° to 4°C .
4. Aspirate and retain the supernatant on ice.
5. Resuspend the pellet in 35 ml of YHM and repeat steps 2 to 4.
6. Combine the two supernatants and centrifuge 30 min at $25,000 \times g$, 0° to 4°C .
7. Resuspend the light mitochondrial pellet in 6 ml of YHM using 10 gentle strokes of the pestle of the small volume Dounce homogenizer.

Separate peroxisomes in Nycodenz gradient

8. Using a two-chamber gradient maker (or Gradient Master) prepare 10.5-ml linear gradients from equal volumes of the 15% and 42.5 % Nycodenz in tubes for the vertical rotor.

Alternatively, a continuous gradient can be generated from a discontinuous one by diffusion. Layer equal volumes of 15%, 25%, 35%, and 45% (w/v) Nycodenz and let stand for 16 hr at 4°C .

9. Using a syringe and metal cannula, underlayer the gradient with a cushion of 0.5 ml of 50% Nycodenz.
10. Layer the yeast light mitochondrial pellet (step 7) on top to fill the tube.
11. Ultracentrifuge 75 min at $174,000 \times g$, 0° to 4°C . Use controlled acceleration and deceleration programs to ensure a smooth reorientation of the gradient. If these are not available turn off the brake below 2000 rpm.

Alternative systems include 15% to 37% Nycodenz for 90 min at $118,000 \times g$ in Beckman 50.2Ti rotor (McAlister-Henn et al., 1995) and 15% to 36% Nycodenz and 42% cushion, 1 hr at $100,000 \times g$ in a Sorvall 865B vertical rotor (Thieringer et al., 1991).

12. Collect the gradient in 0.5-ml fractions, dense-end-first, using a gradient unloader.
13. Dilute the fraction(s) with ≥ 2 vol of YHM and centrifuge 20 min at $30,000 \times g$, 0° to 4°C .
14. Perform a protein assay (APPENDIX 3B) on an aliquot of the pellet(s) resuspended in 1 to 2 ml of YHM or other suitable medium, then adjust the volume to 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20° or -80°C ; always check that any measurements are unaffected by the storage conditions by comparing with those made on fresh material.

ISOLATION OF PEROXISOMES FROM CULTURED CELLS (HepG2) USING A PREFORMED CONTINUOUS NYCODENZ GRADIENT

The cultured cells most widely used in peroxisome studies are the human hepatoblastoma line HepG2, and various skin fibroblast lines that are used in investigations into Zellweger syndrome. Both of these lines are grown routinely as a monolayer culture. In view of the fragility of peroxisomes from cultured cells, homogenization techniques such as nitrogen cavitation (Schrader et al., 1994) or the ball-bearing homogenizer (Watkins et al., 1991) devised by Balch and Rothman (1985) are to be preferred. If these are not available, a standard Potter-Elvehjem homogenizer (Cohen et al., 1992) may be used (see Commentary for more information). The following protocol is adapted from Cohen et al. (1992) and Watkins et al. (1991). The buffer used for all the solutions below is the same as that described for rat liver (i.e., MOPS) but Tris and HEPES are also commonly used with cultured cells. Some workers (Aikawa et al., 1991; Wanders et al., 1991; Cohen et al.,

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3.5.9

1992) use a post-nuclear supernatant rather than a light mitochondrial fraction—in these cases a larger volume gradient may be necessary (see annotations to the corresponding steps below).

Materials

Suspension of cells ($\sim 10^8$ cells) in 3 to 5 ml of homogenization medium (HM; see recipe)

Homogenization medium (HM; see recipe)

10% (w/v) Nycodenz in homogenization medium (HM; see recipe)

40% and 50% (w/v) Nycodenz in high-density diluent (HD; see recipe)

Protease inhibitors (optional; see recipe) added to any or all solutions at concentrations indicated in recipe

Ball-bearing homogenizer (see Balch and Rothman, 1985, for details of construction)

Low-speed centrifuge with swinging-bucket rotor to accommodate 10- to 20-ml tubes

Dounce homogenizer (5- to 10-ml, Wheaton type B)

High-speed centrifuge with fixed-angle rotor to accommodate 10- to 14-ml tubes (e.g., Sorvall SE12)

Gradient maker: two-chamber or Gradient Master (Accurate Chemical)

5-ml syringe with metal cannula (1-mm i.d.)

Ultracentrifuge with vertical rotor (e.g., Beckman VTi65.1 or Sorvall 65V13) with appropriate sealable tubes (~ 13 -ml)

Gradient unloader for dense-end-first collection (tube-puncturing device or thin metal cannula connected to a peristaltic pump)

Additional reagents and equipment for protein assay (*APPENDIX 3B*)

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Isolate the light mitochondrial fraction

1. Homogenize the cells using five passes from syringe to syringe in the ball-bearing homogenizer.

Alternatives include a nitrogen cavitation vessel (e.g. Artisan Industries) or a Potter-Elvehjem homogenizer (clearance ~ 0.09 mm), 10-ml working volume. With nitrogen cavitation, use 400 psi for 10 min; with the Potter-Elvehjem homogenizer use 50 up-and-down strokes of the pestle at 1000 rpm.

2. Check by phase-contrast microscopy that cell breakage is $\geq 90\%$.
3. Centrifuge the homogenate 5 min at $500 \times g$, 0° to 4°C , in the low-speed centrifuge.
4. Aspirate the supernatant and retain on ice.
5. Resuspend the pellet in HM using 4 to 5 strokes of the pestle of the Dounce homogenizer and repeat steps 3, 4, and 5.
6. Centrifuge the combined supernatants 10 min at $6000 \times g$, 0° to 4°C , in the high-speed centrifuge.
7. Aspirate and recentrifuge the supernatant 15 min at $20,000 \times g$, 0° to 4°C .
8. Aspirate and discard the supernatant and resuspend the light mitochondrial pellet in 3 to 4 ml of HM using 3 to 4 gentle strokes of the pestle of the Dounce homogenizer.

Separate the peroxisomes in Nycodenz gradient

9. Using the two-chamber gradient maker (or Gradient Master) prepare 10-ml linear gradients from equal volumes of the 10% and 40% Nycodenz in tubes for the vertical rotor.

Alternatively a continuous gradient can be generated from a discontinuous one by diffusion. Layer equal volumes of 10%, 20%, 30% and 40% (w/v) Nycodenz and let stand for 16 hr at 4°C.

If a post-nuclear supernatant is used rather than a light mitochondrial pellet, then prepare 34-ml gradients.

10. Using the syringe and metal cannula, underlayer the gradient with 0.5 ml of 50% Nycodenz.
11. Layer ~2 ml of the pellet suspension from step 8 on top of the gradient, to fill the tube.

Alternatively use 4 to 5 ml of a post-nuclear supernatant on the larger gradients.

12. Centrifuge 25 to 35 min at $75,000 \times g$, 0° to 4°C . Use a controlled acceleration and deceleration programs to ensure a smooth reorientation of the gradient. If these are not available turn off the brake below 2000 rpm.

For fibroblasts, Aikawa et al. (1991) used a 15% to 30% Nycodenz gradient and 1 hr at $305,000 \times g$ in the same rotor.

13. Collect the gradient in 0.75-ml fractions, dense-end-first, using a gradient unloader.

The peroxisomes band close to the bottom of the tube.

14. Dilute the fraction(s) with ≥ 2 vol of HM and centrifuge 20 min at $30,000 \times g$, 0° to 4°C .

15. Perform a protein assay (*APPENDIX 3B*) on an aliquot of the pellet(s) resuspended in 1 to 2 ml of HM or other suitable medium, then adjust the volume to 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20°C or -80°C ; always check that any measurements made are unaffected by the storage conditions by comparing with those made on fresh material.

ASSAY FOR ENDOPLASMIC RETICULUM MARKER ENZYME NADPH-CYTOCHROME *c* REDUCTASE

NADPH cytochrome *c* reductase is an important oxidoreductase linked to the oxidative status of the biosynthetic pathways present in the endoplasmic reticulum. Most methods measure the production of reduced cytochrome *c* by monitoring the absorbance at 550 nm. Sometimes NADH is used as an alternative substrate to NADPH, in which case it is normal to include an inhibitor of the mitochondrial oxidation pathway—normally rotenone—to which the endoplasmic reticulum enzyme is insensitive. Indeed, the inclusion of rotenone in the assay is not uncommon when using NADPH as the substrate, to avoid any interference from mitochondrial electron transport. The method is taken from Graham (1993) and adapted from Williams and Kamin (1962).

SUPPORT PROTOCOL

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Materials

Assay buffer (see recipe)
25 mg/ml cytochrome *c* in assay buffer (prepare fresh; keep on ice)
10 mM EDTA (dilute 1 ml 100 mM EDTA stock to 10 ml with H₂O)
1 mg/ml rotenone in ethanol (store up to 1 month at 4°C)
2 mg/ml NADPH in assay buffer (prepare fresh; keep on ice away from light)
Recording spectrophotometer (visible wavelength) with 1-ml cuvettes

1. Bring the assay buffer to room temperature and carry out all operations at this temperature.
2. Adjust the chart recorder to give a 0.2 absorbance unit full-scale deflection.
3. In a 1-ml cuvette, add 50 µl of cytochrome *c*, 10 µl 10 mM EDTA, and 10 µl of 1 mg/ml rotenone to 1 ml of assay buffer.
4. Add up to 50 µl of sample and mix well.

Neither Nycodenz nor iodixanol interfere with this enzyme at the concentrations that are likely to occur in the assay mixture. If the gradient fractions need to be concentrated, they should be diluted with 2 vol of HM, sedimented by centrifuging 20 min at 35,000 × g, and resuspended in a smaller volume of HM.

5. Record the absorbance at 550 nm until the baseline is steady, then add 0.1 ml of NADPH.

A double-beam recording spectrophotometer is ideal, but, as single-beam instruments are more commonly available, these steps are designed for such an instrument.

6. Mix well and continue to record the absorbance until a linear increase in value can be measured over a period of 1 to 2 min.
7. Calculate the activity of the enzyme (µmol cytochrome *c* reduced/min) by measuring the slope of the trace.

*The molar extinction coefficient of reduced cytochrome *c* is 27,000.*

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Assay buffer

Dissolve 0.71 g Na₂HPO₄ in 100 ml water (50 mM final). Dissolve 0.68 g KH₂PO₄ in another 100 ml water (50 mM final). Add KH₂PO₄ to Na₂HPO₄ to reach pH 7.7. Store up to 1 to 2 days at 4°C.

Diluent medium (DM)

To 100 ml H₂O add:

12 ml 100 mM disodium EDTA (6 mM final)
60 ml 100 mM MOPS (see recipe; 30 mM final)
1.2 ml ethanol (0.6% v/v final)
Adjust to pH 7.2 with 1 M NaOH
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

High-density diluent (HD)

To 100 ml H₂O add:

- 2 ml 100 mM disodium EDTA (1 mM final)
- 10 ml 100 mM MOPS (see recipe; 5 mM final)
- 0.2 ml ethanol (0.1% v/v final)
- Adjust to pH 7.4 with 1 M NaOH
- Add H₂O to 200 ml
- Store up to 1 to 2 days at 4°C

Homogenization medium (HM)

To 100 ml H₂O add:

- 17.1 g sucrose (0.25 M final)
- 2 ml 100 mM disodium EDTA (1 mM final)
- 10 ml 100 mM MOPS (see recipe; 5 mM final)
- 0.2 ml ethanol (0.1% v/v final)
- Adjust to pH 7.2 with 1 M NaOH
- Add H₂O to 200 ml
- Store up to 1 to 2 days at 4°C

Iodixanol gradient solutions A, B, and C

These gradient solutions are prepared by mixing the following stock solutions at the volume ratios listed below:

- OptiPrep (60% w/v iodixanol; available from Life Technologies, Accurate Chemical and Scientific, Sigma, and Aldrich)
- Diluent medium (DM; see recipe)
- 1 M sucrose (see recipe)

Gradient solution A (50% w/v iodixanol):

- 5 vol OptiPrep
- 0.6 vol DM
- 0.4 vol 1 M sucrose

Gradient solution B (40% w/v iodixanol):

- 4 vol OptiPrep
- 0.6 vol DM
- 0.7 vol 1 M sucrose
- 0.7 vol H₂O

Gradient solution C (20% w/v iodixanol):

- 2 vol OptiPrep
- 0.6 vol DM
- 1.1 vol 1 M sucrose
- 2.3 vol H₂O

MES, 100 mM

- 4.16 g 2-(*N*-morpholino)ethanesulfonic acid (MES)
- H₂O to 200 ml
- Store up to 2 to 3 months at -20°C

MOPS, 100 mM

- 4.18 g 3-(*N*-morpholino)propanesulfonic acid (MOPS)
- H₂O to 200 ml
- Store 2 to 3 months at -20°C

Protease inhibitors

Add the following to solutions as required so that the final concentrations are: 1 mM PMSF and 2.0 µg/ml for each of the other inhibitors. Also see *APPENDIX 1B* for general information on these compounds. All of these stock solutions may be stored in aliquots up to 2 to 3 months at -20°C , except PMSF which should be stored at 4°C .

PMSF: Dissolve 0.348 g phenylmethylsulfonyl fluoride (PMSF; 200 mM final) in 10 ml of dried propan-2-ol or ethanol.

Antipain: Dissolve 10 mg antipain (1 mg/ml final) in 10 ml of 10% (v/v) dimethyl sulfoxide (DMSO).

Aprotinin: Dissolve 10 mg antipain (1 mg/ml final) in 10 ml water.

Leupeptin: Dissolve 10 mg leupeptin (1 mg/ml final) in 10 ml of 10% (v/v) dimethylsulfoxide (DMSO).

Sucrose, 1 M

34.2 g sucrose (1 M final)

Add H_2O to 100 ml

Store up to 1 to 2 days at 4°C

Yeast high-density diluent (YHD)

To 100 ml H_2O add:

8.55 g sucrose (0.125 M final)

15 mg KCl (1 mM final)

2 ml 100 mM disodium EDTA (1 mM final)

10 ml 100 mM MES (see recipe; 5 mM final)

0.2 ml ethanol (0.1% v/v final)

Adjust to pH 6.0 with 1 M NaOH

Add H_2O to 200 ml

Store up to 1 to 2 days at 4°C

Yeast homogenization medium (YHM)

To 100 ml H_2O add:

21.9 g sorbitol (0.6 M final)

15 mg KCl (1 mM final)

2 ml 100 mM disodium EDTA (1 mM final)

10 ml 100 mM MES (see recipe; 5 mM final)

0.2 ml ethanol (0.1% v/v final)

Adjust to pH 6.0 with 1 M NaOH

Add H_2O to 200 ml

Store up to 1 to 2 days at 4°C

Yeast low-density diluent (YLD)

To 100 ml H_2O add:

17.1 g sucrose (0.25 M final)

15 mg KCl (1 mM final)

2 ml 100 mM disodium EDTA (1 mM final)

10 ml 100 mM MES (see recipe; 5 mM final)

0.2 ml ethanol (0.1% v/v final)

Adjust to pH 6.0 with 1 M NaOH

Add H_2O to 200 ml

Store up to 1 to 2 days at 4°C

COMMENTARY

Background information

Differential centrifugation

As with most subcellular organelles, methods for the isolation of peroxisomes have come largely from work with rat liver. With the exception of nuclei and rough endoplasmic reticulum (RER), peroxisomes are the densest of the major subcellular organelles. Peroxisomes are also one of the major components of the light mitochondrial fraction, which is obtained from a homogenate by differential centrifugation. The “classical” differential centrifugation scheme involves four steps: 600 to 1000 × *g* for 10 min (nuclear pellet); 3000 to 4000 × *g* for 10 min (heavy mitochondrial pellet); 15,000 to 25,000 × *g* for 20 min (light mitochondrial fraction); and 50,000 to 100,000 × *g* for 45 min (microsomal pellet). There is considerable variation in the actual RCFs used for each step, and sometimes the isolation of the heavy mitochondrial fraction is omitted. Moreover since the size of peroxisomes is quite diverse (0.2 to 1.0 μm), and as there is evidence for subpopulations of these organelles (Schrader et al., 1994; van Roermund et al., 1995), it is not unusual for the homogenate to be centrifuged at 2000 × *g* for 10 min, followed by 22,000 to 25,000 × *g* for 20 min, in order to pellet as many of the peroxisomes as possible. Unavoidably, some of the more rapidly sedimenting microsomes will also be pelleted. This situation is exacerbated if the RCF is increased; Lyons et al. (1991) used 41,000 × *g* for 30 min to pellet the peroxisomes from a hepatoma homogenate. Although in this case most of the microsomes formed a layer on top of the main organelle pellet, which could be recovered separately, increased microsomal contamination of the light mitochondrial pellet is inevitable with this technique. Some protocols, notably those for cultured cells (e.g., Aikawa et al.,

1991; Wanders et al., 1991; Cohen et al., 1992) often use a post-nuclear supernatant rather than a light mitochondrial fraction; in these cases all of the endoplasmic reticulum (ER) will also be present. This unavoidable contamination of the peroxisome-containing fraction by vesicles from the ER is a significant factor, since this influences the choice of density gradient medium.

Density gradient centrifugation

The densities of the major components of the light mitochondrial fraction in a variety of media are given in Table 3.5.1. In iodinated density gradient media such as Nycodenz or iodixanol, the density of peroxisomes is only slightly lower than that in sucrose; on the other hand the densities of the other organelles—mitochondria, lysosomes and Golgi membranes—are significantly higher in sucrose (Graham et al., 1994). The limiting membrane of peroxisomes is freely permeable to small solute molecules; these organelles therefore do not behave as osmotically sensitive particles in low-molecular mass solutes. Hence their observed density is relatively little affected by the osmolarity of the suspending solution and reflects only the macromolecular composition of the particle. The observed density of the mitochondria, lysosomes, and ER vesicles, on the other hand, includes the enclosed water component and is consequently dependent on the osmolarity of the gradient.

Although sucrose gradients are still occasionally used to purify peroxisomes from yeast (e.g., Crane et al., 1994), in the case of mammalian tissues and cells they have been totally superseded by iodinated density gradient media. In sucrose gradients, peroxisomes are significantly contaminated by lysosomes, and this can only be effectively reduced, in the case of rat liver, if the animals are injected with Triton

Table 3.5.1 Density of Organelles of the Light Mitochondrial Fraction in Different Gradient Media

Organelle	Density (g/ml)			
	Sucrose	Nycodenz	Iodixanol	Percoll
Endoplasmic reticulum	1.06-1.23	1.05-1.16	1.03-1.13	1.03-1.06
Golgi membranes	1.05-1.12	1.03-1.08	1.03-1.06	1.03-1.05
Lysosomes	1.19-1.21	1.12-1.15	1.11-1.14	1.06-1.12
Mitochondria	1.17-1.21	1.13-1.16	1.13-1.15	1.05-1.08
Peroxisomes	1.18-1.23	1.17-1.20	1.16-1.19	1.04-1.06

WR1339 to lower the density of the lysosomes (Leighton et al., 1968). Although contamination by mitochondria is less marked, the densest of these organelles consistently overlap the lighter peroxisomes. Consequently, the lower densities of both mitochondria and lysosomes in iodinated density gradient media make these media ideally suited to the purification of peroxisomes. Since about 1985, Nycodenz has been the gradient medium of choice, and the use of preformed gradients in vertical rotors has become an almost standard technique. Vertical rotors, with their short sedimentation path lengths, allow efficient and rapid separations of organelles. Moreover, the hydrostatic pressures in vertical rotors (and low-angle fixed-angle rotors) are much lower than those in swinging-bucket rotors, thus minimizing any potential deleterious effects on the organelles. Although the various published methods using Nycodenz show some minor differences in the density range of the gradient, generally they run from ~1.10 to 1.19 g/ml and include a cushion of density 1.22 to 1.26 g/ml to prevent any peroxisomes reaching the wall of the tube. This is essential for vertical rotors. The centrifugation conditions show the largest discrepancy, varying from $30,000 \times g$ for 75 min (Kase and Björkhem, 1989; Appelqvist et al., 1990) to $309,000 \times g$ for 60 min (Aikawa et al., 1991). Without exception, the gradients are top-loaded with the sample, and many gradients are centrifuged below $60,000 \times g$ for <1 hr. At these relatively low RCFs and times, any endoplasmic reticulum vesicles will probably not reach their equilibrium density banding position, and this may virtually eliminate any potential contamination of the peroxisomes.

Although inverse gradients of sucrose are often used to balance the osmolarity of Nycodenz gradients, at concentrations above 30% w/v (~1.16 g/ml) any Nycodenz solution is hyperosmotic. Iodixanol solutions on the other hand can be made isoosmotic at all densities (Graham et al., 1994); consequently mitochondria and lysosomes have significantly lower densities (Table 3.5.1) in iodixanol than in Nycodenz, and the mitochondria in particular are easier to resolve from peroxisomes. Moreover, the latter are also isolated under isoosmotic conditions. Most organelle separations with iodixanol are carried out in preformed gradients. Although the formation of self-generated gradients of iodixanol is normally carried out at RCFs above $300,000 \times g$, gradients with a relatively shallow median section, which are ideal for peroxisome purification (see Antici-

pated Results), can be formed at RCFs of $180,000 \times g$, and although the centrifugation time maybe 1.5 to 3 hr (depending on rotor type), the method has the considerable merits of ease of sample preparation and high reproducibility (Graham et al., 1994).

Peroxisomes can also be isolated under isoosmotic conditions in Percoll gradients. Probably because Percoll is a colloidal suspension of silica, rather than a true solute, mammalian peroxisomes behave rather differently: they exhibit a much lower density than in sucrose, Nycodenz, or iodixanol, and they are generally well resolved from both mitochondria or lysosomes (see Table 3.5.1). However, why the density of the major organelles in iodinated density gradient media (lysosomes < mitochondria < peroxisomes) should be essentially reversed in Percoll is not clear. In some nonmammalian systems, however, for example the filamentous hemiascomycete *Ashbya gossypii* (Maeting, et al., 1999), peroxisomes are denser than mitochondria in Percoll.

Although the formation of Percoll gradients has the great merit of self-generation at low RCFs and centrifugation times, a major problem with these gradients is that endoplasmic reticulum vesicles in the light mitochondrial fraction from mammalian tissues always copurify with the peroxisomes (see Table 3.5.1), as evinced by the coincidence of catalase and glucose-6-phosphatase profiles (see for example Schepers et al., 1989). One other problem that is rarely considered is the potential contamination from Golgi membranes in Percoll gradients. In the case of liver in particular, a large proportion of the Golgi will sediment in the LMP. According to Schweizer et al. (1991) the Golgi membranes also band at a low density in Percoll, as they do in iodixanol and Nycodenz (see Table 3.5.1). In Percoll, therefore, they are likely to contaminate the peroxisomes, while in iodixanol (and Nycodenz), Golgi membranes band at the opposite end of the gradient from the peroxisomes.

Moreover, before spectrophotometric assays for peroxisomes (and other organelles) can be carried out, it is usually necessary to remove the Percoll by centrifugation, because of the light-scattering properties of this medium. During this procedure the peroxisomes will also sediment and they are recovered as a loose layer above the Percoll pellet. However Osmundsen (1982) established that this procedure accounted for an approximately 50% loss of the organelles. As long as the organelles are at a

sufficiently high concentration in the gradient fractions, removal of the medium is generally not a strict requirement for either Nycodenz or iodixanol gradients. If the peroxisomes do need to be concentrated or if the medium does interfere with some subsequent procedure, then a simple dilution of the fraction(s) with buffer to reduce the density, followed by pelleting of the organelles, will give high recoveries.

Critical Parameters and Troubleshooting

Homogenization

Only cultured cells may provide a significant homogenization problem. Many monolayer cells can be disrupted in an isoosmotic sucrose medium using relatively mild liquid shear conditions; this minimizes any damage to peroxisomes and loss of intra-organelle components. Watkins et al. (1991) observed that the standard liquid shear techniques of Dounce or Potter-Elvehjem homogenization led to the release of soluble peroxisomal macromolecules from HepG2 cells, and stressed the need to use the more gentle disruptive forces provided by the ball-bearing device of Balch and Rothman (1985). Nevertheless, other workers using the same cells have used up to 50 strokes of the pestle of a Potter-Elvehjem homogenizer (Wanders et al., 1991; Cohen et al., 1992). Nitrogen cavitation is another alternative and was used by Völkl and Fahimi (1985). If only Dounce or Potter-Elvehjem homogenizers are available, a homogenization medium of 0.25 M sucrose/1 mM EDTA/10 mM triethanolamine-acetic acid, pH 7.4 (Marsh et al., 1987), combined with no more than 10 strokes of the pestle of a tight-fitting Dounce homogenizer, has been found to be widely applicable to monolayer cells (and fibroblasts in particular). For a more complete discussion of these problems see Evans (1992) and Graham (1997).

Differential centrifugation

The aim of the homogenization should be to devise conditions such that $\geq 90\%$ of cells are disrupted with the minimum release of peroxisomal markers into the medium. To check this out, all of the differential centrifugation fractions, including the microsomes and the cytosol ($100,000 \times g/45$ min pellet and supernatant), should be assayed for organelle markers and proteins (see Anticipated Results). For tissues or cells other than those described in this unit, always carry out a more thorough differential centrifugation (e.g., $600 \times g/10$ min, $3000 \times$

$g/10$ min, $15,000 \times g/10$ min; $25,000 \times g/10$ min, $40,000 \times g/10$ min) in order to devise the optimal protocol.

Density gradient centrifugation

For a tissue or cell other than one of those described in this unit, it may also be necessary to modulate some of the gradient and/or centrifugation conditions in the light of experience. For example, try making the gradient more or less dense at the bottom to take account of organelles that may be correspondingly more or less dense. Modifications will also need to be made if the type of rotor described in the protocol is not available. If a swinging-bucket or fixed-angle rotor is substituted for a recommended vertical rotor, then the centrifugation times will have to be increased to take account of the longer sedimentation path lengths.

The preparation of preformed continuous gradients using a two-chamber gradient maker requires considerable practice to achieve highly reproducible gradients, and some of the important points in operating such devices are covered by Dobrota and Hinton (1992). A Gradient Master, which involves simply layering the densest and lightest solutions in the centrifuge tube, provides a more expensive but reproducible alternative. If neither of these are available, then a discontinuous gradient constructed from three or four solutions, spanning the required density range, can be prepared and allowed to diffuse, usually overnight at 4°C . Note that the time required for complete linearity to be achieved will depend on the tube diameter and the volume of each layer. Always collect a blank gradient and check (using refractive index) that the gradient is indeed linear and covers the appropriate density range.

In self-generated gradients of iodixanol (Alternate Protocol 1), the light mitochondrial fraction is mixed with OptiPrep to a uniform starting concentration of 25% iodixanol; the gradient forms and the particles move to their banding density during the centrifugation. It is important that the gradient generated be relatively shallow in the middle of the gradient ($\rho = 1.14$ to 1.16 g/ml) to achieve a satisfactory linear separation between the mitochondria and peroxisomes (see Anticipated Results).

Enzyme and other assays

Prior to the execution of an enzyme assay (or other measurement), it is common practice to remove the gradient medium from each gradient fraction by dilution with 2 to 4 volumes of a low-density medium, followed by centrifugation to pellet the organelles and resuspension in

the same low-density medium. The latter is often the homogenization medium, or, if it is isoosmotic, a medium used for the subsequent assay. This practice eliminates any possible interference of the gradient medium on the subsequent procedure(s), and it is described in the final steps of each Basic Protocol. In many instances, however, the sample volumes used in the assay are sufficiently small (as in the Support Protocol) that the gradient solute is adequately diluted so as not to cause any interference in the assay, even at the highest density. Thus, so long as the concentration of organelles in the gradient fraction is high enough for the enzyme activity to be detectable, the assay may be performed directly without washing. If, however, either of these requirements are not met, then dilution, pelleting and resuspension of the pellet in a smaller volume are unavoidable.

In order to determine specific activities of measured enzymes, it is also necessary to estimate the protein content of fractions. Nycodenz and iodixanol interfere with methods using the Folin-Ciocalteu reagent; however, if small volumes ($\leq 100 \mu\text{l}$) of fraction are used in a 1- to 2-ml assay volume, the concentration of gradient solute in the assay solution is $< 10\%$ (w/v) and the interference is marginal. Methods based on Coomassie blue are generally unaffected by any concentration of gradient solute.

The only significant interference of common peroxisome functional assays by Nycodenz (and by implication by iodixanol too) is phytanic acid α -oxidation (Singh et al., 1993).

Anticipated Results

Basic Protocol 1

The expected distribution of the major organelle enzyme markers in the nuclear, heavy

mitochondrial, light mitochondrial, microsomal, and cytosolic fractions from a rat liver homogenate is given in Table 3.5.2. If the first $750 \times g$ is omitted, the figures for the percent protein and percent enzyme content of the $3500 \times g$ pellet will be the sum of the nuclear and light mitochondrial fractions. If the actual RCFs and times used are different from those in Basic Protocol 1, then the data will also be similarly different. Nevertheless, the overall pattern of enzyme content should remain the same. In the case of HepG2 cells, the percent of the total catalase in the light mitochondrial fraction is reported (Watkins et al., 1991) at 32%, and the mitochondria tend to be more prevalent in the heavy mitochondrial fraction (48%) compared to the nuclear fraction (16%). Organelles from a yeast homogenate also conform to this overall pattern.

Basic Protocol 2

Fractions 5 to 14 of the gradient (~ 10 ml) contain $\sim 85\%$ of the total catalase activity recovered from the gradient (Fig. 3.5.1). There is no detectable contamination from mitochondria, lysosomes, or endoplasmic reticulum in these fractions whatsoever.

Alternate Protocol 1

The bottom seven fractions of the gradient (~ 5 ml) contain nearly 90% of the total catalase activity recovered from the gradient (Fig. 3.5.2). There is no detectable contamination from mitochondria, lysosomes, or endoplasmic reticulum (not shown), which co-bands with the lysosomes in this gradient.

Basic Protocol 3

There will be some small differences in the precise banding position of the peroxisomes (in terms of the distance from the bottom of the tube) which reflect the density profile of the

Table 3.5.2 Protein and Enzyme Distribution in Rat Liver Differential Centrifugation Fractions^{a,b}

Fraction	Percent (relative sp. act.)				
	Protein	Catalase	SDH	NADPH cyt <i>c</i> red	AP
Nuc	30-35	20-25 (<1.0)	50-60 (<2.0)	20-25 (<1.5)	20-25 (<2.0)
H Mit	15-25	7.5-12.5 (<0.5)	20-25 (>3.0)	5-7.5 (<0.2)	10-12.5 (<1.0)
L Mit	7.5-12.5	20-25 (>3.0)	5-10 (<2.0)	5-10 (<1.0)	20-25 (>3.0)
Mic	12.5-17.5	1-2 (<0.2)	10-15 (<0.5)	50-55 (>3.0)	2-5 (<0.2)
Cytosol	25-30	25-30 (<1.0)	0	5-10 (<0.5)	20-25 (<1.5)

^aFigures are percent of protein and enzyme in each fraction and figures in parenthesis are relative specific activity of enzymes (percent enzyme/percent protein).

^bAbbreviations: AP, acid phosphatase; H Mit, heavy mitochondrial; L Mit, light mitochondrial; Mic, microsomal; NADPH cyt *c* red, NADPH cytochrome *c* reductase; Nuc, nuclear; SDH, succinate dehydrogenase.

gradient, the concentration of Nycodenz cushion (if present), and the centrifugation conditions. Nevertheless there will be an overall similarity of organelle banding, with the peroxisome peak being very close to the bottom

of the gradient. The example given in Figure 3.5.3 is from Wilcke and Alexson (1994) who used a 25% to 50% Nycodenz gradient, a 60% cushion, and $60,000 \times g$ for 35 min in a vertical rotor. Approximately 70% of the total catalase

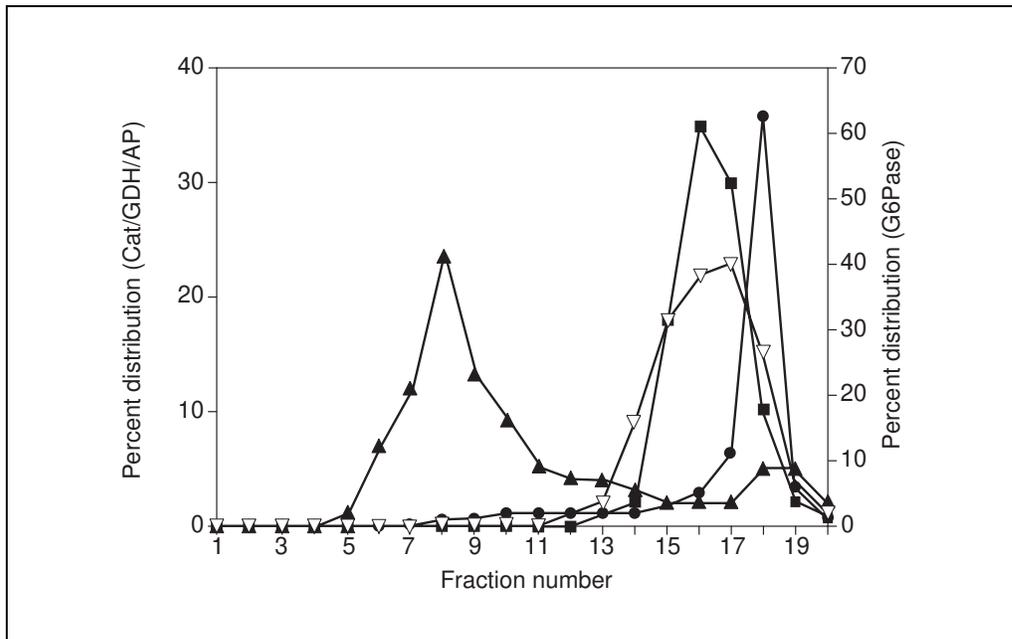


Figure 3.5.1 Isolation of peroxisomes in a preformed continuous gradient of iodixanol in a fixed-angle rotor. Fraction 1 is the dense end of the gradient. Distribution of enzymes: GDH, glutamate dehydrogenase (filled squares); Cat, catalase (filled triangles); AP, acid phosphatase (open inverted triangles); G6Pase, glucose-6-phosphatase (filled circles). Data is adapted from Van Veldhoven et al. (1996) with permission of Academic Press.

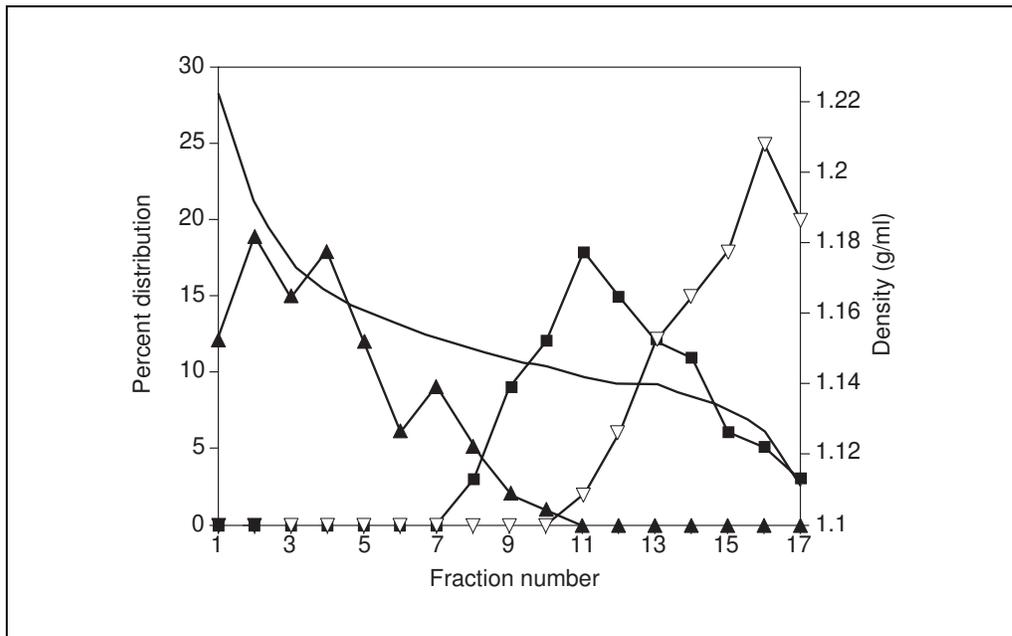


Figure 3.5.2 Isolation of peroxisomes in a self-generated gradient of iodixanol in a 12-ml fixed-angle rotor. Fraction 1 is the dense end of gradient. Distribution of enzymes: Cat, catalase (filled triangles); SDH, succinate dehydrogenase (filled squares); β Gal, β -galactosidase (open inverted triangles). Data is adapted from Graham et al. (1994) with permission of Academic Press.

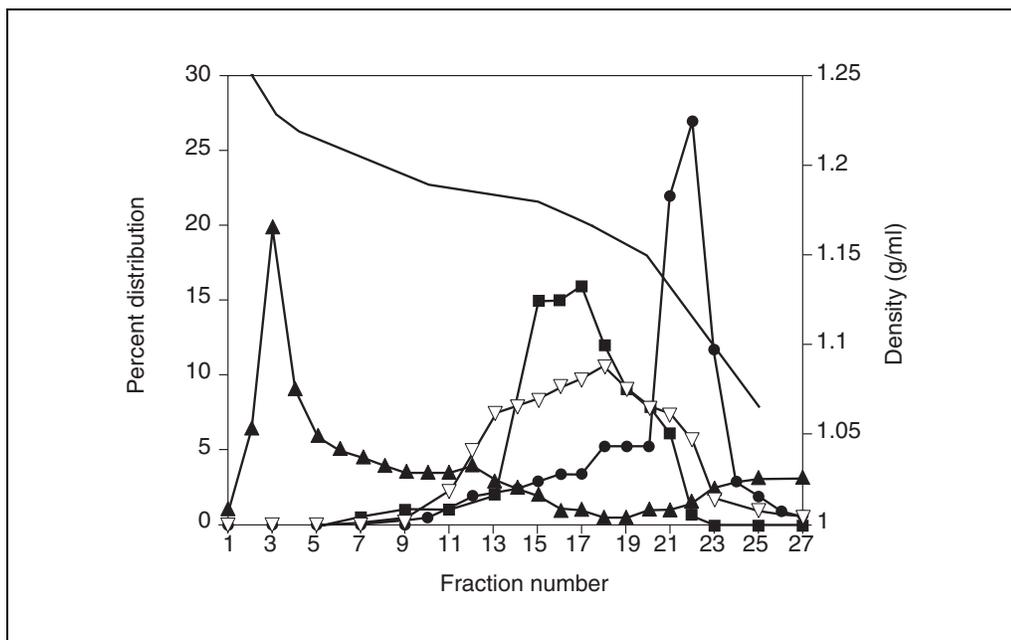


Figure 3.5.3 Isolation of peroxisomes in a 25% to 50% (w/v) Nycodenz gradient ($60,000 \times g$ for 35 min) in a vertical rotor. Fraction 1 is the dense end of the gradient. Distribution of enzymes: Cat, catalase (filled triangles); Cytox, cytochrome oxidase (filled squares); AP, acid phosphatase (open inverted triangles); Est, esterase (filled circles; endoplasmic reticulum marker). Data is adapted from Wilcke and Alexson (1994) with permission of European Journal of Biochemistry.

in the gradient is recovered from the first eleven fractions, with essentially no contamination from any other organelle. Compared to iodixanol, there is a greater tendency for some of the lighter peroxisomes to overlap the denser lysosomes and mitochondria.

Alternate Protocol 2

Because density barriers are less discriminating than continuous gradients, it is difficult to choose a single density that will give both good yields and lack of contamination by other organelles. Nevertheless the 30% Nycodenz barrier devised by Ghosh and Hajra (1986) should give a yield of ~50% of the catalase from the light mitochondrial fraction, with <2% of the lysosomes, mitochondria, and endoplasmic reticulum.

Basic Protocol 4

In this yeast protocol, ~55% of the total catalase is located in the bottom half of the gradient with no detectable contamination from mitochondria (Watkins et al., 1998). Unlike the mammalian systems, however, the rest of the lighter catalase-containing particles form a second well defined peak, which co-bands with the mitochondria. Moreover the median banding densities of all of the particles in Nycodenz gradients appear significantly lighter than their

mammalian counterparts. Peroxisomes have a median density of ~1.16 g/ml and that of the mitochondria is as low as 1.12 g/ml (Thieringer et al., 1991).

Basic Protocol 5

The density of the organelles from HepG2 cells is almost identical to that of those from rat liver. Approximately 55% of the total catalase bands in the lowest third of the gradient; this peroxisome band is devoid of succinate dehydrogenase, but contains ~8% of the total ER in the gradient (Watkins et al., 1991). The overall distribution of enzyme markers is, however, essentially identical to that of Basic Protocol 3.

Time Considerations

There are no points at which any of the basic protocols can be discontinued. The light mitochondrial fraction produced in Basic Protocol 1 must be used immediately for any subsequent gradient separation. Preformed gradients should be made up during Basic Protocol 1 and kept on ice until required. If they are prepared at room temperature, they must be given time to cool down to 4°C.

Basic Protocol 1 should be accomplished in 2 to 2.5 hr. If the preformed iodixanol or Nycodenz gradients have been made up either the previous night (diffusion of discontinuous gra-

dients) or during Basic Protocol 1, Basic Protocols 2 and 3 should take ~3 hr. Alternate Protocol 1 will also take ~3 hr (depending on the rotor), but the preparations prior to centrifugation are considerably simpler and shorter. Basic Protocol 3 should take no more than 2 hr. Because of the ~4 hr spheroplast preparation time, Basic Protocol 4 will require in total ~7 hr. Basic Protocol 5 should be completed within 3.5 hr.

With experience and practice it may be unnecessary to unload the gradients in a series of equal fractions; instead the peroxisome band might be removed using a Pasteur pipet or syringe—this will save at least 15 min per tube.

Functional assays should be carried out as soon as possible after the preparation, but simple marker enzyme assays (such as described in this unit and in *UNIT 3.4*) can be carried out on material stored at 0° to 4°C for up to 16 hr without much loss of activity. These assays and compositional measurements may also be carried out on material frozen to -20° or -80°C. There are, however, no general rules regarding the suitability of a particular storage regime, and it is strongly recommended that the validity of results on stored material be checked by comparison with those obtained with the fresh organelles. Any concentration of the membrane fractions and/or removal of gradient medium must be carried out prior to freezing.

All the solutions can be made up ahead of time and stored at 4°C for 1 to 2 days or frozen to -20°C for longer periods. But note that any enzymes, as well as PMSF or other protease inhibitors, should be added immediately prior to use.

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Isolation of Lysosomes from Tissues and Cells by Differential and Density Gradient Centrifugation

Lysosomes are widely studied organelles; they are an integral part of the complex traffic of membrane vesicles between intracellular and surface membranes. Most notably, they are the site of degradation of macromolecules which have been brought into the cell by endocytosis. Most of the biochemical and structural analysis of lysosomes has been carried out on organelles from rat liver. A Percoll gradient method for this still widely-used tissue is described in this unit (see Basic Protocol 1). In the italicized annotations to this protocol, there are some comments regarding the application to other mammalian tissues (i.e., brain and kidney). More recently, the study of lysosomes as part of the endocytic and synthetic processes has been performed on organelles from a wide range of cultured cells. Because of the diversity of homogenization procedures and minor variations in the Percoll centrifugation regime used for cultured cells, it is not practical to present each cell type in a detailed protocol. A second procedure (see Basic Protocol 2) describes the isolation of lysosomes from HL-60 cells and the italicized annotations, a few of the many variants. The Commentary also presents some of the protocol variations used for cultured cells.

Since lysosomes, mitochondria, and peroxisomes have similar and overlapping densities in sucrose gradients, in the past, density perturbation of the lysosomes by Triton WR1339 loading was a prerequisite to achieving satisfactory purification in these gradients. With the advent of low-osmolarity Percoll and Nycodenz gradient media, the use of sucrose gradients declined and they are little used today, being generally regarded as unsatisfactory for lysosome purification. Nevertheless, to obtain the highest yields and purities of lysosomes, density perturbation can improve the resolution of Percoll gradients. This approach is presented in Alternate Protocol 1. Alternate Protocol 2 describes the use of a discontinuous gradient of Nycodenz for rat liver lysosomes. None of these systems are recommended for concomitant purification of the other major organelles in the light mitochondrial fraction (mitochondria and peroxisomes); this is best carried out by flotation of the light mitochondrial fraction through a continuous iodixanol gradient, described in Alternate Protocol 3.

After collecting the banded material from the gradients, the success of the fractionation is routinely measured by assaying for various marker enzymes; acid phosphatase (see Support Protocol 1) or a glycosidase such as β -galactosidase (UNIT 3.4) are commonly used for lysosomes. For peroxisomes, mitochondria, and endoplasmic reticulum, respectively, catalase, succinate dehydrogenase (UNIT 3.4), and NADPH-cytochrome *c* reductase (UNIT 3.5) may be used as markers.

IMPORTANT NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

ISOLATION OF LYOSOMES FROM RAT LIVER USING A SELF-GENERATED PERCOLL GRADIENT

From a mammalian tissue homogenate, the lysosomes are always purified from a light mitochondrial fraction (LMF), this is also occasionally true of cultured cell homogenates. Routinely, lower relative centrifugal forces (RCFs) are required to produce the LMF for lysosome purification than for peroxisome purification (UNIT 3.5). A medium containing

BASIC PROTOCOL 1

Subcellular Fractionation and Isolation of Organelles

3.6.1

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buffered 0.25 M sucrose and 1 mM EDTA is routinely used both for the homogenization and as the osmotic balancer for the Percoll gradient.

The LMF is either mixed with a Percoll stock solution or layered on top of a uniform concentration of Percoll, normally in the range 25% to 40% (v/v), and most commonly centrifuged in a fixed-angle rotor for 30 to 90 min at 20,000 to 35,000 $\times g$. A gradient is generated by the centrifugal field and the lysosomes are the densest organelle in this system. The normal mode of harvesting the gradient is either tube puncture (for thin-walled centrifuge tubes only) or aspiration from the bottom of the gradient. In a technique requiring practice and experience, the principal lysosome band can be removed using a syringe attached to a long metal cannula. See Commentary for more information on the manner in which the lysosomes are banded in these gradients. The protocol below is adapted from Jonas (1986) and Symons and Jonas (1987).

Percoll must normally be removed from gradient fractions before any spectrophotometric assay for marker enzymes can be carried out, because of the light-scattering properties of the medium. It is also advisable to remove the medium before carrying out any electrophoresis.

While isolation from liver is the focus of this protocol, the use of both brain (Caimi et al., 1989) and kidney (Ohshita et al., 1992) tissues are described in the annotations where appropriate.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

NOTE: The following protocol is suitable for a single rat liver of wet weight 10 to 12 g.

Materials

- 150- to 200-g Sprague-Dawley rats
- Homogenization medium (HM; see recipe)
- Percoll stock solution (see recipe)

- Dissecting equipment
- ~40- and ~5 ml Dounce homogenizers with loose-fitting Wheaton type B pestles (e.g., Kontes)
- Low-speed centrifuge with swinging-bucket rotor to take 50-ml tubes
- Sorvall RC series centrifuge and SE-12 and SS-34 rotors or equivalents
- Gradient unloader: thin metal cannula connected to a peristaltic pump
- Ultracentrifuge with fixed-angle rotor (e.g., Beckman 50 Ti or 70.1 Ti or Sorvall T-865.1)

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all of the solutions.

Isolate and homogenize liver

1. Starve the animal overnight.
2. Sacrifice the animal by cervical dislocation or decapitation.
This must be supervised or carried out by an experienced animal technician.
3. Open the abdominal cavity and remove the liver to a chilled beaker containing ~20 ml of homogenization medium (HM).
4. Decant the medium and finely mince the liver using scissors.

The pieces of liver should be no more than ~25 mm³.

5. Suspend the mince in ~40 ml of HM and homogenize the liver using 15 to 20 strokes of the type B pestle of the 40-ml Dounce homogenizer.

If only a smaller-volume Dounce homogenizer is available, carry out the homogenization in two batches. Brain tissue should be homogenized in 3 vol of HM containing a higher sucrose concentration (0.32 M) using a 50-ml Potter-Elvehjem homogenizer (0.2 mm clearance) with five gentle strokes at 500 rpm. For kidney tissue use 9 vol of HM (0.25 M sucrose) in a Potter-Elvehjem homogenizer.

Isolate light mitochondrial fraction

6. Centrifuge the homogenate 10 min at $750 \times g$, 0° to 4°C , in a low-speed centrifuge with a swinging-bucket rotor.
7. Decant and retain the supernatant.
8. Crudely resuspend the pellet in 20 ml of HM using a glass rod. Complete the resuspension using 3 to 4 gentle strokes of the type B pestle of the Dounce homogenizer
9. Repeat steps 6 and 7.
10. Combine the supernatants and transfer to ~50-ml high-speed centrifuge tubes.

This is the post-nuclear supernatant (PNS).

11. Centrifuge 10 min at $20,000 \times g$ in Sorvall RC series centrifuge and SS-34 or equivalent fixed-angle rotor, 0° to 4°C .
12. Decant and discard the supernatant
13. Suspend the light mitochondrial pellet (LMP) in ~40 ml HM using a glass rod followed by 3 to 4 gentle strokes of the pestle of the Dounce homogenizer.
14. Centrifuge 10 min at $20,000 \times g$, 4°C , and discard the supernatant.
15. Resuspend the LMP in ~6 ml HM using a glass rod. Complete resuspension with 3 to 4 gentle strokes of the pestle of the 5 ml Dounce homogenizer.

The homogenate (light mitochondrial fraction; LMF) should contain ~25 mg/ml protein.

Purify lysosomes

16. Mix 4.5 ml of Percoll stock solution with 5.5 ml LMF from step 15.

This is equivalent to ~40% (v/v) Percoll.

17. Transfer 10 ml of the Percoll/LMF mixture to each high-speed centrifuge tube and centrifuge 90 min at $35,000 \times g$, 0° to 4°C , in a Sorvall SE-12 or equivalent fixed-angle rotor.

The gradient can also be generated in an ultracentrifuge fixed-angle rotor. Such a rotor is required to remove the Percoll particles from the gradient fractions (see step 19).

Because the gradient reorients in the tube during deceleration from 2,000 rpm to rest, use a slow deceleration during the braking phase so deceleration occurs smoothly and disturbance of the gradient and the banded material is avoided.

For brain lysosomes, layer 4 ml of LMF over 36 ml of 27% (v/v) Percoll and centrifuge 90 min at $20,000 \times g$. Kidney lysosomes: mix LMF with Percoll stock to 40% (v/v) Percoll and centrifuge 30 min at $47,000 \times g$.

18. Harvest the gradient (preferably dense-end-first) in ~1 ml fractions from the top using a gradient unloader; the lysosomes band in the bottom 1 to 2 ml.

Alternatively, a syringe (1 to 2 ml) with metal cannula (i.d. 1 mm) can be used to collect equal-volume fractions from the top or bottom of the gradient. Thin-walled tubes can be unloaded by tube puncture.

19. Ultracentrifuge the fractions 1 hr at $100,000 \times g$, 0° to 4°C , and collect the turbid layer of lysosomes just above the Percoll pellet.

20. Perform metabolic assays for lysosomes (see Support Protocols 1 and 2), peroxisomes (UNIT 3.4), mitochondria (UNIT 3.4), or endoplasmic reticulum (UNIT 3.5) as soon as possible upon preparation.

If necessary dilute the lysosomes to 1 to 5 mg protein/ml with HM.

Some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20°C or -80°C ; however, always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with results obtained from fresh material.

ALTERNATE PROTOCOL 1

ISOLATION OF LYOSOMES FROM RAT LIVER USING DENSITY PERTURBATION AND A SELF-GENERATED PERCOLL GRADIENT

After intraperitoneal injection of the carbohydrate polymer dextran, the macromolecule is endocytosed by the liver and delivered to the lysosomes, thereby increasing their density. On the other hand, if the post-nuclear supernatant of a liver homogenate is made 1 mM with respect to Ca^{2+} , the mitochondria take up water and become considerably less dense. This bimodal density perturbation approach was devised by Arai et al. (1991) to improve the yield and resolution of lysosomes from mitochondria in Percoll gradients. These dextran-perturbed lysosomes are sometimes called dextranosomes. The following method is adapted from Arai et al. (1991).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Additional Materials (also see Basic Protocol 1)

100 mg/ml dextran solution (see recipe)
Homogenization medium (HM; see recipe) without EDTA
100 mM CaCl_2 (see recipe)
Percoll stock solution (see recipe) without EDTA
2-ml syringe and 23-G needle
Beckman 70.1 Ti rotor

1. Inject rats intraperitoneally with 1 mg dextran per gram of body weight using a 2-ml syringe and 23-G needle.

This must be carried out by a properly trained and licensed operative.

2. Sacrifice the animal by decapitation or cervical dislocation.
3. Blanch the liver by perfusion from the portal vein with 20 to 30 ml ice-cold HM.
4. Prepare the PNS (see Basic Protocol 1, steps 3 to 10).
5. Measure the volume of PNS; add 0.01 vol of 100 mM CaCl_2 , and incubate 5 min at 37°C .
6. Cool the suspension back to 0° to 4°C and prepare the LMF (see Basic Protocol 1, steps 11 to 15).

- Mix 5.5 ml of Percoll stock solution with 4.5 ml of the LMF and ultracentrifuge 15 min at $60,000 \times g$ in a 70.1 Ti or equivalent fixed-angle rotor, 0° to 4°C .

This is equivalent to ~50% (v/v) Percoll.

- Collect the gradient and process the fractions as described (see Basic Protocol 1, steps 18 to 20).

The dextranosomes band close to the bottom of the gradient.

ISOLATION OF LYSOSOMES FROM A RAT LIVER LIGHT MITOCHONDRIAL FRACTION IN A DISCONTINUOUS GRADIENT OF NYCODENZ

**ALTERNATE
PROTOCOL 2**

The discontinuous metrizamide gradient devised by Wattiaux and Wattiaux-De Coninck (1983) has been superseded by a Nycodenz gradient covering the same density range (Olsson et al., 1989). The light mitochondrial fraction is adjusted to a high density and layered under the gradient; bottom-loading has been shown (Wattiaux and Wattiaux-De Coninck, 1983) to be preferable for isolation of lysosomes in this system.

As Nycodenz is a true solute, the gradient fractions do not require centrifugation at $100,000 \times g$ (as for Percoll) to remove the gradient medium; if necessary the organelles can be pelleted 15 min at $20,000 \times g$ after dilution of the fractions with 2 vol of HM.

Additional Materials (also see Basic Protocol 1)

45% Nycodenz stock solution (see recipe)

Ultracentrifuge with swinging-bucket rotor to take ~38-ml tubes (e.g., Beckman SW28, Sorvall AH629, or equivalent)

Gradient unloader: 1- to 2-ml syringe attached to metal cannula (i.d. 1 mm)

- Prepare LMF as described (see Basic Protocol 1, steps 1 to 15). To keep the sample volume to a minimum, resuspend the LMP in 3 to 4 ml of HM.
- To 1 vol of LMF add 2 vol 45% Nycodenz stock solution.
- Prepare 19%, 24%, 26%, and 30% Nycodenz by dilution of 45% Nycodenz stock solution in HM.
- Transfer 8 ml of 19% Nycodenz to a tube for the SW-28 rotor (or equivalent), and, using a syringe and metal cannula, underlayer 7 ml each of the other three gradient solutions in order of increasing density. Underlayer 9 ml of LMF in the bottom of the tube.
- Ultracentrifuge 2 hr at $95,000 \times g$, 0° to 4°C .
- Harvest the lysosomes from the 19%/24% interface using a syringe and metal cannula.
- If required, dilute the fraction with ≥ 2 vol of HM and centrifuge 20 min at $30,000 \times g$, 0° to 4°C .
- Resuspend the pellet in HM to 1 to 5 mg protein/ml.
- Process the fractions as described (see Basic Protocol 1, step 20).

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20°C or -80°C ; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with results obtained with fresh material.

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3.6.5

FRACTIONATION OF A RAT LIVER LIGHT MITOCHONDRIAL FRACTION IN A CONTINUOUS IODIXANOL GRADIENT

Occasionally it is necessary to at least partially purify the other organelles in the light mitochondrial fraction (as well as the lysosomes), as, for example, when the localization of a particular component or function is being determined (i.e., a gradient is being used analytically rather than preparatively). In this case, none of the gradients described in Basic Protocol 1 or Alternate Protocols 1 and 2 are really suited to this task, because mitochondria, peroxisomes, and endoplasmic reticulum tend to copurify (particularly in Percoll gradients). The continuous iodixanol gradient described in this protocol overcomes this problem to a large extent. Bottom-loading of the sample in a dense medium is described, but top loading can be used.

Additional Materials (also see Basic Protocol 1)

OptiPrep diluent (see recipe)
OptiPrep (Accurate Chemical)
Dense gradient unloading liquid (e.g., Maxidens; Lipotek, U.K.)
Two-chamber gradient maker or Gradient Master
Gradient unloader
Ultracentrifuge with Beckman SW28.1 or equivalent swinging bucket rotor

1. Prepare the LMF (see Basic Protocol 1, steps 1 to 15), but suspend the final pellet in 3.5 ml HM.
2. Prepare 50% (w/v) iodixanol stock by combining 5 vol of OptiPrep (60% iodixanol) and 1 vol OptiPrep diluent. Mix 3.0 ml of LMF with 4.5 ml of 50% iodixanol stock by repeated inversion.

NOTE: Do not shake the tube so vigorously as to create foaming.

If the sample is going to be loaded on top of the gradient rather than beneath it, then omit this step.

3. Prepare a linear iodixanol gradient in the 17-ml centrifuge tubes from 6 ml each of 19% and 27% (w/v) iodixanol.

If the gradient is prepared with a Gradient Master use an angle of 80°, 20 rpm, and a time of 1 min 50 sec.

4. Underlayer the gradient with 3 ml of the LMF/iodixanol from step 2, using a syringe and metal cannula.
5. Overlay the gradient with 1 ml of HM.

Alternatively underlayer the gradient with 1 ml of 30% iodixanol (3 vol iodixanol stock and 2 vol HM) and then layer 3 ml of LMF on top of the gradient.

6. Ultracentrifuge 2 hr at $110,000 \times g$, 0° to 4°C. Collect the gradient in 1-ml fractions, low-density end first by upward displacement with a dense liquid (e.g., Maxidens).

The lysosomes band close to the top of the gradient.

7. If required, dilute the fraction with 2 vol of HM and centrifuge 20 min at $30,000 \times g$, 0° to 4°C.
8. Resuspend the pellet in HM to 1 to 5 mg protein/ml.
9. Process the fractions as described (see Basic Protocol 1, step 20).

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20° or -80°C; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with results obtained with fresh material.

ISOLATION OF LYSOSOMES FROM HUMAN HL-60 CULTURED CELLS USING A SELF-GENERATED PERCOLL GRADIENT

BASIC
PROTOCOL 2

This is an example of the use of a post-nuclear supernatant from a cultured cell homogenate. While similar homogenization media and centrifugation and analysis protocols, with minor modifications, have been applied to the isolation of lysosomes from a variety of cultured cells, the form of homogenization that has been used is extremely variable. The protocol below has been adapted from the method of Carlsson and Fukuda (1992), as these authors used the ball-bearing homogenizer of Balch and Rothman (1985), which causes very little damage to subcellular organelles. The homogenization procedures used for cultured cells are too diverse to be covered comprehensively in this text (see Graham, 1997 for more information), but a few alternatives are suggested in the italicized annotations and discussed more fully in the Commentary. Whatever homogenization method is used, the aim should be to produce ~90% cell breakage using the minimum shearing forces.

Materials

- HL-60 cells (ATCC#CCL-240) in homogenization medium
- Phosphate-buffered saline (PBS; *APPENDIX 2A*)
- Homogenization medium (HM; see recipe)
- 10% (w/v) bovine serum albumin (BSA)
- Percoll stock solution
- 10% (w/v) Nonidet-40 (NP-40) in water
- Ball-bearing cell homogenizer (see Balch and Rothman, 1985, for more details)
- Low-speed centrifuge with swinging-bucket rotor to take 10-ml tubes
- Sorvall high-speed centrifuge with SE-12 and SS-34 or equivalent fixed-angle rotors
- Beckman ultracentrifuge with 50Ti, 70Ti, T865 (Sorvall), or equivalent fixed-angle rotor
- Gradient unloader: thin metal cannula connected to a peristaltic pump

NOTE: An ultracentrifuge rotor is required to pellet Percoll (step 12), but gradient formation (step 10) can be carried out in a high-speed centrifuge or ultracentrifuge.

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all of the solutions.

1. Wash $1-3 \times 10^8$ HL-60 cells once with 5 ml phosphate buffered saline (PBS), and again with 5 ml of HM, pelleting cells by centrifugation at $500 \times g$ for 10 min.

In some methods the EDTA is omitted from the homogenization medium (and the Percoll diluent). The inclusion of EDTA is dependent on the subsequent procedures to be done on purified lysosomes, and how well cells homogenize (some cells will not homogenize without EDTA). An alternative medium in which 10 mM triethanolamine-acetic acid, pH 7.4 replaces the HEPES buffer has been found to be suitable to many monolayer cultures (Marsh et al., 1987).

2. Suspend the cells in 3 ml of HM.
3. Homogenize in the ball-bearing homogenizer using 5 passes.

See Balch and Rothman (1985), for more details on the use of ball-bearing homogenizers. A Dounce (Finley and Kornfeld, 1994; Lipman et al., 1990; Muno et al., 1990) or Potter-Elvehjem homogenizer (Akasaki et al., 1993), or nitrogen cavitation (Kelly et al., 1989) may be used if the ball-bearing homogenizer is not available.

4. Rinse the homogenizer with 0.5 ml of HM and add to the homogenate.

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5. Pellet the nuclei, cell debris and any unbroken cells by centrifuging at $800 \times g$ for 10 min in a low-speed centrifuge.
6. Decant or aspirate the supernatant and keep on ice.
7. Suspend the nuclear pellet in 1 ml of HM (use gentle vortexing). Repeat steps 5 and 6.
8. Combine the supernatants, adding adding HM to a final total volume of 3.5 ml if necessary.
9. Mix 3 ml of supernatant with 0.18 ml 10% bovine serum albumin (BSA), 1.0 ml of Percoll stock solution, and HM to a final volume of 4.5 ml.

The final Percoll concentration is 20%.

In some methods the post-nuclear fraction is layered over the Percoll solution and other Percoll concentrations that have been used are ~27% and 35% (v/v).

10. Ultracentrifuge 30 min at $36,000 \times g$ in a Beckman 50 Ti rotor or equivalent fixed-angle rotor, 0° to 4°C .

Centrifugation conditions vary from 60 min at $15,000 \times g$ to 40 min at $62,500 \times g$ and the gradient volume may be as high as 35 ml.

The density profile required to achieve high resolution will depend on cell type and HM composition. Centrifugation conditions required to produce this profile will depend on rotor type.

11. Collect the gradient dense-end-first (0.4-ml fractions) using a gradient unloader.

The lysosomes band close to the bottom of the gradient.

A thin metal cannula connected to a peristaltic pump can be used as a gradient unloader.

12. Add 10% Nonidet-40 (NP-40) to each gradient fraction (final concentration 0.5%) before pelleting the Percoll by ultracentrifuging 1 to 2 hr at $100,000 \times g$, 0° to 4°C .

By adding a nonionic detergent to solubilize the organelles before the Percoll is pelleted, very high recoveries of lysosomal proteins may be obtained. If the aim, however, is to recover intact organelles, the detergent must be omitted.

13. Perform metabolic assays for lysosomes (see Support Protocols 1 and 2), peroxisomes (UNIT 3.4), mitochondria (UNIT 3.4), or endoplasmic reticulum (UNIT 3.5) as soon as possible upon preparation.

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20° or -80°C ; always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with results obtained with fresh material.

SUPPORT PROTOCOL 1

ASSAY FOR ACID PHOSPHATASE

Acid phosphatase is an established marker for lysosomes. The assay is simple to execute and uses *p*-nitrophenyl phosphate as a substrate. The released nitrophenol is measured spectrophotometrically in an alkaline solution. *p*-Nitrophenyl phosphate is quite stable under alkaline conditions, thus if the reaction is terminated by addition of NaOH, background hydrolysis is negligible. Because of the ubiquity of nonspecific phosphatases, glycosidases such as β -galactosidase (e.g., Graham, 1997; UNIT 3.4) or β -*N*-acetylglucosaminidase (e.g., Berg et al., 1985; Support Protocol 2) are increasingly used today as markers.

Sample handling depends on the type of gradient and the concentration of lysosomes (and other organelles) in the fractions (see Commentary). Ideally, the protocol should be carried out on gradient fractions from which the gradient solute or Percoll has been removed and the gradient fractions suspended in 0.25 M sucrose, 10 mM HEPES/NaOH, pH 7.4 or equivalent.

Materials

Assay buffer I (see recipe)
Substrate solution I (see recipe)
Gradient fractions (see Basic Protocol 1 or 2 or Alternate Protocol 1 or 2)
0.25 M NaOH

1. Prepare assay solution by mixing equal volumes of assay buffer I and substrate solution I. Add 50 μ l of each gradient fraction (containing \sim 20 μ g protein) to separate microcentrifuge tubes containing 0.2 ml of assay solution. Perform the assay in duplicate.
2. Prepare a blank for each fraction by adding 0.2 ml of assay solution to 0.6 ml of 0.25 M NaOH before adding 50 μ l gradient fraction.

As long as the protein content of each assay is approximately the same, a blank for each fraction may not be necessary; a single blank containing buffer instead of sample may suffice for many gradients. This simple approach cannot be adopted for Percoll-containing fractions.

3. Incubate assay tubes at 37°C for 30 min.
4. Add 0.6 ml of 0.25 M NaOH to each test sample to stop the reaction.
5. Remove any precipitate by microcentrifuging 1 to 2 min at maximum speed.
6. Using plastic cuvettes, measure the absorbance of the assay reactions at 410 nm against the appropriate blank.

The molar extinction coefficient of nitrophenol is 9620 $\text{cm}^{-1}\text{M}^{-1}$.

ASSAY FOR β -N-ACETYLGLUCOSAMINIDASE

This sensitive fluorometric method is based on the release of 4-methylumbelliferone from 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide at an acid pH followed by determination of the fluorescence of the aglycone at pH 10 or 11. It may be necessary to remove protein by precipitation with trichloroacetic acid before measuring the fluorescence. A less sensitive spectrophotometric method, which uses the *p*-nitrophenyl derivative, is also available (see β -galactosidase assay in UNIT 3.4).

Sample handling depends on the type of gradient and the concentration of lysosomes (and other organelles) in the fraction (see Commentary). Ideally, the protocols should be carried out on fractions from which the gradient solute or Percoll has been removed; the gradient fractions are resuspended in 0.25 M sucrose, 10 mM HEPES/NaOH, pH 7.4, or equivalent.

This method is adapted from Barrett and Heath (1977).

Materials

Assay buffer II (see recipe)
Substrate solution II (see recipe)
Bicarbonate buffer (see recipe)
1.2 mM (21 mg/100 ml) 4-methylumbelliferone, prepare fresh
Fluorimeter and appropriate cuvettes

SUPPORT PROTOCOL 2

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1. Mix 0.5 ml each of assay buffer II and substrate solution II with 0.45 ml water.
2. Add 50 μ l sample.

It is strongly advised that some preliminary experimentation be carried out to determine the optimum range of protein concentrations that will provide a satisfactory fluorescent emission, detectable by the available instrument and falling on the standard curve (see step 5).

3. Prepare a blank for each fraction by repeating step 1, adding 3 ml bicarbonate buffer, and then adding 50 μ l of sample.

Unless the samples are clarified by the addition of TCA and sedimentation prior to fluorescent measurement (step 6), light scattering in the sample may require a separate blank for each sample. However, if the protein content of each clarified assay reaction is approximately the same, a blank for each fraction may be avoided, in which case a single blank containing 0.5 ml assay buffer, substrate solution, and water (and no sample) may suffice.

4. Incubate 30 to 60 min at 37°C.
5. Prepare a standard curve by diluting 1.2 mM 4-methylumbelliferone with water to make 1 ml standards from 1.2 to 12 nmol and adding 0.5 ml assay buffer II to each.
6. Add 3 ml bicarbonate buffer to all sample and standard reactions (not blanks). Measure the fluorescence emission at 448 nm using an excitation wavelength of 360 nm.

If it is necessary to clarify the samples, add 1.5 ml of 3.3% (w/v) TCA to each tube. Let stand 5 min, then centrifuge 2 min in a microcentrifuge, to remove precipitate. To 2 ml of supernatant add 2 ml bicarbonate buffer and measure fluorescence.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Assay buffer I

To 100 ml H₂O add:
2.94 g sodium acetate (180 mM final)
Adjust to pH 5.0 with glacial acetic acid
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

Assay buffer II

To 100 ml H₂O add:
11.52 g citric acid (0.3 M final)
17.64 g trisodium citrate dihydrate (0.3 M final)
3.5 g NaCl (0.3 M final)
H₂O to 200 ml
Store 1 to 2 days at 4°C

Bicarbonate buffer

To 250 ml H₂O add:
26.5 sodium carbonate (0.5 M final)
21 g sodium bicarbonate (0.5 M final)
H₂O to 500 ml
The pH should be ~9.9
Store 1 to 2 weeks at 4°C

CaCl₂, 100 mM

To 50 ml H₂O add:
1.47 g CaCl₂·2H₂O
Add H₂O to 100 ml
Store up to 3 months at 4°C

Dextran solution, 100 mg/ml

To 50 ml H₂O add:
0.85 g NaCl
10 g dextran (M_r = 60,000 to 90,000)
Add H₂O to 100 ml
Store up to 1 to 2 days at 4°C

HEPES, 1 M and 100 mM

23.83 g N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)
Adjust volume to 100 ml with H₂O for 1 M and to 1000 ml for 100 mM HEPES.

Homogenization medium (HM)

To 100 ml H₂O add:
17.1 g sucrose (0.25 M final)
2 ml 100 mM Na₂EDTA (1 mM final)
20 ml 100 mM HEPES (10 mM final; see recipe)
Adjust to pH 7.0 with NaOH
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

Nycodenz stock solution, 45% (w/v)

To 100 ml H₂O add:
90 g Nycodenz (Accurate Chemicals)
2 ml 100 mM Na₂EDTA (1 mM final)
20 ml 100 mM HEPES (10 mM final; see recipe)
Adjust to pH 7.4 with NaOH
Add H₂O to 200 ml
Store up to 3 months at -20°C

OptiPrep diluent

To 50 ml H₂O add:
8.55 g sucrose (0.25 M final)
6 ml 100 mM Na₂EDTA (6 mM final)
12 ml 1 M HEPES (120 mM final; see recipe)
Adjust to pH 7.4 with NaOH
Add H₂O to 100 ml
Store up to 1 to 2 days at 4°C

Percoll diluent

To 75 ml H₂O add:
171 g sucrose (2.5 M final)
20 ml 100 mM Na₂EDTA (10 mM final)
20 ml 1 M HEPES (100 mM final)
Adjust to pH 7.0 with NaOH
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

For protocols where EDTA is omitted, replace with 20 ml water.

Percoll stock solution, 90% (v/v)

Combine 9 vol Percoll and 1 vol Percoll diluent (with or without EDTA depending on protocol). Store up to 1 to 2 days at 4°C.

Substrate solution I

To 100 ml H₂O add:
0.59 g *p*-nitrophenyl phosphate hexahydrate
Store up to 3 months at -20°C

Substrate solution II

To 100 ml H₂O add:
56.8 mg 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (1.5 mM final)
Prepare fresh

COMMENTARY

Background Information

Homogenization and differential centrifugation

For all mammalian tissues, a routine homogenization medium containing 0.25 M sucrose and 1 mM EDTA, usually buffered with HEPES, is used with no special supplements, although for brain tissue the sucrose concentration is invariably increased to 0.32 M (Caimi et al., 1989). This medium has also commonly been used for cultured cells, although sometimes the EDTA is omitted. Dounce homogenization of the cells is widely used, although nitrogen cavitation of human skin fibroblasts was used by Kelly et al. (1989) and a ball-bearing homogenizer (Balch and Rothman, 1985) by Carlsson and Fukuda (1992) for HL-60 cells. As with peroxisomes (*UNIT 3.5*), the gentlest possible homogenization protocol should be adopted, to avoid any potential damage to the organelles.

With tissues such as rat liver, the first stage of the lysosome purification is always the isolation of a light mitochondrial fraction (LMF), which is then further fractionated on some form of density gradient. This is also true of other tissues such as brain (Caimi et al., 1989; Ohshita and Kido, 1995) and kidney (Ohshita et al., 1992). In the case of cultured cells, a post-nuclear supernatant (PNS) is often used as the source of crude lysosomes for gradient centrifugation. This was also used by Oliver et al. (1989) for rat parotid acinar cells. If a LMF is used, then it is normally sedimented from the PNS by centrifuging 10 to 15 min at 18,000 to 20,000 × *g*; slightly lower than that used for the purification of peroxisomes (*UNIT 3.5*) which is routinely 25,000 × *g* for 10 to 15 min. The reason for this may be that the smallest peroxisomes are ~0.2 μm, while the smallest

lysosomes are ~0.4 μm. There are also a few examples of the use of much lower RCFs: 20 min at 12,500 × *g* for chicken liver (Nakabayashi and Ikezawa, 1988) and 20 min at 11,000 × *g* for macrophages (Muno et al., 1990). Since lysosomes contain many degradative enzymes, resuspension of any differential centrifugation pellets must be carried out as gently as possible; indeed the lack of any pellet resuspension is a clear advantage of using a PNS.

Density gradients

Sucrose gradients. Because the density of lysosomes is very similar to that of mitochondria and peroxisomes in sucrose gradients (Table 3.5.1), that method is now regarded as unsatisfactory for lysosome purification. Sucrose gradients can only achieve a partial resolution of lysosomes from other organelles, unless their density is reduced by loading them with Triton WR1339 (Leighton et al., 1968). This involves injection of the experimental animal with the detergent. It is now known that the lysosomes (or tritosomes) that are produced behave abnormally, particularly in regard to their enzyme composition (Wattiaux and Wattiaux-De Coninck, 1983). Therefore, it is now considered more acceptable to use one of the low-osmolarity media to isolate these organelles, because in such media the density of lysosomes is sufficiently different from that of the other organelles (see Table 3.5.1) to permit easy isolation. In iodinated density gradient media such as Nycodenz or iodixanol, lysosomes are less dense than either mitochondria or peroxisomes, while in Percoll they tend to be more dense; however, the reason for this is not clear. Because Percoll can be used as a self-generated gradient at relatively low RCFs (20,000 to 60,000 × *g* is common) and because

a band of lysosomes is invariably formed towards the bottom of the gradient with relatively little contamination from other organelles, the use of this medium is very popular.

Percoll gradients. In the handling of Percoll gradients, the osmolarity of the commercial medium (which is close to zero) has to be adjusted to ~290 mOsm by the addition of 0.1 vol of a solution containing 2.5 M sucrose. Normally this osmotic balancer is simply a 10× solution of the homogenization medium. In this way, the organelles are always exposed to the same isoosmotic medium. Usually, the final 90% (v/v) Percoll stock solution contains 0.25 M sucrose, 10 to 20 mM HEPES/NaOH, pH 7.0 to 7.4, with or without 1 mM EDTA (see recipe in Reagents and Solutions); however, as an alternative, 0.24 M mannitol, 10 mM MOPS/NaOH, pH 7.0 (Pisoni et al., 1987), and 1 mM EDTA, can be used instead.

As with all self-generated gradients, the centrifugation conditions which are used to produce the Percoll gradient control its profile. That is, lower RCFs and shorter centrifugation times will tend to produce sigmoidal gradients which are very shallow in the middle, while higher RCFs and times cause the gradient to become more linear (although a truly linear gradient is difficult to achieve with Percoll). Usually a fixed-angle rotor is used to create the gradient, but there are examples which use a vertical rotor (Kelly et al., 1989; Oliver et al., 1989). Oliver et al. (1989) included a cushion of 2.5 M sucrose, presumably to prevent any silica particles pelleting on to the vertical wall of the tube. RCFs normally vary from 20,000 to 65,000 × *g* and times from 30 to 90 min, although some methods fall outside these ranges. For lower RCFs, it is possible to use a high-speed centrifuge. The starting concentration of the Percoll, normally 20 to 40% (v/v), affects the density range of the gradient. The lower the starting concentration, the nearer the

bottom of the tube the lysosomes will band. Some of the conditions for lysosome separation from cultured cells are summarized in Table 3.6.1.

The PNS or LMF is either simply mixed with the Percoll stock, or layered over a Percoll solution prepared from the Percoll stock and (usually) HM. Overlaying will minimize any contamination of the lysosomes by soluble proteins, which under the given centrifugation conditions will remain at the top of the gradient.

There are relatively few instances of Percoll being used in a non-self-generated gradient mode. Examples include PNS from guinea-pig alveolar cells fractionated on a preformed 0% to 50% Percoll gradient, 1 hr at 19,000 × *g* (12,000 rpm) in a Beckman SW-28 swinging-bucket rotor (Yamaguchi and Kaneda, 1988), and an 8% Percoll barrier used for pelleting the lysosomes from a K562 erythroleukemia cell PNS, 20 min at 19,000 × *g* (Bridges, 1987).

Nycodenz and iodixanol gradients. The discontinuous Nycodenz gradient (Olsson et al., 1989), based on the metrizamide system developed by Wattiaux and Wattiaux-De Coninck (1983), provides a useful alternative to Percoll. Nycodenz was also used by Graham et al. (1990) for isolation of organelles. Lack of a simple self-generated gradient protocol, however, is a disadvantage. Although iodixanol, which is a dimer of Nycodenz, forms self-generated gradients rather more quickly than Nycodenz and can be used to fractionate the organelles from a LMF (Graham et al., 1994), the RCFs required are in excess of 150,000 × *g*, with centrifugation times of 1 to 3 hr (depending on the RCF and the rotor). However, if it is a requirement that the separation system should adequately resolve both the mitochondria and peroxisomes, as well as the lysosomes, then a continuous iodixanol gradient can achieve this aim more effectively than Percoll.

Table 3.6.1 Percoll Centrifugation Conditions for Selected Cultured Cells

Cell type	% Percoll (v/v)	Volume (ml)	RCF/time	Reference
Human fibroblasts	27	35	35,000 × <i>g</i> /60 min	Kelly et al., 1989
	34	10	17,500 × <i>g</i> /35 min	Pisoni et al., 1987
Macrophages	35	7.5	62,500 × <i>g</i> /40 min	Muno et al., 1990
	27	30	15,000 × <i>g</i> /60 min	Lipman et al., 1990
Monkey COS	36	12	35,000 × <i>g</i> /90 min	Finley and Kornfeld, 1994
Rat hepatocytes	27	10	25,000 × <i>g</i> /40 min	Akasaki et al., 1993

Critical Parameters and Troubleshooting

Homogenization

As with all membrane fractionations, the use of cultured cells, rather than intact tissues, may provide problems in devising a suitable homogenization method. Although most monolayer cultured cells seem to homogenize reasonably effectively in the widely-used sucrose/EDTA/HEPES medium, the homogenization medium developed by Marsh et al. (1987) of 0.25 M sucrose, 1 mM EDTA, and 10 mM triethanolamine/10 mM acetic acid, pH 7.4, is more widely applicable (the triethanolamine-acetic acid buffer being the critically important component). This was used for the preparation of lysosomes from human fibroblasts (Pisoni et al., 1987), while for the J774 mouse macrophage-like cells, Lipman et al. (1990) used 0.25 M sucrose, 1 mM EGTA, 1 mM Mg²⁺, 1 mM dithiothreitol, and 20 mM HEPES/NaOH, pH 7.4. If, upon homogenization, the cell homogenate acquires a gelatinous quality, this may be due either to nuclear breakage or to the release of proteins from cytoskeletal elements. Inclusion of 1 mM Mg²⁺ with or without DNase I (μg/ml) and/or 10 mM KCl may ameliorate the problems, although these measures should be regarded as a last resort.

Although a Dounce homogenizer is often used to break cultured cells, it is strongly recommended that the optimal conditions for disrupting the cells be thoroughly investigated. The aim must always be to use the minimum force to achieve ~90% cell breakage; therefore, protocols which use more than 20 strokes of the pestle should be avoided.

For cells that resist the liquid shear forces either of the Balch and Rothman (1985) ball-bearing homogenizer or of Dounce or Potter-Elvehjem homogenizers, nitrogen cavitation is probably the best option, as an isoosmotic sucrose medium can always be used. Standard conditions are 300 to 600 psi for 10 to 15 min, although Kelly et al. (1989) reported the use of much lower pressures. For a more complete discussion of these problems see Evans (1992) and Graham (1997).

Differential centrifugation

The aim of the homogenization should be to devise conditions such that 90% of cells are disrupted with the minimum possible release of organelle markers into the medium. To determine organelle integrity, all of the differential centrifugation fractions, including the mi-

croosomes and the cytosol (45 min, 100,000 × *g* pellet and supernatant) should be assayed for organelle markers and proteins (see Anticipated Results). For a tissue or cell other than those described in this unit, always carry out a more thorough differential centrifugation (e.g., 10 min at 600 × *g*, 10 min at 3000 × *g*, 10 min at 15,000 × *g*, 10 min at 25,000 × *g*, and 10 min at 40,000 × *g*) in order to devise the optimal protocol.

Density gradient centrifugation

For a tissue or cell other than those described in this unit, it may also be necessary to modulate some of the gradient and/or centrifugation conditions in the light of experience, for example, making the gradient more or less dense at the bottom to take account of organelles which may be correspondingly more or less dense. Modifications will also need to be made if the type of rotor described in this unit (see Basic Protocol 1) is not available. The sedimentation path length of the rotor will influence the shape of the density gradient profile—the longer the path length, the more shallow the gradient will be in the middle, and the steeper it will be at both ends (under the same centrifugation conditions). With Percoll, the density profile of the gradient can be checked by using colored density marker beads, which are available from Amersham-Pharmacia Biotech.

The preparation of preformed continuous gradients of iodixanol using a two-chamber gradient maker requires considerable practice to achieve highly reproducible gradients. Some of the important points in operating such devices are covered by Dobrota and Hinton (1992). A Gradient Master, which involves simply layering the densest and lightest solutions in the centrifuge tube, provides a more expensive but reproducible alternative. If neither of these is available, then a discontinuous gradient constructed from three or four solutions spanning the required density range can be prepared and allowed to diffuse, usually overnight at 4°C. Note that the time required to achieve complete linearity will depend on the tube diameter and the volume of each layer. Always collect a blank gradient and check (using refractive index) that the gradient is indeed linear and covers the appropriate density range.

Enzyme and other assays

Prior to the execution of an enzyme assay (or other measurement), it is common practice to remove Percoll, Nycodenz, or iodixanol from each gradient fraction. In the case of

Percoll, the silica colloid is normally pelleted from the gradient fraction at $100,000 \times g$, while the other two media are removed in the supernatant after dilution of the fraction with 2 to 4 vol of a low-density medium and centrifugation of the organelles 15 to 20 min at $\sim 20,000 \times g$. These practices eliminate any possible interference of the gradient medium with the subsequent procedure(s) and are described in the final steps of the appropriate protocols. Osmundsen (1982) warned, however, that at least with peroxisomes, organelles may be lost into the Percoll pellet and that this loss may be as much as 50% of the total.

If the sample volumes used in an enzyme assay are sufficiently small (see Supports Protocol 1 and 2), the gradient solute may be adequately diluted so as not to cause any interference in the assay, even at the highest density. Thus, so long as the concentration of organelles in the gradient fraction is high enough for the enzyme activity to be detectable, the assay may be performed directly without removal. If, however, either of these requirements are not met, then dilution, pelleting, and resuspension of the pellet in a smaller volume are unavoidable. If Percoll is not removed from the gradient fraction, a blank containing the sample is essential.

In order to determine specific activities of measured enzymes, it is also necessary to estimate the protein content of fractions. Percoll, Nycodenz, and iodixanol interfere with methods using the Folin-Ciocalteu reagent, although, if by using small volumes of Nycodenz or iodixanol-containing fractions ($<100 \mu\text{l}$) in a 1 to 2 ml assay volume, the concentration of gradient solute in the assay solution is $\leq 10\%$, the interference is marginal. Methods based on Coomassie blue are generally unaffected by any concentration of these gradient media, while Percoll-containing fractions may be used so long an appropriate Percoll blank is used.

Anticipated Results

Basic Protocol 1

Differential centrifugation. Table 3.6.2 provides an analysis of the enzyme content of three principal differential centrifugation fractions. In particular it should be noted that values for acid phosphatase in the $20,000 \times g$ supernatant, in excess of those in Table 3.6.2 might indicate that there is considerable lysosomal breakage during the homogenization. There will be some variation depending on the type of tissue or cell, and it is interesting that for brain tissue Caimi et al. (1989) reported only 4% of the acid phosphatase in a $17,500 \times g$ supernatant. The authors used a very mild homogenization method using only 5 very slow strokes of the pestle of a Potter-Elvehjem homogenizer.

Density gradient. In Percoll in particular, the density of the lysosomes (Table 3.5.1) reveals a considerable heterogeneity, with the result that those lysosomes banding at a lower density in Percoll gradients overlap other organelles and are not recoverable in anything approaching a pure form. The profile of the lysosomal enzyme markers in the gradient, while always demonstrating this broad range of density banding, varies with the gradient-forming conditions and the enzyme. In the gradient system described in this protocol (Symons and Jonas, 1987) the β -hexosaminidase of rat liver lysosomes shows only one clear band in the bottom 2 ml of the gradient, while the enzyme is broadly distributed at a low level in the top 8 ml of the gradient; nevertheless, this represents $\sim 40\%$ of the total enzyme in the gradient (Fig. 3.6.1).

In the system of Nakabayashi and Ikezawa (1988), on the other hand, the lysosomal enzymes from chicken liver showed a very pronounced biphasic distribution (Fig. 3.6.2); moreover *N*-acetyl- β -D-glucosaminidase was concentrated in the dense band, while β -D-

Table 3.6.2 Enzyme Content in Differential Centrifugation Fractions

Enzyme/protein	Enzyme content (% of total in homogenate)		
	$750 \times g$ pellet	$20,000 \times g$ pellet	$20,000 \times g$ supernatant
Acid phosphatase	20-25	30-35	35-40
Catalase	20-25	35-40	25-30
NADPH-cyt <i>c</i> reductase	15-20	5-10	70-80
Protein	25-30	15-20	55-60
Succinate dehydrogenase	40-50	40-50	5-10

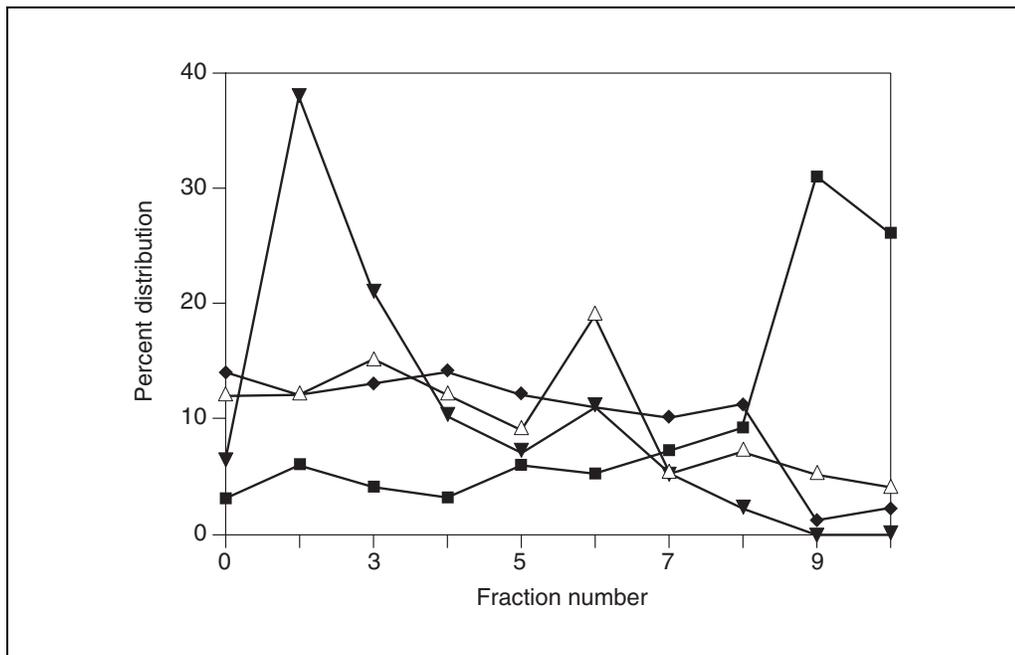


Figure 3.6.1 Isolation of rat liver lysosomes in a Percoll gradient: distribution of enzyme markers. Rat liver LMF in 40% Percoll was centrifuged for 90 min at $35,000 \times g$, 4°C . The gradient was collected low-density end first (1-ml fractions). β -hexosaminidase (filled squares); cytochrome oxidase (filled triangles); catalase (filled-diamonds); and glucose-6-phosphatase (filled inverted triangles). Data adapted from Symons and Jonas (1987) with kind permission.

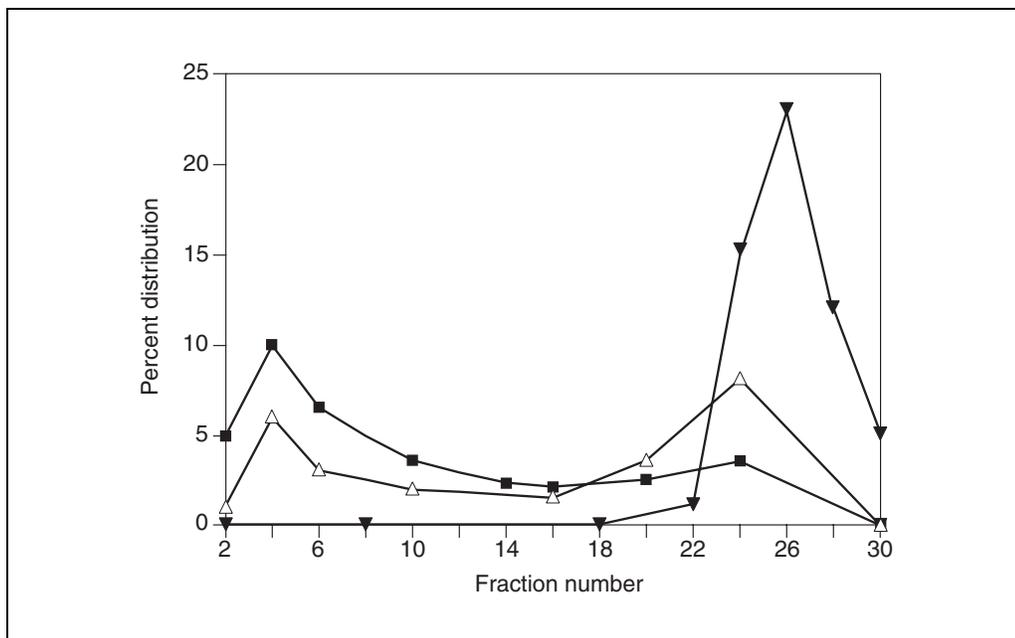


Figure 3.6.2 Isolation of chicken liver lysosomes in a Percoll gradient: distribution of enzyme markers. Chicken liver LMF in 36% Percoll was centrifuged 30 min for $25,000 \times g$, 4°C . The gradient was collected dense-end first. *N*-acetyl- β -glucosaminidase (filled squares); β -galactosidase (open triangles) and succinate dehydrogenase (filled inverted triangles). Data adapted from Nakabayashi and Ikezawa (1988) with kind permission.

galactosidase predominated in the light band. The actual enzyme profiles may be dictated by the gradient density profile, which in turn is controlled by the centrifugation conditions. At a low RCF and short centrifugation time (Nakabayashi and Ikezawa, 1988) the density profile is more sigmoidal (shallow in the middle), while at higher RCFs and longer times the profile will be more linear (Symons and Jonas, 1987).

Although there is clear heterogeneity of the lysosome population in Percoll gradients, it is most likely that the production of two distinct bands is an artifact of the gradient density profile. This was elegantly demonstrated by Draye et al. (1987), who banded rat liver lysosomes in a Percoll gradient and discovered that when the enzyme marker (*N*-acetyl- β -glucosaminidase) was plotted as a relative concentration (concentration in gradient fraction/theoretical concentration if homogeneously distributed through the gradient) versus gradient volume, the typical biphasic profile was obtained (Fig. 3.6.3). When the data is replotted as frequency (enzyme activity/density interval of fraction) versus density, a single asymmetrical peak is obtained (Fig. 3.6.4). The authors confirmed this by banding the lysosomes in a linear gradient and obtained the same single asymmetrical peak.

The overall yield of lysosomes (as a percentage of the homogenate) in the Symons and Jonas (1987) gradient is ~20%, although for most Percoll gradients, the figure is closer to 15%. The relative specific activity (RSA) of lysosomal enzyme markers (specific activity in the fraction/specific activity in the homogenate) is ~60.

Alternate Protocol 1

When the lysosomes are density-perturbed with dextran, 70% to 80% of the total *N*-acetyl- β -glucosaminidase in the gradient is recovered in a single very sharp band in the bottom two fractions, and all of the CaCl_2 -swollen mitochondria are at the top of the gradient (Arai et al., 1991). The RSA of lysosomal markers (~100) is accordingly higher than in Basic Protocol 1.

Alternate Protocol 2

The relative specific activity of lysosomal enzyme markers is ~100 (Olsson et al., 1989) in the 19%/24% Nycodenz interfacial material, while that for enzyme markers of mitochondria, peroxisomes, and endoplasmic reticulum is <0.5, showing a high degree of purification of the lysosomes. Typical yields of lysosomes (as a percent of the total homogenate) are ~10%. Thus, while the yield is lower than with the

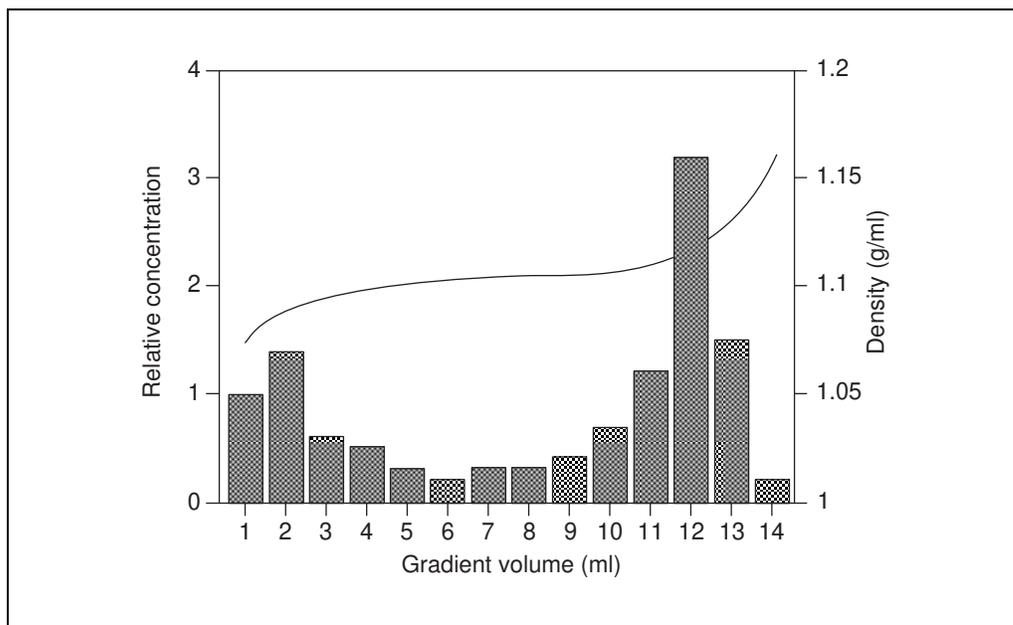


Figure 3.6.3 Biphasic banding of lysosomes in Percoll gradient. The bars show the relative concentration (concentration in gradient fraction/theoretical concentration if homogeneously distributed through the gradient) of *N*-acetyl- β -glucosaminidase plotted against gradient volume. The continuous line is the density profile. Data from Draye et al. (1987) with kind permission.

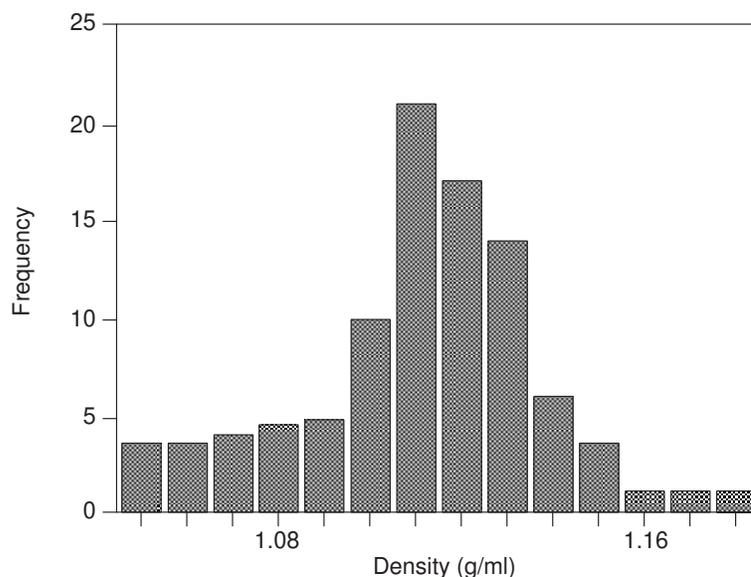


Figure 3.6.4 Real density heterogeneity of lysosomes in Percoll gradient. The data from Figure 3.6.3 were replotted as frequency (enzyme activity divided by density interval of fraction) versus density. Data from Draye et al (1987) with kind permission.

native lysosomes on a Percoll gradient, the purity is as high as that of dextranosomes.

Alternate Protocol 3

A typical separation of the organelles from the LMF on a continuous iodixanol gradient is shown in Figure 3.6.5. The lysosomes band at the top of the gradient and there is a concomitant separation of the mitochondria and peroxisomes in the lower density region of the gradient. Although the iodixanol gradient was designed primarily as an analytical gradient, it could be used preparatively for lysosomes.

Basic Protocol 2

Figure 3.6.6 shows the very satisfactory separation of lysosomes from plasma membrane (leukosialin) and Golgi membranes (galactosyl transferase) in the Percoll gradient (Carlsson and Fukuda, 1992). Approximately 50% of the total *N*-acetyl- β -hexosaminidase is recoverable in the bottom four fractions of the gradient. The mitochondria (not shown) band with the plasma and Golgi membranes.

Time Considerations

There are no points at which either of the basic protocols can be discontinued and then resumed. The light mitochondrial fraction produced in Basic Protocol 1 must be used immediately for any subsequent gradient separation.

The preformed gradients in Alternate Protocols 2 and 3 should be made up during the preparation of the LMF in Basic Protocol 1, and kept on ice until required. If they are prepared at room temperature they must be given time to cool down to 4°C. The continuous iodixanol gradient of Alternate Protocol 3 can also be made from a discontinuous gradient the previous day and stored overnight at 4°C.

Basic Protocol 1 and Alternate Protocol 1 should be accomplished in ~4 hr. Because of the shorter density gradient centrifugation time, Basic Protocol 2 will take ~3.5 hr. As long as the gradients for Alternate Protocols 2 and 3 have been prepared ahead of time, these protocols will take ~4.5 hr. These values assume the use of a Percoll gradient. Protocols will take more or less time if one of the alternative gradients is chosen.

With experience and practice it may be unnecessary to unload the gradients in a series of equal fractions. Instead, the lysosome band might be removed using a Pasteur pipet or syringe and this will save ≥ 15 min per tube.

Functional assays should be carried out as soon as possible after the preparation, but for simple marker enzyme assay (such as described in this unit, and *UNIT 3.4*) material can be stored at 0° to 4°C for up to 16 hr without much loss of activity. These assays and compositional measurements may also be carried out on ma-

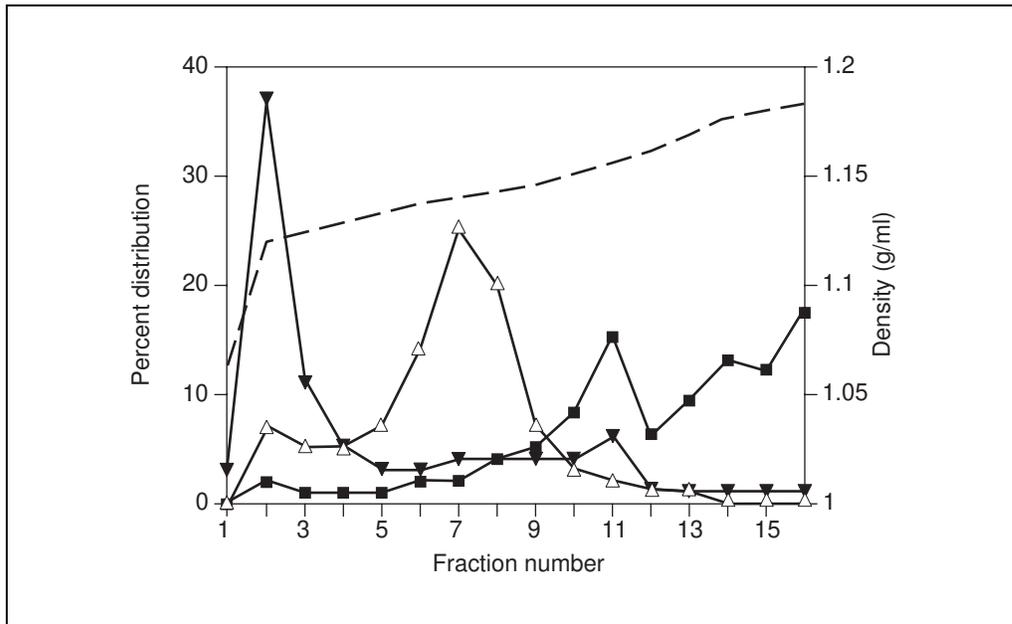


Figure 3.6.5 Fractionation of a rat liver LMF in a continuous iodixanol gradient. Rat liver LMF was centrifuged through a 19% to 27% iodixanol gradient for 2 hr at $110,000 \times g$, 4°C . Gradient collected in 1 ml fractions. β -galactosidase (filled inverted triangles); succinate dehydrogenase (open triangles), and catalase (filled squares). The discontinuous line is the density profile.

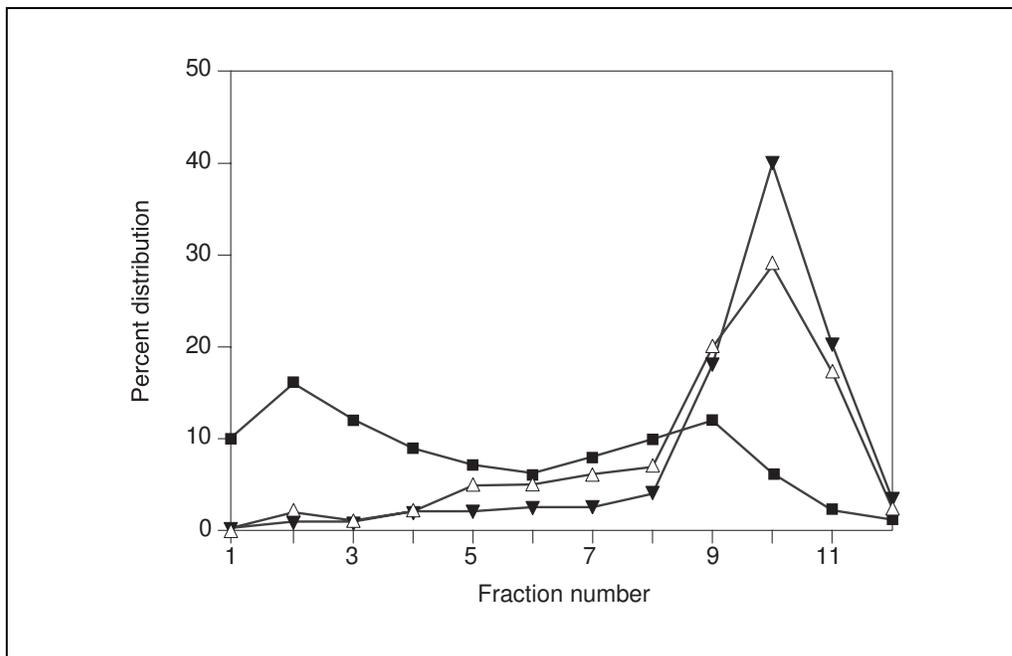


Figure 3.6.6 Isolation of HL-60 cell lysosomes in a Percoll gradient. PNS was layered over 20% Percoll and centrifuged 30 min at $36,000 \times g$, 4°C . The gradient was collected dense-end first in 0.38 ml fractions. β -N-acetylhexosaminidase (filled squares); galactosyl transferase (open triangles) and leukosialin (filled inverted triangles). Data from Carlsson and Fukuda (1992) with kind permission.

terial frozen to -20°C or -80°C . There are, however, no general rules regarding the suitability of a particular storage regime, and it is strongly recommended that the validity of results on stored material be checked by comparison with results obtained with the fresh organelles. Any concentration of the membrane fractions and/or removal of gradient medium must be carried out prior to freezing.

All of the solutions can be made up ahead of time and stored at 4°C for 1 to 2 days, or frozen to -20°C for longer periods, but note that any enzymes and PMSF (or other protease inhibitors) should be added immediately prior to use.

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Overview of Subcellular Fractionation Procedures for the Yeast *Saccharomyces cerevisiae*

The budding yeast *Saccharomyces cerevisiae* is an excellent model organism for the study of eukaryotic cell biology. This unicellular organism shares many features with higher eukaryotic cells including all of the major membrane-bounded subcellular organelles. Accumulating evidence indicates that many cellular processes are mechanistically conserved between yeast and other eukaryotes. Despite the cellular complexity of *S. cerevisiae*, its haploid genome is only 3.5 times larger than that of *E. coli* and has been completely sequenced (Cherry et al., 1997; Clayton et al., 1997; Mewes et al., 1997). This incredible wealth of genetic information, together with powerful genetic and molecular biology techniques, has made *S. cerevisiae* an extremely valuable organism in eukaryotic cell biology (Botstein et al., 1997; Winzeler et al., 1999). The subcellular fractionation techniques for *S. cerevisiae* discussed in this unit are critical to the investigation of the molecular mechanisms of complex cellular functions.

The basic methods used to isolate and characterize organelles and organelle-derived membranes from *S. cerevisiae* are very similar to those used for animal cells and cells from other higher eukaryotes (see UNITS 3.1-3.6). This unit provides an overview of centrifugation-based fractionation procedures used to separate the subcellular components of *S. cerevisiae* according to their size and/or buoyant densities. The critical steps include: (1) cell growth under appropriate conditions, (2) preparation of the cell lysate with minimal damage to organelle integrity, (3) selection of a fractionation protocol that separates the subcellular components of interest into distinct sets of fractions, and (4) identification and characterization of the membranes/organelles in the resulting fractions.

Yeast cells are cultured using relatively simple, rapid, and inexpensive techniques (see UNIT 1.6). Vigorous strains grown in rich medium at 30°C have a doubling time of ~90 min. Yeast are very versatile. They can be maintained in either their haploid or diploid state and grown under either aerobic and anaerobic conditions. The abundance of certain enzymes

and organelles that are subject to metabolic regulation can be manipulated by altering the nutrient composition of the medium. In contrast to animal cells, yeast cells have a thick cell wall that must be disrupted or removed to generate cell lysates. The released subcellular components are then subjected to one or more centrifugation steps, and their distribution among the resulting fractions is determined through their biochemical and morphological characteristics.

Disruption of the cell wall by shearing with glass beads is a rapid and effective method to prepare yeast cell homogenates for the isolation of cytosol or plasma membranes. However, glass-bead lysis is not suitable for most subcellular fractionation experiments, because strong mechanical forces damage the intracellular organelles and can disrupt critical protein and membrane interactions. To minimize the disruption of subcellular integrity, yeast cells are usually enzymatically converted to spheroplasts (yeast cells lacking cell walls) prior to cell lysis. For example, yeast cells can be incubated with enzyme preparations such as Zymolyase 100T (Seikagaku America, ICN), which destabilizes cell walls by cleaving the β -glucan linkages. Subsequently, the spheroplasts are lysed under iso- or hypoosmotic conditions, usually in combination with moderate mechanical force (e.g., several strokes in a Dounce homogenizer).

The most appropriate subcellular fractionation procedure depends on the goals of the particular experiment and the desired levels of organelle yield, purity, and function. Analytical protocols are primarily used to evaluate the subcellular location of one or more proteins. These techniques are designed to obtain discernible differences in the distribution of two or more organelles, while organelle purity, yield, and function are of secondary importance. Ideally, such procedures provide a relatively rapid and simple means to fractionate subcellular components and to subsequently compare the distribution of a protein of interest with the distribution of various organelle membranes. Preparative procedures are generally used to isolate specific organelles, mem-

branes, and/or cytosol. These protocols have a greater emphasis on purity, yield, and/or function than do most analytical protocols. For example, when preparing organelles for reconstitution of a cellular process in a cell-free system, the primary requirement is that the organelle(s) of interest remain functional, while organelle yield and purity are important secondary goals. In contrast, the analysis of the protein or lipid composition of a particular organelle requires that the preparation be of very high purity. If the isolated subcellular components will be fractionated further (e.g., for the separation of mitochondrial inner and outer membranes), and/or used in multiple subsequent experiments, then high yields become especially important.

The following sections provide an overview of several key techniques used during yeast subcellular fractionation experiments. The critical parameters, merits, and limitations of various procedures are discussed to facilitate the development and optimization of centrifugation-based fractionation strategies for yeast. Topics include yeast cell growth, cell lysate preparation, differential velocity centrifugation, density gradient centrifugation, and the analysis of subcellular fractions.

YEAST STRAIN SELECTION AND GROWTH CONDITIONS

Yeast Strains

All references to “yeast” within this unit refer to the budding yeast *S. cerevisiae*, unless otherwise indicated. Many of the protocols developed for *S. cerevisiae* are not directly applicable to other yeast (e.g., *Schizosaccharomyces pombe*) without significant modification. *S. cerevisiae* protocols can be applied to many different strains; however, variations among strains and growth conditions may require adjustments to a given protocol.

The desired *S. cerevisiae* strains can be generated using a variety of genetic and molecular biology techniques (Sherman, 1991). In addition, published strains can be obtained from centralized sources such as the Yeast Genetic Stock Center (<http://www.atcc.org/searchengine/ygsc.html>) or from the investigators that first generated the strain and described it in the literature. In many cases, a limited set of strains is used most frequently by a given laboratory or a group of investigators in a common field. If the strain used in a published protocol is available and compatible with the goals of the experiment, using it can minimize the need for protocol modifications. All

other things being equal, it is usually best to use the healthiest strain and the most optimal growth conditions that meet the goals of the experiment.

For many subcellular fractionation procedures, it is best to use a strain that is deficient in vacuolar protease activity in order to minimize problems with proteolysis. The three major vacuolar proteases are proteinase A (PrA), proteinase B (PrB), and carboxypeptidase Y (CPY), which are encoded by the *PEP4*, *PRB1*, and *PRC1* genes, respectively (Jones, 1991a,b; Van Den Hazel et al., 1996). These proteases, as well as most other vacuolar hydrolases, are first synthesized as inactive precursors and are activated by the cleavage of their pro domain upon arrival in the acidic, protease-rich environment of the vacuole (Klionsky and Emr, 1989; Horazdovsky et al., 1995). The minimal requirement for low vacuolar protease activity is the deletion or inactivation of PrA, because this proteinase self-activates under acidic conditions and subsequently initiates the activation cascade for PrB, CPY, and multiple other vacuolar hydrolases (Zubenko et al., 1983; Jones, 1990, 1991a,b). Protease activity levels can be further reduced by eliminating PrB and CPY expression, as these proteases can become activated in the absence of PrA activity under certain conditions (Jones, 1991a,b; Romanos et al., 1992; Westphal et al., 1996).

Cell Culture Conditions

Most *S. cerevisiae* strains can be cultured with relatively little difficulty and expense (see *UNIT 1.6*; Sherman, 1991). Unless there are special nutritional indications, the medium of choice is a rich broth, such as yeast extract/peptone/dextrose (YPD; *UNIT 1.6*), that promotes rapid growth. Minimal or synthetic medium supplemented with dextrose (SD) and the appropriate amino acids is frequently used if plasmid maintenance is required. Alternate carbon sources can be used to enhance organelle function and proliferation (e.g., peroxisomes, mitochondria) or to regulate the expression of proteins under the control of certain inducible or repressible promoters. The growth of strains carrying mutations in the adenine deficiency genes *ADE1* or *ADE2* can be improved by adding adenine hemisulfate at 100 mg/liter, which minimizes the accumulation of the red metabolic intermediates. (The pH of the medium should be adjusted to 6.0 with NaOH after the addition of adenine hemisulfate.)

The optimal temperature for culturing yeast is 30°C, unless strains sensitive to high or low temperature are being used. Yeast growth is most vigorous under aerobic conditions. Adequate aeration is achieved by culturing yeast in an Erlenmeyer flask (filled to no more than one-fourth capacity) on a rotary shaker. If extensive foaming occurs, the cultures can be safely treated with a standard antifoam solution. Smaller cultures (2 to 10 ml) can be grown in tubes, which should be secured to a rotary shaker at an angle to allow greater movement of the culture. Fermenters can be used to culture the many cells required for large-scale preparations.

To ensure that a culture consists of a homogeneous population of vigorously growing cells, yeast should be cultured for ≥ 16 to 24 hr prior to the start of an experiment. This is often best achieved by inoculating a small volume (~5 to 10 ml) of rich medium with a single yeast colony from a fresh plate. Incubation of this initial culture, sometimes referred to as a preculture, allows viable cells to emerge from the stationary phase and enter the growth phase. Once the cell number has doubled at least twice (two generations), the preculture is used to inoculate a larger volume of medium with cells that will be cultured for an extended period (usually overnight) prior to the start of the experiment. If the culture surpasses its target cell density during the incubation period and its growth rate begins to slow, it is best to dilute the culture and allow the cells to grow for several hours (at least two generations) prior to harvesting.

Monitoring Growth Phase and Cell Titer

For most applications, yeast cells are harvested by centrifugation during their mid- to late-logarithmic growth phase. During this time, the cells are growing and dividing vigorously, and have reached a concentration that permits a reasonable yield. In addition, rapidly dividing cells have lower protease levels and thinner cell walls that are more susceptible to disruption than cells exiting or approaching stationary phase.

The cell concentration that correlates with the mid-logarithmic growth phase can vary considerably for different strains and culture conditions. For example, mutant strains with slower growth kinetics or strains grown in minimal medium will reach the logarithmic growth and stationary phases at significantly

lower cell concentrations than heartier wild-type strains or strains grown in rich medium. Thus, growth curves should be generated for each strain and each set of culture conditions.

The approximate cell concentration of a culture can be determined using a microscope and hemacytometer (*UNIT 1.1*) or by measuring the optical density of the suspension at 600 nm (OD_{600} ; *UNIT 1.6*). Although the latter technique provides only an estimate of the cell concentration, it is rapid and simple. The OD_{600} measurement of a culture, also referred to as the absorbance (A_{600}), is proportional to the number of cells and their size.

The relationship between the OD_{600} value, viable cell number, and growth phase is dependent on the strain and culture conditions. To generate a standard curve for each strain and culture condition to be used, remove samples of the culture at regular intervals throughout its growth and measure the OD_{600} value. The most linear range of the relationship between OD_{600} values and cell number is at OD_{600} values between 0.1 and 0.3; thus, it is usually recommended to measure a diluted culture sample (e.g., 1:5 to 1:10 dilution). Next, determine the corresponding viable cell number by plating appropriate dilutions onto YPAD plates (*UNIT 1.6*).

In general, each 0.1 OD_{600} unit corresponds to $\sim 2\text{--}4 \times 10^6$ cells/ml for haploid cells and $0.5\text{--}2 \times 10^6$ cells/ml for diploid cells. An OD_{600} unit of haploid cells, defined as 1 ml of culture with an OD_{600} value of 1, typically represents 3×10^7 cells. In many cases, a haploid yeast culture (grown with appropriate preculturing as described above) will reach its mid-logarithmic growth phase when its OD_{600} value is between 0.4 and 0.9. However, these values are estimates, and it is strongly recommended that the correlation between the OD_{600} value, cell density, and growth phase be determined empirically for each strain and growth condition to be used.

An unusually rapid increase in the OD_{600} of a yeast culture, as well as an unpleasant odor, may indicate that the culture is contaminated with bacteria. Contamination can be confirmed by inspection with a phase-contrast microscope.

PREPARATION OF YEAST CELL LYSATES

The preparation of the cell homogenate is a crucial step in any subcellular fractionation procedure. The major aim of a homogenization

technique is to reproducibly achieve a high degree of cell breakage with a minimum of damage to the organelles or organelle membranes of interest. To analyze yeast organelle membranes, the cell must be stressed sufficiently to cause the disruption of the cell wall and plasma membrane to release the cytosol or the other subcellular components in an intact, dispersed state. Ideally, the cells are disrupted in such a way that the subcellular compartments and membranes: (1) retain their normal functional and structural integrity, (2) are freely suspended in the homogenate, and (3) are in a state that is compatible with the goals and techniques of the subsequent experiments.

The extent of cell lysis and the quality of a yeast cell homogenate depend largely on the culture conditions, cell lysis and homogenization techniques, and lysis buffer. Culture conditions have been discussed above (see Yeast Strain Selection and Growth Conditions), and should be standardized to ensure optimal and reproducible results. In addition, the experimental conditions to which cells are subjected (e.g., extended incubations on ice, treatment with energy poisons) should be noted and standardized.

All lysis and homogenization techniques cause some degree of membrane fragmentation, which reduces organelle integrity and exacerbates the preexisting problem of organelle heterogeneity. Some organelle membranes will rupture and release their luminal contents; subsequently, the damaged membranes can also reseal and trap inappropriate constituents. The release of hydrolytic enzymes from the vacuole/lysosome can result in extensive protein degradation, while the release of DNA from nuclei increases the viscosity of the suspension and promotes aggregation. Moreover, the normal interactions between organelles and cytoskeletal elements, as well as the formation of aggregates during the homogenization process, can cause difficulties in the preparation of an optimal homogenate. Realistically, homogenization procedures are an imperfect compromise between complete homogenization and minimal damage to intracellular components.

Cell Lysis with Glass Beads

Mechanical disruption of the cell wall by shearing with glass beads is a simple and rapid method to prepare homogenates for the isolation of cytosol or plasma membranes (see Table 3.7.1). This technique is very flexible, as it

can be applied to multiple small cultures using a vortex mixer, as well as to very large cultures using a Bead Beater (BioSpec). However, glass-bead lysis is not suitable for most subcellular fractionation experiments, as the strong mechanical forces damage the intracellular organelles and can disrupt critical protein and membrane interactions.

Spheroplast Preparation and Lysis

The optimal lysates for most subcellular fractionation experiments are prepared by enzymatically removing the yeast cell wall prior to lysis (see Table 3.7.1). Yeast cells lacking cell walls, referred to as spheroplasts, are fragile and can be lysed with far more gentle methods than can whole yeast cells. Thus, the preparation of lysates from spheroplasts causes much less damage to organelles, organelle membranes, protein complexes, and other intracellular components than does glass-bead lysis.

A number of hydrolytic enzyme preparations that rapidly and efficiently destabilize yeast cell walls are available. The key enzyme in these preparations is a glucanase that hydrolyzes the bonds between β 1-3-linked glucans, the primary structural components of yeast cell walls. In addition, the enzymatic preparations have an alkaline protease activity that plays a more minor role in cell wall digestion. Hydrolysis of the cell walls is enhanced by treatment with dithiothreitol (DTT) or 2-mercaptoethanol (2-ME). These sulfhydryl reagents break the disulfide bonds within cell wall proteins, thereby giving the β 1-3-glucanase greater access to the glucan linkages. It is often beneficial to determine the optimal digestion conditions empirically in a small-scale experiment, as they can vary considerably with different yeast strains, culture conditions, and enzyme preparations.

The most widely used enzyme preparation is Zymolyase 100T (Seikagaku America, ICN), a highly purified β 1-3-glucanase preparation derived from cultured filtrates of *Arthro-bacter luteus*. Zymolyase 20T is also used, but its specific activity is one-fifth that of Zymolyase 100T. Several investigators use lyticase preparations to minimize costs, because it can be isolated from cultures of *Oerskovia xanthineolytica* using a straightforward protocol (Scott and Schekman, 1980) or purchased (Sigma). The use of crude enzyme preparations such as β -glucuronidase is usually not recommended for subcellular fractionation ex-

Table 3.7.1 Yeast Cell Lysis Techniques: Advantages and Disadvantages

Method	Advantages	Disadvantages
Glass-bead lysis	<p>Rapid, efficient homogenization</p> <p>Can be applied to many small cultures (using a vortex mixer) or large cultures (using a Bead Beater)</p> <p>Glass beads are inexpensive and easy to prepare</p> <p>Does not require an incubation step prior to cell lysis</p> <p>Plasma membrane is not exposed to hydrolytic enzymes for extended times</p>	<p>Extensive damage to organelles and organelle membranes</p> <p>Agitation generates heat (increased proteolysis) and foam (due to protein denaturation)</p> <p>Can disrupt membrane and protein interactions</p> <p>Lysis efficiency is less reproducible than spheroplast lysis</p>
Spheroplast preparation and lysis	<p>Minimal damage to organelles and protein complexes, as spheroplasts readily lyse when exposed to osmotic and/or mechanical stress</p> <p>Spheroplast lysis is highly efficient and reproducible</p> <p>Multiple methods for lysing spheroplasts: DEAE-facilitated isoosmotic lysis (the most gentle method), exposure to hypoosmotic conditions (osmotic shock), Dounce homogenization, and/or multiple passes through a syringe needle or pipet tip</p> <p>The degree of lysis and homogenization can be easily adjusted (e.g., by changing number of strokes or pestle clearance when using a Dounce homogenizer)</p> <p>High-quality glucanase preparations are readily available (e.g., Zymolyase 100T)</p> <p>Lyticase is very economical if prepared in the laboratory</p>	<p>Takes longer than glass-bead lysis</p> <p>Extended incubation time (30-60 min) before cell lysis can conflict with the goals and steps of some experiments</p> <p>Spheroplasts are fragile; osmotic support and gentle handling are required to prevent premature lysis</p> <p>Enzyme preparations used to digest cell wall can damage some plasma membrane proteins</p> <p>Highly purified glucanase preparations (e.g., Zymolyase 100T) are relatively expensive</p> <p>Lyticase is more economical than Zymolyase, but time and effort are required to prepare it</p>

periments, since they have much higher protease levels than do Zymolyase or lyticase preparations. For example, β -glucuronidase preparations (“snail-gut juice”), which are isolated from the alimentary canal of the Roman snail *Helix pomatia*, can have at least thirty different enzyme activities. Although Zymolyase and lyticase are highly purified preparations, even they can contain residual protease, phosphatase, and nuclease activities; thus, it is always important to wash the spheroplasts well prior to cell lysis.

Spheroplasts are very sensitive to mechanical and osmotic stress. They must be suspended in an isotonic solution during and after the digestion procedure to prevent lysis. The nonmetabolizable sugar sorbitol is frequently used at concentrations of 1.0 to 1.2 M (up to 1.5 M for particularly unstable strains) to provide osmotic support. In addition, spheroplasts

must be handled with care to prevent premature cell lysis.

Spheroplasts can be lysed using several techniques. In many procedures, the spheroplasts are resuspended in a hypoosmotic solution, which causes them to swell and potentially lyse. A dramatic and rapid decrease in osmotic support is sufficient to promote cell lysis; however, it also promotes the lysis of intracellular organelles. To disrupt the plasma membrane with a minimum of damage to subcellular components, mild hypoosmotic conditions are used in combination with moderate homogenization techniques—e.g., glass/glass (Dounce) or glass/Teflon (Potter-Elvehjem) homogenization, or passage through a small-gauge syringe needle or pipet tip. Even milder conditions with very little osmotic and mechanical stress are required to maintain the integrity of a high percentage of very fragile

organelles (such as vacuoles). Cell lysis under isoosmotic conditions can be facilitated by incubating the spheroplasts with a cationic polymer such as DEAE-dextran, which interacts with the negatively charged phospholipids in the plasma membrane. Treatment with triethanolamine/acetic acid, pH 7, also improves cell lysis efficiency under mild hypotonic conditions.

Lysis Buffers

The optimal lysis buffer facilitates cell lysis and simultaneously protects the structural and functional integrity of the organelles, organelle membranes, and protein complexes of interest. Ideally, a lysis buffer should be isoosmotic to the organelles within the homogenate; however, most lysis buffers are hypoosmotic to some degree to facilitate efficient cell lysis. In some cases, the osmolarity of the buffer can be adjusted after cell lysis to protect organelles from extended osmotic stress. In addition to minimizing damage to subcellular components, the lysis buffer should interfere as little as possible with subsequent fractionation steps and biochemical assays.

The most appropriate buffer composition depends on the cell lysis method and the purpose for which the lysate is being prepared. Standard buffers consist of several components, including: (1) a suitable organic buffer to maintain a physiological pH, (2) sorbitol or another osmotic balancer to stabilize the organelle membranes, (3) salts to provide the appropriate ionic strength, and (4) a cocktail of protease inhibitors to minimize the damage caused by hydrolytic enzymes. Most lysis buffers include the cation chelator EDTA, as Mg^{2+} serves as a cofactor for several proteases and can promote membrane aggregation at high concentrations. However, in a few cases, Mg^{2+} is added to the lysis buffers to stabilize the composition, membrane association, and function of certain protein complexes and enzymes.

Protein degradation can be a severe problem during subcellular fractionation experiments. Subcellular components are inevitably exposed to degradative enzymes upon cell lysis and homogenization, as these manipulations disrupt the *in vivo* regulation and compartmentalization of hydrolase activities. The primary source of active degradative enzymes in cell lysates is usually the vacuole, which is an acidic lysosome-like compartment. A cocktail of protease inhibitors with a broad speci-

ficity range should be included in the lysis buffer and all subsequent solutions (even when using a vacuolar protease-deficient strain). Because protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) are unstable in aqueous solutions, they should be added just before the solutions are to be used and should be replenished at multiple steps during the fractionation process. In addition, the lysis buffer and all other solutions and equipment required to process the lysate should be pre-chilled to 0° to 4°C before and during their use (unless otherwise indicated). Damage caused by hydrolytic enzymes can be minimized further by (1) working quickly to limit the potential exposure time to active hydrolases, (2) separating material of interest from the remaining cell lysate as soon as possible, (3) maintaining the integrity of the vacuole, and/or (4) using mutant yeast strains that lack most vacuolar hydrolase activities (e.g., *pep4Δprb1Δ* yeast strains).

It is also critical to be aware that different lysis buffers can destabilize protein-protein interactions to varying degrees and promote the release of sensitive peripheral membrane proteins. The destabilization of protein-protein interactions can disrupt the function, composition, and other biochemical characteristics of subcellular components. In addition, the release of peripheral membrane proteins can change both the absolute and the relative densities of organelles and organelle membranes. It can be particularly difficult to determine the subcellular localization of a peripheral membrane protein, since these proteins can dissociate from their resident membranes during the fractionation procedure and thus cofractionate with soluble cytoplasmic proteins.

The buffer composition often needs to be optimized for a given purpose, since even a slight change can have dramatic effects. Certain buffer characteristics can be either advantages or disadvantages depending on the goals of the experiment. For example, the use of buffers with relatively high ionic strengths minimizes protein and membrane aggregation during cell lysis and homogenization; however, these buffers also tend to destabilize certain protein-protein interactions and promote the release of peripheral membrane proteins. Similarly, inclusion of triethanolamine in the lysis buffer facilitates spheroplast lysis under very mild hypoosmotic conditions, but it can also destabilize some protein-protein interactions.

ISOLATION OF SUBCELLULAR FRACTIONS: PROCEDURAL OPTIONS

Subcellular fractionation procedures capitalize on the differing physical properties of individual organelle and membrane types. The most versatile fractionation procedures are based on centrifugation techniques, which effectively exploit the differences in size, shape, and/or density of distinct organelles and membranes (see Table 3.7.2 and the text below). A number of more-specialized fractionation protocols use gel filtration, immunoisolation, or electrophoresis to separate certain subcellular components according to their size, surface antigen composition, or surface charge, respectively (see Table 3.7.3). However, even these alternate procedures usually begin with centrifugation-based steps to enrich the starting material with the subcellular components of interest and to remove some of the undesirable components.

The most appropriate fractionation procedure will depend on the primary goals of the particular experiment. Parameters to consider include: (1) the properties of the organelles/membranes of interest (e.g., their abundance, fragility, size, density), (2) the properties of the other subcellular components that need to be separated from the organelles/membranes of interest, (3) the required yield, (4) the required purity, (5) the degree to which certain contaminants are harmful, (6) the need for organelle integrity and function, (7) the need to maintain the association of peripheral membrane proteins with their resident compartments, and (8) the need to maintain enzymatic activity levels. The importance of each of these factors should be prioritized for a given experiment, since some desirable qualities will need to be sacrificed to meet the most important goals. For example, the yield of a given organelle within a fraction will generally decrease as its purity increases. Thus, fractionation procedures often need to be evaluated and modified to meet particular requirements.

Centrifugation-Based Subcellular Fractionation Techniques

There are three primary centrifugation procedures used for subcellular fractionation experiments: (1) differential centrifugation by velocity, (2) equilibrium density gradient centrifugation, and (3) rate-zonal gradient centrifugation. Centrifugation-based fractiona-

tion procedures separate organelles and membranes according to their sedimentation velocities and/or buoyant densities. These techniques are very versatile, since the sedimentation velocities and densities of subcellular components are determined both by their intrinsic properties and the characteristics of the surrounding medium. Some of the merits and limitations of these procedures are summarized in Table 3.7.2. Several excellent reviews on the theory and practice of centrifugation-based fractionation techniques are also available (Dobrota and Hinton, 1992; Evans, 1992; Hinton and Mullock, 1997).

The absolute densities of subcellular components, as well as their densities relative to one another, can vary with the composition of the surrounding solution. One cause of this variability is the semipermeable nature of membranes. While the mass of the membrane (i.e., the lipids and integral membrane proteins) and the nondiffusible components within the lumen remain constant, the mass of the water and diffusible solutes within the organelle can change as the diffusible molecules move into or out of the organelle to reach equilibrium. Also, the volume of the organelle will change as it swells with water in a hypoosmotic solution, shrinks in a hyperosmotic solution, or bursts due to osmotic or mechanical stress. The mass of an organelle is also dependent on the amounts of associated peripheral membrane proteins and macromolecular complexes (e.g., ribosomes), which can vary in different buffers and density media. For example, if a density medium destabilizes protein-protein interactions, peripheral membrane proteins may be released from their resident membranes, and the mass and density of the organelle will decrease.

The relative sedimentation velocities of subcellular components are primarily determined by the mass and shape of the components and by their density relative to that of the surrounding medium. If the organelles have significantly higher densities than the surrounding medium, their relative sedimentation velocities will be principally determined by their mass, so that larger organelles will have a faster sedimentation velocity than will smaller ones. Shape usually plays a more minor role. However, if the organelles are subjected to centrifugation in a density gradient, their sedimentation velocities will decrease as the density of the surrounding medium approaches their own density. The movement of

Table 3.7.2 Centrifugation-Based Subcellular Fractionation Techniques: Advantages and Disadvantages

Method	Advantages	Disadvantages
Differential centrifugation (basis: sedimentation velocity ^a , primarily size)	<p>Simple and rapid method to enrich membranes in fractions corresponding to broad size classes (Fig. 3.7.1)</p> <p>Effective separation of membranes with large size differences (see Table 3.7.4)</p> <p>Relatively high yield</p> <p>Initial low-speed centrifugation permits removal of unbroken cells and large aggregates</p> <p>Excellent preparation step for further fractionation: can enrich components of interest, remove unwanted material, and concentrate membranes from large lysate volumes</p> <p>Provides a rapid means to determine the potential subcellular location of a protein of interest (Table 3.7.4)</p>	<p>Low resolution and purity^a</p> <p>Poor separation of membranes of similar sizes</p> <p>Pellets containing larger subcellular components will be contaminated with smaller ones^a</p> <p>Pelleting of organelles and membranes can cause aggregation and structural damage (minimized by pelleting on a cushion)</p> <p>Isolation of particular membrane types requires further fractionation steps</p>
Equilibrium density gradient (basis: density)	<p>Extremely versatile: well suited for both preparative and analytical fractionation experiments</p> <p>Relative densities of organelles/membranes can vary in different gradient media (high flexibility and versatility)</p> <p>Gradients can be either continuous or discontinuous</p> <p>Not sensitive to changes in centrifugation times as long as equilibrium status is reached (good for overnight centrifugations)</p>	<p>Extended centrifugation times may be detrimental to labile organelles and enzyme activities (centrifugation times can be minimized by using a vertical tube rotor)</p> <p>Sample size should be relatively low to maximize resolution and minimize aggregation</p> <p>Very dense organelles/membranes may be exposed to hyperosmotic conditions (especially in sucrose gradients)</p>
Rate-zonal (velocity) gradient (basis: sedimentation velocity and density ^b)	<p>Multiparameter separation based on size, shape, and density</p> <p>Well suited for the separation of multiple subcellular components of different sizes</p> <p>Superior to differential centrifugation for separation of components based on size and sedimentation velocities, since sample is layered on top of the gradient</p> <p>Results vary with centrifugation time and gradient composition and shape (high flexibility)</p> <p>Shorter centrifugation runs than for equilibrium gradients (lower risk of damage to subcellular components)</p>	<p>Results depend on centrifugation times and gradient shape and composition (can be more difficult to design and optimize)</p> <p>Less well suited for organelle/membrane purification unless procedure also includes equilibrium gradient fractionation step</p> <p>Sample size should be relatively low to maximize resolution and minimize aggregation</p> <p>Very large or dense components may be exposed to hyperosmotic conditions</p>

^aRelative sedimentation velocity in a buffer of low density and viscosity is primarily determined by size. Shape and density play a more minor role. The resolution is quite low because components are suspended throughout the medium prior to centrifugation, and thus have a range of migration distances to the bottom of the tube.

^bRelative sedimentation velocity and final banding position in a nonequilibrium density gradient is determined by both particle size and density relative to the surrounding medium. Size is the primary determinant of sedimentation velocity when the density of the component is significantly greater than the surrounding medium. As the component approaches medium equal to its own density, the particle velocity will decrease. If the particle reaches medium with a density equal to its own, the particle will stop, and its final position within the gradient will be based on its buoyant density alone, irrespective of size or shape.

Table 3.7.3 Several Noncentrifugal Fractionation Techniques: Advantages and Disadvantages

Method	Advantages	Disadvantages
Gel filtration (basis: size and shape)	Excellent for purification of small spherical vesicles (<200 nm) from larger or irregularly shaped organelles High resolution	Not suited for fractionation of larger or irregularly shaped organelles/vesicles Poor separation of different vesicle types with similar sizes
Affinity isolation (basis: surface epitope)	Highly selective for compartment or membrane that has appropriate surface epitope (high purity) Relatively gentle, facilitates isolation of intact organelles	Highly specialized; may need extensive optimization Not well suited for large-scale preparations Limited by antibody specificity and avidity (controls for nonspecific interactions are critical) Limited by epitope accessibility
Electrophoresis (basis: surface charge and size)	Can be used after gel filtration to separate different vesicle types that are similar in size, shape, and density	Requires specialized, expensive equipment Not suitable for larger organelles Rarely used for yeast organelles (purification of mammalian endosomes has been the most common application)

a subcellular component will stop once the density of the surrounding medium is equal to its own density; in this case, the position of the component within the gradient is based on its density, irrespective of size.

The fractionation of subcellular components is never perfect or complete. Centrifugation-based fractionation procedures are limited by the fact that different organelle or membrane populations can have similar sizes and/or densities. In addition, members of a given organelle population vary to some degree both in mass and density. Both of these complications (i.e., the differences within an individual population and the similarities between different populations) are exacerbated by the organelle/membrane fragmentation caused by lysate preparation and fractionation procedures.

Differential centrifugation

Differential centrifugation protocols rapidly fractionate cell lysates into broad size classes through sequential centrifugation steps of increasing force and duration (Fig. 3.7.1). This technique is also referred to as differential pelleting. Because the subcellular components have a substantially higher density than the surrounding buffer, their relative sedimentation velocities are primarily determined by their mass; larger organelles and membranes will pellet faster and at lower centrifugal forces

than will smaller organelles. Some of the merits and limitations of differential centrifugation are summarized in Table 3.7.2.

Differential centrifugation is particularly useful to generate fractions that are significantly enriched for the component(s) of interest. The use of enriched fractions (instead of crude cell lysates) facilitates subsequent purification or analysis by density gradient centrifugation and other fractionation techniques. Differential centrifugation is also a convenient way to assess the potential subcellular locations of a protein of interest.

The resolution of size separation by differential centrifugation is quite low, because subcellular components are randomly dispersed throughout the medium prior to centrifugation. Thus, the resulting separation of subcellular components is based both on their sedimentation velocity (which is proportional to their mass) and on their migration distance to the bottom of the centrifugation tube (a random characteristic). Small organelles with short migration distances (and relatively slow sedimentation velocities) will pellet with larger organelles that have faster sedimentation velocities. In other words, a pellet intended to consist of relatively large organelles will also contain smaller organelles that happened to be close to the bottom of the centrifugation tube. Some of the smaller organelles found in low-speed pellets can be removed by resuspending

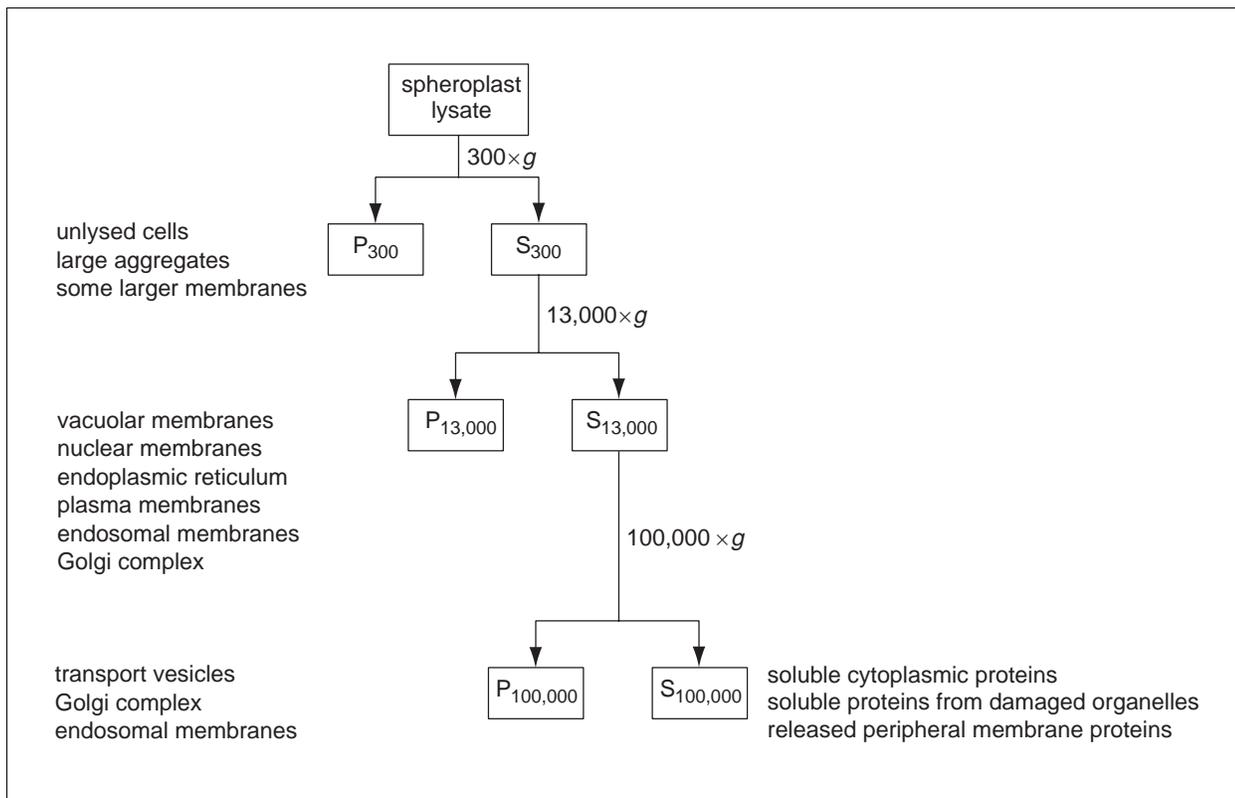


Figure 3.7.1 A procedure for differential centrifugation of yeast cells (see Table 3.7.4 for further details).

the pellet in fresh medium and repeating the centrifugation step, a technique often referred to as “washing” the pellet; however, this step also increases the likelihood of organelle damage.

A typical differential centrifugation scheme is presented in Figure 3.7.1. First, the yeast cell lysates are subjected to a low centrifugal force (e.g., $300 \times g$) to pellet any unlysed cells and large aggregates. This centrifugation step is often referred to as a “clearing spin.” The cleared lysate (S_{300}) is then subjected to sequential centrifugation steps to generate a $13,000 \times g$ pellet ($P_{13,000}$), a $100,000 \times g$ pellet ($P_{100,000}$), and a $100,000 \times g$ supernatant ($S_{100,000}$). The resulting distribution of subcellular components is summarized in Table 3.7.4. The $P_{13,000}$ fraction primarily contains larger organelles and membranes (e.g., plasma membrane, vacuole, endoplasmic reticulum, mitochondria, nuclei, peroxisomes), while the $P_{100,000}$ fraction contains smaller compartments (e.g., Golgi, transport vesicles). Heterogeneous or moderate-sized organelles can distribute between the two fractions (e.g., endosomal membranes). The $S_{100,000}$ fraction contains soluble cytoplasmic proteins, soluble proteins released from the

lumen of damaged organelles, and released peripheral membrane proteins.

Equilibrium density gradient centrifugation

Subcellular components can be separated according to their buoyant densities by centrifugation to equilibrium in a density gradient. This technique is referred to as equilibrium density gradient centrifugation or isopycnic gradient centrifugation. In response to centrifugation, a given organelle or membrane will migrate within the gradient until it reaches the region of the gradient that matches its own buoyant density. The subcellular component will then remain at this position for the duration of the centrifugation irrespective of its size, as there is no net force exerted on a particle that is suspended in a liquid of its own density (Archimedes’ principle). Some of the merits and limitations of equilibrium density gradient centrifugation are summarized in Table 3.7.2.

Equilibrium density gradient centrifugation techniques are very versatile and are well suited for both analytical and preparative procedures. Often the material to be separated by equilibrium density gradient centrifugation is first subjected to differential centrifugation

Table 3.7.4 Distribution of Organelle Membranes after Cleared Yeast Spheroplast Lysate (S₃₀₀) is Subjected to Sequential Centrifugation at 13,000 × *g* and 100,000 × *g*^a

Subcellular component	P _{13,000}	P _{100,000}
Endosomal membranes ^b	~30%-70%	~30%-70%
Endoplasmic reticulum ^c	>95%	
Golgi (<i>cis</i> cisternae)	~30%-60%	~40%-70%
Golgi (medial and <i>trans</i> cisternae)	~10%-30%	~70%-90%
Mitochondria	>95%	
Nuclei ^c	>95%	
Peroxisomes (oleate induced)	>95%	
Plasma membrane ^c	>95%	
Transport vesicles		>95%
Vacuolar membranes	>95%	

^aStrain: SEY6210. Selected references: Gaynor et al. (1994); Marcusson et al. (1994); Cereghino et al. (1995); Zinser and Daum (1995); Rieder and Emr (1997); Babst et al. (1998).

^bThe endosomal membrane system is thought to consist of at least two distinct compartment types. The later prevacuolar endosomes appear to pellet at lower speeds than the earlier post-Golgi endosomal compartments.

^cUp to 50% of these membranes may pellet during the clearing centrifugation step at 300 × *g*. Yields can be improved by resuspending and recentrifuging the 300 × *g* pellet material, but some loss is unavoidable.

and is thus enriched for components of the appropriate size. The combination of differential centrifugation and equilibrium density gradient centrifugation allows subcellular components to be fractionated according to both mass and buoyant density. The material to be fractionated can be loaded on the top, bottom, or middle of the density gradient, since the components will sediment or float until they reach the appropriate region of the gradient. (Loading a gradient at the bottom, however, may damage sensitive organelles if the gradient is hyperosmotic at the dense regions of the gradient.)

Rate-zonal gradient centrifugation

Rate-zonal gradient centrifugation is a non-equilibrium method in which subcellular components loaded on top of a density gradient move into enriched regions within the gradient. This technique is also referred to as velocity gradient centrifugation. The absolute and relative banding positions of subcellular components are primarily determined by each component's mass, the density of the component relative to the surrounding medium, the centrifugation time (duration), and the composition of the gradient. The mass is the primary determinant of the sedimentation velocity (and thus of position) when the density of the component is significantly greater than that of the surrounding medium. As the component ap-

proaches medium equal to its own density, its velocity will decrease. If the particle reaches medium with a density equal to its own, it will stop, and its final position within the gradient will be based on its buoyant density alone, irrespective of its mass or shape.

Like equilibrium density gradient centrifugation techniques, rate-zonal density gradient centrifugation techniques are very versatile and well suited for both analytical and preparative procedures. The separation between different subcellular components can be manipulated empirically to some extent by changing the length of centrifugation, the density limits of the gradient, or the gradient medium. Often the material to be separated by rate-zonal density gradient centrifugation is first subjected to differential centrifugation and is thus enriched for components of the appropriate size. Rate-zonal centrifugation procedures are particularly useful to fractionate subcellular components within a relatively short time, since the rate-zonal centrifugation times are typically much shorter than density equilibrium centrifugation times.

Continuous Versus Discontinuous Density Gradients

The density gradients used for density gradient centrifugation procedures can be either continuous or discontinuous. Some of the mer-

Table 3.7.5 Advantages and Disadvantages of Continuous and Discontinuous Density Gradients

Density gradients	Advantages	Disadvantages
Continuous gradient	<p>Especially well suited for the separation of multiple organelle/membrane types</p> <p>Very versatile: useful for both analytical and preparative fractionation procedures</p> <p>Appropriate for both equilibrium and velocity density gradients</p> <p>Higher resolution than step gradients</p> <p>Less sensitive to overloading than step gradients</p>	<p>Fractions containing organelles of interest are usually not identifiable without monitoring distribution over several fractions</p> <p>Material of interest may be spread over several fractions and may require concentration</p> <p>Preparation is more time consuming</p>
Discontinuous gradient (step gradient)	<p>Gradient preparation is simple and rapid</p> <p>Especially well suited for preparative fractionation procedures to isolate a subcellular component of interest</p> <p>Concentrated organelle/membrane layers are often visible and may have distinguishing characteristics (e.g., color, “fluffiness”)</p> <p>Subcellular components of interest can be rapidly harvested by collecting fractions at the appropriate region of the gradient. (All gradient fractions should be collected and examined to assess the success of the experiment.)</p>	<p>Lower resolution than continuous gradients</p> <p>Preliminary experiments using continuous density gradients are usually required to determine optimal densities</p> <p>Accumulation of membranes/organelles at an interface can block movement of other membranes/organelles</p> <p>Sample quantities loaded on gradients must be relatively low to minimize aggregation at interfaces</p> <p>Generally less well suited for analytical purposes (e.g., separation of multiple organelle types on a single gradient)</p>

its and limitations of continuous and discontinuous density gradients are summarized in Table 3.7.5.

In continuous density gradients, the density of the medium changes gradually along the length of the gradient tube. Such gradients are particularly useful for equilibrium or rate-zonal centrifugation to analyze a complex mixture of subcellular components, some of which may have unique but overlapping distributions along the density gradient. The relationship between the density of the gradient medium and the positions of given densities along the gradient, sometimes referred to as the shape of the gradient, can be linear or nonlinear. The optimal shape depends on the densities of the key components that need to be separated from each other.

Discontinuous density gradients, also referred to as step gradients, consist of two or more layers of gradient media with significantly different densities. The interfaces between the layers should be very sharp; therefore, discontinuous gradients must be prepared just before use. Discontinuous density gradients are most frequently used for preparative procedures (e.g., the purification of a subcellular component of interest) but have also been

used for analytical experiments. In most cases, the appropriate density and volume of the gradient layers (or steps) are determined by first examining the distribution of the subcellular components within a continuous density gradient. For preparative procedures, discontinuous density gradients are often designed in such a way that the components of interest accumulate in a small and easily identifiable region of the gradient (e.g., an interface between two layers), while other components either do not reach this region or pass through it.

Gradient Preparation

The accurate and reproducible preparation of density gradients is critical to the success of density gradient fractionation procedures. Continuous density gradients are often generated using a gradient maker that mixes two solutions of different densities in the appropriate proportions. Alternatively, continuous density gradients can be formed by preparing a multilayer step gradient and allowing the density medium to diffuse for several hours to eliminate the interfaces. Regardless of whether a continuous gradient is prepared with a gradient maker or by diffusion, it is best to analyze the density distributions of at least two test

gradients to ensure that the densities are as planned and are reproducible. A refractometer is the best way to accurately measure the densities of media that are true solutions (e.g., sucrose, Nycodenz, iodixinol).

Discontinuous gradients are prepared just before use by sequentially adding layers of gradient media with significantly different densities to the centrifugation tube. Some investigators prefer to start with the most dense layer, then gently add the subsequent layer of lower density on top (overlying). Other investigators find it easier to add the least-dense layer first and then introduce layers of increasing density underneath it, using a very long, slim cannula or needle (underlying). With either technique, it is critical that the interface between the layers remains sharp. If mixing between layers becomes apparent during gradient preparation, the gradient should be discarded and a new one should be made.

Gradient Media

The optimal density gradient medium should facilitate the separation of the subcellular components of interest into distinct sets of fractions. It should also protect the functional and structural integrity of the organelles and membranes, as well as the appropriate protein-protein and protein-membrane interactions, to the greatest extent possible.

The characteristics of several different density gradient media are summarized in Table 3.7.6. To generate the required density range of the gradient medium, the primary constituent is dissolved or suspended in a buffered solution at varying concentrations. Some density gradient media also contain sucrose to provide osmotic support for organelle membranes, a cocktail of protease inhibitors to minimize damage due to hydrolytic enzymes, and occasionally salts to provide the appropriate ionic strength.

The density gradient media listed in Table 3.7.6 are all very soluble in aqueous buffers and are nontoxic, chemically inert, transparent to visible light, and available at high purity. In addition, each of the media has one or more (but not all) of the following desirable characteristics: (1) has a low viscosity-to-density ratio to minimize the required centrifugation time and/or force, (2) has a low osmolarity-to-density ratio to minimize exposure of dense organelles to hyperosmotic conditions, (3) is transparent to UV light, (4) is inexpensive, (5) is not metabolized by yeast cells, (6) can be

used to form gradients with osmolarity levels that support organelle membranes and vary by <10% across the gradient, (7) does not interfere with subsequent analyses of the resulting fractions, and (8) can be easily removed from isolated subcellular components if required.

For several decades, sucrose density gradients have been the most common method for separating organelles on the basis of both size and density. More recently, density gradient solutions prepared with nonionic iodinated compounds (e.g., metrizamide, Nycodenz, iodixinol) have increased in popularity, since these solutions have lower viscosity and osmolarity levels than do sucrose solutions of equal density. Density gradients prepared from sucrose or nonionic iodinated compounds are well suited for analytical experiments designed to examine the distribution of multiple organelles and membranes along the same gradient, as well as for preparative experiments used to isolate a particular organelle or membrane type at higher yield and purity levels. In contrast, density gradients prepared from Percoll or Ficoll suspensions are primarily used for preparative procedures. Ficoll consists of highly branched polymers of sucrose and epichlorhydrin, while Percoll consists of colloidal silica beads coated with polyvinylpyrrolidone. Percoll and Ficoll suspensions both have low osmolarity and viscosity levels; in fact, sucrose is usually added to the suspensions to provide the required osmotic support. Density gradients prepared with sorbitol or glycerol, or with D₂O instead of H₂O as the solvent, are used less frequently.

The most appropriate density gradient composition depends on the purpose of the experiment. For example, certain protein-protein interactions will be more stable in one medium than in another. In addition, the absolute densities of organelles and membranes, as well as their densities relative to each other, depend on both the intrinsic characteristics of the organelles and the properties of the surrounding gradient medium. Important factors include differential levels of membrane permeability to gradient solutes, osmolarity of the density medium relative to the organelles, and degree of peripheral protein loss. Thus, subcellular components that are unresolvable in one gradient medium may be resolvable in another.

Ideally, the entire density gradient should be isoosmotic to the organelles within the homogenate. Hyperosmotic solutions tend to draw water out of organelles, and thus can

Table 3.7.6 Characteristics of Several Density Gradient Media: Advantages and Disadvantages

Medium	Advantages	Disadvantages
Sucrose	<p>Most common medium, used for many years</p> <p>Inexpensive and available at high purity</p> <p>Many applications (preparative and analytical)</p> <p>Does not absorb UV light</p> <p>Differential membrane permeability to sucrose^a</p> <p>Removed by centrifugation, dialysis, or ultrafiltration</p>	<p>High osmotic-pressure-to-density ratio</p> <p>Moderate to high viscosity</p> <p>Can disrupt some protein and membrane interactions</p> <p>Can pass through organelle membranes to some extent^a</p> <p>Interferes with some enzyme assays</p>
Nycodenz, iodixinol ^b	<p>Exert moderate osmotic pressure (less than sucrose)</p> <p>Isoosmotic density gradients can be generated with osmotic balancers (e.g., sucrose, sorbitol, NaCl)</p> <p>Low to moderate viscosity</p> <p>Many applications (preparative and analytical)</p> <p>Iodixinol gradients can be formed through centrifugation</p> <p>Do not penetrate organelle membranes</p> <p>Removed by centrifugation, dialysis, or ultrafiltration</p>	<p>Moderate osmotic-pressure-to-density ratio</p> <p>Relatively expensive</p> <p>Can disrupt some protein and membrane interactions</p> <p>Moderate viscosity at high concentrations</p> <p>Absorb UV light</p> <p>Interfere with some enzyme assays</p>
Ficoll 400 ^c	<p>Very low osmotic pressure at concentrations <25%</p> <p>Does not penetrate organelle membranes</p> <p>Generally used for preparative procedures</p> <p>Usually does not interfere with enzyme assays</p>	<p>Osmotic pressure increases rapidly at concentrations >25%</p> <p>High osmotic pressure at concentrations >30%</p> <p>High viscosity (longer centrifugation times)</p> <p>Cannot be removed by dialysis or ultrafiltration</p>
Percoll ^d	<p>Exerts essentially no osmotic pressure (required osmotic support is usually provided by adding sucrose)</p> <p>Very low viscosity (shorter centrifugation times)</p> <p>Continuous density gradients form during centrifugation</p> <p>Gradient shape is sensitive to changes in rotor geometry, centrifugation speed, and duration (offers flexibility)</p> <p>Permits simple, rapid isolation of larger organelles</p> <p>Does not penetrate organelle membranes</p>	<p>Adheres to membranes and is difficult to remove</p> <p>Cannot be removed by dialysis or ultrafiltration</p> <p>Absorbs UV light and interferes with some assays</p> <p>Gradient shape usually changes during centrifugation</p> <p>Gradient shape is sensitive to changes in centrifugation speed, time, and rotor (more difficult to design and reproduce)</p> <p>Generally not useful for evaluating multiple organelles</p> <p>Not suitable for small organelles or membrane vesicles</p>

continued

change their size, shape, and density. Exposure to hyperosmotic conditions can also promote the loss of protein coats and other peripheral membrane proteins, thereby altering the composition, function, and density of the affected organelle membranes. In addition, organelles suspended in hyperosmotic solutions are more susceptible to lysis if subsequently transferred to a buffer of lower osmolarity. Organelles can also be damaged through exposure to hypoos-

motric conditions, which can induce organelle swelling, membrane discontinuities, and organelle lysis. However, hypoosmotic conditions are rarely a practical problem during gradient fractionation, since osmotic balancers such as sorbitol or sucrose can be added to gradient solutions to increase their osmolarity.

Information booklets provided by the manufacturers of gradient media can serve as an excellent resource for details on gradient media

Table 3.7.6 Characteristics of Several Density Gradient Media: Advantages and Disadvantages, continued

Medium	Advantages	Disadvantages
Sorbitol	Advantages are similar to those of sucrose Unlike sucrose, sorbitol is not metabolizable Slightly less membrane permeable than sucrose ^a	Not used as frequently as sucrose More expensive than sucrose Other disadvantages are similar to those of sucrose
Glycerol	Used for separating large protein complexes Freely penetrates membranes (organelle/vesicle density primarily determined by membrane composition) ^a Removable by centrifugation, dialysis, or ultrafiltration	Rarely the best medium for membrane separations Very high viscosity (longer centrifugation times) Freely penetrates membranes (high banding densities) Interferes with some enzyme assays
D ₂ O solvent ^e	Lower osmolarity and viscosity levels than with H ₂ O-based media of equal density Extent of exchange between D ⁺ /OD ⁻ and H ⁺ /OH ⁻ molecules varies with membrane composition, changing their relative densities ^a	Expensive and benefits can be insignificant D ⁺ /OD ⁻ molecules can exchange with membrane- and protein-associated H ⁺ /OH ⁻ molecules, increasing the density of components

^aThe absolute densities of subcellular organelles/membranes, as well as their densities relative to each other, can change in different gradient media (e.g., because of differential levels of membrane permeability to the solute, osmolarity of the medium, or peripheral protein loss). Thus, components of interest that have overlapping densities in one medium can have differentiable densities in another gradient medium.

^bNycodenz (Nycomed Pharma) and iodixinol (Optiprep, Nycomed Pharma) are nonionic iodinated density compounds with similar characteristics. Nycodenz is a nonionic derivative of triiodobenzoic acid with three aliphatic side chains. Iodixinol is essentially a dimeric form of Nycodenz, and thus has a higher density-to-osmolarity ratio. These nonionic compounds have largely replaced the nonionic medium metramizide, as well as the ionic iodinated density compounds.

^cFicoll 400 is a neutral, highly branched polymer of sucrose and epichlorhydrin (average mol. wt. 400,000).

^dPercoll consists of colloidal silica beads coated with polyvinylpyrrolidone (PVP).

^ePreparation of gradient density media in heavy water (D₂O; 1.1 g/ml) instead of regular water (1.0 g/ml).

characteristics, gradient preparation techniques, and subcellular fractionation procedures. For example, Nycomed Pharma has several booklets that discuss Nycodenz and Optiprep (iodixinol) gradient media, centrifugation-based fractionation procedures, and several cellular and subcellular fractionation protocols. While the focus of these texts is on mammalian systems, many of the principles are also applicable to yeast fractionation experiments.

ANALYSIS OF SUBCELLULAR FRACTIONS

The specialized functions of subcellular organelles and their membranes are reflected by their unique protein compositions, enzymatic activities, and morphologies. These characteristics can be used to follow the distribution of particular organelles and membranes during the subcellular fractionation procedure, to characterize the resulting fractions, and to analyze the success of an experiment. The most

common method used to monitor the distribution and integrity of particular organelles and membranes after subcellular fractionation is to analyze the distribution of their characteristic marker proteins and enzyme activities (Tables 3.7.7 and 3.7.8). To establish the purity and yield of an organelle preparation or the success of an analytical fractionation experiment, it is of central importance to determine the levels of the components of interest, as well as the levels of the potential contaminants. The distribution and integrity of fractionated organelles and membranes can also be characterized by examining their morphologies, functions, and overall protein compositions.

Marker Proteins and Enzymatic Activities

An optimal marker is a well-characterized protein and/or enzyme activity that (1) is associated with a single subcellular compartment or membrane type, (2) remains tightly associated with its resident compartment/membrane

Table 3.7.7 Marker Proteins Used to Identify Fractionated Subcellular Components

Subcellular fraction	Markers (and corresponding genes) ^{a,b}	Selected references ^c
<i>Plasma membrane:</i>		
	Gas1p (<i>GAS1</i>)	Nuoffer et al., 1991. <i>MCB</i> 11:27
	H ⁺ -ATPase (<i>PM11</i>)	Goffeau and Dufour, 1988, <i>Methods Enzymol.</i> 157:528; Serrano, 1988. <i>Methods Enzymol.</i> 157:533
	Syntaxin complex (<i>SSO1</i> , <i>SSO2</i>)	Aalto et al., 1993. <i>EMBO J.</i> 12:4095
	Chitin synthetases (<i>CHS1</i> , <i>CHS2</i> , <i>CHS3</i>); also associated with chitosomes/endosomes	Chuang and Schekman, 1996. <i>JCB</i> 135:597; Ziman et al., 1996. <i>MBC</i> 7:1909; Ziman et al., 1998. <i>MBC</i> 9:1565
	α1-3-Glucan synthase (<i>GLS1</i> , <i>GLS2</i>)	Mazur et al., 1995. <i>MCB</i> 15:5671
	PM receptors and transport proteins	Andre, 1995. <i>Yeast</i> 11:1575; van der Rest et al., 1995. <i>Microbiol. Rev.</i> 59:304
	a-factor receptor (<i>STE3</i> , α strains); transient	Davis et al., 1993. <i>JCB</i> 127:53
	α-factor receptor (<i>STE2</i> , a strains); transient	Blumer et al., 1988. <i>JBC</i> 263:10836
<i>Endosomes:</i>		
Membrane	Pep12p (<i>PEP12</i>)	Becherer et al., 1996. <i>MBC</i> 7:579
	a-factor receptor (<i>STE3</i> , α strains); transient	Davis et al., 1993. <i>JCB</i> 127:53; Wendland and Emr, 1998. <i>JCB</i> 141:71
	α-factor receptor (<i>STE2</i> , a strains); transient	Blumer et al., 1988. <i>JBC</i> 263:10836; Wendland and Emr, 1998. <i>JCB</i> 141:71
Lumen	Exogenous [³⁵ S]α-factor (MFα1); transient	Singer and Riezman, 1990. <i>JCB</i> 110:1911; Dulic et al., 1991. <i>Methods Enzymol.</i> 194:697; Wendland and Emr, 1998. <i>JCB</i> 141:71
<i>Golgi complex:</i>		
Membrane, <i>cis</i>	α1-6-Mannosyltransferase (<i>OCH1</i>); also in ER	Nakayama et al., 1992. <i>EMBO J.</i> 11:2511; Gaynor et al., 1994. <i>JCB</i> 127:653
Membrane, medial	Guanosine diphosphatase (<i>GDA1</i>)	Vowels and Payne, 1998. <i>MBC</i> 9:1351
	α1-3-Mannosyltransferase (<i>MNN1</i>)	Cunningham and Wickner, 1989. <i>Yeast</i> 5:25
	α1-2-Mannosyltransferase (<i>KRE1</i>)	Lussier et al., 1995. <i>JCB</i> 131:913
Membrane, <i>trans</i> ^d	Endoprotease yscF (<i>KEX2</i>)	Redding et al., 1991. <i>JCB</i> 113:527; Cooper and Bussey, 1992. <i>JCB</i> 119:1459
	Carboxypeptidase ysc-α (<i>KEX1</i>)	Bryant and Boyd, 1993. <i>J Cell Sci.</i> 106:815
	Dipeptidyl aminopeptidase A (<i>STE13</i>)	Bryant and Boyd, 1993. <i>J Cell Sci.</i> 106:815; Nothwehr et al., 1993. <i>JCB</i> 121:1197
	Vacuolar protein receptor (<i>VPS10</i>)	Marcusson et al., 1994. <i>Cell</i> 77:579; Cereghino et al., 1995. <i>MBC</i> 6:1089
<i>Peroxisomes:</i>		
Membrane	ABC transporters (e.g., <i>PXA1</i> , <i>PXA2</i>)	Hettema et al., 1996. <i>EMBO J.</i> 15:3813; Shani and Valle, 1998. <i>Methods Enzymol.</i> 292:753
	Peroxisins (e.g., <i>PEX3</i> , <i>PEX13</i>)	Hohfeld et al., 1991. <i>JCB</i> 114:1167; Gould et al., 1996. <i>JCB</i> 135:86; Waterham and Cregg, 1997. <i>Bioassays</i> 19:57
Matrix	Peroxisomal enzymes: e.g., acyl-CoA oxidase (<i>POX1</i>), malate dehydrogenase (<i>MDH3</i>), catalase A (<i>CTA1</i>), 3-ketoacyl-CoA thiolase (<i>POT1</i>), enoyl-CoA hydratase (<i>EDH1</i> , <i>EDH2</i>), 3-hydroxy-acyl-CoA epimerase (<i>FOX2</i>), citrate synthase (<i>CIT2</i>)	Dmochowska et al., 1990. <i>Gene</i> 88:247; Kispal and Srere, 1991. <i>Arch. Biochem. Biophys.</i> 286:132; Kunau and Hartig, 1992. <i>Antonie van Leeuwenhoek</i> 62:63; Singh et al., 1992. <i>MCB</i> 12:5593; Kunau et al., 1993. <i>Biochimie</i> 75:209; Subramani, 1993. <i>Annu. Rev. Cell Biol.</i> 9:445; Erdmann and Kunau, 1994. <i>Yeast</i> 10:1173; McAlister-Henn et al., 1995. <i>JBC</i> 270:21220; Geisbrecht et al., 1998. <i>JBC</i> 273:33184; Gurvitz et al., 1998. <i>JBC</i> 273:31366

continued

Table 3.7.7 Marker Proteins Used to Identify Fractionated Subcellular Components, continued

Subcellular fraction	Markers (and corresponding genes) ^{a,b}	Selected references ^c
<i>Mitochondria:</i>		
Outer membrane	Porin (<i>POR1, POR2</i>) ^e Outer membrane translocase (e.g., <i>TOM72, TOM20, TOM40, TOM7</i>) ^f	De Pinto et al., 1987. <i>BBA</i> 894:109 Lithgow et al., 1995. <i>Trends Biochem. Sci.</i> 20:98; Pfanner et al., 1997. <i>Annu. Rev. Cell Devel. Biol.</i> 13:25; Pfanner and Meijer, 1997. <i>Curr. Biol.</i> 7:R100; Kunkele et al., 1998. <i>Cell</i> 93:1009
Inner membrane	OM45p (<i>OM45</i>) Cytochrome oxidase (e.g., <i>COX1, COX2, COX3, COX4</i>) ^f Succinate dehydrogenase (e.g., <i>SDH3, SDH4</i>) ^f Mitochondrial H ⁺ -ATPase (e.g., <i>ATP5, ATP9</i>) ^f Inner membrane translocase (e.g., <i>TOM17, TOM23, TOM54</i>) ^{e,f}	Yaffe et al., 1989. <i>JBC</i> 264:21091 Taanman and Capaldi, 1995. <i>Eur. J. Biochem.</i> 227:22481; Geier et al., 1997. <i>FEBS Lett.</i> 412:296 Daignan-Fornier et al., 1994. <i>JBC</i> 269:15469; Oyedotun and Lemire, 1996. <i>MCB</i> 16:31382; Bullis and Lemire, 1997. <i>JBC</i> 272:6543 Jean-Francois et al., 1988. <i>BBA</i> 933:223; Uh et al., 1990. <i>JBC</i> 265:19047 Lithgow et al., 1995. <i>TBS</i> 20:98; Pfanner et al., 1997. <i>Annu. Rev. Cell Devel. Biol.</i> 13:25; Pfanner and Meijer, 1997. <i>Curr. Biol.</i> 7:R100; Blom et al., 1998. <i>MCB</i> 18:309
Intermembrane space	Inner membrane protease (<i>IMP1, IMP2</i>) Cytochrome <i>b2</i> (<i>CYB2</i>) and cytochrome <i>c</i> peroxidase (<i>CCPI</i>)	Nunnari et al., 1991. <i>EMBO J.</i> 10:1997; Schneider, 1991. <i>MCB</i> 34:401 Mathews and Wittenberg, 1979. <i>JBC</i> 254:5991; Daum et al., 1982. <i>JBC</i> 257:13028; Daum et al., 1982. <i>JBC</i> 257:13075; Brown and Trumpower, 1995. <i>J. Bacteriol.</i> 177:1380
Matrix	Fumarase (<i>FUM1</i>) and aconitase (<i>ACO1</i>); both also in cytoplasm	Daum et al., 1982. <i>JBC</i> 257:13075; Gangloff et al., 1990. <i>MCB</i> 10:3551; Stein et al., 1994. <i>MCB</i> 14:4770
<i>Nucleus:</i>		
Matrix	DNA, histones, other DNA-binding proteins	Fukuma et al., 1994. <i>Yeast</i> 10:319; Patterton et al., 1998. <i>JBC</i> 273:7268
Nucleolus	38-kDa yeast fibrillarin (<i>NOPI</i>)	Aris and Blobel, 1988. <i>JCB</i> 107:17; Henriquez et al., 1990. <i>JBC</i> 265:2209
Membrane	Nuclear pore components (e.g., <i>POM152</i>) ^f Membrane proteins (e.g., <i>SPO7, NEM1</i>); also in ER	Wozniak et al., 1994. <i>JCB</i> 125:31 Siniosoglou et al., 1998. <i>EMBO J.</i> 17:6449
<i>Vacuoles:</i>		
Membrane	Alkaline phosphatase (<i>PHO8</i>) ^e Dipeptidyl aminopeptidase B (<i>DAP2</i>)	Klionsky and Emr, 1989. <i>EMBO J.</i> 8:2241; Horazdovsky et al., 1995. <i>Curr. Opin. Cell Biol.</i> 7:544 Roberts et al., 1989. <i>JCB</i> 108:1363; Klionsky et al., 1990. <i>Microbiol. Rev.</i> 54:266
Lumen	Vacuolar SNARE (<i>VAM3</i>) V-ATPase ^f : e.g., 100-kDa subunit (<i>VPH1</i>) ^e , 60- and 69-kDa subunits (<i>VMA1, VMA2</i>) ^{e,g} Vacuolar enzymes: e.g., carboxypeptidase Y (<i>PRC1</i>) ^e , proteinase A (<i>PEP4</i>), proteinase B (<i>PRB1</i>), aminopeptidase I (<i>LAP4</i>), carboxypeptidase S (<i>CPS1</i>) ^h	Darsow et al., 1997. <i>JCB</i> 138:517 Kane et al., 1992. <i>JBC</i> 267:442; Manolson et al., 1992. <i>JBC</i> 267:14294; Nelson and Klionsky, 1996. <i>Experientia</i> 52:1101 Jones, 1990. <i>Methods Enzymol.</i> 185:372; Klionsky et al., 1990. <i>Microbiol. Rev.</i> 54:266; Klionsky et al., 1992. <i>JCB</i> 119:287; Spormann et al., 1992. <i>JBC</i> 267:8021; Horazdovsky et al., 1995. <i>Curr. Opin. Cell Biol.</i> 7:544; Van Den Hazel et al., 1996. <i>Yeast</i> 12:1; Odorizzi et al., 1998. <i>Cell</i> 95:847

continued

Table 3.7.7 Marker Proteins Used to Identify Fractionated Subcellular Components, continued

Subcellular fraction	Markers (and corresponding genes) ^{a,b}	Selected references ^c
<i>Endoplasmic reticulum:</i>		
Membrane	Dolichol P-mannosyltransferase (<i>DPM1</i>) ^e	Orlean et al., 1988. <i>JBC</i> 263:17499; Forsee et al., 1997. <i>Eur. J. Biochem.</i> 244:953
	Dolichol P-glucose synthetase (<i>ALG5</i>)	Heesen et al., 1994. <i>Eur. J. Biochem.</i> 224:71
	NAPDH-cytochrome P-450 reductase (<i>NCPI</i>)	Venkateswarlu et al., 1998. <i>JBC</i> 273:4492
	HMG-CoA reductase (<i>HMG1, HMG2</i>)	Hampton and Rine, 1994. <i>JCB</i> 125:299
	Protein translation complex (e.g., <i>SEC61, SEC62, SSSI, SSH1</i>) ^f	Corsi and Schekman, 1996. <i>JBC</i> 271:30299; Lyman and Schekman, 1996. <i>Experientia</i> 52:1042
Lumen	Oligosaccharyl transferase (e.g., <i>WBP1, OST1-5, STT3, SWP1</i>)	te Heesen et al., 1993. <i>EMBO J.</i> 12:279; Silberstein and Gilmore, 1996. <i>FASEB J.</i> 10:849; Karaoglu et al., 1997. <i>JBC</i> 272:32513
	Ribosome and translocation complex (RER) ^f	Sanderson and Meyer, 1991. <i>JBC</i> 266:13423; Planta and Mager, 1998. <i>Yeast</i> 14:471
	Mannosyltransferase (e.g., <i>PMT1-4</i>) BiP (<i>KAR2</i>)	Gentzsch and Tanner, 1996. <i>EMBO J.</i> 15:5752 Normington et al., 1989. <i>Cell</i> 57:1223; Rose et al., 1989. <i>Cell</i> 57:1211
Cytoplasm	Protein disulfide isomerase (<i>PDI1</i>)	LaMantia and Lennarz, 1993. <i>Cell</i> 74:899
	3-Phosphoglycerate kinase (<i>PGK1</i>) ⁱ	Perkins et al., 1983. <i>Biochem. J.</i> 121:199
	Glycerol-3-P-dehydrogenase (<i>GPD1</i>)	Albertyn et al., 1994. <i>MCB</i> 14:4135
	Glucose-6-P-dehydrogenase (<i>ZWF1</i>) ⁱ	Nogae and Johnston, 1990. <i>Gene</i> 96:161
<i>Transport vesicles:</i>		
ER-Golgi	COPII proteins (e.g., <i>EMP24, SEC23^g, SEC24^g</i>); Emp24p is also in ER	Schimmoller et al., 1995. <i>EMBO J.</i> 14:1329; Schekman and Orci, 1996. <i>Science</i> 271:1526; Matsuoka et al., 1998. <i>Cell</i> 93:263
Golgi-ER	COPI proteins (e.g., <i>COPI, RET2, RET3</i>) ^g	Letourneur et al., 1994. <i>Cell</i> 79:1199; Gaynor et al., 1998. <i>BBA</i> 1404:33
Intra-Golgi	Sec7p (<i>SEC7</i>) ^g	Franzusoff et al., 1991. <i>Methods Enzymol.</i> 194:662
Post-Golgi	Clathrin (<i>CHC1, CLC1</i>) ^g	Lemmon et al., 1988. <i>J. Cell Biochem.</i> 36:329; Chu et al., 1996. <i>JBC</i> 271:33123
Secretory (lumen)	SNARE proteins (<i>SNC1, SNC2</i>)	Protopopov et al., 1993. <i>Cell</i> 74:855
	Invertase (<i>SUC2</i>); induced in low glucose	Klionsky et al., 1988. <i>MCB</i> 8:2105
	Acid phosphatase (<i>PHO5</i>); induced in low phosphate	Vogel and Hinnen, 1990. <i>Mol. Microbiol.</i> 4:2013

^aThe examples of membrane-associated proteins are integral membrane proteins unless otherwise indicated.

^bAbbreviations: ER, endoplasmic reticulum; PM, plasma membrane; RER, rough endoplasmic reticulum.

^cJournal abbreviations: *BBA*, *Biochim. Biophys. Acta*; *JBC*, *J. Biol. Chem.*; *JCB*, *J. Cell Biol.*; *MBC*, *Mol. Biol. Cell*; *MCB*, *Mol. Cell Biol.*

^dAlthough these proteins are primarily located in the late Golgi, they also traffic to and from the endosome.

^eSpecific monoclonal antibodies can be purchased from Molecular Probes.

^fComplex of peripheral and integral membrane proteins.

^gPeripheral membrane protein(s).

^hThe processed vacuolar form of carboxypeptidase S is a soluble protein, whereas the precursor form is an integral membrane protein. Note that in protease-deficient mutant yeast, carboxypeptidase S is not processed into its soluble form.

ⁱPolyclonal antiserum can be purchased from Sigma.

throughout the fractionation experiment, and (3) can be detected by immunological or enzymatic techniques. Proteins that associate with more than one organelle can also serve as markers if they are subjected to compartment-specific posttranslational modifications (e.g., glycosylation, propeptide cleavage). An overview of compartment-specific posttranslational modifications is presented in Table 3.7.9.

Examples of marker proteins for various organelles are supplied in Table 3.7.7. The distribution of marker proteins within a series of fractions can be followed using immunoprecipitation (UNIT 7.2) or immunoblotting (UNIT 6.2) techniques. To ensure the highest possible accuracy, it is important to determine the relative protein levels among the fractions within the linear ranges of the detection methods.

Several enzyme activities that are characteristic of specific organelles are listed in Table 3.7.8. In most cases, the use of marker proteins to analyze the distribution of organelles/membranes is now favored over the use of enzyme activities if the appropriate antibodies are available. Enzyme activity levels are more sensitive than are protein levels to differing experimental conditions. For example, enzyme activities can be significantly impaired by the lysis buffer or gradient medium, the loss of a needed cofactor, or partial proteolysis.

Protein Profiles

The proteins found in several different highly purified yeast organelle preparations have been analyzed by SDS-PAGE (reviewed in Zinser and Daum, 1995; UNIT 6.1). The typical protein pattern (or protein profile) can be used to analyze the purity of an organelle preparation. In addition, the apparent molecular weights and isoelectric points of characterized resident proteins have been used to generate theoretical two-dimensional protein distributions for particular organelles (Yeast Protein Database; Hodges et al., 1998). Several two-dimensional profiles of *S. cerevisiae* proteins have been published (Singer-Kruger et al., 1993; Shevchenko et al., 1996; Garrels et al., 1997; Perrot et al., 1999), and it is likely that experimentally derived two-dimensional protein profiles for various organelle preparations will become increasingly available and informative in the near future.

Analysis of Protein Localization

To determine the subcellular localization of a novel protein (or a mutant form of a charac-

terized protein), the distribution of the protein of interest is compared with the distribution of markers (proteins or enzyme activities) that have a well-characterized subcellular localization. The cofractionation of the protein of interest with a marker suggests, but does not prove, that the two components may be associated with the same organelle or membrane *in vivo*. A second method (e.g., immunofluorescence) should be used to confirm the steady-state localization of the protein of interest (Pringle et al., 1991; Hasek and Streiblova, 1996). In contrast, if the protein of interest fractionates away from a given marker protein, one can usually conclude that the two components have different subcellular distributions *in vivo*. However, this generalization does not apply when the protein of interest primarily cofractionates with soluble cytoplasmic marker proteins, because these data alone do not rule out the possibility that the protein is a released peripheral membrane protein or a soluble protein that normally resides within the lumen of an organelle instead of a native cytoplasmic protein.

Analysis of Organelles Along Protein Transport Pathways

The dynamic compartments along the secretory, endocytic, and vacuolar protein transport pathways can be particularly difficult to characterize (especially endosomes and transport vesicles), as they tend to have few proteins or enzyme activities that can serve as reliable steady-state markers. Many of the proteins associated with these organelles are either: (1) cargo molecules that transit along the transport pathways (e.g., newly synthesized membrane proteins, internalized components from the cell surface), (2) proteins that cycle between two or more of the compartments (e.g., cargo receptors, vesicle targeting molecules), or (3) molecules that only transiently associate with the compartment membranes (e.g., small GTPases, coat proteins).

Despite these complications, several techniques can be used to follow the organelles along the secretory, endocytic, and vacuolar protein transport pathways. For example, proteins that are actively retained within a compartment (and retrieved if they escape) have been used as markers for the endoplasmic reticulum (ER), Golgi complex, and plasma membrane, since their steady-state distribution strongly favors a single organelle type (see Table 3.7.7 for examples). In addition, proteins

Table 3.7.8 Enzyme Activities Associated with Subcellular Organelles

Primary location	Marker enzyme activities	Selected references ^a
<i>Endoplasmic reticulum:</i>		
Membrane	NAPDH-cytochrome <i>c</i> reductase	Kubota et al., 1977. <i>J. Biochem.</i> 81:197; Roberts et al., 1991. <i>Methods Enzymol.</i> 194:644
	Dolichyl phosphate mannosyltransferase	Marriott and Tanner, 1979. <i>J. Bacteriol.</i> 139:566; Heesen et al., 1994. <i>Eur. J. Biochem.</i> 224:71
	Translocation activity (rough ER) ^b	Sanderson and Meyer, 1991. <i>JBC</i> 266:13423
<i>Golgi:</i>		
Membrane: <i>cis</i>	α1-6-Mannosyltransferase (Och1p) GDPase	Nakayama et al., 1992. <i>EMBO J.</i> 11:2511 Abeijon et al., 1989. <i>PNAS</i> 86:6935
Membrane: medial	α1-3-Mannosyltransferase (Mnn1p)	Nakajima and Ballou, 1975. <i>PNAS</i> 72:3912
Membrane: <i>trans</i>	Dipeptidyl aminopeptidase A (heat stable) Kex2p	Julius et al., 1984. <i>Cell</i> 36:309; Roberts et al., 1991. <i>Methods Enzymol.</i> 194:644 Brenner et al., 1994. <i>Methods Enzymol.</i> 244:152
<i>Post-Golgi secretory vesicles:</i>		
Lumen	Invertase; induced in low glucose	Goldstein and Lampen, 1975. <i>Methods Enzymol.</i> 42:504; Holcomb et al., 1987. <i>Anal. Biochem.</i> 166:328; Walworth and Novick, 1987. <i>JCB</i> 105:163
	Acid phosphatase; induced in low phosphate	Van Rijn et al., 1972. <i>BBA</i> 268:431; Holcomb et al., 1988. <i>JCB</i> 106:641
<i>Plasma membrane:</i>		
Membrane	H ⁺ -ATPase (vanadate sensitive) ^c	Willsky, 1979. <i>JBC</i> 254:3326; Serrano, 1988. <i>Methods Enzymol.</i> 157:533; Serrano, 1988. <i>BBA</i> 947:1
	Chitin synthetase; also in chitosomes	Bowman and Slayman, 1979. <i>JBC</i> 254:2928
<i>Vacuoles:</i>		
Membrane	Alkaline phosphatase (ALP) α-Mannosidase	Roberts et al., 1991. <i>Methods Enzymol.</i> 194:644 Opheim, 1978. <i>BBA</i> 524:121; Roberts et al., 1991. <i>Methods Enzymol.</i> 194:644
	Dipeptidyl aminopeptidase B (heat sensitive)	Roberts et al., 1991. <i>Methods Enzymol.</i> 194:644
	H ⁺ -ATPase (bafilomycin sensitive) ^c	Roberts et al., 1991. <i>Methods Enzymol.</i> 194:644

continued

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3.7.20

that transit through multiple compartments can serve as transient markers for the transport intermediate of interest. Several techniques used to generate and monitor transient markers are summarized in Table 3.7.10. In some cases, the transit of a limited pool of proteins is followed until it reaches the compartment of interest. In addition, multiple investigators have successfully used temperature-sensitive mutants that block protein transport at specific steps and accumulate cargo molecules within a particular compartment. Multiple temperature-sensitive mutants that inhibit specific transport steps are available for the

secretory pathway (e.g., *sec* mutants), the endocytic pathway (e.g., *end* mutants), and vacuolar protein transport pathway (e.g., *vps* mutants; see Table 3.7.10 for selected references). However, the results derived from mutant cells should only be extrapolated to normal cells after careful evaluation of the mutant cell properties and potential artifacts.

Analysis of Organelle Morphology, Integrity, and Function

The organelles and membranes within subcellular fractions can also be analyzed by electron microscopy. Some organelles, such as

Table 3.7.8 Enzyme Activities Associated with Subcellular Organelles, continued

Primary location	Marker enzyme activities	Selected references ^a
Matrix	Carboxypeptidase Y (CPY)	Jones, 1991. <i>JBC</i> 266:7963
	Carboxypeptidase S (CPS)	Distel et al., 1983. <i>BBA</i> 741:128
	Proteinase A (PrA), proteinase B (PrB)	Jones, 1991. <i>JBC</i> 266:7963
	Aminopeptidase I (API)	Distel et al., 1983. <i>BBA</i> 741:128
<i>Peroxisomes:</i>		
Peripheral membrane	Acyl-CoA oxidase ^b	Dommes et al., 1981. <i>JBC</i> 256:8259; Kionka and Kunau, 1983. <i>J. Bacteriol.</i> 161:153
Matrix	Catalase A	Luck, 1963 (see Literature Cited); Ueda et al., 1991. <i>Methods Enzymol.</i> 188:463
	3-Oxoacyl-CoA thiolase	Kionka and Kunau, 1983. <i>J. Bacteriol.</i> 161:153
	Citrate synthase	Lewin et al., 1990. <i>MCB</i> 10:1399
<i>Mitochondria:</i>		
Inner membrane	Cytochrome <i>c</i> oxidase	Mason et al., 1973. <i>JCB</i> 248:1346; Poyton et al., 1995. <i>Methods Enzymol.</i> 260:97
	Succinate dehydrogenase	Ackrell et al., 1978. <i>Methods Enzymol.</i> 53:466
	H ⁺ -ATPase (oligomycin sensitive) ^c	Roberts et al., 1991. <i>Methods Enzymol.</i> 194:644
Intermembrane space	Cytochrome <i>b</i> 2	Daum et al., 1982. <i>JBC</i> 257:13075
	Cytochrome <i>c</i> peroxidase	Daum et al., 1982. <i>JBC</i> 257:13028
Matrix	Fumarase	Daum et al., 1982. <i>JBC</i> 257:13028
<i>Cytoplasm:</i>		
	Glucose-6-phosphate dehydrogenase	Roberts et al., 1991. <i>Methods Enzymol.</i> 194:644

^aJournal abbreviations: *BBA*, *Biochim. Biophys. Acta*; *JBC*, *J. Biol. Chem.*; *JCB*, *J. Cell Biol.*; *MCB*, *Mol. Cell Biol.*; *PNAS*, *Proc. Natl. Acad. Sci. U.S.A.*

^bPeripheral membrane protein(s).

^cComplex of peripheral and integral membrane proteins.

intact mitochondria, retain recognizable features after their isolation (e.g., Aris and Blobel, 1991; Glick and Pon, 1995). However, most organelles and membranes have similar tubular and/or vesicular appearances after homogenization and fractionation, and thus cannot be identified by their morphology alone. In these cases, analysis by immunocytochemistry, immunoelectron microscopy (UNITS 4.3, 4.4 & 4.6), or other marker-based techniques is advisable (e.g., Byers and Goetsch, 1991; Clark, 1991; Hasek and Streiblova, 1996; Rieder et al., 1996; Hicke et al., 1997; Prescianotto-Baschong and Riezman, 1998).

The structural integrity of an organelle can be monitored by measuring the latency of marker proteins or enzymes that normally reside within the lumen of an organelle. Morphological analysis can also reveal the integ-

rity of certain organelles, especially mitochondria and nuclei. In addition, the integrity and orientation of organelle and vesicle membranes can be examined using protease- and antibody-accessibility experiments (see UNIT 5.4).

The intracellular functions of several organelles have been successfully reconstituted in a test tube (e.g., Daum et al., 1982a,b; Baker et al., 1988; Vida et al., 1990; Lazarow et al., 1991; Brodsky et al., 1993). These in vitro assays are essential tools for studying complex cellular processes, as the conditions and corresponding consequences can be manipulated and analyzed. In addition, in vitro assays can be used to monitor the functional integrity of the organelle of interest and thus the quality of an organelle preparation.

Table 3.7.9 Compartment-Specific Posttranslational Protein Modifications

Compartment	Posttranslational modification
<i>Mitochondria:</i>	
Intermembrane space	Heme attachment Membrane anchor cleavage
Matrix	Leader peptide cleavage
<i>Endoplasmic reticulum:</i>	
Lumen	Signal peptide cleavage Core N-linked ^a oligosaccharide addition Core O-linked ^a oligosaccharide addition Cleavage of glucose from N-linked oligosaccharides
<i>Golgi:</i>	
<i>cis</i> cisternae	Initial α 1-6-linked oligosaccharide addition α 1-6-linked oligosaccharide elongation
Medial cisternae	α 1-3-linked oligosaccharide addition α 1-2-linked oligosaccharide addition Elongation of O-linked oligosaccharides
<i>trans</i> cisternae	Lysine-arginine dibasic endoproteolysis Cleavage by dipeptidyl aminopeptidase A (DPAP-A)
<i>Vacuoles:</i>	
Lumen	Propeptide cleavage and activation of hydrolases

^aN-linked, asparagine-linked; O-linked, serine- or threonine-linked.

Table 3.7.10 Application of Transient Markers to Identify Compartments Along the Secretory, Endocytic, and Vacuolar Protein Transport Pathways^a

General principle	Specific techniques
Follow transit of limited protein pool until it reaches compartment of interest	Briefly label newly synthesized proteins with [³⁵ S]cysteine/methionine and analyze compartments after appropriate chase times Briefly label cell surface proteins with ¹²⁵ I and analyze endocytic compartments after appropriate chase times Follow radiolabeled ligand (e.g., [³⁵ S] α -factor) to analyze endocytic compartments after appropriate chase times Transiently induce/derepress translation of protein known to transit through compartment(s) of interest Localize cargo proteins by analyzing their compartment-specific posttranslational modifications (Table 3.7.9)
Slow or block protein transport at specific step to accumulate cargo proteins in compartment of interest	Block protein transport at specific step along transport pathway using temperature-conditional mutants (e.g., <i>sec</i> , <i>end</i> , <i>vps</i> mutants) Slow protein transport through endosomal system by cooling cells to 15°C

^aSelected references: Schekman (1985); Rothblatt and Schekman (1989); Walworth et al. (1989); Singer and Riezman (1990); Vida et al. (1990); Dulic et al. (1991); Franzusoff et al. (1991); Raths et al. (1993); Horazdovsky et al. (1995); Wuestehube et al. (1996); Hicke et al. (1997); Rieder and Emr (1997).

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This review provides an excellent overview of the theory and design of subcellular fractionation experiments. Other helpful reviews include Evans (1992) and Hinton and Mullock (1997; see above).

Sikorski, R. and Peters, R. 1998. Yeast sites on the net. *Science* 279:1968.

*This review provides information on multiple Internet resources available for *S. cerevisiae*.*

Walworth et al., 1989. See above.

*This older review provides an excellent summary of the subcellular fractionation techniques applied to *S. cerevisiae*, with a focus on organelles along the secretory pathway.*

Zinser and Daum, 1995. See above.

This review provides an excellent summary of the subcellular fractionation techniques applied to S. cerevisiae and the corresponding references.

INTERNET RESOURCES

<http://genome-www.stanford.edu/Saccharomyces/>

Saccharomyces Genome Database (SGD) provides access to the complete sequence of the S. cerevisiae genome, confirmed and predicted open reading frames (ORFs), protein information, and a number of useful Internet links.

<http://www.atcc.org/searchengine/ygsc.html>

Yeast Genetic Stock Center at the ATCC, which can provide many published yeast strains.

<http://www.proteome.com/>

The Yeast Protein Database (Proteome, Inc.) is an excellent source of comprehensive and up-to-date information on S. cerevisiae proteins. The information is derived from the published literature and DNA sequence databases. The protein information provided, including summaries of the protein and gene characteristics and the corresponding literature references, can be searched and categorized in several convenient ways (e.g., according to subcellular location, gene name, or size).

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Isolation of Subcellular Fractions from the Yeast *Saccharomyces cerevisiae*

The refinement of subcellular fractionation techniques for the budding yeast *Saccharomyces cerevisiae* has provided a valuable approach for investigating the molecular mechanisms of complex cellular functions. The protocols presented in this unit cover a range of centrifugation-based procedures used to fractionate subcellular components from *S. cerevisiae* according to their sizes and/or buoyant densities. The key goals and techniques of each protocol are summarized in Table 3.8.1. In addition, many other *S. cerevisiae* subcellular fractionation procedures and the corresponding references are listed in Table 3.8.2. An overview of centrifugation-based fractionation procedures for *S. cerevisiae* is presented in UNIT 3.7.

The basic methods used to isolate and characterize organelles from *S. cerevisiae* are very similar to those used for mammalian cells and other higher eukaryotes. The most appropriate subcellular fractionation procedure will vary with the goals of the particular experiment and the desired levels of organelle yield, purity, and function. The merits, limitations, and critical parameters of various *S. cerevisiae* techniques are discussed in UNIT 3.7 to facilitate the development of successful subcellular fractionation experiments.

Yeast cells can be cultured using relatively simple techniques (UNIT 1.6) and are extremely versatile. For example, the abundance of certain enzymes and organelles that are subject to metabolic regulation can be manipulated by varying the nutrient composition of the medium. The optimal development of mitochondria and peroxisomes induced by growth on lactate or oleate, respectively, is critical to the effective isolation of these organelles (see Basic Protocols 6 and 7). In addition, the availability of mutant strains is also beneficial. For example, the use of a strain that is deficient in vacuolar protease activity can minimize problems with proteolysis.

In contrast to animal cells, yeast cells have a thick cell wall that must be disrupted or removed in order to generate cell lysates. The disruption of the cell wall by shearing with glass beads is a rapid and effective method to prepare yeast cell homogenates for the isolation of plasma membranes (see Basic Protocol 9) and cytosol (see Basic Protocol 10). However, glass bead lysis is not suitable for most subcellular fractionation experiments, since the strong shear forces required to disrupt the cell wall can also damage intracellular organelles and disrupt protein and membrane interactions. To minimize the disruption of subcellular integrity, yeast cells are usually first enzymatically converted to spheroplasts (yeast cells lacking cell walls) prior to cell lysis (see Support Protocol). Unlike intact yeast cells, spheroplasts are fragile and osmotically sensitive; thus they can be lysed under isosmotic or hypoosmotic conditions, usually in combination with moderate shear force (e.g., several strokes in a Dounce glass homogenizer). Multiple enzyme preparations that digest the cell wall by cleaving the critical β -glucan linkages are available, such as Zymolyase 100T (see Support Protocol) and oxalyticase (see Basic Protocol 4).

NOTE: All references to “yeast” within this unit refer to the budding yeast *S. cerevisiae*. Many protocols developed for *S. cerevisiae* are not directly applicable to other yeasts (e.g., *Schizosaccharomyces pombe*) without significant modification. *S. cerevisiae* protocols can be applied to many different *S. cerevisiae* strains; however, variations among strains and growth conditions may require adjustments to a given protocol.

Table 3.8.1 Summary of the Primary Goals and Techniques of the Protocols^a

Protocol	Primary goals	Key techniques
Basic Protocol 1	Separate lysate into a cytosol fraction and several membrane fractions (roughly according to size; see Table 3.7.2, Table 3.7.4, and Figure 3.7.1) Prepare enriched membrane fractions for subsequent procedures	Spheroplast preparation (Support Protocol) Dounce homogenization Sequential centrifugation steps
Support Protocol	Generate spheroplasts (for subsequent lysate preparation)	Cell culture Cell wall digestion (Zymolyase 100T)
Basic Protocol 2	Separate endosomal and Golgi membranes from vacuolar membranes	Spheroplast preparation (Support Protocol) Homogenization and centrifugation (Basic Protocol 1) Equilibrium Nycodenz gradients
Alternate Protocol	Separate endosomal and vacuolar membranes from Golgi membranes	Spheroplast preparation (Support Protocol) Homogenization and centrifugation (Basic Protocol 1) Equilibrium sucrose gradients
Basic Protocol 3	Subfractionate 13,000 × g pellet: separate PM and ER membranes from vacuolar and Golgi membranes	Spheroplast preparation (Support Protocol) Homogenization and centrifugation (Basic Protocol 1) Sucrose step gradients
Basic Protocol 4	Isolate intact vacuoles	Spheroplast preparation (oxylyticase) DEAE-isoosmotic spheroplast lysis Ficoll 400 step gradients
Basic Protocol 5	Isolate intact nuclei	Spheroplast preparation (Zymolyase 100T and Glusulase) Homogenization and differential centrifugation Ficoll 400 step gradients
Basic Protocol 6	Isolate lactate-induced mitochondria	Induction of mitochondria (lactate as carbon source) Spheroplast preparation (Zymolyase 20T) Homogenization and differential centrifugation Nycodenz step gradients
Basic Protocol 7	Isolate oleate-induced peroxisomes	Induction of peroxisomes (oleate as carbon source) Spheroplast preparation (Zymolyase 100T) Homogenization and differential centrifugation Sucrose step gradient
Basic Protocol 8	Isolate ER membranes	Spheroplast preparation (Support Protocol) Homogenization and differential centrifugation Sucrose step gradient
Basic Protocol 9	Isolate plasma membranes	Glass bead lysis Differential centrifugation Sucrose step gradient
Basic Protocol 10	Prepare “active” cytosol (e.g., for functional in vitro assays)	Glass bead lysis Centrifugation

^aAbbreviations: ER, endoplasmic reticulum; PM, plasma membrane.

NOTE: Operations during and after cell lysis should be conducted at 0° to 4°C (unless otherwise indicated). In addition, solutions and equipment to be used during and after cell lysis should be chilled to 0° to 4°C prior to their use (unless otherwise indicated).

NOTE: Phenylmethylsulfonyl fluoride (PMSF) and other protease inhibitors are toxic and should be handled with care. They should be added to solutions just before use.

FRACTIONATION OF SPHEROPLASTS BY DIFFERENTIAL CENTRIFUGATION

**BASIC
PROTOCOL 1**

The protocol presented below is a differential centrifugation protocol that separates subcellular components from yeast spheroplasts into several fractions, roughly according to their size. Differential centrifugation is a component of most subcellular fractionation experiments, since it provides a way to enrich for the organelle or membrane of interest, to deplete undesired components, and/or reduce the sample volume. Differential centrifugation is also used to analyze the potential subcellular location(s) of a protein of interest (see Table 3.7.4).

Yeast cells in their exponential growth phase are first converted to spheroplasts (see Support Protocol, steps 4 to 15). Alternate spheroplast preparation procedures can also be used; e.g., Basic Protocol 4, Basic Protocol 5, or numerous other protocols. The spheroplasts are lysed using a combination of osmotic shock and Dounce homogenization. Subsequently, the lysates are subjected to sequential centrifugation runs at increasing speeds (see Figure 3.7.1). The resulting fractions include the 300 × *g* pellet (P₃₀₀) and supernatant (S₃₀₀), the 13,000 × *g* pellet (P_{13,000}) and supernatant (S_{13,000}), and the 100,000 × *g* pellet (P_{100,000}) and supernatant (S_{100,000}) fractions.

If compatible with the goals of the experiment, protease-deficient strains can be used to minimize protein degradation (e.g., BJ3505, a *pep4 prb1* mutant yeast strain). The cell number required will depend on the goals of the experiment. This protocol is written for relatively small-scale experiments (10 to 40 OD₆₀₀ units of cells) but can be adapted to experiments requiring more cells. When investigating the localization of a protein of interest, it is helpful to use enough cells so that one can set aside an aliquot of each fraction and monitor the yield of each centrifugation step.

Materials

Desired yeast cultures in their exponential growth

Stop solution or spheroplast medium B (see recipe; optional), ice cold

HEPES/potassium acetate (HEPES/KAc) lysis buffer (see recipe), ice cold
500× protease inhibitor cocktails A and B (500× PIC-A and 500× PIC-B; see recipes)

Protease inhibitor stock solutions A (see recipe)

Dounce glass homogenizer, tight-fitting pestle, prechilled to 4°C

1.6-ml microcentrifuge tubes, prechilled to 4°C

Microcentrifuge prechilled to 4°C

1.6-ml polycarbonate tubes for ultracentrifugation at 100,000 × *g*, prechilled on ice

Beckman refrigerated tabletop ultracentrifuge (or equivalent) and rotor (e.g., Beckman TLA100.3 or equivalent)

Additional reagents and equipment for growing yeast cells (see *UNIT 1.6*) and preparation of spheroplasts (see Support Protocol)

**Subcellular
Fractionation and
Isolation of
Organelles**

3.8.3

Prepare and harvest cells

1. Culture desired yeast strains and prepare spheroplasts (Support Protocol, steps 1 to 15).

From this point on, keep samples on ice or at 4°C.

Spheroplasts are fragile and must be handled very gently.

2. Resuspend the spheroplasts at a concentration of 5 to 10 OD₆₀₀ units/ml in ice-cold stop solution, spheroplast medium B, or another solution with the appropriate osmotic support. Resuspend spheroplasts gently by swirling tube by hand, slowly stirring with a glass rod, or slowly pipetting with a wide bore pipet. Do not vortex. Keep suspension on ice until ready for the next step.

The optimal solution will depend on the goals and subsequent steps of the experiment in which the spheroplasts will be used.

Spheroplasts tend to stick to each other and thus can be difficult to resuspend. To facilitate resuspension, initially resuspend spheroplasts in a smaller volume of solution before adding the remaining solution.

Stop solution contains two energy poisons (i.e., NaN₃ and NaF) that are toxic and should be handled with care. The inclusion of NaN₃ and NaF is not appropriate for all purposes (e.g., to isolate organelles for functional studies).

3. To harvest spheroplasts centrifuge 5 min at 1500 × g, 4°C. Aspirate or decant the supernatants.

Use care while removing supernatants, because spheroplast pellets will be relatively loose and fluffy. In addition, the emergence of CO₂ bubbles from the spheroplasts can significantly disrupt the pellets.

Lyse spheroplasts

4. Gently resuspend spheroplasts at a concentration of 5 OD₆₀₀ units/ml in ice-cold HEPES/KAc lysis buffer.

The spheroplasts can also be lysed at higher concentrations (i.e., 5 to 15 OD₆₀₀ units/ml) for large-scale preparations or if it is crucial to minimize the lysate volume.

5. Transfer spheroplast suspension to a Dounce homogenizer that has been prechilled in an ice bucket. Disrupt spheroplasts with 10 up-and-down strokes of a tight-fitting pestle.

Keep the Dounce homogenizer on ice throughout this process. Move the pestle slowly and maintain contact between pestle and suspension to minimize foaming.

6. Transfer lysate to multiple prechilled 1.6-ml microcentrifuge tubes.

It is often convenient to make 1-ml aliquots of the lysate (e.g., each containing 5 OD₆₀₀ cell equivalents). Larger microcentrifuge tubes can also be used; however, the use of 1.6-ml microcentrifuge tubes and a refrigerated microcentrifuge is often faster and more convenient.

In order to monitor recovery levels in the fractions generated by the subsequent microcentrifugation steps, it is often beneficial to set aside an aliquot of the lysate that will not be fractionated (e.g., 1 ml or 5 OD₆₀₀ cell equivalents).

Clear lysates

7. Clear the lysates of unbroken cells, partially disrupted cells, and aggregates by centrifuging 5 min at 300 × g, 4°C.

- Carefully withdraw the supernatant (S_{300}) without disturbing the very loose, fluffy pellet (P_{300}), and transfer to new prechilled 1.6-ml tubes. Record the volumes of the transferred S_{300} .

Usually only ~80% of S_{300} can be harvested from each tube without disturbing the pellets.

In order to monitor the recovery levels in the fractions generated by the subsequent centrifugation steps at $13,000 \times g$ and $100,000 \times g$, it is often beneficial to set aside an aliquot of the S_{300} that will not be fractionated further.

Also, it is critical to record the volume of harvested supernatant at each step (as it is never 100%) in order to follow the yield of each centrifugation step.

- Optional:** Resuspend the P_{300} by pipetting up and down ten times using a micropipettor with a 200- μ l pipet tip. Add HEPES/KAc lysis buffer to a total volume of ~500 μ l and mix with a cut pipet tip or by vortexing. Repeat steps 7 and 8. Pool the first and second S_{300} supernatants.

Alternatively, the sample can be transferred to a clean, chilled Dounce homogenizer and dispersed with several gentle strokes.

This washing step can improve the yield of intracellular membranes and purity of P_{300} fractions by removing some of the smaller organelles trapped within P_{300} pellets. This step is critical if the P_{300} will be retained for further analyses (e.g., to examine ER and plasma membrane distributions, since up to 50% of these membranes can pellet at $300 \times g$; see Table 3.7.4). However, it is not always recommended, since it increases the sample volume, processing time, and likelihood of organelle damage.

The P_{300} pellets not required for subsequent analyses are often discarded since they contain a crude mixture of unlysed and partially disrupted cells, as well as a very heterogeneous mixture of intracellular membranes.

Prepare 13,000 $\times g$ fractions

- Microcentrifuge S_{300} supernatants 10 min at $13,000 \times g$, 4°C .
- Carefully transfer the supernatants ($S_{13,000}$) to prechilled 1.6-ml polycarbonate ultracentrifuge tubes and keep on ice.

In order to monitor the recovery in the fractions generated by the subsequent centrifugation at $100,000 \times g$, it is often beneficial to set aside an aliquot of the $S_{13,000}$ that will not be fractionated further.

- To remove any residual supernatant from the $P_{13,000}$ pellets and tube walls, microcentrifuge the tubes containing the $P_{13,000}$ samples ~30 sec at $13,000 \times g$, 4°C . Carefully remove any remaining $S_{13,000}$ above pellet.

If the residual $S_{13,000}$ has a small volume (e.g., $\leq 10 \mu\text{l}$), discard it; otherwise, add it to the appropriate $S_{13,000}$ fraction.

- Add ice-cold HEPES/KAc lysis buffer (or another appropriate buffer) with freshly added protease inhibitors (PIC-A, PIC-B, protease inhibitor stock solution A) to the $P_{13,000}$ pellets. Briefly mix using a micropipettor or by vortexing. Cap tubes and store on ice. Quickly move on to step 14.

The appropriate buffer and volume will depend on the goals and procedures of subsequent experiments. It is critical, however, that the buffer contain fresh protease inhibitors.

Prepare 100,000 $\times g$ fractions

- Balance the $S_{13,000}$ samples by adding ice-cold HEPES/KAc lysis buffer as needed. Centrifuge $S_{13,000}$ samples 60 min at $100,000 \times g$, 4°C , in a fixed-angle rotor (e.g., 55,000 rpm for the Beckman TLA100.3 rotor).

15. *Optional:* During the 60-min centrifugation, process the P_{13,000} pellets further as needed.

If it is critical to disperse the membranes for subsequent fractionation steps, transfer the suspension to a small chilled Dounce homogenizer and apply several up-and-down strokes until the pellet is disrupted.

16. After ultracentrifugation, carefully transfer the supernatants (S_{100,000}) to clean, pre-chilled microcentrifuge tubes and store on ice.
17. To remove residual supernatant from pellets (P_{100,000}) and tube walls, centrifuge tubes with P_{100,000} pellets ~30 sec at 13,000 × g, 4°C. Carefully remove and discard any remaining supernatant above the P_{100,000} pellet.
18. Add ice-cold HEPES/KAc lysis buffer with freshly added protease inhibitors (or another appropriate buffer) to P_{100,000} pellets. Quickly mix with a pipettor or vortexer. Keep on ice until needed.

The appropriate buffer and volume will depend on the goals and procedures of subsequent experiments. It is critical, however, that the buffer be ice-cold and contain fresh protease inhibitors.

The P_{100,000} pellets are usually very difficult to resuspend. If it is critical to disperse the membranes for subsequent experiments, transfer the suspension to a small chilled Dounce homogenizer and apply several up-and-down strokes until the pellet is disrupted. It also can be helpful to allow the pellets to “soften” by incubating the pellets with buffer on ice. If the membranes do not need to be intact for further fractionation experiments, the pellet can be quickly resuspended in a small volume of buffer (~100 μl) using a water sonication bath.

SUPPORT PROTOCOL

PREPARATION OF YEAST SPHEROPLASTS USING ZYMOLYASE

The preparation of yeast spheroplasts through enzymatic digestion of the cell wall is the first step for most subcellular fractionation experiments. This protocol describes a method to prepare exponentially growing yeast cultures and convert yeast cells into spheroplasts using Zymolyase 100T, a β-glucanase preparation. Digestion of the cell wall is monitored by spectrophotometry and microscopy. This protocol can be adapted to any cell number or strain.

Additional Materials (also see Basic Protocol 1)

- Exponentially growing yeast cell cultures in yeast extract/peptone/dextrose (YPD) medium (see UNIT 1.6) or appropriate medium
- TSD reduction buffer (see recipe)
- Spheroplast medium A (see recipe)
- 5 mg/ml Zymolyase 100T (ICN Immunobiochemicals) in spheroplast medium A, (store in aliquots at -20°C)
- Spheroplast medium B (see recipe), ice cold
- 50-ml glass culture tubes *or* 100-ml Erlenmeyer flasks
- Shaker platform at 30°C (or appropriate growth temperature of the strains being used)
- 250-ml centrifuge bottles
- 50-ml centrifuge tubes
- Centrifuge and rotor (e.g., Sorvall GS-3 rotor)
- 30°C water bath
- Additional reagents and equipment for culturing yeast cells (UNIT 1.6)

Preculture cells

1. In the morning, a day before the experiment, start precultures of the desired yeast strains by inoculating 10 ml YPD (or appropriate medium) with a single yeast colony from a fresh plate.

The cultures can be grown in 50-ml glass culture tubes or 100-ml Erlenmeyer flasks.

If using strains that grow very slowly or that have been stored on a plate at 4°C for more than a few days, it is advised that the preculture be started with a single colony the night before.

2. Culture cells throughout the day while shaking at 225 to 325 rpm at 30°C (or appropriate permissive temperature).
3. In the evening, a day before the experiment, determine cell density of the precultures by measuring the OD₆₀₀.

For the most accurate measurement, dilute the culture at 1:5 or 1:10 in water, measure OD₆₀₀, and then multiply the reading by 5 or 10, respectively. The correlation between the cell number and the OD₆₀₀ is usually most linear between an OD₆₀₀ of 0.1 to 0.3 (see UNIT 3.7).

4. After completing the calculations below, use the appropriate amount of the preculture to inoculate fresh cultures for overnight growth.

The suitable yeast cell concentration is determined by the growth rate of each strain (i.e., the doubling time), the desired incubation time (usually overnight), and the target cell density. For most applications, the target OD₆₀₀ corresponds to the cell density when the cultures are in their mid-logarithmic growth phase. The culture OD₆₀₀ during this rapid growth phase varies with the strain and growth conditions, but is often between 0.6 and 1.0. The required culture volumes will depend on the number of cells needed. It is best to start slightly larger cultures than required in case the cell density does not quite reach the expected level (see calculations below and UNITS 1.6 & 3.7).

- a. Calculate desired culture volume as follows:

minimum culture volume (ml) = (required OD₆₀₀ equivalents of cells/target OD₆₀₀)
× 1.25

Example: If 20 OD₆₀₀ equivalents of a strain are required and the target cell density is at an OD₆₀₀ of 0.8, then a 30-ml culture should be started, since $(20/0.8) \times 1.25 = 31.25$ ml.

- b. Calculate the inoculation cell density (OD₆₀₀):

inoculation OD₆₀₀ = target OD₆₀₀ × 2^(no. of doublings)

Examples: A strain with a doubling time of 2.5 hr should be inoculated at an OD₆₀₀ of ~0.008 in order to reach an OD₆₀₀ of ~1 after 17.5 hr of growth (7 generations), since $1 \times 2^{-4} = 0.008$. A strain with a doubling time of 1.75 hr should be inoculated at an OD₆₀₀ of ~0.001 in order to reach an OD₆₀₀ of ~1 after 17.5 hr of growth (10 generations), since $1 \times 2^{-10} = 0.001$.

5. In the morning of the day that the spheroplasts are needed, measure the OD₆₀₀ of cultures. Continue to culture the cells until they are within their target density range (i.e., mid-logarithmic growth phase).

If the cultures have significantly exceeded the maximum target density (i.e., by >20%), dilute the cultures (e.g., 1:4 to 1:6 dilution in medium) and allow them to grow for at least two more generations before harvesting.

6. Harvest cells by centrifuging 5 min at 4000 × g (e.g., 5000 rpm in a Sorvall GS-3 rotor), room temperature, and carefully remove supernatant.

Prepare spheroplasts

7. Resuspend cells at 5 to 10 OD₆₀₀ units/ml in TSD reduction buffer and incubate 10 min at room temperature.

The DTT treatment facilitates cell wall digestion by breaking disulfide bonds and making the β -glucan linkages more accessible to the β -glucanase activity present in the Zymolyase preparation.

8. Harvest cells by centrifuging 5 min at $\sim 4000 \times g$, room temperature. Aspirate supernatant.
9. Resuspend cells at ~ 20 to 30 OD₆₀₀ units/ml in spheroplast medium A.
10. Remove 10 μ l of each cell suspension and dilute in 990 μ l water. Mix by briefly vortexing. Measure and record the OD₆₀₀ value.

The decrease in the OD₆₀₀ value of the diluted cell suspension will be used to determine the efficiency of cell wall removal. In the absence of mechanical support (provided by the intact cell wall) or osmotic support (provided by sorbitol in the spheroplast medium), yeast cells will lyse and thus will no longer contribute to the optical density.

11. Add Zymolyase 100T (as 5 mg/ml stock) to the cell suspension at a concentration of 1 to 5 μ g per OD₆₀₀ unit of cells. Incubate at 30°C (or appropriate permissive temperature) for ~ 20 min.

The optimal Zymolyase concentration and digestion time depend on the strains and growth conditions. Thus, these parameters should ideally be determined empirically for the specific strains and culture conditions to be used.

12. To monitor the efficiency of cell wall removal, dilute 10 μ l cell suspension into 990 μ l water. Mix by vortexing. After 1 or 2 min, mix again and then measure the OD₆₀₀ value. Continue cell wall digestion until the OD₆₀₀ measurement of the diluted cell suspension is $<5\%$ of the original value (step 9), indicating that over 95% of the cells have been converted to spheroplasts.

The generation of spheroplasts can also be monitored by light microscopy. Spheroplasts will appear as spherical cells with bright halos, while yeast with intact cell walls will have a darker appearance and a slightly more oval shape. Spheroplast lysis can also be observed under the microscope by adding a drop of water to the sample.

Harvest and wash spheroplasts

13. Harvest spheroplasts by centrifuging 5 min at 1500 $\times g$, room temperature.

Spheroplasts are fragile and must be handled very gently.

If working with a very osmotically sensitive strain, the sorbitol concentration can be increased to 1.2 M or even 1.5 M. In addition, the spheroplasts can be pelleted through a cushion of 1.1 M sorbitol/7.5% (v/v) Ficoll 400 to minimize damage during the following wash steps.

14. Aspirate or decant supernatants.

Use care while removing the supernatants, since the spheroplast pellets will be relatively loose and fluffy. In addition, the emergence of CO₂ bubbles from the spheroplasts can significantly disrupt the pellets.

15. Resuspend spheroplasts at 1 to 5 OD₆₀₀ units/ml in spheroplast medium B by gently swirling tube by hand or gently stirring with a glass rod.

Spheroplasts tend to stick to each other and thus can be difficult to resuspend. To facilitate resuspension, initially resuspend spheroplasts in a smaller volume of solution before adding remaining solution.

This washing step is critical to remove contaminating proteases and any other enzymes present in the Zymolyase preparation.

16. Harvest the spheroplasts by centrifuging 5 min at $1500 \times g$, 4°C . Decant or aspirate the supernatant.
17. *Optional*: Repeat steps 15 and 16 to ensure that the Zymolyase has been completely removed from the spheroplast preparation.
18. Resuspend spheroplasts in ice-cold spheroplast medium B, stop solution, or another solution with the appropriate osmotic support. Keep the suspension on ice.

The optimal solution will depend on the goals and subsequent steps of the experiment in which the spheroplasts will be used.

Stop solution contains two energy poisons (NaN_3 and NaF) that are toxic and should be handled with care. The inclusion of NaN_3 and NaF is not appropriate for all purposes (e.g., to isolate organelles for functional studies).

EQUILIBRIUM DENSITY GRADIENT FRACTIONATION USING NYCODENZ

BASIC PROTOCOL 2

In this protocol, equilibrium Nycodenz density gradient centrifugation is used to separate vacuolar membranes from endosomal and late Golgi compartments (Rieder and Emr, 1997). The distribution of subcellular components among the resulting fractions can be analyzed using marker proteins and activities (see UNIT 3.7). The fractions can also be used to analyze the subcellular localization of proteins of interest.

Yeast cells are first converted to spheroplasts (see Support Protocol). The spheroplasts are lysed using a combination of osmotic shock and Dounce homogenization. Subsequently, the lysates are subjected to centrifugation at $300 \times g$ to remove unlysed cells and aggregates. These cleared cell lysates are loaded onto Nycodenz density gradients for overnight centrifugation.

About 15 to 20 OD_{600} units of cells in their mid-logarithmic growth phase are recommended for each gradient. If compatible with the goals of the experiment, protease-deficient strains can be used to minimize protein degradation (e.g., TVY614, a *pep4 prb1 prc1* mutant yeast strain). Since the membranes are not separated from the cytosol in this protocol, use of a protease-deficient strain is particularly advantageous to minimize damage by vacuolar hydrolases released from ruptured vacuoles.

Materials

- 20 OD_{600} units of cells in mid-logarithmic growth phase per gradient
- HEPES/potassium acetate (HEPES/KAc) lysis buffer (see recipe), ice cold
- 50% (w/v) Nycodenz/sorbitol stock solution (see recipe)
- 37%, 31%, 27%, 23%, 20%, 17%, 13%, and 9% (w/v) Nycodenz in HEPES/KAc lysis buffer (see recipe), 4°C
- 500 \times protease inhibitor cocktails A and B (500 \times PIC-A and 500 \times PIC-B; see recipes)
- Protease inhibitor stock solution A (see recipe)
- 100 mg/ml bovine serum albumin (BSA; optional)
- Stop solution (see recipe) or another appropriate buffer, ice cold
- Trimmed 1-ml pipet tips (cut ~5 to 10 mm from the tips to increase the size of the opening)
- Clear 14 \times 89-mm ultracentrifuge tubes (e.g., Ultraclear 14 \times 89-mm tubes or equivalent) and rack
- Centrifuge and microcentrifuge at 4°C
- Dounce homogenizer with a tight-fitting pestle, prechilled

Subcellular Fractionation and Isolation of Organelles

3.8.9

1.6-ml microcentrifuge tubes, prechilled
Refrigerated ultracentrifuge with swinging bucket rotor (e.g., Beckman SW41 Ti or equivalent), 4°C
Refractometer

Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and preparation of spheroplasts (Support Protocol)

Culture cells

1. On the day before the experiment, culture desired yeast strains (Support Protocol, steps 1 to 5).

About 20 OD₆₀₀ units of cells are usually needed for each gradient.

2. In the morning on the day of the experiment, dilute the cultures so that the cells will be ready to harvest in the early afternoon (see Support Protocol, step 4 for sample calculations).

Prepare gradients

3. In the morning on the day of the experiment, prepare gradients with a micropipettor and trimmed 1-ml pipet tips. Add protease inhibitors to each Nycodenz gradient solution. In a 14 × 89-mm ultracentrifuge tube carefully layer the following volumes of Nycodenz gradient media on top of one another in the following order:

1 ml 37%
1.5 ml 31%
1.5 ml 27%
1.5 ml 23%
1.5 ml 20%
1 ml 17%
1 ml 13%
1 ml 9%.

4. Store the gradients undisturbed for 2 to 3 hr at room temperature. After several hours, transfer gradients to 4°C so that they are chilled prior to use.

During the storage time, the concentration differences at the interfaces decrease through diffusion, and the gradient becomes essentially continuous.

Prepare cleared spheroplast lysates (S₃₀₀)

5. Once the cells have reached their target density, prepare spheroplasts (Support Protocol, steps 6 to 15).

Spheroplasts are fragile and must be handled very gently.

6. Resuspend spheroplasts at a concentration of 1 to 5 OD₆₀₀ units/ml in ice-cold stop solution, spheroplast medium B, or another solution with the appropriate osmotic support. Resuspend spheroplasts gently by swirling tube by hand, slowly stirring with a glass rod, or slowly pipetting with a wide bore pipet. Do not vortex. Keep suspension on ice until ready for the next step.

The optimal solution will depend on the goals and subsequent steps of the experiment in which the spheroplasts will be used.

Spheroplasts tend to stick to each other and thus can be difficult to resuspend. To facilitate resuspension, initially resuspend spheroplasts in a smaller volume of solution before adding the remaining solution.

Stop solution contains two energy poisons (NaN₃ and NaF) that are toxic and should be handled with care. The use of NaN₃ and NaF is not appropriate for all purposes (e.g., to isolate organelles for functional studies).

7. Harvest spheroplasts by centrifuging 5 min at $2000 \times g$, 4°C . Aspirate the supernatant.

From this point on, keep the samples on ice or at 4°C . Also, all solutions and equipment should be chilled to 0° to 4°C prior to as well as during use.

8. Resuspend spheroplasts at a concentration of $\sim 15 \text{ OD}_{600}$ units/ml in ice-cold HEPES/KAc lysis buffer with freshly added protease inhibitors (PIC-A, PIC-B, and protease inhibitor stock solutions A).

The spheroplasts are lysed at a higher concentration than in Basic Protocol 1 in order to minimize the load volume for step 13.

9. Transfer the spheroplast suspension to a Dounce homogenizer that has been pre-chilled in an ice bucket. Disrupt the spheroplasts with 10 up-and-down strokes of a tight-fitting pestle.

Keep Dounce homogenizer on ice throughout this process. Move the pestle slowly and maintain contact between pestle and suspension to minimize foaming.

10. Transfer lysate to multiple chilled 1.6-ml microcentrifuge tubes. Dispense convenient (~ 1.35 -ml) aliquots of lysate ($\sim 20 \text{ OD}_{600}$ cell equivalents) into each tube.

Larger centrifugation tubes can also be used; however, use of 1.6-ml microcentrifuge tubes and a refrigerated microcentrifuge is often faster and more convenient.

In order to monitor recovery levels in fractions generated by the subsequent centrifugation steps, it is often beneficial to set aside an aliquot of the lysate that will not be fractionated.

11. Clear the lysates of unbroken cells, partially disrupted cells, and aggregates by centrifuging 5 min at $300 \times g$, 4°C , in a microcentrifuge.

12. Carefully withdraw supernatants (S_{300}), without disturbing the very loose, fluffy pellet (P_{300}), and transfer them to new prechilled 1.6-ml tubes.

Usually only $\sim 80\%$ of the S_{300} can be harvested from each tube without disturbing the pellets.

In order to monitor the recovery levels in the gradient fractions, it is often beneficial to set aside a small aliquot of the S_{300} that will not be fractionated further.

The yield of intracellular membranes can be improved by "washing" the P_{300} pellet; however, this practice increases the sample volume and handling times, and is not recommended for this protocol.

Fractionate lysate (S_{300}) on Nycodenz density gradients

13. Carefully layer supernatant (S_{300}) on the previously prepared gradient (step 4) using trimmed 1-ml pipet tip. Record volume of transferred S_{300} .

14. Balance appropriate pairs of buckets, gradients, and lids by adding a small amount of ice-cold HEPES/KAc lysis buffer to lighter tubes. Place the loaded gradient into prechilled buckets of the swinging bucket rotor.

Slowly add buffer using a Pasteur pipet. Keep the tip near the surface of the gradient, when adding buffer, to minimize disruption of the gradient.

15. Centrifuge the gradients in a Beckman SW41 Ti rotor (or equivalent) 15 to 18 hr at $170,000 \times g$, 4°C , using the slow acceleration and deceleration settings to minimize disruption of the gradients.

Collect and process fractions

16. While the rotor is slowly decelerating, label 14 microcentrifuge tubes for the fractions to be collected from each gradient and place the tubes on ice.

To prepare for manual fraction collection, cut ~ 5 to 10 mm off the tips of 1-ml pipet tips (~ 15 per gradient). Alternatively, an automated fraction collector system can be assembled.

17. After centrifugation is completed, carefully remove gradients from the rotor buckets and place them in a stable rack. Note any apparent membrane aggregation or other characteristics. While processing each gradient, store remaining gradients undisturbed at 4°C.
18. Collect ~14 fractions starting at the top of the gradient, using either a micropipettor and the trimmed 1-ml pipet tips or an automated fraction collector.

If desired, 200 µg of BSA or another well-characterized protein source can be added to increase the protein concentration of the collected fractions.

19. Mix each sample well by vortexing. Centrifuge 15 sec at 13,000 × g, 4°C, to clear the sample droplets from lids. Transfer 30 to 40 µl of each fraction to new microcentrifuge tubes and set aside at room temperature (for step 21).
20. Process fractions as desired.

For example, membranes can be collected by diluting the fractions 2- to 3-fold with ice-cold HEPES/KAc lysis buffer and then subjecting the samples to centrifugation ≥60 min at 100,000 × g, 4°C. The proteins in each fraction can be precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10%, and subsequently analyzed using immunoprecipitation (UNIT 7.2) or immunoblotting (UNIT 6.2) techniques.

21. Determine Nycodenz density of each fraction by measuring refractive index of the 30- to 40-µl samples set aside in step 19.

ALTERNATE PROTOCOL

EQUILIBRIUM DENSITY GRADIENT FRACTIONATION USING SUCROSE

In this protocol, equilibrium sucrose density gradient centrifugation is used to separate vacuolar and endosomal membranes from late Golgi compartments (Becherer et al., 1996). The resulting fractions can be used to analyze the subcellular localization of proteins of interest.

Yeast cells are first converted to spheroplasts (see Support Protocol). The spheroplasts are lysed under hypoosmotic conditions with a glass Dounce homogenizer. Differential centrifugation is used to generate 13,000 × g pellet and supernatant fractions (P_{13,000} and S_{13,000}, respectively). The P_{13,000} and S_{13,000} fractions are subsequently loaded onto sucrose density gradients for overnight centrifugation.

About 15 to 25 OD₆₀₀ units of cells in their mid-logarithmic growth phase are recommended for each gradient set (P_{13,000} and S_{13,000}). If compatible with the goals of the experiment, protease-deficient strains can be used to minimize protein degradation (e.g., TVY614, a *pep4 prb1 prc1* mutant yeast strain). Since the membranes in the S_{13,000} fraction are not separated from the cytosol in this protocol, the use of a protease-deficient strain is particularly advantageous to minimize damage by vacuolar hydrolases released from ruptured vacuoles.

Additional Materials (also see Basic Protocol 2 and Support Protocol)

60% (w/v) sucrose stock solution, pH 6.8 (see recipe)
Sucrose solutions for equilibrium gradients (see recipe)
Refractometer

Prepare spheroplasts and sucrose gradients

1. Culture desired yeast strains (Support Protocol, steps 1 to 5).
2. Aliquot 37%, 34%, 32%, 29%, 27%, 22%, 10% (w/v) sucrose solutions for equilibrium gradients to separate 5-ml centrifuge tubes and add protease inhibitors just before preparing gradients.

The volumes for these aliquots will depend on the number of gradients to be prepared. Always aliquot a bit more than the amount required.

3. In the morning on the day of the fractionation experiment, prepare the sucrose gradients in Ultra-Clear 14 × 89-mm tubes. Carefully layer the following sucrose solutions on top of each other with a trimmed 1-ml pipet tip:

1 ml 60%
1 ml 37%
1.5 ml 34%
2 ml 32%
2 ml 29%
1 ml 27%
1.5 ml 22%
0.5 ml 10% sucrose.

Store, undisturbed at room temperature for 2 to 3 hr and then carefully transfer to 4°C.

4. Harvest ~20 OD₆₀₀ units of cells in their mid-logarithmic growth phase for each gradient by centrifuging 5 min at ~5000 × g, room temperature.
5. Prepare spheroplasts (Support Protocol, steps 6 to 15).

Spheroplasts are fragile and must be handled very gently.

6. Resuspend the spheroplasts at a concentration of 1 to 5 OD₆₀₀ units/ml in ice-cold stop solution, spheroplast medium B, or another solution with the appropriate osmotic support. Resuspend spheroplasts gently by swirling tube by hand, slowly stirring with a glass rod, or slowly pipetting with a wide bore pipet. Do not vortex. Keep suspension on ice until ready for the next step.

The optimal solution will depend on the goals and subsequent steps of the experiment in which the spheroplasts will be used.

Spheroplasts tend to stick to each other and thus can be difficult to resuspend. To facilitate resuspension, initially resuspend spheroplasts in a smaller volume of solution before adding the remaining solution.

Stop solution contains two energy poisons (NaN₃ and NaF) that are toxic and should be handled with care. The use of NaN₃ and NaF is not appropriate for all purposes (e.g., to isolate organelles for some functional studies).

Prepare spheroplast lysates and generate P_{13,000} and S_{13,000} fractions

7. Harvest spheroplasts by centrifuging 5 min at 2000 × g, 4°C. Aspirate the supernatant.

From this point on, keep the samples on ice or at 4°C. Also, all solutions and equipment should be chilled to 0° to 4°C prior to as well as during use.

8. Gently resuspend spheroplasts at a concentration of ~15 OD₆₀₀ units/ml in ice-cold HEPES/KAc lysis buffer with freshly added protease inhibitors.
9. Transfer spheroplast suspension to a Dounce homogenizer that has been prechilled in an ice bucket. Disrupt spheroplasts with 10 up-and-down strokes of a tight-fitting pestle.

Keep the Dounce homogenizer on ice throughout this process. Move the pestle slowly and maintain contact between pestle and suspension to minimize foaming.

10. Transfer lysate to multiple chilled 1.6-ml microcentrifuge tubes.

In order to monitor recovery levels in the fractions generated by the subsequent centrifugation steps, it is often beneficial to set aside an aliquot of the lysate that will not be fractionated.

11. Clear lysate of unbroken cells, partially disrupted cells, and aggregates by microcentrifuging 5 min at $300 \times g$, 4°C .
12. Carefully withdraw supernatant (S_{300}), without disturbing the very loose, fluffy pellets (P_{300}), and transfer to new chilled 1.6-ml microcentrifuge tube.

Usually only ~80% of S_{300} can be harvested from each tube.
13. Microcentrifuge S_{300} supernatant 10 min at $13,000 \times g$, 4°C .
14. Transfer supernatant ($S_{13,000}$) to prechilled 1.6-ml microcentrifuge tube and store on ice.

If desired, $S_{13,000}$ can be subjected to centrifugation 60 min at $100,000 \times g$, 4°C , to generate $P_{100,000}$ and cytosol $S_{100,000}$ fractions. $P_{100,000}$ membranes can then be fractionated on the density gradients. The $S_{13,000}$ fraction is used instead of the $P_{100,000}$ in this protocol, since membranes can be very difficult to resuspend after centrifugation at $100,000 \times g$.
15. To remove any residual supernatant from $P_{13,000}$ pellets and tube, centrifuge the tube containing $P_{13,000}$ sample ~45 sec at $13,000 \times g$, 4°C . Carefully remove any remaining liquid above pellet and discard.
16. Resuspend ~20 OD_{600} unit equivalents of $P_{13,000}$ membranes in ~800 μl ice-cold HEPES/KAc lysis buffer with freshly added protease inhibitors (PIC-A, PIC-B, and protease inhibitor stock solutions A). Pipet the suspension up-and-down at least 20 times to ensure complete dispersion of the $P_{13,000}$ pellet.

Fractionate $P_{13,000}$ and $S_{13,000}$ samples on sucrose density gradients

17. Carefully layer the $P_{13,000}$ and $S_{13,000}$ samples (i.e., products from steps 13 and 15) on separate previously prepared, separate sucrose gradients using trimmed 1-ml pipet tips.
18. Balance the appropriate pairs of gradients by adding a small amount of ice-cold HEPES/KAc lysis buffer to the lighter tubes. Place loaded gradient into the prechilled bucket of the swinging-bucket rotor.
19. Centrifuge the gradient in a Beckman SW41 Ti rotor (or equivalent) 15 to 18 hr at $170,000 \times g$, 4°C , using the slow acceleration and deceleration settings to minimize disruption of the gradients.

Collect and process fractions

20. While the rotor is slowly decelerating, label fourteen 1.6-ml microcentrifuge tubes for the fractions to be collected from each gradient and place tubes on ice.

To prepare for manual fraction collection, cut ~5 to 10 mm from the pointed ends of 1-ml pipet tips (~15 per gradient). Alternatively, an automated fraction collector system can be assembled.
21. After centrifugation is completed, carefully remove gradients from the rotor buckets and place in a stable rack. Note any apparent membrane aggregation or other characteristics. While processing each gradient, store the remaining gradients, undisturbed, at 4°C .
22. Collect ~14 fractions from top of gradient, using either a micropipettor and trimmed 1-ml pipet tips or an automated fraction collector.

If desired, 200 μg of BSA or another well-characterized protein source can be added to increase protein concentration of the collected fractions.

23. Mix each sample well with a vortexer. Centrifuge 15 sec at $13,000 \times g$ to clear the sample droplets from the lids. Transfer 30 to 40 μl of each fraction to new 1.6-ml microcentrifuge tube and set aside at room temperature for step 25.
24. Process remaining fractions as desired.

For example, membranes can be collected by diluting the fractions 3- to 10-fold with ice-cold HEPES/KAc lysis buffer and then subjecting samples to centrifugation ≥ 60 min at $100,000 \times g$, 4°C . The proteins in each fraction can be precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10% (v/v) and subsequently analyzed using immunoprecipitation (UNIT 7.2) or immunoblotting (UNIT 6.2) techniques.
25. Determine sucrose density of each fraction by measuring refractive index of the 30- to 40- μl samples from step 22.

FRACTIONATION OF P_{13,000} MEMBRANES ON SUCROSE STEP GRADIENTS

**BASIC
PROTOCOL 3**

This protocol was designed to analyze the distribution of one or more proteins among vacuoles, plasma membrane (PM), early Golgi, and endoplasmic reticulum (ER) membranes (Gaynor et al., 1994). Yeast cells are converted to spheroplasts with the β -glucanase Zymolyase 100T (see Support Protocol). The spheroplasts are lysed under hypoosmotic conditions with a glass Dounce homogenizer. Differential centrifugation is used to generate a $13,000 \times g$ pellet that is enriched for vacuoles, PM, early Golgi, and ER membranes (P_{13,000}). This membrane pellet is subsequently fractionated on a sucrose density step gradient.

About 30 OD₆₀₀ units of cells in their mid-logarithmic growth phase are recommended for each gradient. If compatible with the goals of the experiment, protease-deficient strains can be used to minimize protein degradation (e.g., TVY614, a *pep4 prb1 prc1* mutant yeast strain).

Materials

- Desired yeast strains (e.g., TVY614, a *pep4 prb1 prc1* mutant yeast strain)
- Stop solution or spheroplast medium B, ice cold (see recipe; or another buffer with the appropriate osmotic support)
- HEPES/potassium acetate (HEPES/KAc) lysis buffer, ice cold (see recipe)
- 500 \times protease inhibitor cocktails A and B (500 \times PIC-A and 500 \times PIC-B, see recipes)
- Protease inhibitor stock solution A (see recipe)
- 1.2 and 1.5 M sucrose gradient solutions in sucrose gradient buffer (see recipe)
- Ultra-clear 11 \times 34-mm centrifuge tubes (e.g., Beckman or equivalent)
- Benchtop refrigerated centrifuge and swinging bucket rotor, 4°C (e.g., Beckman TLS55 or equivalent)
- Dounce homogenizer with a tight-fitting pestle, prechilled
- 1.6-ml microcentrifuge tubes
- Microcentrifuge, 4°C
- Trimmed 1-ml and 200- μl pipet tips (~5 to 10 mm cut from the tips)
- Additional reagents and equipment for growing yeast cells (UNIT 1.6) and preparation of spheroplasts (Support Protocol)

Culture cells and prepare spheroplasts

1. Culture desired yeast strains (Support Protocol, steps 1 to 5).

About 30 OD₆₀₀ units of cells in their mid-logarithmic growth phase are recommended for each gradient.

**Subcellular
Fractionation and
Isolation of
Organelles**

3.8.15

2. Prepare spheroplasts (Support Protocol, steps 6 to 15).

Spheroplasts are fragile and must be handled very gently.

3. Resuspend spheroplasts in ice-cold stop solution, spheroplast medium B, or another solution with the appropriate osmotic support. Resuspend spheroplasts gently by swirling tube by hand, slowly stirring with a glass rod, or slowly pipetting with a wide bore pipet. Do not vortex. Keep suspension on ice until ready for the next step.

The optimal solution will depend on the goals and subsequent steps of the experiment in which the spheroplasts will be used.

Spheroplasts tend to stick to each other and thus can be difficult to resuspend. To facilitate resuspension, initially resuspend spheroplasts in a smaller volume of solution before adding the remaining solution.

Stop solution contains two energy poisons (NaN_3 and NaF) that are toxic and should be handled with care. The use of NaN_3 and NaF is not appropriate for all purposes (e.g., to isolate organelles for some functional studies).

Prepare lysates and generate $P_{13,000}$ membrane pellets

4. Harvest spheroplasts by centrifuging 5 min at $2000 \times g$, 4°C . Aspirate supernatant.

From this point on, keep samples on ice or at 4°C . Also, all solutions and equipment should be chilled to 0° to 4°C prior to as well as during use.

5. Gently resuspend spheroplasts at a concentration of 5 OD_{600} units/ml in ice-cold HEPES/KAc lysis buffer.

6. Transfer spheroplast suspension to a Dounce homogenizer that has been prechilled in an ice bucket. Disrupt spheroplasts with 10 up-and-down strokes with a tight-fitting pestle.

Keep Dounce homogenizer on ice throughout this process. Move pestle slowly and maintain contact between pestle and suspension to minimize foaming.

7. Transfer lysate to multiple chilled 1.6-ml microcentrifuge tubes.

To monitor recovery levels in the fractions generated by subsequent centrifugation steps, it is often beneficial to set aside an aliquot of lysate that will not be fractionated (e.g., 1 ml or 5 OD_{600} cell equivalents).

8. Clear lysates of unbroken cells, partially disrupted cells, and aggregates by centrifuging 5 min at $300 \times g$, 4°C .

9. Carefully withdraw supernatant (S_{300}), without disturbing the very loose, fluffy pellets (P_{300}), and transfer to new chilled 1.6-ml tube.

Usually only ~80% of the S_{300} can be harvested from each tube.

10. Resuspend P_{300} by pipetting up-and-down 10 times using a micropipettor and a 200- μl trimmed pipet tip. Add ice-cold HEPES/KAc lysis buffer to a total volume of 1 ml. Mix by pipetting up and down a few more times.

Alternatively, the sample can be transferred to a clean, chilled Dounce homogenizer and dispersed with several gentle strokes.

This washing step can improve the yield of intracellular membranes and the purity of the P_{300} fractions by removing some of the smaller organelles trapped within P_{300} pellets.

11. Repeat steps 8 and 9.

The P_{300} pellets not required for subsequent analyses are often discarded since they contain a crude mixture of unlysed and partially disrupted cells, as well as a very heterogeneous mixture of intracellular membranes.

12. Pool the first and second S_{300} supernatants (from step 9 and 11) and mix. Transfer the pooled supernatant (S_{300}) to prechilled 1.6-ml microcentrifuge tube and place on ice.
13. Microcentrifuge the S_{300} supernatant 10 min at $13,000 \times g$, 4°C .
14. Remove the supernatant ($S_{13,000}$) to new 1.6-ml microcentrifuge tube. Store $S_{13,000}$ on ice until the gradient fractionation of $P_{13,000}$ membranes is in progress (step 20).

Alternatively, the supernatants can be discarded.

If desired, an aliquot (e.g., 5 OD_{600} units equivalents) of the $S_{13,000}$ can then be subjected to centrifugation 60 min at $100,000 \times g$, 4°C , to generate $P_{100,000}$ and cytosol $S_{100,000}$ fractions.

15. To remove any residual supernatant from $P_{13,000}$ pellet, centrifuge tubes containing $P_{13,000}$ samples ~45 sec at $13,000 \times g$, 4°C . Carefully remove any remaining liquid above pellet and discard.
16. Resuspend ~20 OD_{600} units equivalents of $P_{13,000}$ membranes in 200 μl ice-cold HEPES/KAc buffer with freshly added protease inhibitors (PIC-A, PIC-B, and protease inhibitor stock solutions A). Pipet each individual $P_{13,000}$ suspension up-and-down at least 20 times with a micropipettor and 200- μl pipet tip to ensure complete dispersion of $P_{13,000}$ pellet. Then combine pellets and mix by pipetting up-and-down a few more times.

Prepare and centrifuge sucrose step gradients

17. Carefully pipet 800 μl of 1.5 M sucrose step gradient solution into each ultra-clear 11×34 -mm centrifuge tube. Using a trimmed 1-ml pipet tip, carefully layer 800 μl of 1.2 M sucrose step gradient solution on top of the 1.5 M sucrose layer.

Prepare the sucrose gradients just prior to their use so that the interface remains crisp.

If the interface is disturbed during or after preparation of the gradient, a new step gradient will need to be prepared.

18. Carefully layer 180 μl $P_{13,000}$ suspension (from step 16) over the 1.2 M sucrose solution with a trimmed 200- μl pipet tip.
19. Balance appropriate pairs of gradients by adding more of the $P_{13,000}$ suspension to the lighter tube if necessary. Put gradient tubes into prechilled buckets of the swinging bucket rotor.
20. Centrifuge gradients for 1 hr at $85,000 \times g$, 4°C (35,000 rpm for TLS55 rotor), using the slow acceleration and deceleration settings to minimize disruption of the gradients.

Collect and process fractions

21. While the rotor is slowly decelerating, prepare six labeled 1.6-ml microcentrifuge tubes for each gradient and keep on ice. Cut ~5 to 10 mm off the tips of 1-ml pipet tips (i.e., ~6 tips per gradient).
22. Carefully remove tubes from rotor buckets and place in a rack. Place gradients, undisturbed, at 4°C and process one gradient at a time. Make a note of position and appearance of bands and any other visible characteristics.

Two discrete membrane bands should be visible, one in the 1.2 M sucrose layer and one at the interface between the 1.2 M and 1.5 M sucrose layers.

23. Collect the six fractions listed below with a micropipettor and cut 1-ml pipet tips. Store fractions in labeled microcentrifuge tubes on ice.

Fraction 1: the top of gradient to just above the first membrane layer

Fraction 2: first membrane band within the 1.2 M sucrose layer

Fraction 3: sucrose solution between first membrane layer (Fraction 2) and second membrane layer (Fraction 4)

Fraction 4: membrane band at the 1.2 M/1.5 M sucrose interface

Fraction 5: 1.5 M sucrose solution below the interface

Fraction 6: membrane pellet.

The fractions will not have identical volumes.

The vacuolar membranes are found in fractions 1 and 2; Golgi membranes are primarily in fraction 2; and ER membranes and the plasma membrane are split between fractions 2 (~35%) and 4 (~65%).

If ER membranes and/or plasma membranes are the only features of interest, one can simply harvest the two membrane bands and discard the remaining solutions.

24. Increase the volume of each fraction to ~1.4 ml with ice-cold HEPES/KAc lysis buffer and mix.

25. Process fractions as desired.

For example, the membranes can be collected by diluting the fractions 3- to 10-fold with ice-cold HEPES/KAc lysis buffer and then subjecting samples to centrifugation ≥ 50 min at $100,000 \times g$, 4°C . The proteins in each fraction can be precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10% (v/v) and subsequently analyzed using immunoprecipitation (UNIT 7.2) or immunoblotting (UNIT 6.2) techniques.

BASIC PROTOCOL 4

ISOLATION OF INTACT VACUOLES USING FICOLL STEP GRADIENTS

This protocol was designed to purify intact, functional vacuoles from yeast spheroplasts (Haas, 1995). Yeast cells are converted to spheroplasts by incubation with oxalyticase, a β -glucanase preparation. The spheroplasts are lysed under isoosmotic conditions after treatment with the polybase DEAE-dextran. This gentle lysis procedure facilitates the isolation of intact vacuoles. Subsequently, the vacuoles are isolated through flotation on a Ficoll density step gradient.

The use of ~550 to 800 OD₆₀₀ units of cells is recommended for each gradient. If compatible with the goals of the experiment, protease-deficient strains can be used to minimize protein degradation (e.g., TVY1, a *pep4* mutant yeast strain). The protocol is optimized for the yeast strains BJ3505 (a *pep4 prb1* mutant strain, deficient for most vacuolar hydrolase activities) and DKY6281 (a *pho8* mutant strain that does not express vacuolar alkaline phosphatase but has normal vacuolar protease activity levels).

Materials

Desired yeast strain

0.2 \times YPD (UNIT 1.6)

PIPES/DTT buffer (see recipe)

Oxalyticase buffer (see recipe)

Oxalyticase (45,000 U/mg, Enzogenetics; 0.5 to 1.0 mg/gradient) or an alternate β -glucanase enzyme (e.g., Zymolyase 100T; Support Protocol)

15%, 8%, 4%, and 0% (w/v) Ficoll solutions, 4°C (see recipe)

50 \times protease inhibitor cocktail (PIC, see recipe)

0.4 mg/ml dextran solution, freshly made (see recipe)

Protein assay reagent kit (e.g., BioRad; or an alternate technique, see APPENDIX 3B)

250-ml and 2-liter Erlenmeyer flasks
 Shaker platform at 30°C (or appropriate growth temperature)
 Centrifuge and rotor, room temperature and 4°C (e.g., Beckman JA-20 and JA-10 rotors or equivalents)
 500-ml centrifuge bottles (e.g., for Beckman JA-10 rotor or equivalent)
 10-ml glass pipets
 30-ml glass Corex tubes (1 tube per gradient) or equivalent glass centrifugation tubes
 Water bath at 30°C
 Adapters for 30-ml glass Corex tubes (e.g., Beckman JA-20 rotor or equivalent)
 Polyallomer centrifuge tubes, prechilled (e.g., 14 × 89-mm for SW41 Ti rotor)
 Ultracentrifuge and rotor, 4°C (e.g., Beckman SW41 Ti rotor or equivalent)
 200- μ l pipet tips with ~5 to 8 mm trimmed off each tip
 1.6-ml microcentrifuge tubes

Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and determining protein concentration (*APPENDIX 3B*)

Grow cells

1. In the morning, the day before the experiment, start precultures of desired yeast strains by inoculating 20-ml of YPD (or appropriate medium) in 250-ml Erlenmeyer flasks. Culture cells throughout the day at 30°C (or appropriate permissive temperature) while shaking at 225 to 350 rpm.

It is best to start precultures with a single yeast colony from a fresh plate.

If using a strain that grows very slowly or if using a strain that has been stored on a plate at 4°C for more than a few days, it is wise to start the preculture with a single colony the night before.

2. In the evening, the day before the experiment, measure and record the OD_{600} of precultures.
3. Inoculate 1 liter of fresh medium with an appropriate volume of each preculture.

A 1-liter culture is required for each gradient, since 550 to 800 OD_{600} units of cells from a rapidly growing culture (e.g., OD_{600} of 0.6 to 0.8) are needed per gradient. The suitable yeast cell concentration at this inoculation step depends on the growth rate of each strain and length of the incubation time. See Support Protocol, step 3 for sample calculations.

*Example: Haas and coworkers recommend inoculating 1 liter of YPD with 0.25 OD_{600} equivalents of DKY6281 cells or 0.9 OD_{600} equivalents of BJ3505 cells when incubating for 16.5 hr. (Protease-deficient *pep4* mutant strains grow more slowly than the corresponding wild-type strains.)*

4. Culture cells overnight at 30°C (or appropriate permissive temperature) while shaking at 225 to 325 rpm.

Generate spheroplasts with oxalyticase treatment

5. In the morning, the day of the experiment, analyze cell density.

If the OD_{600} is >1.2, dilute cultures to an OD_{600} of 0.2 and allow growth to an OD_{600} of 0.6 to 0.8.

Ideally, cultures should have an OD_{600} of 0.6 to 0.8 to begin the experiment.

6. Harvest cells at an OD_{600} of 0.6 to 0.8 by centrifuging 5 min at 4000 × *g* (5000 rpm in JA-10 rotor), room temperature.

As noted earlier, ~550 to 800 OD_{600} units are needed per vacuole isolation gradient.

7. Decant the supernatant. Resuspend pellet in freshly prepared PIPES/DTT buffer at a concentration of ~ 15 OD₆₀₀ units/ml (i.e., ~ 50 ml of buffer per liter of original culture).
8. Pool or divide cell suspension for each gradient into an individual 500-ml centrifuge bottle (i.e., one bottle of cells per gradient).
9. Incubate the cells for 10 min in a 30°C water bath.
10. Harvest cells by centrifuging 5 min at $4000 \times g$ (5000 rpm in a JA-10 rotor), room temperature.
11. Decant supernatants and gently resuspend each pellet in oxalyticase buffer at a concentration of ~ 40 OD₆₀₀ units/ml (e.g., ~ 15 ml/liter of original culture) using a 10-ml glass pipet.
12. Transfer suspension from each centrifuge bottle into separate, labeled 30-ml glass Corex tubes (i.e., one glass tube per gradient).

It is important to use glass tubes, since they allow for more rapid temperature changes than plastic tubes.
13. Transfer 10 μ l from each cell suspension to a labeled microcentrifuge tube and set aside to use as controls in step 16.
14. Add oxalyticase (or an alternate β -glucanase enzyme) to a concentration of 1800 to 3600 U/ml.

Oxalyticase is often supplied at 45,000 U/mg. The amount of oxalyticase required to efficiently digest the cell wall needs to be determined empirically for each strain and growth condition.

For DKY6281 cells, 1800 U/ml of oxalyticase is usually sufficient to generate spheroplasts efficiently (i.e., ~ 0.04 mg/ml or 0.8 mg for each 1000 OD₆₀₀ units of cells). However, a higher concentration of ~ 2700 U/ml of oxalyticase is recommended for BJ3505 cells (i.e., ~ 0.06 mg/ml or 1.2 mg for each 1000 OD₆₀₀ units of cells).
15. Incubate 25 min in 30°C water bath (or appropriate permissive temperature). Prepare the fresh 0.4 mg/ml dextran solution during the oxalyticase digestion and keep on ice until ready to use.
16. To monitor the efficiency of cell wall removal, dilute 10 μ l of each cell suspension with oxalyticase into 990 μ l water and mix by briefly vortexing. Also, dilute the 10- μ l control samples set aside at step 13. After 1 or 2 min, briefly vortex again and measure OD₆₀₀ values of all samples.

The decrease in OD₆₀₀ values of diluted oxalyticase cell suspension indicates the extent of cell wall removal. In the absence of mechanical support (provided by the intact cell wall) or osmotic support (provided by sorbitol), spheroplasts will lyse and thus will no longer contribute to optical density.

The extent of cell-wall digestion can also be examined by phase-contrast microscopy (Support Protocol, step 11).
17. Continue to incubate at 30°C (or appropriate permissive temperature) until $\geq 95\%$ of cells have been converted to spheroplasts.

The total incubation time for preparation of spheroplasts should be ≤ 60 min.
18. Place 30-ml glass Corex tubes in a JA-20 rotor with appropriate adapters. Harvest spheroplasts by centrifuging 1 min at $800 \times g$ (2500 rpm in a JA-20 rotor), 4°C, followed by centrifugation for 1 min at $1500 \times g$ (3500 rpm in a JA-20 rotor), 4°C.

Spheroplasts are fragile and must be handled very gently.

19. *Optional:* Wash spheroplasts by gently resuspending each pellet in ~15 ml oxalate buffer. Resuspend by gently swirling tube by hand or by gently stirring with a glass rod. Do not vortex. Harvest spheroplasts as in step 18.

Perform dextran-mediated spheroplast lysis

20. Carefully decant supernatant and place tube on ice. Add 2.5 ml 15% Ficoll solution, 4°C, to each glass tube. Resuspend spheroplasts by gently swirling tube by hand or by gently stirring with a glass rod. Do not vortex.
21. Add 100 to 200 μ l freshly made 0.4 mg/ml dextran solution to each tube. Immediately mix by gently swirling tubes by hand or by gently stirring with a glass rod. Do not vortex.

The optimal amount of dextran for each strain needs to be determined empirically. (For DKY6281 strains, 100 μ l per tube is recommended, while 180 to 200 μ l per tube is recommended for BJ3505.)

22. Place tube on ice for 2 min to allow dextran to bind to the plasma membrane.
23. Disrupt the cell membrane by placing tube in a 30°C water bath for 60 to 75 sec, then immediately place tube on ice.
24. Examine cell lysates by phase contrast microscopy. Do not repeat the heat-shock step.

Haas (1995) has found that lysates with one free-floating vacuole per 5 to 10 unlysed cells seem to yield the most vacuoles.

Prepare Ficoll step gradient

25. Transfer cell lysate from the glass tube to prechilled 14 \times 89-mm polyallomer centrifuge tube and keep on ice.
26. Carefully layer 3 ml 8% Ficoll solution and 3.5 ml 4% Ficoll solution over cell lysate. Fill tube with 0% Ficoll solution (i.e., up to ~5 mm from top of tube).
27. Balance tubes using 0% Ficoll solution and place in prechilled ultracentrifuge rotor buckets (e.g., Beckman SW41 Ti).
28. Centrifuge 90 min at 110,000 \times g (30,000 rpm for SW41 Ti rotor), 4°C. Use the slow acceleration and deceleration rate settings.

To minimize mixing of the layers, allow the rotor to coast to a stop after slowing to 1000 rpm.

Collect and assess fractions

29. First, carefully remove top layer consisting of lipid with trimmed 200- μ l tip and place in a microcentrifuge tube on ice (or discard).

Remember to change the tip after each pipetting step.

The vacuoles primarily accumulate in the interface between the 4% Ficoll and 0% Ficoll layers.

30. Carefully remove the vacuolar fraction from the 0% Ficoll/4% Ficoll interface using trimmed 200- μ l tip and place in a microcentrifuge tube on ice.

Avoid harvesting any of the 4% Ficoll layer, since Ficoll can interfere with subsequent steps. Collect a maximum of ~600 μ l to avoid overdiluting the sample and to minimize Ficoll contamination.

31. Check for organelle integrity and purity by phase-contrast microscopy. Carefully mix 2 μ l vacuole preparation with 10 μ l 15% Ficoll solution for viewing.

The vacuoles will appear as bright disks on a darker background, while lipid bodies that often associate with vacuolar membranes will appear as smaller dark spheres.

32. Determine protein concentration using 10 μ l of each vacuole preparation and the BioRad protein assay reagent kit (or an alternate technique, see *APPENDIX 3B*).

Each sample of ~600 μ l should have a protein concentration of ~0.25 to 0.9 mg/ml.

Vacuoles that will be used for functional assays should not be stored on ice for more than a few hours. Vacuoles remain functional in in vitro vacuole-vacuole fusion assays for up to several weeks if stored at -80°C as described in Haas (1995).

ISOLATION OF INTACT NUCLEI WITH FICOLL STEP GRADIENTS

This protocol was designed to isolate intact nuclei from yeast spheroplasts (Dove et al., 1998). Yeast cells are converted to spheroplasts by incubation with two β -glucanase preparations, Glusulase and Zymolyase 100T. The spheroplasts are lysed under hypoosmotic conditions. The lysate is cleared of unbroken cells and aggregates by centrifugation. Subsequently, the nuclei are isolated by fractionation on two sequential Ficoll step gradients.

The protocol is written for the preparation of nuclei from 6 liters of yeast culture in the exponential growth phase (i.e., $\sim 3 \times 10^{11}$ cells), but can be scaled up or down as needed. It has been optimized for the haploid strain BJ2168, which is deficient for vacuolar protease activity. BJ5465, another protease-deficient strain, also yields good results. Other strains can also be used; however, the yields will vary from strain to strain, and the protocol may need to be optimized (Aris and Blobel, 1991; Dove et al., 1998). If the nuclei will be used for functional studies, a recovery incubation after spheroplast preparation is needed for optimal activity.

Materials

Desired yeast strain
YPD medium (*UNIT 1.6*; or appropriate growth medium)
Sterile H_2O , ice cold
Pretreatment buffer (see recipe)
1.1 M sorbitol solution (see recipe), ice cold
Glusulase (NEN Life Science Products)
10 mg/ml Zymolyase 100 T (or 100,000 U/g yeast lytic enzyme can also be used; ICN)
Spheroplast recovery medium (optional; see recipe)
1000 \times protease inhibitor cocktail-D (1000 \times PIC-D; see recipe)
1000 \times protease inhibitor cocktail-W (1000 \times PIC-W; see recipe)
Ficoll cushion solution (see recipe), 4°C
20%, 30%, 40%, and 50% (w/v) Ficoll lysis solutions with 1 \times PIC (see recipe)
1 \times and 2 \times PM buffer (see recipe), 4°C
PSM 1 solution (see recipe)
TE/SDS solution (see recipe; optional)
Shaker platform in incubator at 30°C (or appropriate permissive temperature)
250-ml and 2-liter Erlenmeyer flasks
250-ml centrifuge bottles
Hemocytometer
Sorvall GSA rotor or equivalent; prechill to 4°C
1.6-ml microcentrifuge tubes
 30°C water bath
50-ml polycarbonate centrifuge tubes (e.g., Oakridge or equivalent)
Swinging bucket rotor (e.g., Sorvall HB-4 rotor or equivalent), prechill to 4°C

Homogenizer (e.g., Potter-Elvehjem or a 40-ml glass Dounce homogenizer with a loose-fitting pestle), prechilled
Beckman Ultra-Clear 25 × 89-mm centrifuge tubes (or equivalent), prechill to 4°C
Beckman SW28 rotor (or equivalent), 2°C
20-ml syringes with 16-G needles
Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and determining protein concentration (*APPENDIX 3B*)

Grow cells

1. In the morning, 2 days before the experiment, start precultures of desired yeast strains by inoculating 5 ml YPD (or appropriate medium) with a single yeast colony from a fresh plate.
2. Culture cells throughout the day at 30°C (or appropriate permissive temperature) while shaking at 225 to 350 rpm.
3. In the evening, measure and record the OD₆₀₀ of precultures. Use 5-ml preculture to inoculate 20 ml of growth medium in 250-ml Erlenmeyer flasks to yield a culture with an ~1 OD₆₀₀ unit by the following morning.

The suitable yeast cell concentration at this inoculation step depends on the growth rate of each strain and length of incubation time. See Support Protocol, step 3 for sample calculations.

The doubling time of BJ2168 and BJ5465 in YPD at 30°C is ~110 min.

4. Culture cells overnight at 30°C (or appropriate permissive temperature) while shaking at 225 to 350 rpm.
5. In the morning, the day before the experiment, measure and record the OD₆₀₀ of precultures. Use the 20-ml precultures to inoculate 50 ml of growth medium in 250-ml Erlenmeyer flasks so that the culture will reach an OD₆₀₀ of 0.5 to 1.0 by the late afternoon.
6. Measure OD₆₀₀ of the culture in the evening. Prewarm six 1-liter volumes of YPD to 30°C. Add appropriate volume of 50-ml preculture to each of six 1-liter volumes of YPD so that the cultures will reach an OD₆₀₀ of 0.6 to 0.7 by morning. Culture cells overnight at 30°C while shaking at 225 to 350 rpm.

Harvest cells

7. Measure OD₆₀₀ of 1-liter cultures in the morning of the experiment. Harvest cells when cultures reach an OD₆₀₀ of 0.6 to 0.7.

If cultures exceed an OD₆₀₀ of 0.8, dilute cultures to ~0.15 and continue growth until cultures reach an OD₆₀₀ of 0.6 to 0.7.

8. Chill cells by placing flasks in an ice-water bath with occasional swirling.
9. Transfer cells to 250-ml centrifuge bottles and harvest by centrifuging 5 min at ~4000 × g (Sorvall GSA rotor at 5000 rpm), 4°C.

If a Pellicon (Millipore) or similar cell concentrator is available, it is convenient to concentrate cells before centrifugation.

10. Decant supernatant and place tubes on ice. Resuspend cell pellets in ice-cold water to a total of 200 ml.

11. Transfer cell suspension to a single, preweighed 250-ml centrifuge bottle. Centrifuge for 5 min at $\sim 4000 \times g$ (Sorvall GSA rotor at 5000 rpm), 4°C.
12. Decant supernatant and aspirate remaining supernatant. Determine wet weight of cell pellet.

The cell pellet usually weighs ~9 g.

Pretreat cells

13. Resuspend pellet in freshly-prepared pretreatment buffer, using 4 ml for every gram of wet cell pellet (e.g., add 36 ml pretreatment buffer to a 9-g cell pellet). Incubate 10 min at room temperature with intermittent swirling.
14. Add ice-cold water to a final volume of 250 ml and mix well. Transfer 10 μ l to a microcentrifuge tube and store on ice for step 16.
15. Centrifuge 5 min at $\sim 4000 \times g$ (Sorvall GSA rotor at 5000 rpm), 4°C.
16. During centrifugation, determine the cell yield (i.e., cell number) using a hemacytometer. Dilute the 10- μ l sample in 990 μ l water to facilitate counting.

BJ2168 typically yields 1.6×10^{10} cells/g. The number of cells per gram varies with different strains, but is usually between 1.2 and 1.7×10^{10} cells/g.

17. Resuspend cells in ~ 200 ml ice-cold 1.1 M sorbitol solution. Centrifuge 5 min at $\sim 4000 \times g$ (Sorvall GSA rotor at 5000 rpm), 4°C.
18. Decant supernatant and aspirate remaining supernatant. For every 1×10^{11} cells, add 25 ml 1.1 M sorbitol solution (e.g., if there are 3×10^{11} cells, add 75 ml sorbitol solution). Disperse well by pipetting.
19. Remove 100 μ l of cell suspension and transfer to a microcentrifuge tube. Store on ice until step 22.

This sample will be used as a control when monitoring cell wall integrity.

Prepare spheroplasts

20. To digest cell wall, add 1.5 ml Glusulase (at concentration supplied by manufacturer) and 0.3 ml of 10 mg/ml Zymolyase 100T for every 1×10^{11} cells (e.g., if there are 3×10^{11} cells in 75 ml of sorbitol solution, add 4.5 ml Glusulase and 0.3 ml 10 mg/ml Zymolyase 100T). Mix cell suspension with a glass rod and loosely cap the bottle.
21. Incubate 1.5 to 2.5 hr in a 30°C water bath with gentle swirling. Monitor the digestion microscopically (under 400 \times magnification) every 15 to 20 min. Continue digesting until cells are round and clumped cells separate.

After ~1 hr of digestion, cells should be spherical and show extensive clumping. After further digestion, the spheroplasts will separate.

22. To monitor the efficiency of cell wall removal, dilute 10 μ l of each cell suspension into 990 μ l water and mix by briefly vortexing. Also dilute the 9.4- μ l samples stored at step 19 to serve as controls. After 1 or 2 min, briefly vortex again and measure the OD₆₀₀ values.

The decrease in the OD₆₀₀ values of the digested cell suspensions indicate the extent of cell wall removal. In the absence of mechanical support (provided by the intact cell wall) or osmotic support (provided by sorbitol), spheroplasts will lyse and thus will no longer contribute to the optical density.

Wash spheroplasts with sorbitol

23. During cell-wall digestion and subsequent steps, prepare first set of Ficoll step gradients as time permits (see step 39).
24. *Optional:* If the nuclei will be used for functional studies, a recovery incubation is needed for optimal activity. Incubate spheroplasts in prewarmed spheroplast recovery medium for 30 min at 30°C with gentle swirling.

Other growth media supplemented with sorbitol at a final concentration of 1 to 1.5 M can be used as well.

Optimum recovery conditions should be determined empirically for each application.

Chill and wash spheroplasts

25. Divide spheroplast suspension into 50-ml polycarbonate centrifuge tubes adding 1×10^{11} cells per tube. Store on ice for 10 min to chill the cells.
26. Centrifuge in a swinging-bucket rotor for 5 min at $\sim 4000 \times g$ (e.g., Sorvall HB-4 rotor at 5000 rpm), 4°C. Aspirate supernatants.
27. Gently resuspend each pellet with ~ 25 ml ice-cold 1.1 M sorbitol solution. Use a glass rod to carefully resuspend pellet. Do not vortex.
28. Centrifuge in a swinging-bucket rotor 5 min at $\sim 4000 \times g$ (e.g., Sorvall HB-4 rotor at 5000 rpm), 4°C. Aspirate supernatant.
29. Resuspend each pellet with ~ 20 ml ice-cold 1.1 M sorbitol solution. Use a glass rod to carefully resuspend pellet. Do not vortex.

Prepare cushion solution and filter cells

30. Add 20 μ l of each 1000 \times PIC-W and PIC-D to a 6-ml aliquot of Ficoll cushion solution and vortex. Carefully load 6 ml Ficoll cushion solution under spheroplast suspension using a Pasteur pipet or a syringe with a long needle or canula. Repeat for each tube.

To load the Ficoll cushion solution, slowly move the pipet, needle, or canula through the lysate until the tip is at the bottom of the tube. Slowly expel 6 ml of the Ficoll cushion solution. Carefully remove the pipet, needle, or canula by lifting straight up.

31. Centrifuge spheroplasts through cushion solution 10 min at $\sim 4000 \times g$ (e.g., Sorvall HB-4 rotor at 5000 rpm), 4°C. Aspirate supernatants and store wet pellets on ice.

Isolate enriched nuclear fraction

32. To lyse spheroplasts, add 25 ml of 20% Ficoll solution (room temperature) to each pellet. Quickly disperse pellet with a 25-ml pipet.

For optimal lysis, it is important that the Ficoll solution is at room temperature, not 4°C.

33. Immediately transfer suspension to a prechilled motorized Potter-Elvehjem homogenizer. Lyse cells with 5 strokes and a fast-spinning pestle using a motorized drive.

Alternatively, cells can be lysed in a prechilled 40-ml Dounce homogenizer. Use 20 slow strokes with a loose-fitting pestle (3 to 4 min total).

34. Immediately transfer lysate to a 50-ml polycarbonate centrifuge tube that is submerged to the neck in ice.

Work quickly so that each pellet is processed within 3 to 5 min.

35. Chill lysates on ice for 10 to 15 min. Examine lysates by microscopy (400× magnification).

The spheroplasts should be completely disrupted. The nuclei will be relatively small, round, and dark. The edges may look slightly ruffled and the nucleolus may appear as a dark crescent. Released vacuoles will look larger and refractile.

36. Centrifuge lysate in a precooled swinging-bucket rotor for 5 min at 13,000 × g (e.g., Sorvall HB-4 rotor at 9000 rpm), 2°C.

37. Carefully transfer supernatant into new, prechilled 50-ml polycarbonate centrifuge tubes without transferring any of the loose pellet. Centrifuge supernatant 10 min at 13,000 × g (e.g., 9000 rpm in a Sorvall HB-4 rotor), 2°C.

After centrifugation, a tight pellet should be visible.

38. Do not transfer supernatant; place tube on ice until step 40.

Prepare Ficoll step gradients

39. Prepare the first set of Ficoll step gradients as time permits during previous steps. Warm the 50%, 40%, and 30% Ficoll solutions to ~25°C. Transfer ~20 ml of each solution into a separate 50-ml tube. Add PIC-W and PIC-D solutions and vortex.

40. Place a 25 × 89-mm ultracentrifuge tube and support base on a balance. Tare. Slowly pipet 6.5 g of 50% Ficoll solution to the bottom of the tube. Place the first tube on ice and repeat for the remaining tubes.

It is important to chill each Ficoll layer for a few minutes before adding another layer. At lower temperatures Ficoll increases in viscosity and it becomes less likely that addition of the next layer will disrupt the layer below.

41. Add 6.5 g of 40% Ficoll solution to each tube as in step 40. Chill tubes on ice.

42. Add 6.5 g of 30% Ficoll solution to each tube as in step 40. Chill gradients >10 min on ice before use.

One gradient is needed for every 1×10^{11} cells. Typically, three gradients are needed for a 6-liter preparation.

Ficoll solutions are very viscous so it is more accurate to aliquot by weight.

Purify and collect nuclei

43. Carefully layer ~25 ml supernatant from each tube (from step 38) onto each of the chilled Ficoll gradients. Do not transfer any of the pellet.

44. Fill and balance tubes with cold 1× PM buffer as necessary. Centrifuge the gradients 60 min at 58,400 × g (18,000 rpm in a Beckman SW28 rotor), 2°C.

45. During the last 30 min of centrifugation, prepare second set of Ficoll step gradients as in steps 39 to 42, except use 5.5 g of Ficoll solution. Chill on ice for ≥10 min before using gradients.

Typically, two gradients are needed for a 6-liter preparation.

46. After centrifugation (step 44), inspect gradients and note any visible characteristics.

Bands containing nuclei are usually visible at the 30%/40% and 40%/50% Ficoll interfaces. (Nuclei are also found throughout the 40% Ficoll layer.) A layer of white film should be visible at the top of the gradient. A thick layer should be visible at the 20%/30% interface. A small, halo-like pellet should occur at the bottom.

47. Collect nuclei with a 20-ml syringe and a 16-G needle. Insert needle through tube wall just below the 40%/50% Ficoll interface. With needle tip in the center of tube and the beveled edge facing up, carefully remove ~8 ml while slowly moving needle back and forth.

Cold Ficoll solutions are quite viscous, so collection should be done smoothly and slowly. The 8-ml volume will include the 40% Ficoll layer and some of the 50% Ficoll layer. Avoid the 30% Ficoll layer.

If an enriched (but less pure) preparation of nuclei is sufficient for the purposes of the experiment, one can collect ~6 ml from each gradient and omit the second Ficoll gradient. However, this is not recommended when purity is of importance.

48. Remove needle and slowly expel harvested nuclear suspension into a 150-ml glass beaker chilled on ice.
49. Repeat steps 46 to 48 for each gradient and pool harvested nuclei in the 150-ml beaker.
50. Dilute suspension with 1 volume of cold 1× PM buffer by gently stirring with a glass rod.
51. Carefully layer harvested nuclear suspension over two prechilled second set Ficoll step gradients (prepared in step 45). Fill and balance tubes with cold 1× PM buffer plus PIC as necessary.
52. Centrifuge gradients in a 60 min at $58,400 \times g$ (18,000 rpm in a Beckman SW28 rotor), 2°C.
53. After centrifugation, collect ~10 ml from each gradient as described in step 47.
54. Pool harvested nuclei in a 50-ml tube on ice. Mix by stirring gently with a glass rod.
55. Transfer 50 μ l nuclear preparation to a chilled microcentrifuge tube for protein determination. Store on ice until step 57.

If the nuclei are not needed right away, aliquot harvested nuclei while still in Ficoll solution, freeze in liquid nitrogen, and store at -70°C.

56. To remove Ficoll solution, dilute nuclei in 10 vol cold 1× PM buffer plus PIC. Mix by gently stirring with a glass rod. Pellet nuclei by centrifuging 10 min at $10,000 \times g$, 4°C. Process nuclei as desired.

For most purposes (e.g., TCA protein precipitation, SDS-PAGE analysis, functional assays), the Ficoll will need to be removed from harvested nuclei.

Determine protein concentration

57. To determine protein concentration, dilute 50 μ l of harvested nuclei in 10 volumes PSM 1 solution and mix well. Centrifuge 30 min at $12,000 \times g$, 4°C. Resuspend pellet in 50 μ l of water or TE/SDS solution. Determine protein concentration using a modified Lowry procedure or Bradford assay (e.g., APPENDIX 3B).

ISOLATION OF LACTATE-INDUCED MITOCHONDRIA USING NYCODENZ STEP GRADIENTS

This protocol is designed to isolate intact, functional mitochondria from lactate-grown D273-10B spheroplasts (Glick and Pon, 1995). Yeast cells are precultured in a glucose medium for a day and then transferred to a lactate medium to encourage optimal development of mitochondria. After ~15 hr in lactate medium, the cells are converted to spheroplasts using the β -glucanase preparation Zymolyase 20T and lysed by osmotic shock. Differential centrifugation is used to generate a mitochondria-enriched pellet. This

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pellet is subsequently fractionated on a Nycodenz density step gradient to purify the mitochondria.

This protocol calls for four 1-liter cultures of lactate-grown D273-10B cells, but can be scaled-up or scaled-down as needed. Cells other than lactate-grown D273-10B cells can be used; however, the mitochondrial density and abundance will vary with strain and growth conditions, and thus the protocol may need to be optimized. If the mitochondria will be used for functional studies, a recovery incubation after spheroplast preparation may be needed for optimal activity.

Materials

- D273-10B cells (e.g., or desired yeast strain)
- Semi-synthetic lactate medium (see recipe)
- TSD reduction buffer (see recipe)
- Zymolyase 20T (e.g., ~75 mg)
- Buffer A: 1.2 M sorbitol/20 mM potassium phosphate, pH 7.4 (see *APPENDIX 2A* for phosphate buffer), room temperature
- Semi-synthetic lactate medium (see recipe) supplemented with 1.2 M sorbitol (optional)
- 200 mM phenylmethylsulfonyl fluoride (PMSF; 34.5 mg/ml) in absolute ethanol; prepare fresh
- Buffer B: 0.6 M sorbitol/20 mM potassium MES (pH 6.0), ice cold
- 0.6% (w/v) SDS solution
- 2× Buffer B (see recipe)
- Buffer C: 0.6 M sorbitol/20 mM potassium HEPES (pH 7.4), ice cold
- 18% and 14.5 % (w/v) Nycodenz solution (see recipe), prechilled
- 100 mg/ml fatty-acid free bovine serum albumin (BSA)
- 200-ml Erlenmeyer flask
- Platform shaker, 30°C (or permissive growth temperature)
- Sorvall GS-3 rotor (or equivalent)
- 250-ml centrifuge bottles (e.g., Sorvall GS-3 rotor or equivalent)
- 30°C water bath
- 1.6-ml microcentrifuge tubes
- 40- and 1-ml glass Dounce homogenizers with a tight-fitting pestles (or Teflon homogenizer), 4°C
- 40-ml centrifuge tubes (e.g., for Sorvall SS-34 rotor or equivalent)
- Sorvall SS-34 rotor (or equivalent)
- Clear ultracentrifuge tubes (e.g., Beckman SW-41 14 × 89-mm Ultra-Clear centrifuge tubes)
- Beckman ultracentrifuge with a SW-41 rotor (or equivalent)
- Cut 1-ml pipet tips
- Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and determining protein concentration (*APPENDIX 3B*)

Grow D273-10B cells and induce mitochondrial development

1. In the evening, 2 days before fractionation experiment, start a preculture of D273-10B cells (or desired yeast strain) by inoculating 40 ml semi-synthetic lactate medium in a 200-ml Erlenmeyer flask.

It is best to use a single colony from a fresh plate.

2. Culture cells for ~24 hr at 30°C while shaking at 225 to 325 rpm.

Good aeration is critical for optimal cell growth and proliferation of mitochondria.

3. In the evening of the day before fractionation experiment, use 40-ml stationary-phase preculture to inoculate 4 liters semi-synthetic lactate medium (e.g., use 10 ml preculture per 1-liter culture).
4. Culture cells at 30°C overnight with good aeration until OD₆₀₀ value is ~3.

For strain D273-10B, cells should be grown for ~15 hr.

Harvest cells

5. In the morning, on the day of the experiment, check OD₆₀₀ value of cultures. Allow cells to grow until OD₆₀₀ reaches ~3.
6. Harvest cells by centrifuging 5 min at 4000 × g (5000 rpm in a Sorvall GS-3 rotor), room temperature, and removing the supernatant.
7. Resuspend cells in a total volume of 120 ml sterile water. Transfer suspension to a preweighed 250-ml centrifuge bottle.
8. Pellet cells by centrifuging 5 min at 2000 × g (3500 rpm in a Sorvall GS-3 rotor), room temperature.
9. Decant supernatant and weigh the wet pellet. Determine the wet weight of pellet by subtracting bottle weight.

Four liters of a D273-10B culture at an OD₆₀₀ of 3.0 usually yields ~30 g of packed cells.

Prepare spheroplasts

10. Resuspend cells in 40 ml freshly prepared reduction buffer.
11. Incubate cell suspension for 15 min in a 30°C water bath with gentle shaking. During this incubation, weigh out the required amount of Zymolyase 20T to add 2.5 mg Zymolyase 20 T per gram of packed cells.

Alternatively, 0.5 mg Zymolyase 100T per gram of packed cells can be used.

12. Pellet cells by centrifuging 5 min at 2000 × g (3500 rpm in a Sorvall GS-3 rotor), room temperature. Remove supernatant.
 13. Gently resuspend pellet in buffer A, using 2 ml buffer A per gram of packed cells (e.g., 60 ml buffer A for a 30-g cell pellet).
 14. Remove 10 μl suspension and store in a 1.6-ml microcentrifuge tube on ice until step 18.
 15. Pellet cells by centrifuging 5 min at 2000 × g, room temperature. During centrifugation, dissolve weighed Zymolyase 20T in buffer A at a concentration of 1.25 mg/ml.
- If using Zymolyase 100T, dissolve it in buffer A at a concentration of 0.25 mg/ml.*
16. Decant supernatant and resuspend pellet in Zymolyase solution.
 17. Incubate suspension for 30 min in a 30°C water bath with gentle shaking.
 18. After 30 min, monitor the efficiency of cell wall removal. Dilute 10 μl of each cell suspension into 990 μl water and mix by briefly vortexing. Also dilute the 10-μl samples stored after step 14 to serve as controls. After 1 or 2 min, briefly vortex again and measure OD₆₀₀ values.

The decrease in OD₆₀₀ values of diluted cell suspension indicates the extent of cell wall removal. In the absence of mechanical support (provided by the intact cell wall) or osmotic support (provided by sorbitol), spheroplasts will lyse and thus will no longer contribute to the optical density.

The extent of cell-wall digestion can also be examined by phase-contrast microscopy as described in Support Protocol, step 11.

19. Continue incubation until >95% of cells have been converted to spheroplasts.

Wash spheroplasts

20. Centrifuge spheroplasts 5 min at $2000 \times g$ (3500 rpm in a Sorvall GS-3), 4°C. Remove supernatant.
21. Gently resuspend spheroplast pellet in 30 ml buffer A. Pellet spheroplasts by centrifuging 5 min at $2000 \times g$ (Sorvall GS-3 rotor, 3500 rpm), 4°C.
22. Wash the spheroplasts again by repeating step 21.
23. *Optional:* Incubate washed spheroplasts for 30 to 60 min at 30°C in semi-synthetic lactate medium supplemented with 1.2 M sorbitol (final concentration). Shake gently. Pellet spheroplasts (step 21).

Optimal recovery conditions should be determined empirically for each application.

Other growth media supplemented with sorbitol to a final concentration of 1 to 1.5 M can be used.

A recovery period after cell wall removal is essential for optimal translation/transcription activity.

24. Prepare buffer B by adding 0.5 ml of 200 mM PMSF drop-wise to 200 ml of ice-cold buffer B while stirring. Filter through Whatman paper into a flask on ice.

It is important to keep the suspensions, solutions, and equipment at 0° to 4°C from this step on.

Prepare spheroplast lysates

25. Resuspend spheroplasts in 40 ml ice-cold buffer B with PMSF. Divide suspension into two equal portions and keep on ice.
26. Homogenize each portion in an ice-cold 40-ml glass Dounce homogenizer, using 15 strokes with a tight-fitting pestle.

Keep Dounce homogenizer on ice throughout this process. Move pestle slowly and maintain contact between pestle and suspension to minimize foaming.

27. Combine lysates in a prechilled beaker on ice. Add buffer B with PMSF to a total volume of 100 ml.

Collect mitochondria

28. Transfer lysate to four 40-ml centrifuge tubes. Centrifuge 5 min at $1500 \times g$ (3500 rpm in a Sorvall SS-34 rotor), 4°C.
29. Carefully transfer supernatant to a prechilled beaker on ice, being careful to avoid the loose pellet. Store supernatants on ice until step 33.
30. Resuspend each pellet in 10 ml buffer B with PMSF. Combine suspensions in a single tube, swirl to mix, and then divide into two 20-ml portions.
31. Homogenize each 20-ml suspension in the 40-ml glass Dounce homogenizer, using 15 strokes with a tight-fitting pestle, without introducing air.
32. Combine lysates in a prechilled beaker on ice. Add buffer B with PMSF to a total volume of 100 ml.
33. Transfer lysate to four new 40-ml centrifuge tubes. Centrifuge 5 min at $1500 \times g$, 4°C.

34. Transfer supernatant to the beaker containing the stored supernatants (step 28), being careful to avoid the loose pellets. Discard pellets.

Isolate enriched mitochondrial fraction

35. Divide supernatants between six 40-ml centrifuge tubes. Centrifuge 10 min at $12,000 \times g$ (10,000 rpm in Sorvall SS-34 rotor), 4°C .
36. Decant supernatants and resuspend each pellet in 7 ml ice-cold buffer B (without PMSF) and combine into two tubes.
37. Homogenize each suspension (gently, but thoroughly) in a clean, prechilled 40-ml glass Dounce homogenizer, using 5 to 10 slow strokes with a tight-fitting pestle.
38. Transfer suspensions to two clean 40-ml tubes. Centrifuge 5 min at $1500 \times g$, 4°C .
39. Transfer supernatants into two fresh 40-ml centrifuge tubes, avoiding the loose pellets. Centrifuge 10 min at $12,000 \times g$ (10,000 rpm in a Sorvall SS-34 rotor), 4°C .
40. Decant supernatant and gently resuspend pellets in 0.5 ml ice-cold buffer B (without PMSF) using a prechilled 1-ml glass Dounce homogenizer or Teflon homogenizer. Transfer suspensions to 1.6-ml microcentrifuge tubes and store on ice.

These suspensions are crude preparations that are enriched for mitochondria.

The mitochondria prepared from lactate-grown D273-10B cells usually have a dark brown color but may also look orange or pink.

41. Estimate protein concentration by measuring A_{280} of 10 μl of crude mitochondrial suspension diluted in 990 μl of 0.6% SDS. Mix 10 μl buffer B with 990 μl of 0.6% SDS to use as a reference standard.

An A_{280} value of 0.21 corresponds to ~ 10 mg/ml protein in the undiluted suspension. A 4-liter culture typically yields 120 mg of crude mitochondrial protein.

If no further purification is desired, dilute the crude mitochondrial suspension to 15 ml with ice-cold buffer C. Centrifuge 10 min at $12,000 \times g$, 4°C . Gently resuspend crude mitochondrial pellet to the desired concentration in ice-cold buffer C.

Purify mitochondria

42. Centrifuge crude mitochondrial suspension generated in step 40 for 5 min at $3000 \times g$, 4°C , in a microcentrifuge, to remove aggregated material. Transfer supernatant to new microcentrifuge tube and store on ice.
43. Prepare two Nycodenz step gradients just before use. Transfer 5 ml prechilled 18% Nycodenz solution to each clear ultracentrifuge tube. Carefully add 5 ml prechilled 14.5% Nycodenz solution, taking care to obtain a sharp interface. Keep gradients on ice.

Two Nycodenz gradients are usually sufficient for purifying mitochondria obtained from 4 liters of cells. For optimal separation, the amount of material loaded on each 10-ml gradient should not exceed 50 to 75 mg protein (as measured in step 41).

44. Gently layer ~ 1 ml crude mitochondrial preparation from step 42 onto each gradient.
45. Ultracentrifuge 30 min at $284,000 \times g$ (40,000 rpm in Beckman SW-41 rotor), 2°C , using the slow acceleration and deceleration settings.
46. After centrifugation is completed, carefully remove gradient from the rotor buckets and place them in a stable rack. Note any apparent membrane aggregations and other characteristics. While processing each gradient, store remaining gradients undisturbed at 4°C .

The purified mitochondria should collect at the 18%/14.5% Nycodenz interface and form a brown band. The mitochondrial layer may also appear to be orange or pink.

Collect mitochondria

47. First remove buffer layer and most of the 14.5% Nycodenz layer using cut 1-ml pipet tips. Harvest mitochondrial layer with a cut 1-ml pipet tip and transfer to a 50-ml tube on ice.

Change pipet tips with each pipetting step to minimize contamination.

Alternatively, the mitochondrial layer can be recovered using a syringe with a 19-G needle. Place a piece of clear tape on each tube at the 18%/14.5% Nycodenz interface and puncture the side of each tube to collect mitochondria.

48. Pool harvested mitochondria and dilute to 15 ml with buffer C. Centrifuge 10 min at $12,000 \times g$, 4°C .
49. Discard supernatant and gently resuspend pellet in 0.5 ml ice-cold buffer C (0.25 ml buffer C per Nycodenz gradient), using a glass Dounce or Teflon homogenizer to resuspend mitochondria completely. Store suspension in a microcentrifuge tube on ice.

Determine protein concentration

50. To determine approximate protein concentration, dilute 10 μl purified mitochondrial suspension from step 49 in 0.5 ml buffer C. Centrifuge 3 min at $12,000 \times g$, 4°C .

This centrifugation step is required to remove any residual Nycodenz from the mitochondrial suspension, since Nycodenz absorbs ultraviolet light and will distort the A_{280} measurements.

51. Discard supernatant, resuspend pellet in 1.0 ml of 0.6% SDS, and measure the A_{280} .

For gradient-purified mitochondria, an A_{280} value of 0.12 corresponds to a protein concentration of ~ 10 mg/ml in the undiluted suspension.

52. To store mitochondria, dilute mitochondria with a combination of cold buffer C and cold buffer C plus BSA to yield final concentrations of 25 mg/ml mitochondrial protein and 10 mg/ml BSA. Aliquot, freeze in liquid nitrogen, and store at -70°C or -80°C . Just before use, rapidly thaw the mitochondrial suspension in a 30°C water bath and immediately place on ice.

Alternatively, mitochondria can be frozen in a DMSO-mannitol solution as described by Yaffe (1991).

BASIC PROTOCOL 7

ISOLATION OF OLEATE-INDUCED PEROXISOMES USING SUCROSE STEP GRADIENTS

This protocol was designed to purify intact, functional peroxisomes from the wild-type yeast strain D273-10B after growth in oleate medium (Distel et al., 1996). Yeast cells are precultured in glucose medium for two days and then transferred to oleate medium to induce the proliferation and growth of peroxisomes. After 12 to 18 hr in oleate medium, they are converted to spheroplasts using the β -glucanase preparation Zymolyase 100T and lysed by osmotic shock. Differential centrifugation is used to generate a peroxisome-enriched pellet. This pellet is subsequently fractionated on a sucrose density step gradient to purify the peroxisomes.

The protocol presented here calls for a 1-liter culture of D273-10B cells in oleate medium, but can be scaled up or down as needed. The extent of peroxisome proliferation in response

to growth on oleate medium can vary significantly among different *S. cerevisiae* strains. If using a strain other than D273-10B, it is wise to conduct a pilot experiment and monitor peroxisome induction using peroxisomal enzyme activity assays and electron microscopy. If the peroxisomes will be used for functional studies, a recovery incubation after spheroplast preparation may be needed for optimal activity.

Materials

Rich growth medium (see recipe)
Yeast strain D273-10B (or another strain of interest)
Peroxisome induction medium (see recipe)
TSD reduction buffer (see recipe)
1.2 M sorbitol/phosphate solution (see recipe)
100,000 U/g Zymolyase 100T (ICN Biochemicals)
1.2 M sorbitol in MES buffer (see recipe for buffer), 4°C
MES buffer (see recipe), 4°C
0.65 M sorbitol/MES solution (see recipe), ice cold
20%, 30%, 40%, 44%, 46%, and 60% (w/w) sucrose/MES solution: ultrapure sucrose in MES buffer (see recipe), 4°C

100-ml and 2-liter flasks
50-ml centrifuge tubes
250-ml centrifuge bottles
1.6-ml microcentrifuge tubes
Sorvall GS-3 rotor
Centrifuge and Sorvall SS-34 rotor (or equivalent)
Dounce homogenizer with a loose-fitting pestle (optional), chilled
Beckman polyallomer Quick-seal tubes (25 × 89-mm) or equivalent
Vertical rotor (Beckman VTi 50 or equivalent), 4°C
30°C water bath
Syringe and wide-gauge needle

Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and determining protein concentration (*APPENDIX 3B*)

Preculture cells

1. Three to four days before fractionation, inoculate 10 ml of rich growth medium in a 100-ml flask with a single colony of desired yeast strain from a fresh plate. Culture cells overnight at 28°C with vigorous shaking.
2. Measure OD₆₀₀ the following morning. Use preculture to inoculate 10 ml of rich growth medium (in a new 100-ml flask) to an OD₆₀₀ of 0.15. Culture cells during the day at 28°C with vigorous shaking.
3. Once culture reaches an OD₆₀₀ of 1.0 to 1.5, inoculate 10 ml of rich growth medium (in a new 100-ml flask) with cells to an OD₆₀₀ of ~0.010 to 0.015 (i.e., a 1:100 dilution). Incubate at 28°C overnight with vigorous shaking.
4. *Optional:* Repeat steps 2 and 3.

Extended preculturing will generate a relatively homogeneous population of healthy, rapidly dividing cells that will respond well when transferred to the peroxisome proliferation medium; however, the third day of preculturing is usually not necessary.

Culture and harvest cells

5. In the morning, the day before the fractionation experiment, measure the OD₆₀₀ of the 100-ml preculture from step 3. Inoculate 300 ml of rich growth medium (in a

2-liter flask) to an OD_{600} of 0.15. Culture cells at 28°C with vigorous shaking until culture reaches an OD_{600} of 1.0.

6. Once the culture reaches an OD_{600} of 1.0, harvest cells by centrifuging 5 min at $\sim 4000 \times g$ (4900 rpm in Sorvall GS-3 rotor), room temperature.
7. Decant supernatant and resuspend cells in 10 ml induction medium. Transfer 5 ml suspension to a flask containing 1 liter induction medium. Incubate overnight at 28°C with vigorous shaking.

Prepare cells to generate spheroplasts

8. In the morning of the day of fractionation experiment, harvest cells by centrifuging 5 min at $4000 \times g$, room temperature.

For best results, harvest cells 12 to 18 hr after the shift to induction medium.

9. Decant supernatant and resuspend cells in a total of ~ 100 ml sterile water. Pellet cells by centrifuging 5 min at $\sim 4000 \times g$, room temperature.
10. Decant supernatant and resuspend cells in a total of 40 ml sterile water. Transfer suspension to a single preweighed 50-ml centrifuge tube. Pellet cells by centrifuging 5 min at $\sim 4000 \times g$, room temperature.
11. Decant supernatant and aspirate any remaining liquid. Weigh centrifuge tubes containing pellets to determine the wet weight of cell pellet.
12. Resuspend cells in freshly prepared TSD reduction buffer at 0.125 g/ml (i.e., add 8 ml TSD reduction buffer per gram wet cell pellet). Incubate 15 min at 30°C with gentle shaking.
13. Harvest cells by centrifuging 5 min at $\sim 3000 \times g$ (e.g., 5000 rpm in Sorvall SS-34 rotor), room temperature.

Generate spheroplasts

14. Decant supernatant and resuspend cells with ~ 30 ml 1.2 M sorbitol/phosphate buffer. Harvest cells by centrifuging 5 min at $\sim 3000 \times g$, room temperature.
15. Decant supernatant and resuspend cells to 0.125 g/ml in 1.2 M sorbitol/phosphate buffer. Transfer 10 μ l cell suspension to a 1.6-ml microcentrifuge tube and store on ice until step 17.
16. Add Zymolyase 100T to a final concentration of 1 mg enzyme per gram of wet cell pellet. Incubate 20 min at 30°C with occasional gentle agitation.
17. After 20 min, monitor cell wall digestion. Dilute 10 μ l of each cell suspension into 990 μ l water and mix by briefly vortexing. Also dilute the 10- μ l samples stored after step 15 to serve as controls. After 1 or 2 min, briefly vortex again and measure the OD_{600} values.

The decrease in OD_{600} values of diluted cell suspension indicates the extent of cell wall removal. In the absence of mechanical support (provided by the intact cell wall) or osmotic support (provided by sorbitol), spheroplasts will lyse and thus will no longer contribute to the optical density.

The extent of cell-wall digestion can also be examined by phase-contrast microscopy as described in Support Protocol, step 11.

18. Continue incubation until $>95\%$ of cells have been converted to spheroplasts, which usually takes 30 to 60 min. Harvest spheroplasts by centrifuging 5 min at $\sim 1000 \times g$ (e.g., 3000 rpm in Sorvall SS-34 rotor), room temperature.

19. Decant supernatant and carefully resuspend pellet in ice-cold 1.2 M sorbitol/MES buffer by swirling and gently mixing with a glass rod. Do not vortex, as spheroplasts are fragile.

All subsequent steps are performed at 4°C with prechilled solutions and equipment.

20. Pellet spheroplasts by centrifuging 5 min at $1000 \times g$, 4°C. Transfer cell suspension to a preweighed centrifuge tube. Repeat steps 19 and 20.

21. *Optional:* Incubate spheroplasts 30 to 60 min at 28° to 30°C in rich growth medium supplemented with sorbitol to a final concentration of 1.2 M. Shake gently. Pellet spheroplasts by centrifuging 5 min at $\sim 1000 \times g$, 4°C.

Optimal recovery conditions should be determined empirically for each application.

Other growth media supplemented with sorbitol to a final concentration of 1 to 1.5 M can be used.

22. Repeat steps 19 and 20.

Isolate enriched peroxisome fraction

23. Decant supernatant and weigh wet pellet. Gently resuspend spheroplasts in ice-cold 1.2 M sorbitol/MES buffer to a concentration of 0.125 g/ml (i.e., add 8 ml of solution per g wet cell pellet determined in step 11). Resuspend with a glass rod. Do not vortex.

24. To generate hypoosmotic conditions, slowly add ice-cold MES buffer with freshly added PMSF dropwise to spheroplast suspension while gently stirring until the final sorbitol concentration is 0.65 M.

The required volume ice-cold MES buffer will need to be calculated based on the volume of 1.2 M sorbitol/MES added in step 23.

25. Monitor the degree of cell lysis microscopically.

If >20% of the spheroplasts remain intact after several minutes, induce further lysis with a few gentle strokes in a chilled Dounce homogenizer with a loose-fitting pestle.

26. Centrifuge homogenate 10 min at $2000 \times g$ (e.g., 4100 rpm in Sorvall SS-34), 4°C, to remove unlysed cells and large aggregates. Transfer supernatant to a new tube and store on ice until step 28.

27. Using a pipet, resuspend pellet in ice-cold 0.65 M sorbitol/MES solution with PMSF to a concentration of 0.125 g/ml. Centrifuge 10 min at $2000 \times g$, 4°C.

28. Pool supernatants from steps 26 and 27 in a 50-ml centrifuge tube prechilled on ice.

29. Pellet peroxisomes (as well as mitochondria and other organelles) from pooled supernatants by centrifuging 30 min at $20,000 \times g$ (e.g., 13,000 rpm in Sorvall SS-34 rotor), 4°C.

30. After 30-min centrifugation, aspirate supernatant. Carefully resuspend the organelle pellet in 2 ml cold 30% sucrose/MES solution and store organelle suspension on ice.

In order to minimize damage to the fragile peroxisomes, resuspend pellet using a glass rod or a pipet with a wide tip opening.

Determine protein concentration

31. Determine approximate protein concentration of organelle suspension by any standard method (e.g., APPENDIX 3B).

32. Add cold 30% sucrose/MES solution to organelle suspension to reach a final protein concentration of ~ 5 mg/ml. Carefully mix with a glass rod or a pipet with a wide tip opening.

Purify peroxisomes

33. Carefully prepare sucrose step gradients in Quick-seal centrifuge tubes by layering 5 ml of 60% sucrose/MES solution, 12 ml of 46% sucrose/MES solution, 12 ml of 44% sucrose/MES solution, and 5 ml of 40% sucrose/MES solution. Cool on ice for ≥ 10 min.

It is important that gradients be prepared <30 min before use and interfaces between sucrose layers are sharp.

One gradient will be needed for every 2 ml of organelle suspension.

34. Apply 2 ml suspension from step 30 to each sucrose step gradient. Fill and balance tubes with cold 20% sucrose/MES solution and carefully seal tubes.
35. Centrifuge 2.5 hr at $34,500 \times g$, 4°C , in a vertical rotor (Beckman VTi 50 or equivalent), using the slow acceleration and deceleration modes.
36. After centrifugation is completed, carefully remove gradient from the rotor buckets and place them in a stable rack. Note any apparent membrane aggregations and other characteristics. While processing each gradient, store remaining gradients undisturbed at 4°C .

Two bands will be visible in the gradient: (1) a narrow band at the 60%/46% sucrose interface consisting primarily of peroxisomes, and (2) a broader band at the 46%/44% sucrose interface consisting primarily of mitochondria.

37. Collect peroxisomes at the 60%/46% sucrose interface using a syringe with a wide-gauge needle.

Alternatively, collect 2-ml fractions with an automated collector or a cut 1-ml pipet tip. Ideally the membrane layers at the 60%/46% and 46%/44% sucrose interfaces should each be in a single fraction.

If the peroxisomes are not immediately needed, the sucrose fraction(s) can be rapidly frozen in liquid nitrogen and stored at -80°C .

BASIC PROTOCOL 8

ISOLATION OF ENDOPLASMIC RETICULUM USING SUCROSE STEP GRADIENTS

This protocol was designed to isolate functional ER membranes from yeast spheroplasts (Wuestehube and Schekman, 1992). Yeast cells are converted to spheroplasts with the β -glucanase Zymolyase 100T as in Support Protocol. The cells are lysed under hypoosmotic conditions with a motor-driven Potter-Elvehjem homogenizer (or a glass Dounce homogenizer). Differential centrifugation is used to generate a pellet that is enriched for ER membranes. This pellet is subsequently fractionated on a sucrose density step gradient to isolate ER membranes.

The protocol presented here calls for 5000 OD_{600} units of cells in mid-logarithmic growth phase, but it can be scaled-up or scaled-down as needed. If compatible with the goals of the experiment, protease-deficient strains can be used to minimize protein degradation (e.g., TVY614, a *pep4 prb1 prc1* mutant yeast strain).

Materials

- Desired yeast strain ($\sim 5000 \text{OD}_{600}$ units of cells in exponential growth phase)
- HEPES lysis buffer (see recipe), 4°C
- 1 M DTT
- Protease inhibitor stock solutions B (see recipe)
- 1.5 M and 1.2 M sucrose/HEPES solution (see recipe)

Isolation of Subcellular Fractions from Yeast

3.8.36

250-ml centrifugation bottles
Motor-driven Potter-Elvehjem homogenizer (or a glass Dounce homogenizer)
1.6-ml centrifuge tubes
1.6-ml polycarbonate ultracentrifuge microcentrifuge tubes, prechilled
Sorvall GS-3 rotor
Trimmed 1-ml and 200- μ l pipet tips (i.e., ~5 to 10 mm cut from the tips)
4-ml Dounce homogenizer, prechilled
Beckman Ultraclear 11 \times 34-mm centrifuge tubes (or equivalent), prechilled
Swinging bucket rotor (e.g., Beckman SW 50.1 or equivalent), 4°C
Additional reagents and equipment for growing yeast cells (*UNIT 1.6*), spheroplast preparation (see Support Protocol), and Lowry assay for proteins (*APPENDIX 3B*)

Culture cells and prepare spheroplasts

1. Culture desired yeast strains as described in Support Protocol, steps 1 to 4 (also see *UNIT 1.6*).
2. Harvest ~5000 OD₆₀₀ units of cells in mid-logarithmic growth phase by centrifuging 5 min at ~4000 \times g (5000 rpm in Sorvall GS-3 rotor), room temperature.
3. Prepare spheroplasts as described in Support Protocol, steps 5 to 15.

Alternatively, spheroplasts can be prepared as in Basic Protocol 4 or by other methods.

Spheroplasts are fragile and must be handled very gently.

4. Resuspend spheroplasts in ~100 ml ice-cold cold stop solution, spheroplast medium B, or another solution with the appropriate osmotic support. Resuspend spheroplasts gently by swirling tube by hand, slowly stirring with a glass rod, or slowly pipetting with a wide bore pipet. Do not vortex. Keep suspension on ice until ready for the next step.

The optimal solution will depend on the goals and subsequent steps of the experiment in which the spheroplasts will be used.

Spheroplasts tend to stick to each other and thus can be difficult to resuspend. To facilitate resuspension, initially resuspend spheroplasts in a smaller volume of solution before adding the remaining solution.

Spheroplasts can be stored at -80°C. Resuspend at 1000 OD₆₀₀ units/ml in 0.7 mM sorbitol/20 mM HEPES (pH 7.4). Slowly freeze to -80°C in an insulated container and then store at -80°C.

Prepare spheroplast lysates

5. Harvest spheroplasts by centrifuging 5 min at 1500 \times g, 4°C, and aspirate supernatant.

From this point on, keep samples on ice or at 4°C. Also, all solutions and equipment should be chilled to 0° to 4°C prior to as well as during use.

6. Resuspend spheroplasts at a concentration of 1000 OD₆₀₀ units/ml in ice-cold HEPES lysis buffer with freshly-added DTT and protease inhibitors.
7. Prepare lysate with a motor-driven Potter-Elvehjem homogenizer (i.e., 4 to 10 strokes). Keep lysate at 4°C.

Alternatively, spheroplasts can be disrupted with 10 up-and-down strokes of a tight-fitting pestle in an ice-cold Dounce homogenizer. Keep Dounce homogenizer on ice throughout this process. Move pestle slowly and maintain contact between pestle and suspension to minimize foaming.

8. Inspect 10 μ l of the lysate by phase microscopy.

Intact spheroplasts have characteristic bright halos that are easily distinguished from lysed spheroplast and spheroplast fragments.

Usually, >95% lysis is achieved.

9. Transfer lysate to multiple chilled 1.6-ml centrifuge tubes.
10. Clear lysate of unbroken cells, partially disrupted cells, and aggregates by microcentrifuging 10 min at $1000 \times g$, 4°C .
11. Carefully withdraw supernatants (S_{1000}) and transfer to new chilled tubes, taking care not to disrupt loose pellets (P_{1000}).
12. Resuspend P_{1000} in ~ 2.5 ml ice-cold HEPES lysis buffer. Repeat homogenization and low-speed centrifugation (steps 7 to 10).

This second homogenization step can improve the yield of intracellular membranes by $\sim 30\%$.

Prepare $P_{27,000}$ membrane pellets

13. Pool the first and second S_{1000} supernatants (steps 11 and 12). Mix and transfer the pooled supernatants (S_{1000}) to polycarbonate ultracentrifuge tubes, chilled on ice.

When not required for subsequent analyses, the P_{1000} pellets are usually discarded since they contain a crude mixture of unlysed and partially disrupted cells, as well as a very heterogeneous mixture of intracellular membranes.

14. Centrifuge the S_{1000} supernatants 10 min at $27,000 \times g$, 4°C . Remove the supernatants ($S_{27,000}$).

The $S_{27,000}$ can either be saved for analysis or discarded.

15. To remove any residual supernatant from $P_{27,000}$ pellets, centrifuge tubes containing $P_{27,000}$ samples ~ 45 sec at $13,000 \times g$, 4°C . Carefully remove any remaining liquid above pellet and discard.
16. Resuspend $P_{27,000}$ membranes in 1.0 ml HEPES lysis buffer (~ 5000 OD₆₀₀ equivalents per ml). Use five strokes in a Dounce homogenizer to ensure complete dispersion of $P_{27,000}$ pellet.

Prepare and run sucrose step gradients

17. Prepare four sucrose step gradients just prior to use so that the interface remains crisp. Carefully pipet 1.0 ml of 1.5 M sucrose/HEPES solution into each 11×34 -mm ultracentrifuge tube. Using a trimmed 1-ml pipet tip, carefully layer 1.0 ml of 1.2 M sucrose/HEPES solution on top of the 1.5 M sucrose layer.

If the interface is disturbed during or after preparation of gradient, a new step gradient will need to be prepared.

18. Carefully layer $P_{27,000}$ suspension (step 16) over the four sucrose gradients using a trimmed 200- μ l pipet tip.

Distribute the $P_{27,000}$ suspension among the four gradients so that they are loaded with similar volumes. Up to 0.5 ml of suspension can be loaded on each gradient.

19. Balance the appropriate pairs of gradients by adding ice-cold lysis buffer as necessary. Put gradient tubes into the prechilled buckets of the swinging bucket rotor (e.g., SW 50.1 rotor or equivalent).

20. Centrifuge gradient 1 hr at $100,000 \times g$, 4°C , using the slow acceleration and deceleration settings to minimize disruption of gradients.

Collect and process fractions

21. Carefully remove tubes from the rotor buckets and place in a rack. Make a note of the position and appearance of bands and any other visible characteristics.

Two discrete membrane bands should be visible, one in the 1.2 M sucrose layer and one at the interface between the 1.2 M and 1.5 M sucrose layers.

22. Collect ER membranes at the 1.2 M/1.5 M sucrose interface. First remove the load layer and most of the 1.2 M sucrose layer by aspiration. Then collect the band at the interface.

Alternatively, all of the gradient can be harvested as described in Basic Protocol 3, step 23.

23. Dilute ER membrane suspension 10-fold in ice-cold HEPES lysis buffer.
24. Harvest membranes by centrifuging 10 min at $27,000 \times g$, 4°C . Remove supernatant and process pellet fractions as desired.
25. Determine the protein concentration with the Lowry assay (*APPENDIX 3B*) after resuspending a small amount of the preparation in 1% SDS.

Typically, this protocol yields ~7 mg of protein per 5000 OD_{600} units of cells.

ISOLATION OF PLASMA MEMBRANES FROM WHOLE YEAST CELLS USING SUCROSE STEP GRADIENTS

This protocol is an efficient method to isolate plasma membranes from intact yeast cells (Panaretou and Piper, 1996). Yeast cells are rapidly disrupted by vortexing with glass beads. The resulting lysate is fractionated by differential centrifugation to generate a pellet enriched for plasma membrane. The plasma membrane-enriched pellet is then fractionated on a sucrose density step gradient.

The protocol presented here calls for a 1-liter culture of yeast cells in exponential growth phase, but it can be scaled-up or -down as needed. If compatible with the goals of the experiment, it is often beneficial to use a strain that is deficient in vacuolar protease activity to minimize protein degradation (e.g., TVY1, a *pep4* mutant yeast strain), especially when working with heat-shocked cells.

Materials

- 1-liter culture of desired yeast cells in exponential growth phase
- 0.4 M, 1.1 M, 1.65 M, 2.25 M sucrose/imidazole solutions (see recipe), 4°C
- Protease inhibitor stock solutions C (see recipe)
- Breakage buffer (see recipe), 4°C
- Plasma membrane storage buffer (optional; see recipe)
- 250-ml centrifuge bottles
- 50-ml polycarbonate centrifuge tubes
- Acid-washed glass beads (0.45-mm diameter; see recipe), prechilled to 4°C
- Sorvall GS-3 rotor
- Sorvall SS-34 rotor (or equivalent) and appropriate centrifugation tubes, 4°C
- 14×89 -mm Beckman Ultra-clear centrifugation tubes
- Beckman SW41 or SW40Ti rotor (or equivalent), 4°C
- Beckman 50 Ti rotor (or equivalent)
- Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and culture preparation (Support Protocol)

BASIC PROTOCOL 9

Subcellular Fractionation and Isolation of Organelles

3.8.39

Grow cells

1. Prepare a 1-liter yeast culture in exponential growth phase (see Support Protocol, steps 1 to 4).
2. Harvest cells by centrifuging 5 min at $5000 \times g$ (5000 rpm in Sorvall GS-3 rotor), 4°C .

All subsequent steps should be performed at 4°C with prechilled solutions and equipment.

3. Decant supernatant and resuspend cells in 80 ml cold 0.4 M sucrose/imidazole solution with freshly added protease inhibitors.
4. Transfer cell suspension to two 50-ml polycarbonate centrifuge tubes. Centrifuge 10 min at $5000 \times g$ (e.g., 6500 rpm in Sorvall SS-34 rotor), 4°C .

Perform glass-bead lysis

5. Decant supernatant and add two times the pellet volume of prechilled glass beads to pellets. Add sufficient cold 0.4 M sucrose/imidazole solution to just cover pellet and glass beads.
6. Vortex 5 min at maximum speed. Keep lysate cold by alternating between 30 sec of vortexing followed by 30 to 60 sec in an ice-water bath.

Ice can be used instead of an ice-water bath; however, an ice-water bath will cool the lysates more efficiently.

7. Monitor degree of cell breakage under a microscope.

The percentage of lysed cells should be between 50% and 80%. If the lysis efficiency is significantly below 50%, repeat step 6.

8. Place tubes on ice ~2 min to allow glass beads to settle and cool the lysate. Transfer supernatant to a new tube and store on ice.
9. Add ~2 times volume of cold 0.4 M sucrose/imidazole solution to glass beads. Vortex for 30 sec. Allow glass beads to settle while the tube is on ice.
10. Remove supernatant and pool with the supernatant collected in step 8. Repeat steps 9 and 10.

Isolate enriched plasma membrane fraction

11. Clear the pooled lysate of unbroken cells, cell wall debris, and aggregates by centrifuging 20 min at $530 \times g$ (2150 rpm in Sorvall SS-34 rotor), 4°C .
12. During the centrifugation step, prepare two sucrose step gradients in 14×89 -mm Beckman Ultra-clear centrifugation tubes. Add 4 ml of 2.25 M sucrose/imidazole solution to each tube. Then carefully overlay 4 ml of 1.65 M sucrose/imidazole solution, followed by 4 ml of 1.1 M sucrose solution. Chill gradients on ice until ready for use.

Crisp interfaces are essential to the success of the experiment.

13. Transfer supernatant from step 11 to fresh centrifuge tubes. Centrifuge 30 min at $22,000 \times g$ (13,500 rpm in Sorvall SS-34 rotor), 4°C , to obtain a pellet that includes plasma membranes (as well as nuclei, mitochondria, and vacuoles).
14. Carefully remove supernatant and resuspend pellet in 2 ml cold breakage buffer by vortexing for 30 sec at slow-medium speed.

The use of <2 ml breakage buffer is not recommended, as membranes will tend to clump.

Purify plasma membranes

15. Carefully layer 1 ml resuspended membranes onto each of the two sucrose step gradients (prepared in step 12). Balance tubes with breakage buffer.
16. Centrifuge 14 hr at $80,000 \times g$ ($\sim 22,000$ rpm in Beckman SW-41 rotor), 4°C .
Alternatively, centrifuge for 6 hr at $284,000 \times g$ ($\sim 40,000$ rpm in Beckman SW-41 rotor).
17. After centrifugation is completed, carefully remove gradient from the rotor buckets and place them in a stable rack. Note any apparent membrane aggregations and other characteristics. While processing each gradient, store remaining gradients undisturbed at 4°C .
A layer primarily consisting of plasma membrane should form at the 2.25 M/1.65 M sucrose solution interface; this band represents up to two-thirds of the plasma membranes loaded onto the gradient. The remaining plasma membranes will colocalize with mitochondria at the 1.65 M/1.10 M sucrose solution interface.
18. Collect membranes at the 2.25 M/1.65 M sucrose interface using a Pasteur pipet. Dilute membranes with 4 vol cold breakage buffer and store on ice.
If desired, the other fractions can also be harvested and analyzed.
19. Transfer suspensions to centrifuge tubes. Pellet membranes by centrifuging 40 min at $30,000 \times g$ ($\sim 17,000$ rpm in Beckman 50 Ti rotor), 4°C .
20. Decant supernatant and resuspend pellet in desired buffer for immediate use.
For storage, resuspend the pellet in plasma membrane storage buffer and store at -80°C .
21. *Optional*: Determine protein concentration by indirect means (*APPENDIX 3B*).

PREPARATION OF CYTOSOL FROM WHOLE YEAST CELLS

This protocol is designed to prepare cytosol from intact yeast cells (Haas, 1995). Yeast cells are rapidly disrupted by vortexing with glass beads. The resulting lysate is subjected to high-speed centrifugation to separate the cytosol from membrane components.

The protocol presented here calls for 2000 OD_{600} units of yeast cells grown to a concentration of 1 to 2 OD_{600} units/ml, but it can be scaled up or down as needed. If compatible with the goals of the experiment, it is often beneficial to use a strain that is deficient in vacuolar protease activity to minimize protein degradation (e.g., TVY1, a *pep4* mutant yeast strain).

Materials

- Desired yeast strains
- YPD (*UNIT 1.6* or appropriate growth medium)
- PIPES lysis buffer (see recipe), prechilled
- 50 \times protease inhibitor cocktail (50 \times PIC; see recipe) or equivalent
- Acid-washed glass beads (~ 0.45 -mm diameter; BDH or Sigma; see recipe), prechilled to 4°C
- Methylene blue
- BioRad protein assay reagent kit (or equivalent)
- 250-ml and 2-liter Erlenmeyer flasks
- Platform shaker, 30°C (or appropriate permissive temperature)
- Centrifuge rotors (e.g., Beckman JA-10 and JA-20 rotors or equivalents)
- 500-ml centrifuge bottles
- 30-ml glass Corex tubes (1 tube per cytosol preparation), prechilled

**BASIC
PROTOCOL 10**

**Subcellular
Fractionation and
Isolation of
Organelles**

3.8.41

Ice-water bath
Adapters for 30-ml glass Corex tubes (for Beckman JA-20 rotor or equivalent)
Ultracentrifuge tubes, 4°C
Ultracentrifuge rotor (Beckman TLA 100.2 rotor or equivalent)
Tabletop ultracentrifuge, 4°C
Microcentrifuge tubes, prechilled
Additional reagents and equipment for growing yeast cells (*UNIT 1.6*) and determining protein concentration (*APPENDIX 3B*)

Grow cells

1. In the morning, the day before the experiment, start precultures of desired yeast strains by inoculating 20-ml of YPD (or appropriate medium) in 250-ml Erlenmeyer flasks. Grow throughout the day at 30°C (or appropriate permissive temperature) shaking at 225 to 335 rpm.

It is best to start with a single yeast colony from a fresh plate.

If using a strain that grows very slowly or if using a strain that has been stored on a plate at 4°C for more than a few days, it is wise to start the preculture with a single colony the night before.

2. In the evening, the day before the experiment, measure and record the OD₆₀₀ values of precultures.
3. Inoculate two 1-liter cultures (in 2-liter Erlenmeyer flasks) with the appropriate volume of each preculture.

Cultures of 1 to 2 liters are recommended for each strain, since 2000 OD₆₀₀ units of cells at 1 to 2 OD₆₀₀ units/ml are needed for each batch of cytosol. The suitable yeast cell concentration at this step depends on the growth rate of each strain and the length of incubation time. See Support Protocol step 3 for sample calculations.

For some purposes, cytosol can be prepared from yeast cell cultures with a higher cell density (OD₆₀₀ of 2 to 6); however, cytosol preparations from lower-density cultures (OD₆₀₀ 1 to 2) generally seem to have higher activities as determined by vacuolar fusion assays (Haas, 1995).

4. Culture cells overnight at 30°C (or appropriate permissive temperature) while shaking at 225 to 325 rpm. In the morning, the day of experiment, analyze cell density.

Ideally the cultures should have an OD₆₀₀ of 1 to 2 to begin the experiment.

5. Harvest 2000 OD₆₀₀ units of cells in 500-ml centrifugation bottles by centrifuging 5 min at 4400 × *g* (5000 rpm in JA-10 rotor), room temperature. Decant and discard supernatants.

Prepare cell lysates using glass beads

6. Resuspend pellets in a total of 50 ml prechilled PIPES lysis buffer (50 ml per 2000 OD₆₀₀ units of cells). Transfer cells to smaller centrifuge tubes and vortex at maximum speed for 15 sec. Transfer cell suspensions to a single 500-ml centrifugation bottle.
7. Harvest the washed cells by centrifuging 5 min at 4400 × *g* (e.g., 5000 rpm in JA-10 rotor), room temperature. Decant and discard supernatants.
8. Resuspend cells in 2 ml prechilled PIPES lysis buffer (2 ml per 2000 OD₆₀₀ units of cells). Add 80 μl 50× PIC to each 2-ml suspension and mix by vortexing.
9. Transfer the cell suspension into a prechilled, labeled 30-ml glass Corex tube (one tube/2000 OD₆₀₀ units suspension). Add 3 g prechilled acid-washed glass beads. Seal tubes tightly with Parafilm and keep tubes 2 min in an ice-water bath.

10. Vortex at maximum speed for 30 sec. Then immediately return to ice-water bath for 1 min. Repeat 15 times for each tube.

Ice can be used instead of an ice-water bath; however, an ice-water bath will cool the lysate more efficiently.

11. Monitor the degree of cell lysis by phase-contrast microscopy. Dilute 10 μ l cell lysate in 90 μ l PIPES lysis buffer. Add \sim 5 μ l methylene blue and mix. View lysate to determine the percentage of cells that stain with the reagent.

The stained cells have been lysed. Ideally, 80% of the cells should be lysed. If <50% of cells are lysed, repeat step 10.

Prepare cytosol

12. Clear lysate of unlysed cells, larger aggregates, and glass beads by centrifuging 10 min at $3000 \times g$ (5000 rpm in JA-20 rotor with adapters for the Corex tubes), 4°C.

13. Carefully transfer supernatant to prechilled ultracentrifuge tubes (\sim 1.2 ml per tube).

If required, balance tubes using cold PIPES lysis buffer.

14. Centrifuge samples 1 hr at $150,000 \times g$ (65,000 rpm in Beckman TLA 100.2 rotor), 4°C.

15. Carefully remove and discard the lipid layer visible on top of each sample. Then transfer supernatant to prechilled microcentrifuge tubes, being careful not to include any of the pellet.

16. Determine protein concentration of each cytosol sample using the BioRad protein assay reagent kit (or equivalent; see APPENDIX 3B). Prepare several dilutions of cytosol in water (e.g., 1:10 to 1:100; 10 μ l total).

The cytosol preparations usually have protein concentrations of 10 to 30 mg/ml.

*If the cytosol is not needed immediately, aliquot cytosol in prechilled tubes (e.g., 100 μ l per 0.6-ml microcentrifuge tube). Quickly freeze the samples in liquid nitrogen and store at -80°C . Haas and coworkers (1995) have found that cytosol can be stored for months at -80°C and still support an *in vitro* vacuole-vacuole fusion assay.*

REAGENTS AND SOLUTIONS

Use double-distilled water and Ultrapure reagents in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Acid-washed glass beads

For yeast glass bead lysis, it is best to use glass beads between 0.4 and 0.5 mm (BDH or Sigma). Soak the glass beads in concentrated nitric acid or 1 M hydrochloric acid for 2 hr in a fume hood (use caution; wear safety goggles, nitrile gloves, and a long lab coat). During incubation, carefully mix beads a few times using a long glass stirring rod. Wash beads extensively with deionized water. Continue washing until the water has the same pH before and after addition to the beads (it is helpful to use a filter apparatus for the washing). Transfer beads to a glass dish or metal tray. Place them in a vacuum oven at \sim 200°C. Stir a few times to ensure that all of the beads will dry completely. Once the beads are completely dry and have cooled to room temperature, store them in a glass bottle at 4°C.

Breakage buffer

2 mM EDTA

25 mM imidazole-HCl, pH 7.0

Store up to a few weeks at 4°C

Just before use, add PMSF and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) to a final concentration of 1 mM each. Also add pepstatin A to a final concentration of 2.5 µg/ml (see recipe for protease inhibitor stock solutions C).

Keep buffer at 4°C, not 0°C, to prevent precipitation of protease inhibitors.

Buffer B, 2×

1.2 M sorbitol

40 mM potassium MES, pH 6.0

Filter sterilize

Store up to several weeks at 4°C

Dextran solution, 0.4 mg/ml (Basic Protocol 4)

Dissolve 2 mg dextran in 5 ml of 15% Ficoll solution (see recipe). Mix by vortexing. Prepare fresh and keep on ice until ready to use.

Less than 0.3 ml is required for each gradient.

Ficoll cushion solution (Basic Protocol 5)

5.5 g sorbitol

1.25 g Ficoll 400

H₂O to 25 ml

Prepare fresh and store at 0° and 4°C until used

Ficoll gradient buffer (Basic Protocol 4)

10 mM PIPES/KOH, pH 6.8

200 mM sorbitol

Store a few weeks at 4°C

Add 50× PIC and PMSF (1 mM; see recipes) just before use

Ficoll solutions

0% Ficoll solution: Ficoll gradient buffer (see recipe) without Ficoll. Add 50× protease inhibitor cocktail (50× PIC; see recipe) and 1 mM PMSF (from 100 mM stock; see recipe for protease inhibitor stock solutions) just before use.

4% (w/v) Ficoll solution: Dissolve 4 g Ficoll 400 in 50 ml Ficoll gradient buffer (see recipe); adjust final volume to 100 ml with Ficoll gradient buffer. Add 50× PIC (see recipe) and 1 mM PMSF (from 100 mM stock; see recipe for protease inhibitor stock solutions) just before use.

8% (w/v) Ficoll solution: Dissolve 8 g Ficoll 400 in 50 ml Ficoll gradient buffer (see recipe); adjust final volume to 100 ml with Ficoll gradient buffer. Add 50× PIC (see recipe) and 1 mM PMSF (from 100 mM stock; see recipe for protease inhibitor stock solutions) just before use.

15% (w/v) Ficoll solution: Dissolve 15 g Ficoll 400 in 50 ml Ficoll gradient buffer (see recipe); adjust final volume to 100 ml with Ficoll gradient buffer. Add 50× PIC (see recipe) and 1 mM PMSF (from 100 mM stock; see recipe for protease inhibitor stock solutions) just before use.

Prepare solutions on the day before the experiment (or earlier) since Ficoll takes a long time to dissolve. To minimize clumping, slowly add the indicated amount of Ficoll 400 to the Ficoll gradient buffer while stirring. Continue to stir the Ficoll solutions without heat. Once Ficoll has dissolved, adjust final volume of each solution to 100 ml with Ficoll gradient buffer. Sterilize by filtration. Store up to a few weeks at 4°C.

Ficoll lysis solutions (Basic Protocol 5)

50% (w/v) Ficoll lysis solution. Heat 100 ml 2× PM buffer (see recipe) and 30 ml water in a 400-ml beaker to ~60°C (not boiling). Slowly add 100.0 g Ficoll 400 with continuous, rapid stirring over a period of 5 to 10 min. Keep solution hot (but avoid boiling) and cover with a glass petri dish. Continue moderate stirring and gentle heating until Ficoll has completely dissolved (often takes ~1 hr). Allow the solution to cool to room temperature. Transfer Ficoll solution to a clean glass 250-ml cylinder. Use multiple washes with water to transfer all of Ficoll from beaker to cylinder. Once all of the bubbles have reached the surface, add water to cylinder to a final volume of 200 ml. Carefully seal cylinder with Parafilm and tape. Mix on a rotator until Ficoll stock is completely homogeneous (30 to 60 min). Aliquot into five sterile, plastic 50-ml tubes and store at –20°C. Remember to mix well after thawing. Warm to room temperature and add 1000× protease inhibitor cocktails (1000× PICs; see recipe) PIC-W and PIC-D at 1:1000 (1× final) just before use.

It is critical to prepare the 50% Ficoll lysis stock solution accurately.

20% (w/v) Ficoll lysis solution. Mix 40.0 ml of 50% Ficoll lysis solution (see recipe) and 30 ml of 2× PM buffer (see recipe), and add water to 100 ml. Warm to room temperature and add 1000× protease inhibitor cocktails (1000× PICs; see recipe) PIC-W and PIC-D at 1:1000 (1× final) just before use.

30% (w/v) Ficoll lysis solution. Mix 27.0 ml of 50% Ficoll lysis solution (see above) and 9.0 ml of 2× PM buffer (see recipe), and add water to 45 ml. Warm to room temperature and add 1000× protease inhibitor cocktails (1000× PICs; see recipe) PIC-W and PIC-D at 1:1000 (1× final) just before use.

40% (w/v) Ficoll lysis solution. Mix 36.0 ml of 50% Ficoll lysis solution (see above) and 4.5 ml of 2× PM buffer (see recipe), and add water to 45 ml. Warm to room temperature and add 1000× protease inhibitor cocktails (1000× PICs; see recipe) PIC-W and PIC-D at 1:1000 (1× final) just before use.

HEPES/KAc lysis buffer (Basic Protocols 1, 2, 3, and Alternate Protocol)

20 mM HEPES/KOH, pH 6.8
50 mM potassium acetate
200 mM sorbitol
1 mM EDTA

Autoclave and store up to 1 month at 4°C

Just before use, aliquot the desired amount of HEPES/KAc lysis buffer to a tube. Add 500× protease inhibitor cocktails A and B (PIC-A and PIC-B, see recipes) to a final concentration of 1×. Add 1 mM PMSF and 5 µg/ml α-macroglobulin (final concentrations; protease inhibitor cocktail stock solutions A, see recipe). If desired, add DTT to 1 mM but omit α-macroglobulin.

HEPES lysis buffer (Basic Protocol 8)

20 mM HEPES/KOH, pH 6.8
50 mM potassium acetate
100 mM sorbitol
2 mM EDTA

Autoclave and store up to 1 month at 4°C

Add DTT and protease inhibitors just before use at final concentrations of 1 mM DTT, 1 mM PMSF, 1 µg/ml pepstatin, and 1 µg/ml leupeptin (see recipe for protease inhibitor stock solutions B).

MES buffer (Basic Protocol 7)

5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.5
1 mM EDTA
1 mM KCl
Store up to 1 month at 4°C

Nycodenz solutions for equilibrium gradients (Basic Protocol 2)

Prepare the following solutions by diluting the 50% (w/v) Nycodenz/sorbitol stock solution (see recipe) with the appropriate volume of HEPES/KAc lysis buffer (see recipe): 37%, 31%, 27%, 23%, 20%, 17%, 13%, and 9% (w/v) Nycodenz solutions. Just before use, aliquot the desired volume of each Nycodenz solution to individual 15-ml tubes. Add 500× protease inhibitor cocktails A and B (PIC-A and PIC-B, see recipes) to a final concentration of 1×. Add 1 mM PMSF and 5 μg/ml α-macroglobulin (final concentrations; protease inhibitor cocktail stock solutions A, see recipe). If desired, add DTT to 1 mM but omit α-macroglobulin.

Alternatively, each solution can be prepared in a manner similar to 60% sucrose stock solution (see recipe).

Nycodenz stock solution, 50% (w/v) (Basic Protocol 6)

Dissolve 50% (w/v) Nycodenz (Life Technologies) in sterile water by slowly adding powder while stirring. Store several months at –20°C.

It may take several hours for the Nycodenz to dissolve completely.

Nycodenz/sorbitol stock solution, 50% (w/v)

50% (w/v) Nycodenz powder
50 mM HEPES/KOH, pH 6.8
1 mM EDTA
200 mM sorbitol
Aliquot and store several weeks at –20°C

Prepare well in advance because Nycodenz powder can take several hours to dissolve. To minimize formation of clumps, stir while adding Nycodenz powder.

Nycodenz step gradient solutions

18% (w/v) Nycodenz step gradient solution: Mix 10 ml of 2× buffer B (see recipe) with 7.2 ml of 50% Nycodenz stock solution (see recipe). Adjust final volume to 20 ml with water. Prepare fresh. Add PMSF to 1 mM final concentration just before use.

14.5% Nycodenz step gradient solution: Mix 10 ml of 2× buffer B (see recipe) with 5.8 ml of 50% Nycodenz stock solution (see recipe). Adjust final volume to 20 ml with water. Prepare fresh. Add PMSF to 1 mM final concentration just before use.

Oxalyticase buffer (Basic Protocol 4)

7.5 ml 1 M potassium phosphate buffer, pH 7.5 (APPENDIX 2A)
22.5 ml 4 M sorbitol
120 ml 0.2× YPD medium (UNIT 1.6)
Store up to a few weeks at 4°C

Final concentrations are 50 mM potassium phosphate, pH 7.5, 0.6 M sorbitol, and 0.16× YPD. About 15 ml is required for each gradient.

Peroxisome induction medium (Basic Protocol 7)

0.5% (w/v) Bacto peptone
0.3% (w/v) yeast extract
0.12% (w/v) oleic acid
0.2% (v/v) Tween 40

continued

0.5% (w/v) KH_2PO_4

Adjust to pH 6.0 with NaOH

Autoclave

Store up to several weeks at room temperature

At least 1.1 liter of induction medium will be needed per strain.

This medium is best prepared within a few days of use.

PIPES/DTT buffer (Basic Protocol 4)

Prepare 100 mM PIPES/KOH (pH 9.4) and 1 M DTT stock solutions in sterile water at room temperature. Mix 10 ml of 1 M PIPES/KOH (pH 9.4), 1 ml of 1 M DTT, and water for a final volume of 100 ml. Prepare fresh before use.

About 50 ml is required for each gradient.

PIPES lysis buffer (Basic Protocol 10)

20 mM PIPES/KOH (pH 6.8)

250 mM sorbitol

100 mM potassium acetate

50 mM KCl

5 mM MgCl_2

Store several weeks at 4°C

Just before use, add DTT, PMSF, and 50× PIC to final concentrations of 2 mM, 1 mM, and 2× respectively

Prepare for sterile components or filter sterilize.

PM buffer, 2× and 1× (Basic Protocol 5)

For 2×:

2.5 ml 1 M K_2HPO_4

5.5 ml 1 M KH_2PO_4

0.4 ml 1 M MgCl_2

H_2O to 200 ml

Filter sterilize

Store up to several weeks at 4°C

For 1×: Dilute 2× PM buffer 1:1 with water. Chill to 4°C. Add 1000× protease inhibitor cocktails PIC-W and PIC-D (see recipe) at 1:1000 just before use.

PMSF, 200 mM

Dissolve 34.5 mg phenylmethylsulfonyl fluoride (PMSF) in 9 ml absolute ethanol.

Prepare fresh or store aliquots at -20°C for a few weeks.

Pretreatment buffer (Basic Protocol 5)

100 mM Tris-Cl, pH 9.4

50 mM DTT

5 mM EDTA, pH 9.0

Prepare fresh at room temperature from sterile stock solutions

Protease inhibitor cocktail (PIC), 50× (Basic Protocols 4 and 10)

10 µl leupeptin (0.5 mg/ml in water)

50 µl 1,10-phenanthroline (500 mM in ethanol)

25 µl pepstatin A (1 mg/ml in methanol)

50 µl Pefabloc (100 mM in water)

865 µl sterile water

Mix thoroughly, dispense into 1-ml aliquots, and store several months at -80°C

Dilute 1:50 or 1:25 in the indicated buffers just before use

Protease inhibitor cocktails (PICs), 500× and 1000× (Basic Protocols 1, 2, 3, and Alternate Protocol)

500× PIC-A: Dissolve 5 mg/ml antipain, 1 mg/ml leupeptin, and 1 mg/ml aprotinin in water. Prepare 1-ml aliquots and store several months at -20°C .

500× PIC-B: Dissolve 5 mg/ml pepstatin A and 5 mg/ml chymostatin in methanol. Prepare 1-ml aliquots and store several months at -20°C .

1000× PIC-D: Dissolve 880 mg phenylmethylsulfonyl fluoride (PMSF; final concentration 0.5 M), 10 mg pepstatin A, and 10 mg chymostatin in 10 ml dimethylsulfoxide (DMSO). Prepare ten 1-ml aliquots and store up to several months at -20°C .

1000× PIC-W: Dissolve 1.5 g benzamidine (final concentration 1 M), 131 mg ϵ -aminocaproic acid (final concentration 1 M), 5 mg bestatin, and 5 mg leupeptin in 10 ml water. Prepare ten 1-ml aliquots and store up to several months at -20°C .

Dilute PIC solutions at 1:1000 in the indicated buffers solutions just before use. Add PIC-D below the surface of the solution while rapidly stirring to minimize precipitation.

Protease inhibitor cocktail solutions A

5 mg/ml α -macroglobulin in H_2O (optional; omit if using DTT)

200 mM PMSF in absolute ethanol (200×)

Store a few weeks at -20°C

Protease inhibitor cocktail solutions B

100 mM PMSF in absolute ethanol (100×)

1 mg/ml pepstatin in methanol (1000×)

1 mg/ml leupeptin in water (1000×)

Store a few weeks at -20°C

Protease inhibitor stock solutions C (Basic Protocol 9)

Prepare 100 mM PMSF in absolute ethanol

Prepare 100 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) in absolute ethanol

Prepare 2.5 mg/ml pepstatin A in methanol (1000×)

Store protease inhibitor stocks in aliquots up to several months at -20°C

PSM 1 solution (Basic Protocol 5)

1.6 ml 1 M K_2HPO_4

2.4 ml 1 M KH_2PO_4

0.2 ml 1 M MgCl_2

17.12 g ultrapure sucrose (final concentration 250 mM)

H_2O to 200 ml

Filter sterilize

Store up to several weeks at 4°C

Rich growth medium (Basic Protocol 7)

0.67% (w/v) yeast nitrogen base without amino acids

0.1% (w/v) yeast extract

0.3% (w/v) glucose

Autoclave. Add required amino acids (usually to a final concentration of 20 $\mu\text{g}/\text{ml}$) once medium has cooled. Store up to a few weeks at room temperature.

At least 350 ml of growth medium will be needed per strain.

Semi-synthetic lactate medium (Basic Protocol 6)

In a total volume of 2.5 liters, mix:

15 g yeast extract

2.5 g glucose

2.5 g CaCl₂·2H₂O

2.5 g NaCl

3 g MgCl₂·2H₂O

5 g KH₂PO₄

5 g NH₄Cl

220 ml 90% (w/v) DL-lactic acid

40 g NaOH

Adjust volume to 5 liters with water

Autoclave

Store up to several weeks at room temperature

This medium is best prepared within a few days of use.

1.2 M Sorbitol/phosphate solution (Basic Protocol 7)

50 mM potassium phosphate, pH 7.5 (*APPENDIX 3B*)

1 mM EDTA, pH 7.5

Prepare with sterile stock solutions, autoclave, or filter sterilize

Store several months at 4°C

Sorbitol solution, 1.1 M (Basic Protocol 5)

Dissolve 50 g ultrapure sorbitol in water and dilute to 250 ml. Autoclave. Store up to a few months at 4°C.

Spheroplast medium A, pH 7.5 (Support Protocol)

1× yeast nitrogen base (YNB; Difco)

2% (w/v) glucose

1× amino acids

1 M sorbitol

20 mM Tris·Cl, pH 7.5

Store up to a few weeks at room temperature

Prepare from sterile stock solutions or filter sterilize.

Spheroplast medium B (Support Protocol, optional for other protocols)

1× yeast nitrogen base (YNB; Difco)

2% (w/v) glucose

1× amino acids

1 M sorbitol

Store up to a few weeks at room temperature

Spheroplast recovery medium (Basic Protocol 5, optional)

1 M sorbitol

1% (w/v) Bacto peptone (Difco)

1% (w/v) glucose

0.5% (w/v) Bacto yeast extract (Difco)

20 mM potassium phosphate, pH 6.5 (*APPENDIX 2A*)

Store up to a few weeks at room temperature

Stop solution (Support Protocol, optional for other protocols)

1 M sorbitol
20 mM NaN₃
20 mM NaF

Prepare shortly before use and chill to 4°C

Stop solution contains two energy poisons (NaN₃ and NaF) that are toxic and should be handled with care. The use of NaN₃ and NaF is not appropriate for all purposes (e.g., to isolate organelles for functional studies).

Sucrose gradient buffer (Basic Protocol 5)

50 mM potassium acetate
20 mM HEPES, pH 6.8
2 mM EDTA

Autoclave

Store up to several weeks at room temperature

Sucrose gradient solutions, 1.2 M and 1.5 M in sucrose gradient buffer (Basic Protocol 3)

Stock solutions: Prepare 1.516 M and 1.213 M sucrose solutions in sucrose gradient buffer (see recipe). Filter sterilize. Store up to several weeks at room temperature. Chill prior to use.

Working solutions: Prepare required amounts of 1.5 M and 1.2 M sucrose solutions on day of the experiment by adding 1 M DTT and 100 mM PMSF to a final concentration of 1 mM each (i.e., for 3 ml of 1.5 M sucrose, add 3 µl 1 M DTT and 30 µl 100 mM PMSF).

Sucrose/HEPES gradient solutions, 1.5 M and 1.2 M (Basic Protocol 8)

Prepare 1.516 M and 1.213 M stock sucrose solutions in HEPES lysis buffer (see recipe). Filter sterilize. Store at room temperature. Chill prior to use. Prepare required amounts of 1.5 M and 1.2 M sucrose solutions just before use by adding 1 M DTT and 100 mM PMSF to a final concentration of 1 mM each (i.e., for 3 ml of 1.5 M sucrose, add 3 µl 1 M DTT and 30 µl 100 mM PMSF).

Sucrose solutions for equilibrium gradients (Alternate Protocol)

Prepare the following sucrose solutions by diluting 60% sucrose stock solution (see recipe) with the appropriate volume of HEPES/KAc lysis solution (see recipe): 37%, 34%, 32%, 29%, 27%, 22%, and 10% (w/v) sucrose solutions. Just before use, aliquot the desired volumes of solutions to individual 15-ml tubes. Add 500× protease inhibitor cocktails A and B (PIC-A and PIC-B, see recipes) to a final concentration of 1×. Add 1 mM PMSF and 5 µg/ml α-macroglobulin (final concentrations; protease inhibitor cocktail stock solutions A, see recipe). If desired, add DTT to 1 mM but omit α-macroglobulin.

Alternatively, each solution can be prepared in a manner similar to 60% sucrose stock solution (see recipe).

Sucrose stock solution, 60% (w/v; Alternate Protocol)

60% (w/v) ultrapure sucrose
20 mM HEPES/KOH, pH 6.8
50 mM potassium acetate
1 mM EDTA
1 mM DTT (optional)

Store up to several months at -20°C

Sucrose/imidazole solutions (Basic Protocol 9)

0.4 M sucrose/imidazole solution: 0.4 M sucrose/2 mM EDTA/25 mM imidazole-HCl (pH 7.0)

1.1 M sucrose/imidazole solution: 1.1 M sucrose/2 mM EDTA/25 mM imidazole-HCl (pH 7.0)

1.65 M sucrose/imidazole solution: 1.65 M sucrose/2 mM EDTA/25 mM imidazole-HCl (pH 7.0)

2.25 M sucrose/imidazole solution: 2.25 M sucrose/2 mM EDTA/25 mM imidazole-HCl (pH 7.0)

Store up to several weeks at 4°C

Prechill to 4°C

Add protease inhibitor stock solutions C (see recipe) just before use

TE/SDS solution (Basic Protocol 5, optional)

10 mM Tris·Cl, pH 8 (APPENDIX 2A)

1 mM EDTA

0.1% (w/v) SDS

Store at up to several months at room temperature

100 mM Tris·SO₄, pH 9.4

Solubilize 12.11 g Tris in water. Adjust pH to 9.4 with sulfuric acid (H₂SO₄). Adjust final volume to 1 liter with water. Autoclave or filter sterilize. Store up to several months at room temperature.

TSD reduction buffer

0.1 M Tris sulfate, pH 9.4 (see recipe)

10 mM DTT added just before use

COMMENTARY

Background Information

A range of centrifugation-based subcellular fractionation procedures have been successfully applied to the yeast *Saccharomyces cerevisiae*. This unit includes several analytical procedures that are primarily designed to analyze the distribution of proteins along the exocytic, endocytic, and biosynthetic protein transport pathways (see Basic Protocols 1 to 3; Alternate Protocol). Preparative protocols for the isolation of yeast nuclei, vacuoles, mitochondria, peroxisomes, endoplasmic reticulum, plasma membrane, and cytosol are also presented (see Basic Protocols 4 to 10). In addition, numerous alternative *S. cerevisiae* subcellular fractionation procedures and the corresponding references are listed in Table 3.8.2.

These centrifugation-based fractionation procedures separate organelles and membranes according to their sedimentation velocities and/or buoyant densities. An overview of yeast subcellular fractionation procedures and the key considerations are presented in UNIT 3.7. In addition, several excellent reviews on yeast subcellular fractionation techniques (Walworth et al., 1989; Zinser and Daum, 1995) and on the

theory and practice of centrifugation-based fractionation techniques (Dobrota and Hinton, 1992; Evans, 1992; Hinton and Mullock, 1997) are available.

Most yeast subcellular fractionation procedures begin with spheroplast preparation (i.e., the enzymatic removal of the yeast cell wall) in order to facilitate cell lysis and minimize damage to cellular components (e.g., see Support Protocol). Lysates can also be prepared by agitating intact yeast cells with glass beads. While glass bead lysis works well for the isolation of plasma membrane and cytosol (see Basic Protocols 9 and 10), this technique is not recommended for other fractionation procedures because the strong shear forces required to break the cell wall also damage subcellular components. Once cell lysates are prepared, most protocols then utilize differential centrifugation (e.g., see Basic Protocol 1) in order to enrich the starting material with the components of interest and to remove some of the undesirable components. Further fractionation is achieved by density gradient centrifugation (see Tables 3.8.1 and 3.8.2). Density gradient separation techniques are especially versatile,

since the sedimentation velocities and densities of subcellular components are determined both by their intrinsic properties and the characteristics of the surrounding medium. Thus, the optimal density gradient medium, density range, and shape will vary depending on which components one is trying to separate.

Preparation of yeast spheroplasts using Zymolyase

Most subcellular fractionation procedures begin with preparation of spheroplasts. Standard spheroplast preparation protocols include a reduction step (i.e., with DTT or 2-mercaptoethanol) to break disulfide bonds, followed by incubation with a glucanase enzyme preparation that disrupts the critical β -glucan linkages of the cell wall. Multiple glucanase preparations are commercially available (e.g., Zymolyase, lyticase). In addition, lyticase can be prepared in the laboratory (Scott and Schekman, 1980).

The conditions for optimal cell wall digestion will vary with the strain and growth conditions and thus often need to be determined empirically. The choice of a spheroplast preparation is primarily a matter of preference and expertise of the lab. Zymolyase and oxalyticase are generally preferred since they are highly purified. In contrast, Glusulase is very effective but is much less pure and consequently, contaminating proteases are more of a concern. In Support Protocol, DTT and Zymolyase treatment are used to prepare spheroplasts. Similar methods are used in Basic Protocols 6 and 7. Oxalyticase is used to digest the cell wall in Basic Protocol 4, while a combination of Zymolyase and Glusulase is used in Basic Protocol 5. In all cases, it is wise to monitor the cell wall digestion process by microscopy and/or optical density (OD₆₀₀) (lysis in water due to osmotic shock) as described in the Support Protocol.

Fractionation of spheroplasts by differential centrifugation

Differential centrifugation protocols rapidly fractionate cell lysates into broad size classes through sequential centrifugation steps with each step increasing in force and duration. The relative sedimentation velocities of the subcellular components are primarily determined by their mass; thus larger organelles and membranes will pellet faster and at lower centrifugal forces than smaller organelles. Differential centrifugation is particularly useful to generate fractions that are significantly enriched for the component(s) of interest and thus facilitates the subsequent fractionation

steps. Differential centrifugation is also a convenient way to assess the potential subcellular locations of a protein of interest.

The resolution of the size separation by differential centrifugation is quite low, since the subcellular components are randomly dispersed throughout the medium prior to centrifugation. While the low-speed and medium-speed pellets will primarily consist of relatively large components, they will contain smaller components that were near the bottom of the tube before the centrifugation began.

Equilibrium density gradient fractionation

Basic Protocol 2, Basic Protocol 3, and Alternate Protocol are used to analyze the distribution of one or more proteins among the dynamic compartments of the exocytic, endocytic, and biosynthetic protein transport pathways. The protocols separate these subcellular components into sets of distinct, yet often overlapping fractions.

Basic Protocol 2 was designed to separate vacuolar membranes from endosomal and late Golgi compartments using equilibrium Nycodenz density gradient centrifugation (Rieder and Emr, 1997). The cleared cell lysate is loaded onto gradients, this minimizes the manipulation of lysate and the potential for membrane aggregation. However, abundant cytosolic proteins and unneeded membrane fractions can increase the potential for contamination and can limit the amount of the organelle of interest that can be loaded on a gradient. Thus it is often beneficial to use differential centrifugation (see Basic Protocol 1) to prepare a sample for density gradient fractionation. Pelleting of the membranes allows the removal of cytosolic proteins and can achieve some separation of membrane-bound organelles. A disadvantage to this approach is that it can be difficult to resuspend pelleted membranes well.

The samples can also be loaded onto the bottom of the density gradient if a concentrated Nycodenz stock is added to the sample to increase its density. Singer and co-workers have been able to differentiate between two different endocytic transport intermediates using Nycodenz floatation gradient (Singer and Riezman, 1990). The purification of endosomes, however, has been difficult. Endosome-enriched fractions contain significant levels of other membranes (e.g., ER and Golgi; Singer-Kruger et al., 1993), and the identification of new endosomal proteins using endosome-enriched fractions has remained elusive.

The equilibrium sucrose density gradient centrifugation protocol (Alternate Protocol) is particularly useful to separate vacuolar and endosomal membranes from late Golgi membranes (monitored using mALP, Pep12p, and Vps10p, respectively; see Table 3.7.9); however, the endosomal and vacuolar membranes overlap significantly (Becherer et al., 1996). In contrast, the Nycodenz gradient presented in Basic Protocol 2 can separate vacuolar membranes from endosomal and late Golgi membranes, but in this case the vacuolar and endosomal membranes overlap (Rieder and Emr, 1997). A similar sucrose equilibrium density gradient has been used to separate plasma membranes, ER membranes, and Golgi membranes into distinct sets of fractions (Bowser and Novick, 1991).

Fractionation of P_{13,000} membranes on sucrose step gradients

Basic Protocol 3 was designed to analyze the distribution of one or more proteins among vacuoles, plasma membrane, early Golgi, and ER membranes (Gaynor et al., 1994). The sucrose step gradient is especially useful to determine whether a protein is associated with ER or Golgi compartments. The protocol is very similar to Basic Protocol 8, but on a smaller scale. A continuous sucrose velocity gradient has also been used to obtain discernible differences in the distribution of Golgi, ER, and vacuolar membranes (Antebi and Fink, 1992).

Isolation of intact vacuoles using Ficoll step gradients

Yeast vacuoles are acidic, lysosome-like compartments that occupy ~25% of the cell volume. These single-membrane-bound organelles harbor numerous hydrolases (e.g., proteases, phosphatases, lipases), as well as metabolites (e.g., basic amino acids, polyphosphates, and *S*-adenosylmethionine). Yeast vacuoles are very fragile and are easily damaged during subcellular fractionation experiments. This has two key consequences: (1) it is difficult to isolate intact vacuoles unless very gentle lysis and fractionation techniques are used (see Basic Protocol 4); and (2) the active hydrolases released from ruptured vacuoles can cause significant problems with protein degradation and other damage to subcellular components. Difficulties with protein degradation by vacuolar enzymes can be minimized by using yeast strains with deficient protease activity (e.g., *pep4 prb1* strains; see UNIT 3.7).

Basic Protocol 4 was designed to isolate intact, functional vacuoles. The spheroplasts

are lysed with a dextran-mediated isoosmotic lysis procedure, a more gentle lysis technique that allows the isolation of intact vacuoles. However, even with this technique, some of the vacuoles inevitably rupture. The vacuoles and vacuolar membranes are isolated by floatation on a Ficoll gradient. The buoyant density of vacuoles is due to their relatively low protein density and the association of lipid particles or lipid storage granules with the vacuolar membrane (Zinser and Daum, 1995).

An alternate protocol for the isolation of vacuoles uses an hypoosmotic spheroplast lysis and two sequential Ficoll gradients (Roberts et al., 1991; Uchida et al., 1988). The hypoosmotic lysis procedure disrupts the integrity of the majority of vacuoles, as indicated by the loss of luminal contents and the relatively small size of the isolated vacuoles (Uchida et al., 1988). However, the vacuoles are primarily right-side-out and still functional in several enzyme and transport assays.

Isolation of intact nuclei with Ficoll step gradients

The yeast nucleus is a relatively large (~1 μm), dense organelle that consists of a crescent-shaped nucleolus, chromatin, RNA, and numerous nuclear proteins surrounded by the nuclear envelope. The nuclear envelope is composed of two membrane bilayers that are interconnected at the nuclear pores. The outer membrane is continuous with portions of the rough ER (RER); indeed the nuclear envelope represents ~30% of the functional RER. Thus it is not possible to completely separate ER membranes and nuclei.

Basic Protocol 5 was designed to isolate intact nuclei from yeast spheroplasts using Ficoll step gradients (Dove et al., 1998). Nuclei have also been isolated using Ficoll-glycerol gradients and sucrose gradients (see Table 3.8.2). For example, Kalinich and co-workers developed a protocol that includes cytochalasin B treatment to minimize the interactions between nucleus and the cytoskeleton, followed by glycerol/Ficoll-glycerol step gradients to isolate the nuclei (Kalinich and Douglas, 1989).

Isolation of lactate-induced mitochondria using Nycodenz step gradients

Mitochondria are double-membrane-bound organelles that play a key role in eukaryotic cell metabolism. The enzymes of the electron transport and oxidative phosphorylation pathways are localized to mitochondria, which also contain mitochondrial DNA and the enzymes re-

quired for its replication, transcription, and translation. Growth of yeast on non-fermentable carbon sources such as lactate allows for optimal development of mitochondria. In contrast, the proliferation of mitochondria is repressed under anaerobic conditions, and the formation of pro-mitochondria is observed instead (Pon et al., 1989).

Basic Protocol 6 was designed to isolate intact mitochondria from lactate-grown D273-10B cells on Nycodenz step gradients. (Glick and Pon, 1995). If using cells other than lactate-grown D273-10B cells, it may be necessary to modify the Nycodenz density gradient procedure, as mitochondrial characteristics can vary between yeast strains. In addition, density of the mitochondria can vary significantly depending on growth conditions of the cells, such as the carbon source, temperature, and growth phase. A continuous Nycodenz gradient fractionation procedure to empirically determine the optimal Nycodenz concentrations for a step density gradient is presented by Glick and Pon (1995). Alternatively, mitochondria can be purified using Percoll gradients (Yaffe, 1991), as well as other gradients (see Table 3.8.2). Isolated mitochondria can be fractionated further into outer membrane, inner membrane, intermembrane space and matrix fractions (see Table 3.8.2).

Isolation of oleate-induced peroxisomes using sucrose step gradients

Peroxisomes are single-membrane-bound organelles that range from 0.1 to 1 μm in diameter. The enzymes of the β oxidation and glyoxylate pathways are localized to peroxisomes, as well as enzymes that generate and degrade hydrogen peroxide. The abundance, size, and protein composition of peroxisomes can vary dramatically in response to the cells' environment. When *S. cerevisiae* are grown under standard conditions on a fermentable carbon source (e.g., glucose), peroxisomes and their proteins are barely detectable. In contrast, when *S. cerevisiae* are cultured on fatty acids (e.g., oleate), the expression of peroxisomal proteins is induced and the peroxisomes proliferate and increase in size (Veenhuis et al., 1987).

Basic Protocol 7 was designed to purify intact peroxisomes from D273-10B cells (Distel et al., 1996). The peroxisomes are induced by growth on oleate medium and purified on sucrose step gradients. Alternatively, peroxisomes can be purified on continuous Nycodenz gradients, Nycodenz step gradients, or

continuous sucrose gradients (see Table 3.8.2). Some researchers have found that peroxisomes purified on continuous Nycodenz gradients have slightly higher activities and lower levels of contamination with mitochondria than peroxisomes purified on sucrose gradients (Kunau et al., 1993). If an especially pure peroxisome preparation is required, peroxisomes can be purified on two sequential gradients, a sucrose gradient followed by a Nycodenz/sucrose gradient (Erdmann and Blobel, 1995).

Isolation of endoplasmic reticulum using sucrose step gradients

The endoplasmic reticulum (ER) is a complex network of compartments that plays a key role in the transport, folding, and post-translational modification of proteins, as well as in lipid metabolism and several other processes. ER compartments cannot be isolated in a completely intact state, and thus ER preparations primarily consist of ER membrane vesicles, often referred to as microsomes. Basic Protocol 8 was developed to isolate ER membranes for an in vitro protein transport assay (Wuestehube and Schekman, 1992). The protocol is very similar to Basic Protocol 3, but on a larger scale. Rough ER membrane vesicles can be separated from smooth ER vesicles using density gradients, since the ribosomes associated with the rough ER membranes increase their density relative to smooth ER membranes (Sanderson and Meyer, 1991).

Isolation of plasma membranes from whole yeast cells using sucrose step gradients

The plasma membrane forms a selective barrier between the cell's surroundings and its interior. Plasma membrane contains many signaling molecules, as well as transport proteins and channels that move molecules in and out of cells. The plasma membrane is very dynamic, due to the fast growth and division rates of yeast and the continuous exocytic and endocytic transport processes. Like ER membranes, plasma membrane preparations consist of vesicles and sheets.

Basic Protocol 9 provides a method to isolate plasma membranes from intact yeast cells after glass bead lysis (Panaretou and Piper, 1996). The use of intact yeast cells instead of spheroplasts has several benefits beyond ease and speed of the technique. Plasma membranes isolated from glass-bead cell lysates tend to have lower levels of ER contamination. In addition, Zymolyase and other β -glucanase preparations are contaminated with low levels

of proteases that could damage cell surface proteins. Indeed, plasma membrane preparations from spheroplasts have a slightly lower density than those prepared from whole cells.

Plasma membranes can also be successfully prepared from yeast spheroplasts, which are osmotically lysed and subjected to density gradient fractionation (see Table 3.8.2). Several of these procedures specifically alter the sedimentation properties of the plasma membrane. For example, a procedure using cationic silica beads that specifically bind to the plasma membrane is well suited for large-scale isolation of plasma membranes (Panaretou and Piper, 1996). In addition, researchers have used the lectin concanavalin A to facilitate the separation of plasma membrane from other cellular components (e.g., Ziman et al., 1996). Concanavalin A interacts with the glycoproteins found on the cell surface, thereby increasing the plasma membrane density and also minimizing its vesiculation.

Preparation of cytosol from whole yeast cells

The preparation of yeast cytosol is critical to the success of *in vitro* functional assays. Basic Protocol 10 is a simple and efficient method to prepare cytosol from yeast cells lysed by agitation with glass beads (Haas, 1995). The resulting lysate is subjected to high-speed centrifugation to separate the cytosol from cell wall fragments and membrane components. This cytosol preparation supports an *in vitro* vacuole-vacuole fusion assay and is likely to support other functional assays as well (Haas, 1995). Cytosol is also frequently prepared from spheroplast lysates (see Table 3.8.2). In addition, a large number of intact yeast cells can be processed by freezing them in liquid nitrogen and then disrupting the cells in a blender or with a mortar and pestle (see Table 3.8.2). Small molecules, such as residual ATP or ions, can be removed from cytosol by passing it over a Sephadex G-25 column (e.g., Wuestehube and Schekman, 1992).

Critical Parameters and Troubleshooting

Subcellular fractionation experiments share several key steps, including, (1) cell growth under appropriate conditions; (2) preparation of cell lysate with minimal damage to subcellular components; (3) selection of a fractionation protocol that separates subcellular components of interest into distinct sets of fractions; and (4) identification and characterization of the membranes/organelles in the resulting frac-

tions. Table 3.8.3 summarizes some of the important factors that are relevant to most subcellular fractionation experiments. Many of these points are discussed in more detail in *UNIT 3.7*. Table 3.8.4 provides a summary of problems that can arise during subcellular fractionation experiments and suggestions for potential solutions. Some key steps that are especially important for the preparative protocols presented in this unit are summarized below.

Isolation of intact vacuoles

In Basic Protocol 4, vacuoles are isolated from spheroplast lysates prepared using a dextran-mediated isoosmotic lysis technique. This lysis procedure is more gentle than hypoosmotic lysis, leaving more of the vacuoles intact. The timing and temperature shifts are critical to the success of this lysis technique. It is important to use glass tubes, since they allow for more rapid temperature changes than plastic tubes. After adding the freshly prepared dextran and swirling to mix, the tubes are placed on ice for 2 min to allow the dextran to bind to the plasma membrane. The optimal amount of dextran for each strain needs to be determined empirically. The cell membrane is disrupted by placing tubes in a 30°C water bath for 60 to 75 sec and the tubes are then immediately placed on ice again. This heat-shock step should not be repeated.

After Ficoll gradient centrifugation, it is important to collect the vacuoles without harvesting the Ficoll solution. Ficoll can be difficult to remove and interferes with many subsequent analyses. If necessary, the Ficoll can be removed by dilution and centrifugation as described in Basic Protocol 5.

Isolation of intact nuclei

For optimal results, the cell cultures need to be in their early log growth phase. The extensive incubation time with glucanase preparations is important to maximize yields. The digestion should be continued until the cells are round and the clumped cells have separated. The lysis buffer should be at room temperature, not 4°C as in most other protocols. Lysis at low temperature will reduce the yield by 50%. However, after the lysis step, the preparation should be kept at 4°C. If the nuclei will be used for functional experiments, it is critical to allow the spheroplasts to recover from the glucanase treatment by incubating in spheroplast recovery medium (or another rich medium supplemented with 1.2 M sorbitol). Optimal conditions for the recovery often need to be determined empirically. For most purposes (e.g.,

Table 3.8.2 Subcellular Fractionation Protocols for *Saccharomyces cerevisiae*

Focus	Primary techniques	Selected references ^a
<i>Endocytic and secretory pathways</i>		
	Sucrose velocity gradient	Antebi and Fink (1992) <i>MBC</i> 3:633; Schimmoller et al. (1995) <i>EMBO J.</i> 14:1329; Schroder et al. (1995) <i>JCB</i> 131:895
	Sucrose equilibrium gradient ^b	Bowser and Novick (1991) <i>JCB</i> 112:1117; Becherer et al. (1996) <i>MBC</i> 7:579
	Sucrose equilibrium gradient, ConA treatment	Chuang and Schekman (1996) <i>JCB</i> 135:597; Ziman et al. (1996) <i>MBC</i> 7:1909; Ziman et al. (1998) <i>MBC</i> 9:1565
	Nycodenz equilibrium gradient ^b	Rieder and Emr (1997) <i>MBC</i> 8:2307
	Nycodenz equilibrium floatation gradient	Singer and Riezman (1990) <i>JCB</i> 110:1911
	Sucrose/D ₂ O step gradient	Singer-Kruger et al. (1993) <i>JBC</i> 268:14376
	Sucrose 2-step gradient ^b	Dean and Pelham (1990) <i>JCB</i> 111:369; Gaynor et al. (1994) <i>JCB</i> 127:653
<i>Organelle isolation</i>		
Nucleus	Sucrose step gradient	Strambio-de-Castillia et al. (1995) <i>JCB</i> 131:19
	Sucrose step gradient ^b	Aris and Blobel (1991) <i>Methods Enzymol.</i> 194:735; Dove et al. (1998) <i>Methods Cell Biol.</i> 53:33; Mann and Mecke (1980) <i>BBA</i> 687:57
	Ficoll/glycerol gradient	Kalinich and Douglas (1989) <i>JBC</i> 264:17979; Chang et al. (1999) <i>Methods Enzymol.</i> 304:76
Nuclear envelope	PVP-sucrose step gradient	Strambio-de-Castillia et al. (1995) <i>JCB</i> 131:19
	Ficoll-sucrose gradient	Mann and Mecke (1982) <i>BBA</i> 687:57; Hurt et al. (1988) <i>Eur. JCB</i> 46:554
Endoplasmic reticulum	Sucrose 2-step gradient ^b	Wuestehube and Schekman (1992) <i>Methods Enzymol.</i> 219:124
	Sucrose multistep gradient	Zinser et al. (1991) <i>J. Bacteriol.</i> 173:2026
	Continuous sucrose gradient (RER and SER)	Sanderson and Meyer (1991) <i>JBC</i> 266:13423
Golgi	Sorbitol gradient	Cleves et al. (1991) <i>Cell</i> 64:789; McGee et al. (1994) <i>J. Bacteriol.</i> 176:6861
	Sucrose/D ₂ O step gradient	Lupashin et al. (1996) <i>JCB</i> 132:277
Vacuoles	Hypoosmotic lysis, two Ficoll step gradients	Uchida et al. (1988) <i>Methods Enzymol.</i> 157:544; Roberts et al. (1991) <i>Methods Enzymol.</i> 194:644
	Isoosmotic lysis, Ficoll step gradient ^b	Haas (1995) <i>Methods Cell Sci.</i> 17:283; Mayer et al. (1996) <i>Cell</i> 85:83
Intravacuolar vesicles	Iodixinol gradient (Optiprep)	Harding et al. (1995) <i>JCB</i> 131:591; Oda et al. (1996) <i>JCB</i> 132:999

continued

Table 3.8.2 Subcellular Fractionation Protocols for *Saccharomyces cerevisiae*, continued

Focus	Primary techniques	Selected references ^a
Mitochondria	Self-forming Percoll gradient Nycodenz step gradients ^b	Yaffe (1991) <i>Methods Enzymol.</i> 194:627 Glick and Pon (1995) <i>Methods Enzymol.</i> 260:213
	Sucrose gradient	Daum et al. (1982) <i>JBC</i> 257:13028; Zinser et al. (1991) <i>J. Bacteriol.</i> 173:2026; Poyton et al. (1995) <i>Methods Enzymol.</i> 260:97; Martin et al. (1998) <i>Anal. Biochem.</i> 265:123
Mitochondrial membranes	Sucrose step gradients (inner membrane)	Jascur (1991) <i>Methods Cell Biol.</i> 34:359
	Continuous sucrose gradients (outer membrane and contact sites)	Pon et al. (1989) <i>JCB</i> 109:2604; Simbeni et al. (1991) <i>JBC</i> 266:10047
	Sucrose step gradients (outer membrane)	Alconada et al. (1995) <i>Methods Enzymol.</i> 260:263
Peroxisomes	Nycodenz/sucrose gradient	Thieringer and Kunau (1991) <i>JBC</i> 266:13110; Kunau et al. (1993) <i>Biochimie</i> 75:209; Erdmann and Blobel (1995) <i>JCB</i> 128:509
	Sucrose step gradient ^b	McCammon et al. (1990) <i>J. Bacteriol.</i> 172:5816; Distel et al. (1996) <i>Methods Mol. Biol.</i> 53:133
	Nycodenz gradient	Lewin et al. (1990) <i>MCB</i> 10:1399
Plasma membrane	Glass bead lysis, sucrose step gradient ^b	Serrano (1988) <i>Methods Enzymol.</i> 157:533; Panaretou and Piper (1996) <i>Methods Mol. Biol.</i> 53:117.
	Cationic bead isolation from spheroplasts	Schmidt et al. (1983) <i>BBA</i> 732:421; Panaretou and Piper (1996) <i>Methods Mol. Biol.</i> 53:117
Post-Golgi secretory vesicles ^c	Sephacryl S-1000	Walworth and Novick (1987) <i>JCB</i> 105:163
	Sephacryl S-1000, electrophoresis	Holcomb et al. (1987) <i>Anal. Biochem.</i> 166:328
ER-to-Golgi vesicles	Sephacryl S-1000, Nycodenz gradients	Harsay and Bretscher (1995) <i>JCB</i> 131:297
	Permeabilized cells, affinity-isolation	Groesch et al. (1990) <i>JCB</i> 111:45; Groesch et al. (1992) <i>Methods Enzymol.</i> 219:137
	Purified membrane and COPII components	Barlowe et al. (1994) <i>Cell</i> 77:895; Yeung et al. (1995) <i>JBC</i> 270:30567
Clathrin-coated vesicles	Permeabilized <i>ypt1^{ts}</i> cells ^c , affinity-isolation	Rexach et al. (1994) <i>JCB</i> 126:1133
	Sephacryl S-1000 column	Mueller and Branton (1984) <i>JCB</i> 98:341
Cell lysates/cytosol	Glass bead lysis of whole cells ^b	Wuestehube and Schekman (1992) <i>Methods Enzymol.</i> 219:124; Haas (1995) <i>Methods Cell Sci.</i> 17:283
	Osmotic lysis of spheroplasts	Cheng et al. (1990) <i>Methods Enzymol.</i> 181:89; Hartley et al. (1996) <i>Methods Mol. Biol.</i> 53:249; Schultz (1999) <i>Methods</i> 17:161
	Disruption of whole cells frozen in liquid nitrogen	Dunn and Wobbe (1993) <i>CPMB UNIT 13.13</i>

^aAbbreviations: *BBA*, Biochimica Biophysica Acta; *CPMB*, Current Protocols in Molecular Biology; *Eur JCB*, European Journal of Cell Biology; *JBC*, Journal of Biological Chemistry; *JCB*, Journal of Cell Biology; *MBC*, Molecular Biology of the Cell; *MCB*, Molecular Cell Biology.

^bThese types of protocols are included in this unit.

^cVesicles are isolated from certain temperature-sensitive mutant yeast strains that accumulate vesicles under nonpermissive conditions.

Table 3.8.3 Critical Parameters Common to Most Subcellular Fractionation Experiments

Procedure	Critical parameters
Strain selection and growth	<p>The use of a vacuolar protease deficient strain can minimize proteolysis</p> <p>If available and compatible with goals of the experiment, use the strain(s) that the protocol was optimized for, as results can vary with strain and growth conditions</p> <p>Inoculate the culture with a single colony from a fresh plate (ideally prepared from frozen stock within the past week)</p> <p>Determine the correlation between the growth phase, OD₆₀₀ value, and viable cell number for the strains, growth conditions, and spectrophotometer in use</p> <p>To generate a homogeneous, active cell population, culture the cells so that they are in their exponential growth phase at least 16 to 24 hr</p> <p>For the purification of peroxisomes and mitochondria, preculture cells in the appropriate induction medium prior to harvesting (i.e., oleate or lactate medium)</p> <p>Harvest cells in their mid-logarithmic growth phase (unless otherwise indicated)</p>
Reagents and equipment	<p>Use Ultrapure (or equivalent) reagents and double-distilled water for all solutions</p> <p>Be sure that glassware is clean and completely free of detergents</p> <p>Prepare reagents and solutions ahead of time (unless otherwise indicated)</p> <p>Bring reagents to the appropriate temperature in advance</p> <p>Label tubes ahead of time to save time and minimize mistakes</p> <p>Reserve critical equipment and prechill as needed</p>
Spheroplast preparation	<p>Start with a homogeneous population of dividing cells (unless otherwise indicated)</p> <p>Optimal conditions may need to be determined for each strain and growth condition in a small-scale pilot experiment</p> <p>Pretreat cells with DTT or 2-ME to break disulfide bonds within the cell wall</p> <p>Provide sufficient osmotic support (usually with 1 to 1.5 M sorbitol)</p> <p>Digest cell wall with Zymolyase 100T or another glucanase preparation</p> <p>Handle the spheroplasts gently (e.g., do not vortex)</p> <p>Monitor cell wall digestion by microscopy or lability in water (Support Protocol)</p> <p>Wash spheroplasts to remove residual glucanase and contaminating enzymes before continuing to the cell lysis step</p>
Glass bead cell lysis	<p>Use acid-washed beads of ~0.45 mm diameter</p> <p>Prechill beads to 4°C</p> <p>Resuspend the cell pellet in buffer before adding glass beads</p> <p>Use ~1:1 ratio of cell suspension to bead volume</p> <p>Cool the lysate every 30 to 45 sec in an ice-water bath</p> <p>Minimize foaming. If agitating the sample with a vortexer, be aware of how speed, pressure, and angle affect foaming</p> <p>Monitor lysis by microscopy (and methylene blue treatment)</p> <p>A Bead Beater is recommended for larger volumes (2 to 200 ml) as there is better heat dissipation and less foaming</p>

continued

Table 3.8.3 Critical Parameters Common to Most Subcellular Fractionation Experiments, continued

Procedure	Critical parameters
Lysate preparation	<p>Keep samples at 0° to 4°C (unless otherwise indicated)</p> <p>Add fresh protease inhibitors to solutions just before use</p> <p>Use a buffer with the appropriate composition and osmotic support (may need to be determined empirically)</p> <p>Use a homogenization technique that balances the need for cell lysis and the need for intact subcellular components</p> <p>Minimize foaming</p> <p>Handle the lysate gently as many subcellular components are fragile (e.g., avoid narrow pipet tips, vigorous pipetting, mixing with a vortexer)</p>
Fractionation	<p>Set aside samples at each step in order to monitor the recovery levels and marker distribution at each step</p> <p>Work as quickly, carefully, and consistently as possible</p> <p>Keep samples at 0° to 4°C (unless otherwise indicated)</p>
Gradient preparation	<p>Test whether gradient medium will disrupt protein interactions of interest</p> <p>Prepare mock gradients and measure their densities to ensure accuracy and reproducibility of gradient preparation</p> <p>Chill the gradients to 4°C before using</p> <p>Prepare step gradients just before use so that interface is sharp</p> <p>Use the slow acceleration and deceleration rate settings. To minimize mixing of layers, allow the rotor to coast to a stop after slowing to 1000 rpm</p>
Gradient harvesting	<p>Note any bands, pellets, and other characteristics before unloading the gradient</p> <p>Save all fractions until confident that they are not needed</p> <p>Save a small amount of each fraction to test its density</p> <p>When unloading one gradient, store the others at 4°C in a safe place</p> <p>If harvesting with a pipettor, change the pipet tip with every fraction</p> <p>If the organelles need to be intact, handle the fractions gently (e.g., avoid narrow pipet tips, vigorous pipetting, mixing with a vortexer)</p> <p>Work as quickly, carefully, and consistently as possible</p>
Analysis of fractions	<p>Determine the distribution and enrichment factors of multiple markers (see Tables 3.7.8 and 3.7.9)</p> <p>Remove gradient medium if it will interfere with subsequent analyses (e.g., by centrifugation)</p> <p>If the organelles need to be intact, handle the fractions gently (e.g., avoid narrow pipet tips, vigorous pipetting, mixing with vortexing)</p> <p>Remember that colocalization of subcellular components in a given fraction does not necessarily mean they colocalize in vivo</p> <p>Optional: examine the fractions for organelle integrity and/or function</p> <p>Ideally, confirm results and interpretations using an alternate technique</p>

Table 3.8.4 Troubleshooting Yeast Fractionation

Problem	Potential causes	Potential solutions ^a
Inefficient cell wall digestion	Presence of older and/or stationary-phase cells (which have thicker cell walls)	Generate growth curves for strains and conditions being used Dilute or restart cultures and allow cells to grow for several generations until the desired growth phase is reached
	Bacterial contamination	Check for contamination by light microscopy If necessary, start a new yeast culture with sterile reagents
	Insufficient enzyme activity levels or incubation times	Purchase and/or prepare a fresh enzyme stock if required Determine the optimal conditions in small-scale experiments (e.g., enzyme concentration, duration of treatment with reducing agent, duration of digestion)
	The use of strains with especially thick, strong cell walls (e.g., mutant cells, cells approaching stationary growth phase)	Determine the optimal conditions in small-scale experiments
Inefficient cell lysis (spheroplasts)	Inefficient cell wall digestion	See above
	Insufficient mechanical force to disrupt spheroplasts and/or too much osmotic support	Increase the homogenization intensity and/or duration (e.g., tighter-fitting pestle; more strokes; different lysis technique; buffer with less osmotic support). However, these steps will tend to increase damage to intracellular components
Inefficient cell lysis (whole cells)	Glass beads not of proper size	Glass beads for cell lysis should be 0.45 to 0.55 mm in diameter
	Cells not resuspended before the addition of the glass beads	Resuspend the cell pellet in the lysis buffer before adding glass beads
	Excessive foaming	Determine the optimal conditions with test samples. Changing the angle of the tube or the vortex used can make a difference
	Insufficient intensity or duration of agitation	Increase the duration of agitation (with intermittent chilling on ice). Determine the optimal conditions with test samples
	Glass bead to cell ratio not optimal	Usually a 1:1 ratio of cell suspension to glass beads works well. The cell suspension is ~1:1 cells to buffer
	Presence of older and/or stationary-phase cells (which have thicker cell walls)	Generate growth curves for strains and conditions being used Dilute or restart cultures and allow cells to grow for several generations until the desired growth phase is reached
	The use of strains with especially thick, strong cell walls (e.g., mutant cells, cells in stationary growth phase)	Determine the optimal conditions in small-scale experiments

continued

Table 3.8.4 Troubleshooting Yeast Fractionation, continued

Problem	Potential causes	Potential solutions ^a
Proteolysis	Exposure to temperatures >4°C after cell lysis	Keep samples at 0° to 4°C as much as possible. Prechill all solutions and equipment to 0° to 4°C. Use ice and ice-water baths as possible
	Inadequate levels or specificity range of protease inhibitors	Increase levels or change composition of protease inhibitor cocktail Add inhibitor cocktail to all solutions during and after cell lysis Add PMSF more than once (as it has a short half-life in aqueous solutions) Prepare fresh protease inhibitor stocks (if older or improperly stored)
	Extended exposure to hydrolytic enzymes	Work as quickly as possible and keep samples ice cold Use a vacuolar protease-deficient strain (e.g., <i>pep4 prb1</i>) Minimize vacuole lysis (especially important with <i>PEP4</i> strains) by using a more gentle homogenization technique, increasing osmotic support in the lysis buffer, and/or treating samples gently throughout the experiment Separate components of interest from proteases as soon as possible (e.g., limit exposure to cytosol containing released vacuolar enzymes)
Poor separation on density gradients	Gradients overloaded	Decrease sample amount loaded onto gradients
	Membrane aggregation Inadequate cell lysis	See below See above
	Gradient density medium, density range and/or shape are not ideal for the application	Determine more optimal conditions in small-scale experiments
Membrane aggregation	Insufficient resuspension of pelleted membranes	Pellet membranes onto a cushion (instead of against tube wall) Minimize the number and/or intensity of pelleting steps Increase intensity and/or duration of resuspension step (e.g., resuspend membranes in a small Dounce homogenizer or by passage through a small pipet tip or needle) Allow resuspended membranes to sit on ice in buffer
		Too much material Omission of a clearing step

*continued***3.8.61**

Table 3.8.4 Troubleshooting Yeast Fractionation, continued

Problem	Potential causes	Potential solutions ^a
Damage to subcellular components	Buffer composition not optimal	Buffer composition can contribute to aggregation (e.g., high Mg ²⁺ levels). Aggregation can be minimized to some extent with the addition of 1-2 mM DTT or 2-ME. EDTA can also be helpful
	Insufficient homogenization	Increase the homogenization intensity and/or duration (e.g., tighter-fitting pestle; more strokes; different lysis technique; buffer with less osmotic support). However, these steps will tend to increase damage to intracellular components
	Release of DNA from nuclei	Minimize damage to nuclei by handling samples gently (see below) If required, add DNase to 10 µg/ml
	Insufficient osmotic support or rapid change in osmotic support	Increase osmotic support. Avoid rapid buffer changes
	High levels of proteolysis	See above
	Loss of peripheral membrane proteins and/or disruption of protein complexes	Use an alternate buffer or gradient medium. Some buffers and gradient media can destabilize protein interactions. Determine the optimal conditions in small-scale experiments
Low recovery of proteins and/or enzyme activities	Repeated pipetting, pelleting, and/or resuspension	Pellet membranes onto a cushion (instead of the tube wall) Handle suspension gently (e.g., cut pipet tip to enlarge the opening, initially resuspend by gently tapping the tube, pipet slowly, etc.) Minimize pelleting steps required
	Homogenization technique not gentle enough	Avoid cell lysis with agitated glass beads Use a more gentle method to lyse and homogenize spheroplasts (e.g., DEAE-facilitated isoosmotic lysis, a Dounce homogenizer with greater clearance) Monitor the appearance of the lysate with a light microscope
	Exposure to active hydrolases	See above
Low recovery of membranes	Damage to membrane integrity and loss of luminal contents	See above
	Loss of peripheral membrane proteins and/or disruption of protein complexes	Use an alternate buffer or gradient medium. Some buffers and gradient media can destabilize protein interactions
	Gradient media may interfere with enzymatic assays and/or liquid scintillation assays	Remove gradient media from fractions with an appropriate technique (e.g., centrifugation, dialysis). If necessary, choose an alternative gradient medium
	Low recovery of membranes	See below

continued

Table 3.8.4 Troubleshooting Yeast Fractionation, continued

Problem	Potential causes	Potential solutions ^a
Low recovery of membranes	Inefficient cell lysis	See above
	Inadequate homogenization	Increase the homogenization intensity and/or duration (e.g., tighter-fitting pestle; more strokes; different lysis technique; buffer with less osmotic support). However, these steps will tend to increase damage to intracellular components Repeat homogenization of low-speed pellet
	Membrane aggregation	See above
	Poor recovery of spheroplasts due to premature spheroplast lysis	Increase osmotic support (up to 1.5 M sorbitol) Handle spheroplasts gently (e.g., pellet spheroplasts onto a cushion or through a cushion, cut pipet tips to increase bore size, resuspend cells by swirling, pipet very slowly and gently, do not vortex)

^aThese potential solutions are not compatible with the goals of all experiments; thus they are only recommended if there are no conflicts with subsequent steps and the desired final result. In most cases, the problems cannot be entirely eliminated. Furthermore, pilot experiments may be needed to determine to optimum conditions for a particular purpose.

TCA protein precipitation, SDS-PAGE analysis, functional assays), Ficoll will need to be removed from the harvested nuclei.

Isolation of lactate-induced mitochondria

Basic Protocol 6 was optimized for lactate-grown D273-10B cells. If using cells other than lactate-grown D273-10B cells, it may be necessary to modify the Nycodenz density gradient procedure (Glick and Pon, 1995). The density of the mitochondria can vary significantly with the strain, growth conditions of the cells—e.g., carbon source, temperature—and growth phase.

Good aeration is critical for optimal cell growth and proliferation of mitochondria. A recovery period after cell wall removal is essential for optimal mitochondrial transcription/translation activity. It is critical that high-quality deionized sorbitol be used in the reagents, since lower quality grades contain trace metal contaminants that can damage mitochondria.

Isolation of oleate-induced peroxisomes

The isolation of intact peroxisomes from *S. cerevisiae* is more difficult than from other yeast strains (e.g., *Pichia*, *Candida*). In *S. cerevisiae*, the peroxisomes do not proliferate as readily and are especially fragile. For optimal peroxisome induction, cells must be dividing

rapidly prior to the shift to oleate medium. Thus, extended preculturing (i.e., 2 to 3 days) is recommended in order to generate a relatively homogeneous population of healthy, rapidly-dividing cells that will respond well when transferred to the oleate medium. The cells should then be harvested 12 to 18 hr after the shift to induction medium for optimal results. If working with a strain other than D273-10B, it is wise to do a pilot experiment and monitor peroxisome induction using peroxisomal enzyme activity assays and electron microscopy.

In order to isolate intact peroxisomes from *S. cerevisiae*, it is critical that all buffers used during the isolation procedure be adjusted to a low pH (5.5 to 6.0; Thieringer and Kunau, 1991). The sucrose step gradients need to be prepared shortly before use and must have a sharp interface between the sucrose layers. Since peroxisomes are especially fragile, the density gradients used for the purification of peroxisomes are often centrifuged in a vertical rotor to decrease the required centrifugation time.

Isolation of endoplasmic reticulum

In order to maximize yield, the pellet from the first 1000 × *g* clearing spin should be homogenized a second time. This second homogenization step can improve the yield of intracellular membranes by ~30%. The sucrose step

gradients need to be prepared shortly before use and must have a sharp interface between the sucrose layers.

Isolation of plasma membranes from whole yeast cells

The plasma membranes are isolated from yeast cells after glass bead lysis. For efficient cell lysis, the ratio of glass beads to cell suspension is important. It is also critical to keep the lysate cold by alternating between vortexing and incubation in an ice-water bath. Ice can be used instead of an ice-water bath; however, an ice-water bath will cool the lysate more efficiently. The step gradients need to be prepared shortly before use and must have a sharp interface between the sucrose layers.

Preparation of cytosol from whole yeast cells

In Basic Protocol 10, cytosol is isolated from yeast cells cultured to an OD₆₀₀ of 1 to 2. Cytosol can also be prepared from yeast cell cultures with a higher cell density (OD₆₀₀ of 2 to 6); however, these cytosol preparations tend to have lower activity levels as determined by *in vitro* vacuolar fusion assays (Haas, 1995). For efficient cell lysis, the ratio of glass beads to cell suspension is important. It is also critical to keep the lysate cold by alternating between vortexing and incubation in an ice-water bath. The tubes need to be sealed tightly with Parafilm. The use of glass tubes allows the lysate to be cooled more rapidly.

A high-speed centrifugation step is used to separate cytosol from cell wall fragments, cellular membranes, and larger protein complexes and assemblies. The lipid layer that forms on top of each sample needs to be carefully removed and discarded. The supernatant should then be transferred to a prechilled microfuge tube with a clean pipet, being careful not to include any of the pelleted material.

Use of protease inhibitors

It is critical to include protease inhibitors in all solutions for every step during and after cell lysis. In most cases a number of different inhibitors are included to ensure that a broad range of protease types are inactivated. The protocols described in this unit require a number of different protease combinations that have been recommended by the authors of published methods. Other combinations may be equally or even more appropriate for a particular application. Also convenient premixed protease inhibitor cocktails are commercially available (e.g., Boehringer-Mannheim, Sigma).

Anticipated Results

The unique protein compositions, enzymatic activities, and morphologies of subcellular organelles can be used to follow their distribution among the resulting fractions and analyze the success of an experiment. The most common method used to monitor the distribution and integrity of particular subcellular components is to analyze the distribution of characteristic marker proteins and enzyme activities (see UNIT 3.7, see Tables 3.7.7 and 3.7.8). The fractionated subcellular components can also be characterized by examining their morphologies, functions, and overall protein compositions. In order to establish the purity and yield of an organelle preparation or the success of an analytical fractionation experiment, it is important to determine the levels of the components of interest, as well as the levels of the potential contaminants.

The fractionation of subcellular components is never perfect or complete. Centrifugation-based fractionation procedures are limited by the fact that different organelle or membrane populations can have similar sizes and/or densities. In addition, members of a given organelle population vary both in mass and density to some degree. Both of these complications are exacerbated by the additional fragmentation caused by the lysate preparation and fractionation procedures.

Fractionation of spheroplasts by differential centrifugation

Basic Protocol 1 is a typical differential centrifugation method used to separate subcellular components into three membrane pellet fractions and a cytosol fraction. A schematic is presented in Figure 3.7.1. First, yeast cell lysates are subjected to a low centrifugal force (e.g., 300 × *g*) to pellet any unlysed cells and large aggregates. The lysate (S₃₀₀) is then subjected to sequential centrifugation at 13,000 × *g* and 100,000 × *g* to generate P_{13,000} and P_{100,000} pellets, respectively. The distribution of subcellular components in the resulting fractions is summarized in Table 3.7.4. The P_{13,000} primarily contains large membrane structures, such as vacuolar membranes, plasma membrane, endoplasmic reticulum, mitochondria, and nuclei, while the P_{100,000} fraction contains Golgi membranes and transport vesicles (Marcusson et al., 1994). Endosomal membranes containing the syntaxin-homolog Pep12p is distributed between the P_{13,000} and P_{100,000} pellets (Becherer et al., 1996). Soluble cytoplasmic proteins, soluble proteins released from the lumen of dam-

aged organelles, and released peripheral membrane proteins are found in the $S_{100,000}$ fraction. The P_{300} pellet will contain significant amounts of the larger organelles (e.g., plasma membrane, endoplasmic reticulum). The P_{300} and $P_{13,000}$ pellets will contain some smaller organelles that happen to be close to the bottom of the centrifuge tube or that are trapped within partially lysed cells and larger membrane structures. Some of these smaller organelles found in low-speed pellets can be removed by resuspending the pellet in fresh medium and repeating the centrifugation step.

Preparation of yeast spheroplasts

The optimal conditions for digesting the cell wall will vary with the strains, growth conditions, and glucanase enzyme preparation. It is wise to monitor the digestion process by microscopy or lability in water (Support Protocol). For most applications, at least 95% of the cells should be converted to spheroplasts within 20 to 40 min.

Equilibrium density gradient fractionation using Nycodenz

Basic Protocol 2 is particularly useful to separate vacuolar and endosomal membranes from late Golgi membranes (monitored using mALP, Pep12p, and Vps10p, respectively; see Table 3.7.9); however, the endosomal and late Golgi membranes overlap significantly (Rieder and Emr, 1997). The vacuolar membranes migrate to the top region of the gradient, while the endosomal and Golgi membranes localize to the more central fractions of the Nycodenz gradient.

Equilibrium density gradient fractionation using sucrose

The Alternate Protocol is particularly useful to separate vacuolar and endosomal membranes from late Golgi membranes (monitored using mALP, Pep12p, and Vps10p, respectively; see Table 3.7.9); however, the endosomal and vacuolar membranes overlap significantly (Becherer et al., 1996). The vacuolar and endosomal membranes migrate to the top fractions of the gradient, while the Golgi membranes localize to the more central fractions of the sucrose gradient.

Fractionation of $P_{13,000}$ membranes

Basic Protocol 3 was designed to analyze the distribution of one or more proteins among vacuoles, plasma membranes, early Golgi, and ER membranes (Gaynor et al., 1994). Six frac-

tions of unequal volume are collected (summarized below). Vacuolar membranes are found in fractions 1 and 2. Golgi membranes are primarily in fraction 2. ER membranes and the plasma membrane vesicles are split between fractions 2 (~35%) and 4 (~65%; Gaynor et al., 1994).

Isolation of intact vacuoles

Basic Protocol 4 was designed to purify intact vacuoles that are functional in an in vitro vacuole-vacuole fusion assay (Haas, 1995). The resulting vacuole preparation is ~50-fold enriched in vacuolar proteins with respect to the total cell protein of the spheroplast lysate. Cytosolic and ER markers are found in the vacuole preparation in only trace amounts. The level of endosome contamination has not been investigated and may be significant.

The yield is <5% of the total vacuoles. Each gradient with material from ~500 OD₆₀₀ cell equivalents yields a ~600- μ l vacuole preparation with a protein concentration of ~0.25 to 0.9 mg/ml. One of the most prominent proteins in the vacuole preparation is Vph1p, a 95-kDa integral membrane protein that is part of the vacuolar ATPase. It is sensitive to proteolysis, so it also can appear as a 75-kDa fragment on SDS-PAGE gels. Isolated vacuoles can be viewed by staining with the vital dyes carboxy-dichlorofluorescein diacetate (CDC-FDA; lumen) or *N*-(3-triethylammoniumpropyl)-4-(diethylamino)phenyl hexatrienylpyridinium dibromide (FM 4-64; membrane) in the presence of ATP (Roberts et al., 1991; Vida and Emr, 1995).

Isolation of intact nuclei

Basic Protocol 5 was designed to isolate intact nuclei from yeast spheroplasts (Dove et al., 1998). The preparation also contains ER membranes, as well as very low levels of plasma membrane, vacuoles, and sometimes mitochondria. The most prominent proteins include the histones and fibrillarin, the 38-kDa nuclear envelope protein. Six liters of yeast culture grown as described usually yield ~75 mg total nuclear protein. Only 2% to 5% of all nuclei are recovered after Ficoll gradients (Aris and Blobel, 1991). Yield and purity of nuclei can vary depending on strain, carbon source, and life stage. Quality of the preparation can be followed by microscopy due to characteristic size and shape of nuclei. Under a light microscope, the nuclei are uniformly gray with slightly rough edges. A small aliquot of the preparation can also be stained with DAPI (4',6-diaminidino-2-phenylindone) to monitor the purification. This

protocol yields primarily intact nuclei. The nuclei are functional in several *in vitro* assays. The nuclei can be fractionated further to isolate nucleoli or nuclear envelope preparations (see Table 3.8.2; Dove et al., 1998).

Isolation of lactate-induced mitochondria

Basic Protocol 6 is designed to isolate intact, functional mitochondria from lactate-grown D273-10B spheroplasts (Glick and Pon, 1995). Cells other than lactate-grown D273-10B cells can be used; however, the mitochondrial density and abundance will vary with the strain and growth conditions, and thus the protocol may need to be optimized. The mitochondria will usually have dark brown color but may also look orange or pink. The gradient-purified mitochondrial preparation usually contains only trace amounts of the marker proteins for vacuoles, nuclei, endoplasmic reticulum, plasma membrane, cytosol, or cytosolic ribosomes. Some of the residual microsomal membranes (that associate with the mitochondrial surface) can be removed with an acidic wash as described in Zinser and Daum (1995). When examined by electron microscopy, the gradient-purified mitochondria preparation primarily consists of mitochondria; however, a minor population of unidentified membranes is present. Common mitochondrial marker proteins include porin (outer membrane), cytochrome *c* (inner membrane), cytochrome *b₂* (intermembrane space), and citrate synthase (matrix). A typical yield from four 1-liter cultures of lactate-grown D273-10B cells is 20 to 25 mg of mitochondrial protein. These mitochondria are suitable for organelle translation studies, *in vitro* protein import studies, and other *in vitro* assays (Daum et al., 1982). If the mitochondria will be used for functional studies, a recovery incubation after spheroplast preparation may be needed for optimal activity.

Isolation of oleate-induced peroxisomes

Basic Protocol 7 was designed to purify intact, functional peroxisomes from the wild-type yeast strain D273-10B after growth in oleate medium (Distel et al., 1996). Strains other than D273-10B can be used; however, it is wise to conduct a pilot experiment and monitor peroxisome induction and isolation steps. Peroxisomal fractions are highly enriched with peroxisomal proteins, such as catalase A, thiolase, and acyl-CoA oxidase. Contamination of the peroxisomal fraction with proteins from mitochondria, vacuoles, ER, and plasma membrane is low. Only a fraction of the initial peroxisome population is recovered after the sucrose density

gradient steps. A substantial proportion of the peroxisomes are unavoidably lost in the low-speed pellet. A 1-liter culture of oleate-grown D273-10B cells typically yields 5 to 15 mg of peroxisomal protein. By electron microscopy, a homogenous population of single-membrane bound organelles should be visible. The isolated peroxisomes are functional for *in vitro* protein-import studies. If the peroxisomes will be used for functional studies, a recovery incubation after spheroplast preparation may be needed for optimal activity.

Isolation of endoplasmic reticulum

Basic Protocol 8 was designed to isolate endoplasmic reticulum (ER) membranes from yeast spheroplasts (Wuestehube and Schekman, 1992). These membranes are functional in *in vitro* protein import and transport studies. The gradients are similar to those used in Basic Protocol 3. The ER membranes distribute in a membrane layer within the 1.2 M sucrose solution and at the 1.2 M/1.5 M sucrose interface. Both of these fractions have significant amounts of plasma membrane vesicles. Since the membrane band in the 1.2 M sucrose solution also contains vacuolar membranes and Golgi membranes, only the ER vesicles at the 1.2 M/1.5 M sucrose interface are harvested. Typically, this protocol yields ~7 mg of protein per 5000 OD₆₀₀ units of cells.

Isolation of plasma membranes

Basic Protocol 9 isolates plasma membranes from yeast cells that are rapidly disrupted by vortexing with glass beads (Panaretou and Piper, 1996). Glass bead lysis protocols typically lyse 50% to 80% of the cells. The isolated plasma membrane preparation consists of vesicles in both the right-side-out and inside-out orientations. Vacuolar markers are usually not detected, while low levels of mitochondrial, ER, and Golgi markers are found. The plasma membrane vesicles isolated from disrupted whole cells tend to have lower levels of ER contamination than plasma membrane vesicles prepared from spheroplasts. Plasma membranes obtained from ruptured spheroplasts tend to have a lower density, presumably due to degradation of surface glycoproteins. The most prominent plasma membrane protein is the 100-kDa PM-ATPase. This Mg²⁺-dependent ATPase activity is inhibited by orthovanadate, while the ATPase activities of mitochondria and vacuoles are inhibited by KNO₃ and bafilomycin A1, respectively. The activity of the plasma membrane ATPase can be sensitive to gradient medium, so protein analy-

sis is recommended in addition to enzymatic analysis.

Preparation of cytosol

Basic Protocol 10 is a simple and efficient method to prepare cytosol from yeast cell lysates prepared by glass bead lysis (Haas, 1995). Glass bead lysis protocols typically lyse 50% to 80% of the cells. The resulting cytosol is enriched 10- to 15-fold over the crude lysate in cytosolic proteins. ~2000 OD₆₀₀ units of cells yield 25 to 60 mg of cytosolic protein at a concentration of 10 to 30 mg/ml.

Cytosol prepared by this technique supports *in vitro* vacuole-vacuole fusion and is likely to be functional in other *in vitro* assays. The cytosol should be tested and titrated for functional activity every time a fresh batch is prepared. Typically, cytosol prepared from cultures in their early- to mid-logarithmic growth phase has a higher specific activity than that prepared from cultures at later growth stages. Small molecules (e.g., ions, ATP) can be removed by passing the cytosol over a G25-Sephadex column.

Time Considerations

For best results, desired yeast strains should be obtained from glycerol stocks (stored at -80°C) and streaked onto fresh plates 2 to 3 days prior to starting the first cultures. Precultures should be started with a single colony from fresh plates 1 to 3 days before the fractionation experiment. Extensive preculturing (i.e., 2 to 3 days) is especially important prior to the induction of peroxisomes (see Basic Protocol 7). A full day should be allowed for each fractionation experiment, especially when doing the experiment for the first time. Some of the experiments will require several more hours on the following day to harvest fractions from gradients subjected to centrifugation overnight (see Basic Protocol 2, Alternate Protocol, and Basic Protocol 9).

It is wise to prepare well for fractionation experiments, since it is critical to work as quickly as possible once the cells have been lysed. Prepare all the solutions ahead of time (unless otherwise indicated) and store at the appropriate temperature. Reserve the necessary equipment and prechill if required. Prelabel tubes to the extent that it is possible. Minimize the number of strains used, especially when doing the experiment for the first time. It is often helpful to do small pilot experiments to determine the optimal experimental conditions for a given strain and growth regimen.

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Key References

Walworth et al., 1989. See above.

This older review provides an excellent summary of the subcellular fractionation techniques applied to S. cerevisiae, with a focus on organelles along the secretory pathway.

Zinser and Daum, 1995. See above.

This review provides an excellent summary of the subcellular fractionation techniques applied to S. cerevisiae and the corresponding references.

Internet Resources

<http://www.proteome.com/>

The Yeast Protein Database (Proteome) is an excellent source of comprehensive and up-to-date information on S. cerevisiae proteins. The information is derived from the published literature and DNA sequence databases. The protein information provided, including summaries of the protein and gene characteristics and the corresponding literature references, can be searched and categorized in several convenient ways (e.g., according to subcellular location, gene name, size).

<http://genome-www.stanford.edu/Saccharomyces/>
Saccharomyces Genome Database (SGD) provides access to the complete sequence of the S. cerevisiae genome, the confirmed and predicted open reading frames (ORFs), protein information, and a number of useful internet links.

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Isolation of Golgi Membranes from Tissues and Cells by Differential and Density Gradient Centrifugation

UNIT 3.9

Golgi membranes from actively secreting tissues form a major defined organelle and are relatively easy to isolate so long as the method of tissue disruption is as mild as possible, such that fragmentation of the Golgi stacks is minimized. As a general rule, if the Golgi membranes retain their *in vivo* tubular structure, they will sediment when centrifuged at 10,000 to 15,000 $\times g$ for 10 to 20 min. They can thus be purified from a light mitochondrial fraction by flotation through a discontinuous sucrose gradient (see Basic Protocol 2). Alternate Protocol 1 adapts this method to a self-generated iodixanol gradient. This may be of particular use for multiple samples because of the simplicity of gradient preparation. In mammalian liver, not only can the homogenization conditions be tailored to the retention of Golgi tubules, but, by inclusion of dextran in the homogenization medium, the entire Golgi stack may be retained so that it sediments at a relative centrifugal force (RCF) as low as 5000 $\times g$ (see Basic Protocol 1). This not only promotes a high recovery of Golgi membranes but also reduces possible contamination by other membrane particles. Isolation of Golgi membranes is facilitated by their having a distinctively low density, lower than any other membrane except possibly the *trans*-Golgi network and some plasma membranes. Thus, even if the Golgi membranes are present in a homogenate in a vesicular form (e.g., those from cultured cells), they can be isolated either in a discontinuous sucrose density gradient (see Basic Protocol 3), a continuous iodixanol gradient (see Alternate Protocol 2), or a self-generated iodixanol gradient (see Basic Protocol 4). Again the latter may be of particular use for multiple samples.

The aim of the protocols described in this unit is to isolate the Golgi in a preparative manner; the unit does not specifically address the requirements for an analytical investigation of the secretory process, which requires the simultaneous fractionation of a series of membrane compartments through a continuous gradient. Strategies for these studies (including the partial resolution of the *cis*-, medial, and *trans*-Golgi domains; *trans*-Golgi network; and various vesicular intermediates) will be given in a future unit. It is worth noting, however, that the protocols for the isolation of Golgi vesicles (particularly Basic Protocol 4 and Alternate Protocol 2) also permit the simultaneous isolation of other vesicular fractions and can therefore also be used in an analytical mode.

This unit also includes a routine method (see Support Protocol) for the assay of the UDP-galactose galactosyltransferase, which, although strictly a *trans*-Golgi enzyme, is the most commonly used marker in any preparative Golgi method.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations for the care and use of laboratory animals.

RAPID ISOLATION OF GOLGI MEMBRANES FROM RAT LIVER USING A SUCROSE DENSITY BARRIER

**BASIC
PROTOCOL 1**

The use of dextran in the homogenization medium and a mechanical shear means of homogenization, as opposed to liquid shear, favors the retention of intact Golgi stacks. This permits the recovery of the Golgi membranes in a relatively low-speed pellet (5000 $\times g$ for 15 min). In the bipartite pellet that is formed, the Golgi forms a quite distinct upper layer, which can be resuspended independently of the much more well-packed material

**Subcellular
Fractionation and
Isolation of
Organelles**

Contributed by John M. Graham

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3.9.1

Supplement 10

(containing nuclei, some plasma membrane sheets, and mitochondria) in the lower layer (Morré et al., 1972). Purification of the Golgi is then achieved using a simple sedimentation on a 1.2 M sucrose density barrier. Because dextran effectively cross-links the Golgi tubules, these membranes cannot be further subfractionated into the *cis*-, medial, and *trans*-Golgi domains unless they are unstacked by hydrolyzing the dextran with a mixture of amylases. The unstacking procedure of Hartel-Schenk et al. (1991) is presented below as an option.

If the presence of dextran is undesirable, then one of the alternative methods described in Basic Protocol 2 and Alternate Protocol 1 should be used. The following protocol is adapted from Morr e et al. (1972).

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Materials

- 150- to 200-g Sprague-Dawley rat
- DHM (see recipe)
- 1.2 M sucrose density barrier (see recipe)
- HM (see recipe), optional
- Crude α -amylase Type X-A from *Aspergillus oryzae* (Sigma Aldrich Co.), optional
- α -Amylase Type VIII-A from barley (Sigma Aldrich Co.), optional
- Dissecting tools, including scissors, razor blade, and forceps
- Polytron homogenizer (Brinkmann Instruments)
- Phase-contrast microscope
- High-speed centrifuge with swinging-bucket rotor and 30- to 50-ml clear plastic tubes
- 10-ml syringes with long metal cannulas (i.d. ~1 mm) or Pasteur pipet attached to an aspirator
- Glass rod
- Ultracentrifuge with swinging-bucket rotor (Beckman SW 28.1, Sorvall AH629, or equivalent) and 17-ml tubes
- Additional reagents and equipment for determining protein concentration (APPENDIX 3B)

NOTE: Protease inhibitors (UNIT 3.4) can be added to any or all solutions.

Isolate and mince liver

1. Starve a 150- to 200-g Sprague-Dawley rat overnight.
2. Sacrifice the animal by cervical dislocation or decapitation.
This must be supervised or carried out by an experienced animal technician.
3. Open the abdominal cavity and remove liver to a chilled beaker containing ~20 ml DHM. Record weight of the liver.
This protocol is suitable for a 10- to 12-g liver.
4. Decant medium and finely mince liver using scissors or a razor blade.
The pieces of liver should be no more than ~25 mm³.

Isolate intact stacked Golgi

5. Suspend liver mince in ~20 ml DHM.

6. Set suspension on ice and homogenize with a Polytron homogenizer set at 10,000 rpm (setting 1) for 40 sec, using 10-sec pulses and 10-sec rests. Check for completeness of tissue disruption by phase-contrast microscopy.
7. Centrifuge 15 min in a high-speed centrifuge at $5000 \times g$, 4°C .
The brake should not be used to decelerate the rotor; rapid changes in rpm may disturb the layering of the pellet.
8. Very carefully remove most of the supernatant using a 10-ml syringe with a long metal cannula or a Pasteur pipet attached to an aspirator. Leave 0.5 to 1.0 ml supernatant.
The supernatant should not be decanted.
The Golgi membranes are contained in the upper (yellow-brown) portion of the bipartite pellet.
9. Resuspend upper portion of pellet in the residual supernatant by very gentle stirring with a glass rod.
Resuspension of the lower part of the pellet, which contains nuclei and whole cells, must be avoided.
10. Transfer resuspended Golgi material to a 10-ml measuring cylinder using the syringe and cannula.
11. Adjust concentration of the suspension with DHM to 6 ml per 10 g liver and layer over 2 vol of 1.2 M sucrose density barrier in a 17-ml tube.
12. Centrifuge 30 min in an ultracentrifuge at $120,000 \times g$, 4°C .
13. Remove Golgi membranes, which collect at the interface, with a fresh syringe and cannula.
14. If required, dilute fraction with ≥ 2 vol DHM (or HM) and centrifuge 20 min at $10,000 \times g$, 4°C .
15. Determine protein concentration (*APPENDIX 3B*) and resuspend pellet in DHM (or HM) to 1 to 5 mg protein/ml.
Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for ≤ 16 hr. Many assays can be carried out on material frozen to -20° or -80°C . Measurements that are made with frozen material should be compared with those made on fresh material to confirm that they are relatively unaffected by storage conditions.

Unstack Golgi (optional)

If a subsequent aim is to attempt to resolve various domains of the Golgi membranes, it is necessary to hydrolyze the dextran in order to unstack the Golgi. The following is adapted from Hartel-Schenk et al. (1991).

16. Incubate 4 to 5 ml Golgi membrane suspension with 3 mg each of crude α -amylase Type X-A from *Aspergillus oryzae* and α -amylase Type III-A from barley at 4°C for 45 min.
17. Use gentle liquid shear to complete the destacking process by repeatedly (5 to 6 times) drawing suspension into and expelling it from a Pasteur pipet (1-mm tip i.d.) or a 10-ml syringe with a long metal cannula.

The success of the destacking process can only be monitored by electron microscopy or by comparing the banding pattern of cis-, medial, and trans-Golgi markers in a suitable density gradient or in some other separative technique before and after treatment.

**ISOLATION OF GOLGI MEMBRANES FROM A RAT LIVER LIGHT
MITOCHONDRIAL FRACTION BY FLOTATION THROUGH A
DISCONTINUOUS SUCROSE GRADIENT**

If the use of dextran to maintain the stacked format of the Golgi apparatus, as described in Basic Protocol 1, is unacceptable, then a light mitochondrial fraction from a liver homogenate can be used to overcome several of the problems associated with the fragmentation of the Golgi. The relatively gentle homogenization technique used in this protocol is aimed at retaining the tubular structure of the Golgi membranes and avoiding excessive fragmentation. In this manner, at least some of the Golgi membranes will sediment at $\sim 15,000 \times g$, thus facilitating the separation procedure. Purification of Golgi vesicles from vesicles produced from other smooth membrane compartments of the cell (plasma membrane, *trans*-Golgi network, smooth endoplasmic reticulum, and some endocytic structures) may be more problematic. In a microsomal fraction the Golgi vesicles will be both denser and lighter than some of the other vesicle populations. By processing the light mitochondrial fraction rather than a microsomal fraction (or some other microsome-containing fraction) these problems are minimized, as the Golgi is the least dense component of this fraction. This method works very satisfactorily for rat liver but its applicability to other tissues (and to cultured cells) will depend on the behavior of the Golgi membranes during homogenization and differential centrifugation. For the method to be suitable for a particular tissue or cell type, a significant amount of a Golgi marker (e.g., galactosyltransferase) should be recovered in the light mitochondrial fraction rather than the microsomes. The method is adapted from Fleischer and Fleischer (1970) and Graham and Winterbourne (1988).

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Materials

- 150- to 200-g Sprague-Dawley rat
- HM (see recipe)
- 2.0, 1.33, 1.2, 1.1, 0.77, and 0.25 M sucrose gradient solutions (see recipe)
- Dissecting tools, including scissors or razor blade
- Potter-Elvehjem homogenizer, ~ 0.09 -mm clearance, 40-ml working volume (Fisher Scientific), with chilled pestle
- Overhead high-torque electric motor, thyristor controlled (Fisher Scientific)
- Low-speed centrifuge with swinging-bucket rotor and 50-ml tubes
- ~ 5 - and ~ 30 -ml Dounce homogenizers (Wheaton) with loose-fitting type-B conical pestles
- High-speed centrifuge with 8×50 -ml fixed-angle rotor (e.g., Sorvall SS34)
- 10-ml syringe and metal cannula
- Refractometer
- Ultracentrifuge with swinging-bucket rotor (Beckman SW 28.1, Sorvall AH629, or equivalent) and 17-ml tubes
- Additional reagents and equipment for determining protein concentration (*APPENDIX 3B*)

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all solutions.

Isolate and mince liver

1. Starve a 150- to 200-g Sprague-Dawley rat overnight.
2. Sacrifice the animal by cervical dislocation or decapitation.
This must be supervised or carried out by an experienced animal technician.
3. Open the abdominal cavity and remove liver to a chilled beaker containing ~20 ml HM.

This protocol is suitable for a 10- to 12-g liver.

4. Decant medium and finely mince liver using scissors or a razor blade.

The pieces of liver should be no more than ~25 mm³.

Homogenize liver

5. Suspend mince in ~40 ml HM and transfer half of this suspension to the glass vessel of a Potter-Elvehjem homogenizer.
6. Attach the cold homogenizer pestle to an overhead high-torque electric motor and homogenize liver mince using four to five up-and-down strokes of the pestle, rotating at ~500 rpm.

The motor should be mounted either to a wall via a G clamp, to the bench, or in a floor-standing cradle. Attachment to a free-standing retort stand is not adequate.

7. Decant homogenate into a beaker on ice and rinse homogenizer with HM, wiping pestle to remove any connective tissue that may be adhering.
8. Repeat the procedure with the other half of the mince.

Prepare light mitochondrial fraction

9. Transfer homogenate to a 50-ml conical tube and centrifuge 10 min at $1000 \times g$, 4°C, in a low-speed centrifuge to pellet nuclei.
10. Decant supernatant and place on ice.
11. Transfer nuclear pellet to an ~30-ml Dounce homogenizer in ~20 ml HM. Use four gentle strokes of the loose-fitting pestle to resuspend pellet.
12. Transfer suspension to a clean 50-ml tube and recentrifuge 10 min at $1000 \times g$, 4°C.
13. Combine supernatants from steps 10 and 12, and centrifuge 10 min at $3000 \times g$, 4°C, to pellet the heavy mitochondria.
14. Transfer supernatant to a clean 50 ml tube and centrifuge 20 min at $17,000 \times g$, 4°C, in a high-speed centrifuge.
15. Aspirate supernatant using a 10-ml syringe and metal cannula, and discard.

Isolate Golgi membranes

16. Resuspend light mitochondrial pellet in 1 to 2 ml HM by vortex mixing, then add 8 ml of 2.0 M sucrose gradient solution (final concentration ~1.55 M). Transfer to an ~5-ml Dounce homogenizer and homogenize using gentle strokes of the loose-fitting pestle.
17. Use a refractometer to check that the refractive index is 1.4080 and adjust, if necessary, with either HM or 2.0 M sucrose.

If a refractometer is not available, check that the suspension will layer beneath the 1.33 M sucrose solution.

18. Transfer 5 ml light mitochondrial suspension to a 17-ml tube and overlay it with the following sucrose gradient solutions:

- 4.0 ml 1.33 M sucrose
- 2 ml 1.2 M sucrose
- 2 ml 1.1 M sucrose
- 2 ml 0.77 M sucrose
- 0.25 M sucrose to fill the tube

19. Centrifuge 1 hr in an ultracentrifuge at $100,000 \times g$, 4°C .

Harvest light Golgi membranes

20. Harvest material that bands at the 0.77 M/1.1 M and 1.1 M/1.2 M sucrose interfaces and any intervening material.

21. If required, dilute fraction with ≥ 2 vol HM and centrifuge 20 min at $10,000 \times g$, 4°C .

22. Determine protein concentration (*APPENDIX 3B*) and resuspend pellet in HM to 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for ≤ 16 hr. Many assays can be carried out on material frozen to -20°C or -80°C . Measurements that are made with frozen material should be compared with those made on fresh material to confirm that they are relatively unaffected by storage conditions.

ALTERNATE PROTOCOL 1

ISOLATION OF GOLGI MEMBRANES FROM A RAT LIVER LIGHT MITOCHONDRIAL FRACTION IN A SELF-GENERATED GRADIENT OF IODIXANOL

In this variation, the light mitochondrial fraction from mammalian liver is suspended in a medium containing 15% iodixanol and centrifuged in a vertical, near-vertical, or low-angle fixed-angle rotor at $>180,000 \times g$ for 1 to 3 hr. The centrifugation conditions are chosen to produce a slightly sigmoidal density profile; the relatively shallow gradient in the middle of the tube is used to provide a large linear separation between the Golgi membranes (low density) and the other organelles (high density). The precise gravitational forces and times will depend on the rotor type. In high-performance vertical rotors, which can produce $\sim 350,000 \times g$, the separation will take only 1 hr. The method is adapted from Graham et al. (1994). It should be applicable to other tissues and cells, although the iodixanol concentration (step 3) may need to be modulated.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Additional Materials (also see *Basic Protocol 2*)

- HME (see recipe), optional
- OptiPrep
- ODB solution (see recipe)
- IWS solution (see recipe)
- High-density cushion (20%, w/v, iodixanol): 2:3 (v/v) IWS/HME (or HM)
- Ultracentrifuge with vertical (e.g., Beckman VTi 65.1, Sorvall 65V13), near-vertical (e.g., Beckman NVT 65) rotor and ~ 11 -ml sealable ultracentrifuge tubes or low-angle fixed-angle rotor ($\sim 20^{\circ}$; e.g., Beckman 50.3Ti) and ~ 5 -ml sealable ultracentrifuge tubes.

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all solutions.

1. Isolate a rat liver, homogenize, and prepare a light mitochondrial fraction as described (see Basic Protocol 2, steps 1 to 15) in either HM or HME.

The sample can be prepared in the presence (HME) or absence (HM) of EDTA (see Background Information, discussion of homogenization).

2. Resuspend light mitochondrial pellet in HME or HM to a total volume of 14 ml.
3. Add 6 ml IWS to the suspension (15% w/v iodixanol final).

EDTA may be omitted from the OptiPrep dilution buffer (ODB) used to make IWS.

4. Transfer 9.5 ml to an ~11-ml sealable ultracentrifuge tube or a 4.5- to 5.0-ml sealable tube.
5. Underlayer suspension with 0.5 ml high-density cushion; omit cushion with fixed-angle rotor.

The cushion prevents the densest organelles (peroxisomes) from pelleting against the wall of the tube and making unloading difficult. This is particularly important if a vertical rotor is used.

6. Fill the tube by layering HME or HM on top, seal the tube, and centrifuge 1 to 3 hr in an ultracentrifuge at 180,000 to 350,000 $\times g$, 4°C.

The precise centrifugation conditions depend on the rotor type and tube sedimentation path length. In a vertical rotor such as the VTi 65.1, 1 hr at 350,000 $\times g$ will be sufficient, while at 180,000 $\times g$, 3 hr will be needed. Longer path-length rotors may need 3 hr at 350,000 $\times g$. Fixed-angle rotors with these tube volumes are not ideal for rapid formation of self-generated gradients because of their relatively long sedimentation path lengths. Many Beckman rotors that are capable of high speeds (e.g., 80 Ti and 90 Ti) can, however, be adapted to accommodate smaller-volume g-Max tubes that have suitable path lengths. Ford et al. (1994) and the Axis-Shield Density Gradient Media Catalog (Axis-Shield, 2000) provide more information on the preparation of self-generated iodixanol gradients.

7. During deceleration below 2000 rpm, either use a programmed deceleration or turn off the brake.
8. Harvest the Golgi membranes, which band near the top of the gradient.

ISOLATION OF GOLGI MEMBRANES FROM CULTURED CELLS BY FLOTATION THROUGH A DISCONTINUOUS SUCROSE GRADIENT

This protocol, devised by Balch et al. (1984), is applicable to any cell homogenate (primarily from cultured cells) in an isoosmotic sucrose medium. The whole homogenate is adjusted to 1.4 M sucrose and layered under a discontinuous sucrose density gradient of 1.2 M and 0.8 M sucrose. During centrifugation, the Golgi membranes, which have a much lower density than those of the other membrane particles (with the exception of the *trans*-Golgi network and possibly the plasma membrane), float upwards to band at the 1.2 M/0.8 M sucrose interface. By including a layer of 1.6 M sucrose beneath the homogenate it is also possible to resolve (at least partially) some of the endoplasmic reticulum (ER) from the other denser organelles (mitochondria, lysosomes, peroxisomes, and nuclei), which will sediment into or through this layer (the smooth ER in particular will remain in the load zone). The advantage of this procedure is not only the speed of preparation but the elimination of differential centrifugation; resuspending the pellets also minimizes any comminution of the subcellular organelles. A similar discontinuous sucrose gradient consisting of 8.4% (0.25 M), 32.5% (0.95 M), and 45.2% (1.33 M) sucrose, with the sample (an 11,000 $\times g$ supernatant) loaded beneath the gradient in ~51% (1.5 M) sucrose, was devised by Macintyre (1992). In Alternate Protocol 2, the use of a continuous iodixanol gradient is described.

BASIC PROTOCOL 3

Subcellular Fractionation and Isolation of Organelles

3.9.7

The homogenization procedure should be as gentle as possible (in a medium containing 0.25 M sucrose) to avoid excessive fragmentation of internal organelles. A ball-bearing device ("cell cracker") of the type designed by Balch and Rothman (1985) is recommended; it certainly causes the least amount of damage to organelles. A tight-fitting Dounce homogenizer is a possible alternative. The method may also be used for a tissue such as rat liver, but volumes will need to be scaled up (see annotations). The following protocol is adapted from Balch et al. (1984).

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Materials

Cultured cells
HM (see recipe)
0.8, 1.2, 1.6, and 2.0 M sucrose gradient solutions (see recipe)

Ball-bearing homogenizer
5-ml syringe and metal cannula
Ultracentrifuge with swinging-bucket rotor (Beckman SW 28.1, Sorvall AH629, or equivalent) and ~17-ml tubes, 4°C

Additional reagents and equipment for determining protein concentration
(*APPENDIX 3B*)

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all solutions.

1. Wash $1\text{--}3 \times 10^8$ cultured cells twice in 5 ml HM, resuspend in 3 ml HM, and homogenize in a ball-bearing homogenizer using five passes.

Alternatively use ten to fifteen strokes of a tight-fitting pestle (0.025- to 0.075-mm clearance) in a Dounce homogenizer.

For a tissue such as rat liver, the liver mince (see Basic Protocol 1, steps 1 to 4) should be suspended in 20 ml HM and homogenized using ten strokes of a loose-fitting pestle in a Dounce homogenizer.

2. Make the homogenate 1.4 M with respect to sucrose by adding 2 vol of 2.0 M sucrose gradient solution.
3. Transfer 6 ml to an ~17-ml tube.
Use ~2.2× this volume (here and in step 4) for rat liver. A larger-volume rotor will also be required (~38-ml tubes).
4. Overlay sample with 6 ml of 1.2 M sucrose gradient solution and 3 ml of 0.8 M sucrose gradient solution.
5. Use a 5-ml syringe and metal cannula to underlay sample with 2 ml of 1.6 M sucrose gradient solution.
6. Centrifuge 2 hr in an ultracentrifuge at $110,000 \times g$, 4°C.
7. Harvest the Golgi band from the 0.8 M/1.2 M sucrose interface.
If required, harvest the ER, which bands within the 1.4 M sucrose layer.
8. If required, dilute fraction(s) with ≥ 2 vol HM and centrifuge 30 min at $100,000 \times g$, 4°C.

9. Determine protein concentration (*APPENDIX 3B*) and resuspend pellet in HM to 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for ≤16 hr. Many assays can be carried out on material frozen to -20°C or -80°C. Measurements that are made with frozen material should be compared with those made on fresh material to confirm that they are relatively unaffected by storage conditions.

ISOLATION OF GOLGI MEMBRANES FROM CULTURED CELLS IN A CONTINUOUS GRADIENT OF IODIXANOL

**ALTERNATE
PROTOCOL 2**

Yang et al. (1997) devised a continuous iodixanol (0% to 26%, w/v) gradient to study the localization of UBC6 ubiquitin-containing protein in COS-7 cells, and Zhang et al. (1998) used a similar gradient (1% to 20%, w/v) to study the localization of presenilins to the ER or Golgi membranes of CHO cells. Sufficient resolution of the Golgi was obtained that these methods could be used to prepare Golgi on a preparative basis. The following protocol is based on these two published methods and may be used in one of three sample-handling modes: (1) layering a postnuclear fraction (or postmitochondrial supernatant) on top, (2) layering a microsomal fraction on top, or (3) layering any fraction (including a homogenate) at the bottom (see annotations to this protocol). The method also allows simultaneous purification of the ER and plasma membrane. See Commentary for more information on the relative advantages and disadvantages of the three types of sample handling.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Additional Materials (also see *Basic Protocol 3*)

Cellular homogenate (see *Basic Protocol 3*, step 1)

25% (w/v) iodixanol: 1:1 (v/v) IWS solution (see recipe) and HM (see recipe)

Low-speed centrifuge with swinging-bucket rotor and 10- to 15-ml tubes, all at 4°C

Ultracentrifuge with fixed-angle rotor and 5- to 10-ml tubes (optional), all at 4°C

5- to 10-ml Dounce homogenizer (Wheaton) with loose-fitting type-B pestle (optional)

Two-chamber gradient maker or Gradient Master (e.g., BioComp Instruments)

Gradient unloader (upward displacement; Axis-Shield)

Refractometer

Additional reagents and equipment for determining protein concentration (*APPENDIX 3B*)

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all solutions.

1. Transfer a cellular homogenate to a 10- to 15-ml centrifuge tube and centrifuge 10 min at $2000 \times g$, 4°C, in a low-speed centrifuge. Save supernatant.

This step should be omitted if the whole homogenate is to be layered under the gradient.

2. *Optional:* Transfer supernatant to a 5- to 10-ml tube and centrifuge 40 min at $100,000 \times g$, 4°C, in the fixed-angle rotor of an ultracentrifuge. Resuspend microsomal pellet in 4 to 8 ml HM using a 5- to 10-ml Dounce homogenizer with a loose-fitting pestle.

This step should be omitted if either the homogenate or the $2000 \times g$ supernatant is used as gradient input.

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3.9.9

3. Prepare a 14-ml continuous gradient from 7 ml each HM and 25% iodixanol solution in a 17-ml tube for a swinging-bucket rotor using a two-chamber gradient maker or Gradient Master.
4. Layer 3 ml of $2000 \times g$ supernatant (step 1) or resuspended microsomes (step 2) on top of the gradient and centrifuge 2 to 3 hr at $200,000 \times g$, 4°C .

Alternatively, the homogenate (or any of the other fractions) can be adjusted to 30% (w/v) iodixanol with IWS solution and layered beneath the gradient.

5. Collect gradient in 0.75-ml fractions using a gradient unloader. Check the density of the fractions with a refractometer.

The Golgi bands between 1.040 and 1.065 g/ml. With practice it is possible to use a syringe or Pasteur pipet to remove the Golgi-containing gradient zone (see Commentary for more details).

6. If required, dilute Golgi fractions with ≥ 2 vol HM and centrifuge 30 min at $100,000 \times g$, 4°C .

7. Determine protein concentration (APPENDIX 3B) and resuspend pellet in HM to 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for ≤ 16 hr. Many assays can be carried out on material frozen to -20°C or -80°C . Measurements that are made with frozen material should be compared with those made on fresh material to confirm that they are relatively unaffected by storage conditions.

BASIC PROTOCOL 4

ISOLATION OF GOLGI MEMBRANES FROM A MICROSOMAL FRACTION OF HEPATOCYTES IN A SELF-GENERATED GRADIENT OF IODIXANOL

Cartwright et al. (1997) developed a rapid method for the fractionation and subfractionation of the smooth and rough endoplasmic reticulum (SER and RER) from a microsomal fraction of rabbit hepatocytes. The method used a self-generated gradient of iodixanol (starting concentration 20%, w/v) at $\sim 350,000 \times g$ for 2 hr in a vertical rotor and was used to study the synthesis of very low density lipoproteins (VLDL) from radiolabeled precursors. Later, Plonné et al. (1999) adapted this method to the simultaneous isolation of Golgi, SER, and RER membranes from both isolated hepatocytes and intact liver. Although it is possible to make the gradient more shallow in the low-density region by increasing the centrifugation time to 3 hr and thus improve the resolution of the Golgi membranes from other intracellular particles, Plonné et al. (1999) preferred an alternative method of generating the gradient from equal volumes of 15% and 20% (w/v) iodixanol without increasing the centrifugation time. Although both layers can contain the microsomal fraction, if it is restricted to the denser 20% iodixanol layer, any residual contaminating soluble proteins remain in this layer and the Golgi membranes float into the soluble-protein-free lower-density region. The following protocol is adapted from Plonné et al. (1999) and uses collagenase-prepared rat hepatocytes as the source material. Some comments regarding the use of rat liver are given in the annotations.

NOTE: With the exception of the Dulbecco's modified Eagle medium with BSA (DMEM/BSA), all solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

Materials

95% O₂/5% CO₂ gas mixture

DMEM/BSA: Dulbecco's modified Eagle medium (*APPENDIX 2A*) with 1.0% (w/v) BSA

Collagenase-prepared rat hepatocytes (Plonné et al., 1999)

PBS (*APPENDIX 2A*), 4°C

10 mM HEPES-NaOH buffer, pH 7.8

HB (see recipe)

SB (see recipe)

OptiPrep (Axis-Shield, Life Technologies, Accurate Chemicals)

30% (w/v) iodixanol: 1:1 (v/v) OptiPrep/SB

15% (w/v) iodixanol: 1:3 (v/v) OptiPrep/SB

Low-speed centrifuge with swinging-bucket rotor and 50-ml conical tubes, all at 4°C

~10-ml Dounce homogenizer (Wheaton) with tight-fitting type-A pestle

High-speed centrifuge with fixed-angle rotor and ~15-ml tubes (e.g., Sorvall SE12), 4°C

Ultracentrifuge with the following, all at 4°C:

Fixed-angle rotor (e.g., Beckman 50Ti, SorvallT865.1) and ~10-ml tubes

Vertical rotor (e.g., Beckman VTi 65.1, Sorvall 65V13), near-vertical rotor (e.g., Beckman NVT 65), with ~11-ml sealable tubes or a low-angle fixed-angle rotor (~20°; e.g., Beckman 50.3Ti) and ~5-ml sealable ultracentrifuge tubes.

~10-ml Dounce homogenizer (Wheaton) with loose-fitting type-B pestle

Gradient unloader (upward displacement; e.g., Axis-Shield)

Additional reagents and equipment for determining protein concentration (*APPENDIX 3B*)

NOTE: Protease inhibitors (*UNIT 3.4*) can be added to any or all solutions.

Homogenize hepatocytes

1. Bubble a 95% O₂/5% CO₂ gas mixture through DMEM/BSA for 20 min, and then suspend collagenase-prepared rat hepatocytes in this medium at 0.5–1 × 10⁷ cells/ml.
2. Incubate ≥30 min at 37°C in a shaking incubator with continuous gassing.
3. Transfer cells to a preweighed 50-ml conical tube and centrifuge 2 min in a low-speed centrifuge at 800 × g, 4°C, to pellet cells. Discard supernatant and record weight of cell pellet.
4. Wash cell pellet twice in ~20 ml PBS at 4°C.
5. Remove all PBS after last wash and resuspend cells in 10 mM HEPES buffer (5 ml/g cells).
6. Allow cells to swell at 4°C for ~10 min and then centrifuge 2 min at 800 × g, 4°C, to pellet cells.
7. Remove and discard 70% of buffer and then add a volume of HB equal to residual supernatant.
8. Resuspend cells and homogenize using 30 strokes of a tight-fitting pestle in an ~10-ml Dounce homogenizer.

For intact liver, isolate and homogenize as described (see Basic Protocol 2, steps 1 to 8) using SB as the homogenization medium.

Isolate microsomal fraction

9. Transfer homogenate to a 15-ml centrifuge tube and add protease inhibitors, if required.
10. Centrifuge homogenate 20 min at $8000 \times g$, 4°C , in a high-speed centrifuge.
11. Transfer supernatant to an ~10-ml tube and centrifuge in the fixed-angle rotor of an ultracentrifuge 40 min at $150,000 \times g$, 4°C , to pellet microsomes.
12. Resuspend microsomes in SB (~2.5 ml/g cells) using the loose-fitting pestle of an ~10-ml Dounce homogenizer.

Purify Golgi

13. Mix 4 vol microsome suspension with 2 vol OptiPrep (20% w/v iodixanol final).
14. Transfer 4.5 ml to an ~11-ml sealable tube for a vertical, near-vertical, or low-angle fixed-angle rotor. Underlayer with 1.8 ml of 30% iodixanol and overlayer with 4.5 ml of 15% iodixanol. Seal tube.

In fixed-angle rotors the 30% iodixanol may be omitted; fill the tube with equal volumes of the two iodixanol solutions.

Fixed-angle rotors with these tube volumes are not ideal for rapid formation of self-generated gradients because of their relatively long sedimentation path lengths. Many Beckman rotors that are capable of high speeds (e.g., 80 Ti and 90 Ti) can, however, be adapted to accommodate smaller-volume g-Max tubes that have suitable path lengths. Ford et al. (1994) and Axis-Shield Density Gradient Media Catalog (Axis-Shield, 2000) provide more information on the preparation of self-generated iodixanol gradients.

15. Centrifuge 2 hr at $350,000 \times g$, 4°C .
16. Use a gradient unloader to unload into 1-ml sample tubes by upward displacement in ~0.5-ml fractions.

The Golgi bands in the top 7 ml of the gradient, smooth ER in the median 8 to 14 ml, and rough ER in the bottom third.
17. If required, dilute Golgi fractions with ≥ 2 vol SB and centrifuge 30 min at $100,000 \times g$, 4°C .
18. Determine protein concentration (APPENDIX 3B) and resuspend pellet SB to 1 to 5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some marker enzyme assays can be carried out after storage at 4°C for ≤ 16 hr. Many assays can be carried out on material frozen to -20°C or -80°C . Measurements that are made with frozen material should be compared with those made on fresh material to confirm that they are relatively unaffected by storage conditions.

SUPPORT PROTOCOL

ASSAY FOR UDP-GALACTOSE GALACTOSYLTRANSFERASE

The domains of the Golgi (*cis*, medial, and *trans*) are involved in the post-translational modification of oligosaccharide residues of glycoproteins and proteoglycans. As such, any of the enzymes that characterize these reactions can be used as markers. They all have their active sites directed towards the cisternal space of the Golgi, and if a glycoprotein is used as an acceptor molecule in the assay, it is normal practice to include a mild detergent in the medium to allow access of the acceptor to the enzyme. The most commonly used marker is UDP-galactose galactosyltransferase, which is really a marker for the *trans*-Golgi, but in total Golgi membrane preparations it is used as a general marker for these membranes.

UDP-galactose galactosyltransferase can be measured using as an acceptor a suitable glycoprotein (normally) that has had its terminal *N*-acetylneuraminic acid and galactose residues removed chemically (Kim et al., 1971). To avoid this rather lengthy procedure, two alternative acceptors are more commonly used today: *N*-acetylglucosamine (the terminal sugar residue, which is the acceptor in vivo) or ovalbumin (a commercially available glycoprotein, which can be used as an artificial acceptor without modification). With *N*-acetylglucosamine as acceptor, the radiolabeled product, *N*-acetylglucosamine, is separated from UDP-galactose by passage through a small Dowex ion-exchange resin (Fleischer et al., 1969); this is a simple but very tedious technique with large numbers of samples. The ovalbumin method is easier to carry out and is described below (Beaufay et al., 1974).

CAUTION: When working with radioactivity (UDP-gal), take appropriate precautions to avoid contamination of the experimenter and the surroundings (*APPENDIX 1D*). Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer.

Materials

10% (w/v) trichloroacetic acid (TCA)
Acceptor solution (see recipe)
UDP-gal solution (see recipe)
2.4-cm filter paper discs
Polystyrene board
Pins

1. Prepare two 2.4-cm filter paper discs per sample (numbered lightly with a pencil).
2. Use pins to attach discs to a polystyrene board, making sure that the discs do not touch the board.
3. Place 250 ml of 10% TCA in a beaker on ice.
4. In a 0.5-ml tube in an ice/water bath, mix 50 μ l acceptor solution with 50 μ l sample and add 10 μ l UDP-gal solution.
5. At time zero and after a 20-min incubation at 37°C, transfer 50 μ l incubation mixture to a filter disc.
6. Plunge disc into the ice-cold 10% TCA.
If the disc is dropped onto the surface of the TCA, the pencilled number is liable to lift off.
7. Leave for 2 to 3 hr, swirling discs occasionally.
8. Wash discs with several changes of distilled water and leave to dry overnight at room temperature.
9. Count radioactivity in any commercial scintillant for non-aqueous samples.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

Acceptor solution

To 5 ml assay buffer (see recipe) add:
55 mg Na₂ATP (10 mM final)
0.5 g ovalbumin (5% final)
Add assay buffer to 10 ml
Prepare fresh

Assay buffer

To 50 ml H₂O add:
0.19 g MnCl₂·4H₂O (10 mM final)
0.23 g 2-mercaptoethanol (30 mM final)
0.2 g Triton X-100 (0.2% v/v final)
1.6 g sodium cacodylate (0.1 M final)
Adjust to pH 6.2 with 1 M HCl
Add H₂O to 100 ml
Store up to 1 month at 4°C

Dextran-containing homogenization medium (DHM)

To 100 ml H₂O add:
34.2 g sucrose (0.5 M final)
2 g dextran (mol. wt. 225,000; 1% final)
1.21 g Tris base (50 mM final)
1.16 g maleic acid (50 mM final)
Adjust pH to 6.4 with 50 mM Tris base or 50 mM maleic acid as required
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

Homogenization medium (HM)

To 100 ml H₂O add:
17.1 g sucrose (0.25 M final)
2 ml 1 M Tris·Cl, pH 7.4 (APPENDIX 2A; 10 mM final)
Adjust to pH 7.4 with 1 M Tris or 1 M HCl if required
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

Homogenization medium with EDTA (HME)

Prepare as for HM (see recipe), but add 2 ml 100 mM Na₂EDTA (APPENDIX 2A; 1 mM final) before adjusting pH. Store 1 to 2 days at 4°C.

Hyperosmotic buffer (HB)

To 100 ml H₂O add:
41 g sucrose (0.6 M final)
20 ml 100 mM HEPES (10 mM final)
Adjust to pH 7.8 with NaOH
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

Iodixanol working stock (IWS) solution

Dilute 5 vol OptiPrep (Axis-Shield, Life Technologies, Accurate Chemicals) with 1 vol ODB (see recipe) to give a final iodixanol concentration of 50% (w/v). Store up to 1-2 days at 4°C.

OptiPrep dilution buffer (ODB)

To 100 ml H₂O add:
17.1 g sucrose (0.25 M final)
12 ml 100 mM Na₂EDTA (6 mM final)
12 ml 1 M Tris·Cl, pH 7.4 (APPENDIX 2A; 60 mM final)
Adjust to pH 7.4 with 1 M Tris or 1 M HCl if required
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

Sucrose density barrier, 1.2 M

To 50 ml H₂O add:
41.0 g sucrose (1.2 M final)
0.61 g Tris base (50 mM final)
0.58 g maleic acid (50 mM final)
Adjust pH to 6.4 with 50 mM Tris base or 50 mM maleic acid
Add H₂O to 100 ml
Store up to 1 to 2 days at 4°C

Sucrose gradient solutions

2.0 M solution:

To 45 ml H₂O add:
69 g sucrose (2.0 M final)
1 ml 1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*; 10 mM final)
Adjust to pH 7.4 with 1 M Tris or 1 M HCl if required
Add H₂O to 100 ml
Store up to 1 to 2 days at 4°C

For other concentrations: Dilute as needed with 10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*). Store up to 1 to 2 days at 4°C.

For 1.33 M sucrose, dilute 1.33:0.67, v/v; for 1.2 M, dilute 1.2:0.8, v/v; for 1.1, dilute 1.1:0.9, v/v; and for 0.77 M, dilute 0.77:1.23, v/v.

Suspension buffer (SB)

To 100 ml H₂O add:
17.1 g sucrose (0.25 M final)
20 ml 100 mM HEPES (10 mM final)
Adjust to pH 7.8 with NaOH
Add H₂O to 200 ml
Store up to 1 to 2 days at 4°C

Uridine-5'-diphospho[6-³H]galactose (UDP-gal) solution

To 5 ml H₂O add:
76 mg uridine 5'-diphosphogalactose, disodium salt (12.5 mM final)
10 µl uridine 5'-diphospho[6-³H]galactose (70 kBq/ml)
Add H₂O to 10 ml
Store up to 1 month at -80°C

COMMENTARY

Background Information

Homogenization

Most published methods describing the isolation of Golgi membranes from mammalian tissues and cells employ a standard buffered sucrose solution as homogenization medium, although there is apparently no rigorous requirement for the presence of some chelating agent such as EDTA. In this respect, the isolation of the Golgi membranes from a light mitochondrial fraction is different from the isolation of other organelles from this fraction (e.g., mitochondria, lysosomes, peroxisomes), where inclusion of EDTA facilitates the recovery of highly purified and functionally compe-

tent organelles. Indeed, the inclusion of EDTA in the homogenization medium is actually detrimental to the isolation of Golgi membranes from a microsomal fraction using the self-generated iodixanol gradient described in Basic Protocol 4 (J.A. Higgins, pers. comm.). Moreover, in those methods that use gradient purification, any fraction containing microsomal material (an actual microsomal fraction, a postnuclear or postmitochondrial supernatant, or a homogenate), the presence of EDTA may also destabilize the ribosomes on the rough endoplasmic reticulum (RER), thus causing these membranes to band anomalously. Such an effect would be particularly undesirable if the density gradient was being used for the simul-

taneous purification of one or more components of the ER system. This is much less of a problem if a light mitochondrial fraction is used as a Golgi source for a density gradient; indeed, inclusion of EDTA will reduce contamination of this fraction by the RER.

The only major exceptions to the use of a standard isoosmotic sucrose homogenization medium are: (1) the method of Morr  et al. (1972), where dextran is included in a slightly hyperosmotic (0.5 M) sucrose medium to promote the maintenance of Golgi stacks (from rat liver) during homogenization; and (2) for the isolation of Golgi from yeast, where it is common to use higher concentrations of sorbitol (~0.8 M) for lysis of spheroplasts (Bowser and Novick, 1991).

Rat liver may be homogenized in either a Potter-Elvehjem (four to five strokes of the pestle rotating at ~500 rpm) or in a loose-fitting Dounce homogenizer (ten to fifteen strokes), although in the method of Morr  et al. (1972) the use of a Polytron homogenizer (or some similar means of providing controlled mechanical shear) is an absolute requirement, as otherwise the stacks of Golgi tend to fragment unpredictably. Hepatocytes are best homogenized in a tight-fitting Dounce homogenizer. For the often smaller volumes of cultured-cell suspensions, a ball-bearing homogenizer ("cell cracker"), repeated passages through a fine needle of a 1- to 2-ml syringe, or a tight-fitting Dounce homogenizer are commonly used techniques. It is strongly recommended that for any cell suspension (either from cultured cells or from a disaggregated tissue), the homogenization procedure be thoroughly investigated so that ≥90% of the cells can be disrupted reproducibly in the shortest time.

Differential centrifugation

Generally, it is only the Golgi membranes from an intact tissue such as mammalian liver

that form highly organized and extensive cytoplasmic structures. When dextran is included in the homogenization medium (Morr  et al., 1972) stacked tubules will sediment along with nuclei and heavy mitochondria at 5000 × g; otherwise, so long as the homogenization procedure is gentle, tubular elements will sediment in the light mitochondrial fraction (10,000 to 15,000 × g). On the other hand, the Golgi from most cultured cells will form fragments and vesicles of a variety of sizes. Hence, in the interest of maximum recovery, it is common to use either a total homogenate or, more commonly, a postnuclear or post-heavy mitochondrial supernatant for gradient fractionation of these cells. Although use of a microsomal fraction (centrifugation of a light mitochondrial supernatant at 100,000 to 150,000 × g for 30 to 40 min) may result in the loss of some of the larger fragments in earlier fractions of a differential centrifugation scheme, the elimination of both the majority of large organelles and the cytosolic proteins may be an advantage. The volume of medium required to suspend the microsomes will also be considerably less than that of the homogenate or postnuclear or post-mitochondrial supernatant, thus facilitating sample and gradient handling. This may be of particular importance with intact tissues or hepatocytes where volumes tend to be large.

Density gradients

In sucrose gradients (Table 3.9.1), the density of Golgi membranes is predominantly in the range of 1.07 to 1.12 g/ml (although densities as low as 1.05 g/ml are possible). Because Golgi membranes are considerably lighter than other organelles in the light mitochondrial fraction (mitochondria, peroxisomes, and lysosomes), which have densities in sucrose >1.16 g/ml, discontinuous gradients for purifying Golgi membranes were successfully developed by a number of workers between 1970 and

Table 3.9.1 Density (g/ml) of Membrane Fractions from Rat Liver in Sucrose and Iodixanol

Particle	Density in sucrose	Density in iodixanol
Nuclei	>1.32	1.23-1.25
Peroxisomes	1.19-1.23	1.18-1.21
Rough endoplasmic reticulum	1.18-1.26	1.14-1.20
Mitochondria	1.17-1.21	1.14-1.16
Lysosomes	1.19-1.21	1.11-1.13
Smooth endoplasmic reticulum	1.10-1.17	1.08-1.12
Golgi	1.07-1.12	1.04-1.07
Plasma membrane	1.07-1.14	1.02-1.03

1980 (e.g., Fleischer and Fleischer, 1970; Ehrenreich et al., 1973). These have formed the basis for most of the technology based on sucrose (e.g., Basic Protocol 2). Later these methods were adapted to the use of the iodinated density gradient medium metrizamide (Graham and Winterbourne, 1988) and more recently to iodixanol (Graham et al., 1994; Yang et al., 1997; Zhang et al., 1998). Because solutions of iodinated density gradient media have a significantly lower osmolarity than those of sucrose, osmotically sensitive subcellular particles exhibit a much lower density in gradients of these media compared to those of sucrose (Table 3.9.1). The density of Golgi fragments in iodixanol, for example, is principally in the range 1.04 to 1.07 g/ml. As in sucrose, there are some variations: Plonné et al. (1999), for example, reported the same density range for most of the Golgi membranes in rat hepatocytes, but a small fraction of galactosyltransferase activity was observed at ~1.10 g/ml.

The variation in density may at least be partly explained by the fact that the Golgi membranes, and indeed the other membranes of the synthetic and secretory systems, are involved in post-translational modification of proteins and lipids that are destined either for incorporation into other membranes or for secretion. The density of these membranes (or of certain domains of these membranes) will be influenced by the density of the macromolecules (and macromolecular complexes) within them. Thus, the presence of particles such as VLDL, which are synthesized by hepatocytes, will tend to make membrane vesicles containing them have a relatively low density, while the presence of heavily glycosylated molecules (carbohydrate is normally very dense) will have the reverse effect. This may not be true of all glycosylated molecules: many proteoglycans, for example, are highly hydrated, giving them a relatively low density in isoosmotic gradients (Ford et al., 1983), but possibly not in a hyperosmotic medium such as sucrose.

The choice of medium may be influenced less by the attainable resolution, which is very satisfactory in both sucrose and iodixanol, than by the ease of handling, notably the commercial availability of iodixanol as a 60% (w/v) solution (OptiPrep) and the avoidance of the need to prepare highly viscous solutions of sucrose. Iodixanol may also be used as a self-generated gradient (Alternate Protocol 1 and Basic Protocol 4). In a self-generated gradient system, the sample is loaded into the centrifuge tube as a dilute suspension; this practice, together with

the absence of any density interfaces, reduces aggregation of particles and promotes high resolution. It is also highly reproducible and facilitates the handling of large numbers of samples. Generation of a suitable density gradient profile depends on the use of an appropriate type of rotor. Vertical, near-vertical, or low-angle fixed-angle rotors can be used. Because the separation of the Golgi from a light mitochondrial fraction (Alternate Protocol 1) requires a gradient containing a relatively shallow median region, g forces as low as $180,000 \times g$ are permissible. For more information on the production and use of self-generated gradients, see Ford et al. (1994) and the Axis-Shield Density Gradient Media Catalog (Axis-Shield, 2000). In any system it may be necessary to modulate the density range of the gradients according to the median density of the Golgi membranes.

By using homogenization conditions that allow the Golgi from rat liver to retain its *in vivo* stacked form, Morr e et al. (1972) were able to use a simple density barrier to purify the intact Golgi from the upper layer of a $5000 \times g$ pellet. Yields also tend to be higher than in other methods that use a light mitochondrial fraction (Basic Protocol 2 and Alternate Protocol 1) and in which the size of the Golgi fragments is variable. The only disadvantage of the method is that because the stacking is maintained by the inclusion of dextran in the homogenization medium, this fraction has to be hydrolyzed enzymatically if further subfractionation into domains is to be attempted (see Basic Protocol 1).

As to the separation of Golgi membrane vesicles from other vesicles of the secretory system and from plasma membrane, the situation is rather less clear with sucrose. Although the density in sucrose gradients of the RER is clearly considerably greater than that of the Golgi (Table 3.9.1), the lighter smooth-ER (SER) vesicles clearly overlap the denser Golgi membranes. The overlap of the Golgi with the ER is less significant in iodixanol gradients (Table 3.9.1), making iodixanol the gradient medium of choice for the isolation of Golgi membranes from a microsomal fraction, post-nuclear (or postmitochondrial) supernatant, or homogenate (see Anticipated Results).

The advantage of using either a whole homogenate or a postnuclear supernatant for the isolation of Golgi membranes is that the response of the Golgi tubules to the homogenization procedure is irrelevant as all of the membranes will be applied to the gradient. If a homogenate is used, then it is certainly advis-

able to adjust its density such that it can be layered under the gradient; if it were layered on top, the presence of rapidly sedimenting nuclei might disturb the gradient. The situation with a postnuclear supernatant or with a microsomal fraction is less critical, and top-loading is permissible. Nevertheless, it is often considered an advantage, especially when isolating the least-dense membrane from much larger populations of denser particles, that bottom loading provides better resolution (Hinton and Mullock, 1997). In this mode the densest particles either remain in or float very slowly into the gradient from the load zone. The disadvantage to this approach is that there is an increase in sample volume when the sample density is adjusted by adding a high-density solution, and this may require that more gradients be set up to accommodate the volume.

In top loading a fraction that also contains the cytosolic fraction (i.e., any fraction except a resuspended microsomal pellet), it is inevitable that soluble proteins will also move into the gradient due to sedimentation and diffusion. If discrimination of cytosolic proteins from any membrane fraction is important, then bottom loading should alleviate this problem as the proteins will tend to remain in the load zone. Although cytoplasmic proteins may also be subsequently removed by pelleting the membranes from the gradient fractions, this additional step may be undesirable.

Use of a pelleted microsomal fraction will not only remove most of the cytosolic proteins (although, unless the pellet is washed, there will always be some residual contamination), it will also permit reduction of the fluid volume. On the other hand, when the pellet is resuspended, liquid shear forces may cause some transient damage to the membrane vesicles and consequent leakage of their contents.

Critical Parameters and Troubleshooting

Homogenization

Homogenization of the commonly used rat liver is covered in these protocols, and the methodology should be applicable to other soft tissue such as kidney. The homogenization procedures are unsatisfactory for hard tissues such as skeletal or cardiac muscle. These tissues require special treatment, and it is strongly recommended that methods that employ severe mechanical shear be avoided, as they tend to cause organelles such as lysosomes to be disrupted. Use of a protease (Nagarse, marketed

by Sigma-Aldrich as Protease Type VII) to soften the tissue allows its subsequent homogenization by more gentle means in a Dounce or Potter-Elvehjem homogenizer. For more information see Bhattacharya et al. (1991) and Graham (1997).

Hepatocytes, CHO cells, and COS-7 cells can be homogenized either in a tight-fitting Dounce homogenizer or a ball-bearing homogenizer using a routine homogenization medium (i.e., 0.25 M sucrose with or without 1 mM EDTA, buffered with either Tris or HEPES). Although most monolayer cultured cells seem to homogenize reasonably effectively in this medium, the medium developed by Marsh et al. (1987), in which the buffer was changed to 10 mM triethanolamine/10 mM acetic acid, pH 7.4, is more widely applicable. The triethanolamine/acetic acid buffer is the critically important component. If, upon homogenization, the cell homogenate acquires a gelatinous quality, this may be due either to nuclear breakage or to the release of proteins from cytoskeletal elements. Inclusion of 1 mM Mg^{2+} with or without DNase I (10 μ g/ml) and/or 10 mM KCl may alleviate the problems.

For cultured cells, the device of choice that seems to provide the least damage to intracellular organelles is the ball-bearing homogenizer, first described by Balch and Rothman (1985). A Dounce homogenizer (or passage through a fine syringe needle) is often used as an acceptable alternative, but for cells other than those described in this unit, it is strongly recommended that the optimal conditions for disrupting the cells be thoroughly investigated. The aim must always be to use the minimum force to achieve ~90% cell breakage, and protocols, for example, that use more than 20 pestle strokes of a Dounce homogenizer should be avoided, if at all possible.

For cells that resist disruption by liquid shear forces, nitrogen cavitation is probably the best option, as an isoosmotic sucrose medium can always be used. Standard conditions are 300 to 600 psi for 10 to 15 min, although Kelly et al. (1989) reported the use of much lower pressures. For a more complete discussion of these problems, see Evans (1992) and Graham (1997).

Ideally the Polytron homogenizer used in Basic Protocol 1 should not be substituted by any other homogenization device. If another device is used, the homogenate should be centrifuged at $5000 \times g$ for 15 min and the supernatant recentrifuged at $100,000 \times g$ for 30 min. The recovery of galactosyltransferase in the

5000 × *g* pellet should be ≥50% of the total (Morré et al., 1972).

Density gradient centrifugation

Use of tissues or cells other than those for which the protocols were designed may require some modification to the density range of gradients; this can only be determined by experimentation. In those protocols in which preformed continuous or discontinuous gradients are used, the use of swinging-bucket rotors with tube volumes other than those recommended in the protocols is permissible so long as the volume ratios of sample and gradient are maintained and the rotors are capable of similar relative centrifugal forces (RCFs). However, use of alternative rotors for self-generated gradients is not recommended unless it is first established that the rotor can be used to form a gradient of the correct density profile (see Anticipated Results). The sedimentation path length and maximum RCF of the rotor are the critical factors; generally speaking, path lengths of >30 mm and RCFs <180,000 *g*_{av} are unacceptable. The gradient-forming capacity of any rotor should be checked by running a blank gradient, fractionating the gradient either by upward displacement or tube puncture, and determining the density of the gradient fractions by refractometry. In the case of iodixanol, density (ρ) and refractive index (η) of solutions produced by diluting OptiPrep with a medium containing 0.25 M sucrose are related by the following equation: $\rho = 3.4713\eta - 3.6393$.

Enzyme and other assays

If the sample volumes from sucrose or iodixanol gradient fractions used in an enzyme assay are sufficiently small (compared to the assay volume), the gradient solute may be adequately diluted so as not to cause any interference in the assay, even at the highest density. Indeed, it is well established that neither iodixanol nor sucrose exhibits any significant inhibition of the standard enzyme markers (Ford et al., 1994). Thus, so long as the concentration of organelles in the gradient fraction is high enough for the enzyme activity to be detectable, the assay may be performed directly without washing. If, however, either of these requirements is not met, or if it is important to suspend the membrane fractions in a medium of constant composition, then dilution, sedimentation, and resuspension of the pellet in a smaller volume of an appropriate medium is the recommended method. In the case of sucrose, dialysis is an acceptable alternative, although any resid-

ual dextran in a gradient fraction from Basic Protocol 1 is not dialyzable. For iodixanol, ultrafiltration using a microcentrifuge cone filter can be used.

In order to determine specific activities of measured enzymes, it is also necessary to estimate the protein content of fractions. Both iodixanol and, to a lesser extent, sucrose interfere with methods using the Folin Ciocalteu reagent. However, if the concentration of gradient solute in the assay solution can be reduced to <10%, i.e., by using small volumes of gradient fractions (≤100 μ l) in a 1- to 2-ml assay volume, the interference is marginal. Methods based on Coomassie blue are generally unaffected by any concentration of these gradient media.

Any electrophoretic and electroblotting technique can also be carried out on sucrose- or iodixanol-containing fractions without prior removal of the medium, again, as long as the concentration of membrane in the sample is sufficiently high for the analytical procedure.

Anticipated Results

Basic Protocol 1

According to the data of Morr e et al. (1972), 40% to 70% of the total *N*-acetylglucosamine galactosyltransferase activity originally in the rat liver homogenate should be recoverable in the material harvested from the top of the 1.2 M sucrose density barrier. The recovery from other tissues or cells will depend very much on the existence of a well-organized Golgi apparatus in the cell and the behavior of the Golgi membranes during homogenization. The fraction purity should be ≥80% with a relative specific activity (ratio of specific activity of *N*-acetylglucosamine galactosyltransferase in the Golgi fraction to that in the homogenate) of ~90.

Basic Protocol 2

The following information is taken from Fleischer and Fleischer (1970). The Golgi membranes at the 0.77 M/1.1 M sucrose interface should contain the highest activity of galactosyltransferase activity (a specific activity of ~120 relative to that of the homogenate), while the slightly denser membranes should have lower activity (~70). Total yield of galactosyltransferase in the two bands is 30% to 35% of that in the homogenate, while only ~0.3% of the total protein is recovered in these two bands.

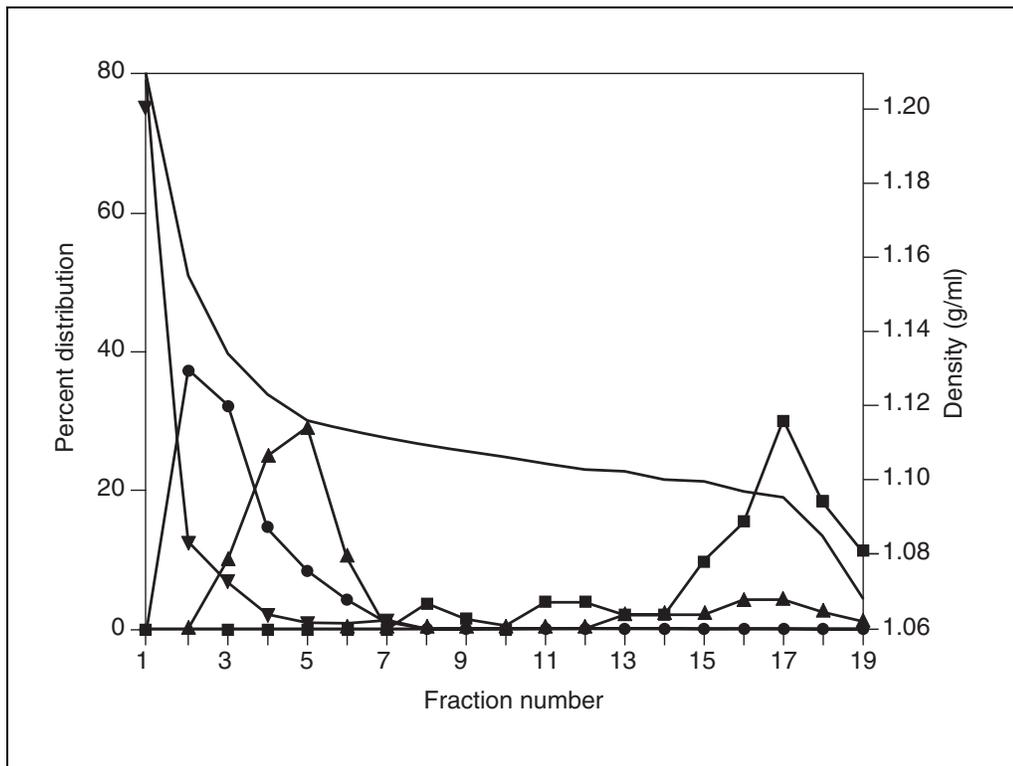


Figure 3.9.1 Fractionation of a light mitochondrial fraction (mouse liver) in a self-generated gradient of iodixanol: percent distribution of enzyme markers (symbols) and density profile (—). Light mitochondrial fraction adjusted to 15% (w/v) iodixanol and centrifuged at $180,000 \times g$ for 3 hr in a 20° fixed-angle rotor. Organelle markers and symbols: catalase for peroxisomes (filled inverted triangle), succinate dehydrogenase for mitochondria (filled circle), β -galactosidase for lysosomes (filled triangle), and galactosyltransferase for Golgi (filled square). Adapted from Graham et al. (1994) with permission from Academic Press.

Alternate Protocol 1

Figure 3.9.1 shows a typical distribution of marker enzymes in the iodixanol gradient. At least 80% of the total galactosyltransferase present in the light mitochondrial fraction from mouse liver should be recovered from the top five fractions. The same fractions should contain <10% of any lysosomal marker (β -galactosidase) and negligible amounts of enzyme markers for other organelles (Graham et al.,

1994). The relative specific activity of galactosyltransferase (specific activity in gradient fraction relative to that in the light mitochondrial fraction) in the peak Golgi fraction should be ~20.

Basic Protocol 3

Some typical recoveries of Golgi (*N*-acetylglucosamine galactosyltransferase) and ER (glucosidase I) markers are given in Table 3.9.2

Table 3.9.2 Marker Enzyme Activity in Isolated Fractions from CHO Cells Processed by Flotation Through a Discontinuous Sucrose Gradient^a

Fraction	Protein recovery (%)	<i>N</i> -Acetylglucosamine galactosyltransferase		Glucosidase I	
		Total activity	Specific activity	Total activity	Specific activity
Postnuclear supernatant	NA ^b	164	2.3	0.6	0.008
Golgi	3.6	70	26.8	0.026	0.01

^aData from Balch et al. (1984).

^bAbbreviation: NA, not applicable.

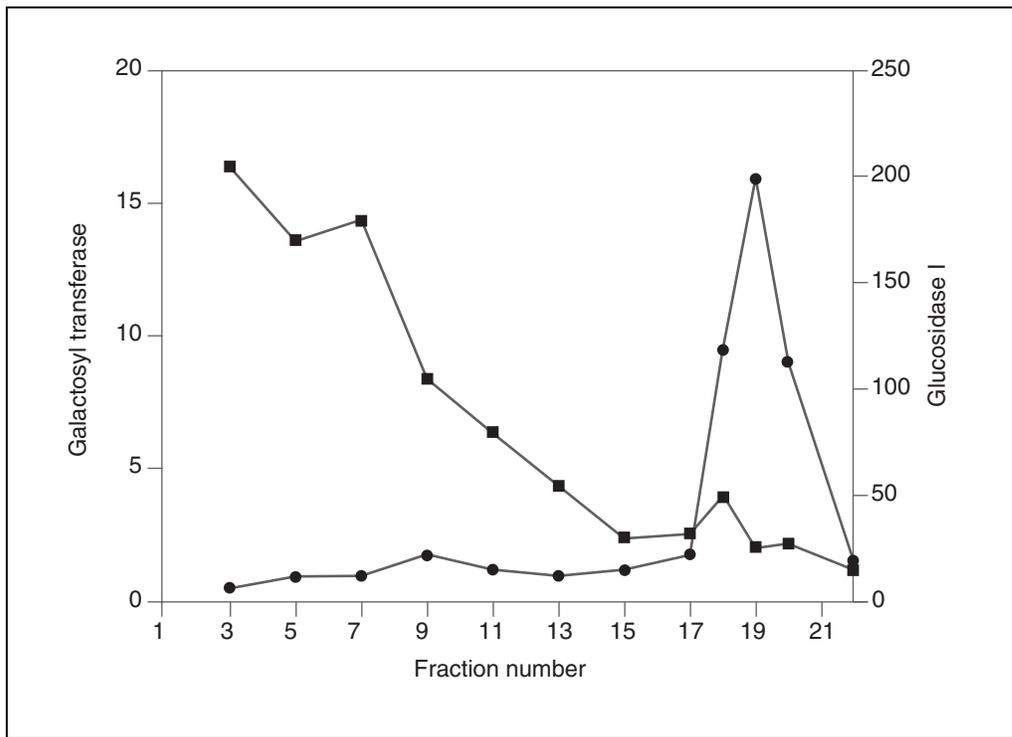


Figure 3.9.2 Fractionation of a homogenate of CHO cells in a 0.8 to 2.0 M discontinuous sucrose gradient: distribution of Golgi (galactosyltransferase) and endoplasmic reticulum (ER; glucosidase I) markers. Gradients were unloaded dense-end first. Activity of galactosyltransferase (filled circle) expressed as $\text{cpm} \times 10^{-2}$; activity of glucosidase I (filled square) expressed as cpm. Adapted from Balch et al. (1984) with permission from Elsevier Science.

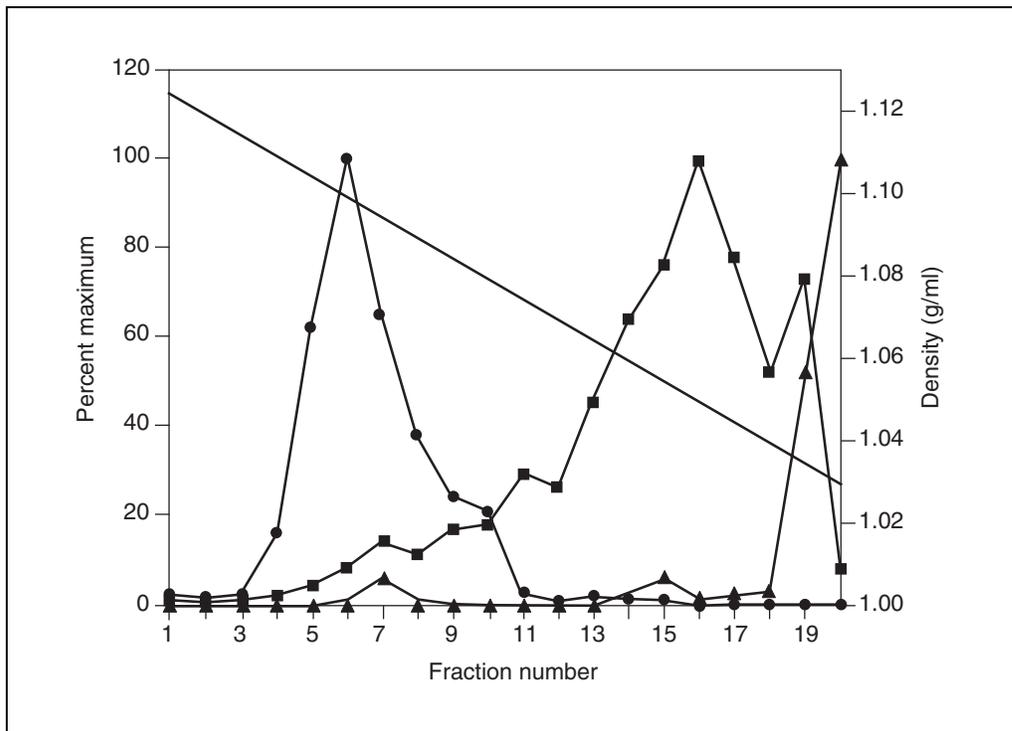


Figure 3.9.3 Fractionation of a postnuclear supernatant from CHO cells on a preformed continuous 0% to 26% (w/v) iodixanol gradient: distribution of markers (symbols) and density profile (—). Activities in each fraction expressed as a percentage of the maximum fraction. Organelle markers and symbols: ribophorin for endoplasmic reticulum (ER; filled circle), galactosyltransferase for Golgi (filled square), and biotinylated surface proteins for plasma membrane (filled triangle). Adapted from Yang et al. (1997) with permission from the American Society for Biochemistry and Molecular Biology.

(Balch et al., 1984). The Golgi fraction contains ~40% of the total *N*-acetylglucosamine galactosyltransferase of the postnuclear supernatant but only ~4% of the glucosidase I. Figure 3.9.2 shows that essentially all of the ER is distributed quite broadly in the bottom half of the gradient, while the Golgi bands sharply at the 0.8 M/1.2 M sucrose interface.

Alternate Protocol 2

Distribution of the Golgi and ER markers (Yang et al., 1997) in the continuous iodixanol gradient (Fig. 3.9.3) is not unlike that observed in the discontinuous sucrose gradient (Basic Protocol 3), but the rather broader distribution of the Golgi may provide some potential for subfractionation of the Golgi into light and dense populations. Moreover, some small contamination of the Golgi by the ER is evident in the sucrose gradient (the specific activity of glucosidase I is indeed slightly raised in the Golgi fraction over the postnuclear supernatant; Table 3.9.2). In the iodixanol gradient, on the other hand, no contamination whatsoever is apparent. If the two lightest fractions containing plasma membrane are discarded, then ≥80% of the galactosyltransferase in the gradient is recovered in a highly purified manner.

Basic Protocol 4

Figure 3.9.4 shows a typical result of the self-generated iodixanol gradient (Plonné et al., 1999): ~80% of the galactosyltransferase is contained in the top seven fractions of the gradient. The system can also be used for the simultaneous isolation of smooth ER (fractions 9 to 13) and rough ER (fractions 15 to 20).

Time Considerations

There are no points at which any of the basic or alternate protocols can be discontinued. The light mitochondrial fraction produced in Basic Protocol 2 must be used immediately for any subsequent gradient separation. Discontinuous gradients may be made up immediately prior to use and underlayered with the dense light mitochondrial fraction (Basic Protocol 2) or homogenate (Basic Protocol 3). Preformed continuous gradients (Alternate Protocol 2) may be made up during the preparation of the postnuclear supernatant and kept on ice until required. If these continuous gradients are prepared at room temperature they must be given time to cool down to 4°C. Alternatively, they can be made from a discontinuous gradient the previous day and stored overnight at 4°C to allow diffusion to occur. Always check that a

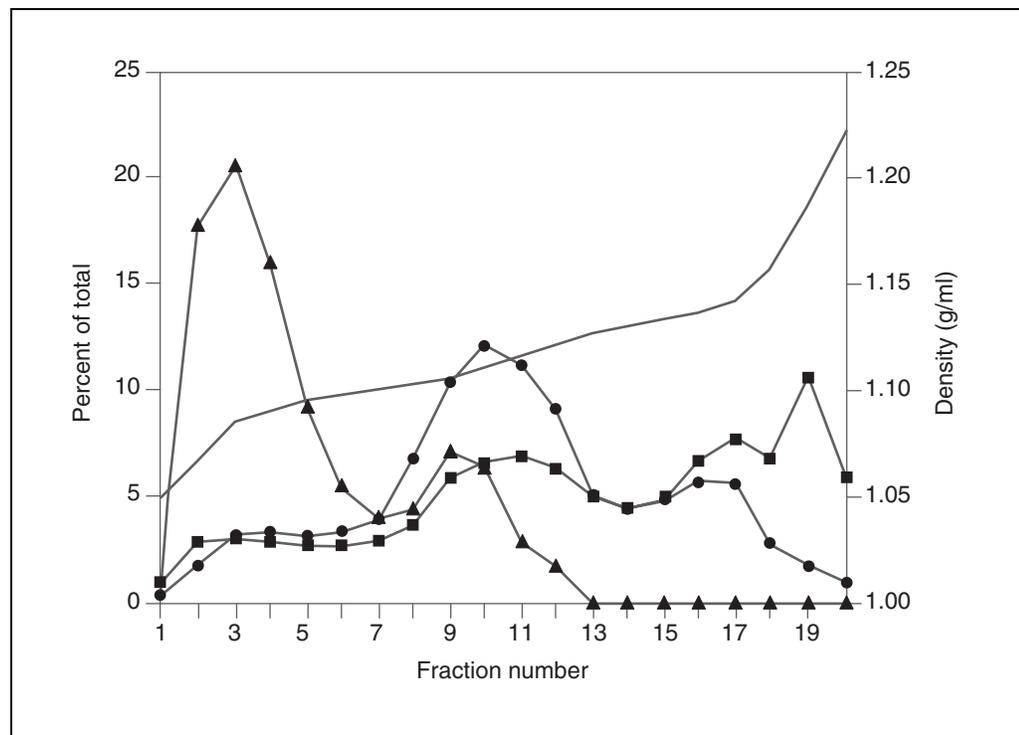


Figure 3.9.4 Fractionation of a rat hepatocyte microsomal fraction in a 15%/20% self-generated iodixanol gradient: percent distribution (symbols) and density profile (—). Centrifugation was carried out in a Beckman VTi 65.1 vertical rotor at $350,000 \times g$ for 2 hr. Organelle markers and symbols: NADPH-cytochrome c reductase for ER (filled circle), galactosyltransferase for Golgi (filled triangle), and protein (filled square). Adapted from Plonné et al. (1999) with permission from Academic Press.

continuous gradient has been formed by measuring the refractive index of fractions from a blank gradient.

Basic Protocol 1 is a rapid procedure and Morré et al. (1972) considered that the livers from as many as ten to twelve rats could be processed in 2 hr. Preparation of the light mitochondrial fraction (Basic Protocol 2), which forms the input for gradients described in the same protocol and in Alternate Protocol 1, should take no more than 1 hr for one to two rat livers. Forming, centrifuging, and harvesting the discontinuous gradients (Basic Protocol 2) will take ~2 hr for two tubes. Tube preparation for self-generated gradients (Alternate Protocol 1) is much faster than is the construction of preformed gradients, but because of the longer acceleration and deceleration times, the overall time will be similar if the centrifugation is carried out at $350,000 \times g$. Basic Protocol 3 will take ~3 hr, as will Alternate Protocol 2 if the shorter centrifugation time is used. If hepatocytes are used, the overall time for Basic Protocol 4 will be ~5 hr, but for liver this will be nearer to 4 hr.

For the first couple of preparations it is probably advisable to unload continuous gradients in a number of equivolume fractions (fraction volume should be ~5% of gradient volume). With experience and practice, however, it will be possible to remove the Golgi-containing fractions with a syringe or Pasteur pipet.

Functional assays should be carried out as soon as possible after the preparation. The Support Protocol should take ~4 hr for 20 to 30 samples. For simple marker enzyme assays (such as described in this unit), material can be stored at 0° to 4°C for ≤16 hr without much loss of activity. These assays and compositional measurements may also be carried out on material frozen to -20° or -80°C. There are, however, no general rules regarding the suitability of a particular storage regime, and it is strongly recommended that the validity of results on stored material be checked by comparison with those made on the fresh organelles. Any concentration of the membrane fractions and/or removal of gradient medium must be carried out prior to freezing.

All solutions, except the acceptor solution (Support Protocol), can be made up ahead of time and stored at 4°C for 1 to 2 days or frozen to -20°C for longer periods (e.g., 5 months). Any enzymes or protease inhibitors should be added immediately prior to use.

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Isolation of Nuclei and Nuclear Membranes From Animal Tissues

UNIT 3.10

The vast majority of methods for the isolation of nuclei and nuclear membranes use soft mammalian tissues (particularly rat liver) as the biological source; however, the protocols described in this unit should be applicable generally to any tissue type, cultured cells, or cells of lower eukaryotes or plants, so long as a suitable homogenization method is available. Generally, a buffered isoosmotic medium containing KCl and MgCl₂ is used to stabilize the nuclei of mammalian tissues. Some of the media used for cultured animal cells and nonmammalian sources are described later in the unit (see Commentary). Purification using a sucrose barrier of 2.3 M sucrose is described (see Basic Protocol 1). The use of such dense sucrose solutions has several disadvantages, notably the high viscosity, meaning that particles sediment very slowly through the medium; therefore, the procedure requires an ultracentrifuge. The high osmolarity may also be detrimental to the intranuclear structure, and certainly the organelles lose water and shrink considerably in size. The use of OptiPrep (iodixanol; Axis-Shield) overcomes both the viscosity and osmolarity problems (see Alternate Protocol 1) and permits the use of a high-speed centrifuge and much shorter centrifugation times. A series of alternate steps are given for plant cell (i.e., wheat germ) nuclei, since the dense layer of iodixanol used in this method is able to resolve nuclei from the denser starch granules (see Commentary for more details).

The centrifugation of any subcellular particle through an aqueous medium tends to lead to loss of some proteins that may be important for proper functioning of the organelle. Although nonaqueous media can overcome this problem, they are generally avoided, since they often pose more problems than they solve. Nuclei are probably the only organelle that might benefit from such an approach, so a method employing a gradient of Nycodenz (Axis-Shield) in ethylene glycol is described (see Alternate Protocol 2).

The isolation of nuclear membranes from purified nuclei has been approached via diametrically opposite strategies. High-ionic-strength media generally give high yields of nuclear membranes, and the hollow membranous spheres that are formed seem well suited to permeability studies (see Basic Protocol 2). Low-ionic-strength media produce highly purified nuclear envelopes, which are particularly good for compositional studies (see Basic Protocol 3).

Two simple chemical methods for estimating DNA and RNA (see Support Protocols 1 and 2) and a fluorometric method for measuring both nucleic acids (see Support Protocol 3) are also provided.

ISOLATION OF NUCLEI FROM RAT-LIVER HOMOGENATE USING A SUCROSE DENSITY BARRIER

**BASIC
PROTOCOL 1**

In this procedure the tissue is homogenized in an isoosmotic medium containing 0.25 M sucrose, 25 mM KCl, 10 mM MgCl₂, and 10 mM Tris-Cl, pH 7.4 (Blobel and Potter, 1966). Variations for other tissues, cultured cells, or nonmammalian material are described elsewhere (see Commentary). Filtration of the homogenate is often included to avoid contamination of the subsequent crude nuclear pellet, produced by centrifugation of the homogenate at 800 × *g* for 10 min, by connective tissue and unbroken cells; the connective tissue may also make resuspension of the pellet difficult. The pellet is then resuspended in ~1.6 M sucrose and layered over 2.3 M sucrose. After centrifugation at 100,000 × *g* for 1 hr, the nuclei form a pellet at the bottom of the tube, while any

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contaminating material remains in the load zone. In some methods (Widnell and Tata, 1964), the lower barrier is eliminated and the nuclei are simply pelleted from the dense crude nuclear suspension. This alternative is given in the annotations to the respective steps.

If the exposure of the nuclei to such a vastly hyperosmotic medium is undesirable, then a method using an isoosmotic discontinuous gradient of iodixanol can be used (see Alternate Protocol 1). These gradients are also much less viscous and the centrifugation can be carried out at much lower RCFs in a high-speed centrifuge. A gradient that is nonaqueous can also be used (see Alternate Protocol 2). The following protocol is adapted from Blobel and Potter (1966).

Materials

150- to 200-g Sprague-Dawley rats
Nuclear isolation medium (NIM; see recipe)
Sucrose density barrier (SDB; see recipe)

Scissors or razor blade
~30-ml Potter-Elvehjem homogenizer with 0.1-mm clearance and loose-fitting pestle
75- μ m pore size nylon gauze or cheesecloth (muslin)
Glass rod
Refrigerated low-speed centrifuge with swinging-bucket rotor and appropriate 50-ml polypropylene or polystyrene tubes
~30-ml Dounce homogenizer with loose-fitting Wheaton Type B pestle
Refractometer
Ultracentrifuge with swinging-bucket rotor (e.g., Beckman SW40Ti, Sorvall TH641) and appropriate 14-ml tubes

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C, and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

NOTE: Protease inhibitors (*UNIT 3.4*) may be added to any or all solutions.

Isolate and homogenize the liver

1. Starve a 150- to 200-g Sprague-Dawley rat overnight.
2. Sacrifice the animal by cervical dislocation or decapitation.
This must be supervised or carried out by an experienced animal technician.
3. Open the abdominal cavity and remove the liver to a chilled beaker containing ~20 ml nuclear isolation medium (NIM).
4. Decant the medium and finely mince the liver using scissors or a razor blade.
The pieces of liver should be no more than ~25 mm³.
5. Suspend the mince in ~40 ml NIM and transfer half of this suspension to the glass vessel of the ~30-ml Potter-Elvehjem homogenizer.

The motor should be mounted either to a wall, via a G-clamp to the bench, or in a floor-standing cradle. Attachment to a free-standing retort stand is not adequate.

6. Attach the precooled pestle to the electric motor and homogenize the liver mince using 4 to 5 up-and-down strokes of the pestle, rotating at ~500 rpm.
7. Decant the homogenate into a beaker on ice. After rinsing the homogenizer with NIM, and wiping the pestle to remove any connective tissue that may be adhering, perform the procedure (i.e., steps 5 to 7) with the other half of the mince.

Prepare crude nuclear pellet

8. Filter the homogenate through a single layer of nylon gauze (pore size of 75 μm), or three layers of cheesecloth (muslin) to remove unbroken cells and any connective tissue.

Do not force the homogenate through the filter; assist filtration only by stirring with a glass rod.

9. Dilute the homogenate with at least an equal volume of NIM.

For cultured cells that have been homogenized in a hypoosmotic medium, add an equal volume of buffer containing 0.5 M sucrose. If there is neither KCl nor MgCl_2 in the homogenate, double the concentration of these salts in the added buffer.

10. Distribute the suspension equally between two 50-ml centrifuge tubes and centrifuge in a low-speed centrifuge with a swinging-bucket rotor 10 min at $800 \times g$, 4°C.
11. Decant the supernatant, discarding as much of the upper, loosely packed brown layer as possible. Resuspend the pellet in 40 ml NIM using the ~30-ml Dounce homogenizer.
12. Repeat the centrifugation as described in step 10.
13. Decant the supernatant and make a crude resuspension of the combined pellets in NIM, using a glass rod, so that the final volume is ~8 ml.

If the crude nuclear suspension itself is to be adjusted to ~2.2 M sucrose and centrifuged without layering over a barrier, do not resuspend the pellets in NIM.

Purify nuclei

14. Add 2 vol sucrose density barrier (SDB) to the crudely resuspended nuclear pellet and mix very thoroughly using the Dounce homogenizer.

Alternatively, resuspend the pellets from step 13 directly in ~9 vol SDB. This critical step is quite difficult because of the viscosity of the SDB. It may be easier to use the Potter-Elvehjem homogenizer rotating at 1000 rpm.

15. Check that the refractive index of the suspension is 1.4117 ± 0.0004 , using a refractometer. Adjust concentration with SDB, if necessary, to attain this refractive index.
16. Transfer 9 ml suspension to a 14-ml ultracentrifuge tube. Underlayer with 2.3 M sucrose to fill the tube. Ultracentrifuge in a swinging-bucket rotor 1 hr at $100,000 \times g$, 4°C.

In the alternative method, simply fill the tubes with the resuspended nuclei and centrifuge in a swinging-bucket or fixed-angle rotor.

17. Remove any material that has floated to the meniscus with a spatula, then decant all the liquid from above the pellet. Leave the tubes inverted for a few minutes to drain as much of the viscous sucrose layer as possible.
18. While the tube is inverted, wipe off as much of the liquid from the walls of the tube as possible, using a tissue wrapped around forceps.

19. Resuspend the pellet in NIM, or other suitable medium compatible with the next process, to ~5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; some enzyme assays can be carried out after storage at 4°C for up to 16 hr. Assays can often be carried out on material frozen to -20° or -80°C. Always check that the measurements made are relatively unaffected by the storage conditions by comparing with those made on fresh material. Note however that thawing a frozen suspension of nuclei may cause release of DNA, and consequently almost irreversible aggregation of the particles.

ALTERNATE PROTOCOL 1

ISOLATION OF NUCLEI FROM ANIMAL OR PLANT (WHEAT GERM) CELLS USING AN IODIXANOL GRADIENT

If elimination of any potential damage to the nucleoprotein complexes is important, then the alternative iodixanol gradient described in this protocol might be relevant. Using these gradients it is possible to isolate nuclei by isopycnic banding in an isoosmotic nonionic environment (Graham et al., 1994; Provost et al., 1996). Because the nuclei retain their normal hydration, their density ($\rho = 1.20$ to 1.22 g/ml) is much lower than in sucrose (>1.32 g/ml). Buoyant density banding thus requires much lower concentrations of iodixanol; consequently the viscosity of the medium is much lower, as are the centrifugation forces and times needed to purify the nuclei. Also, unlike using a sucrose gradient (see Basic Protocol 1), it is not necessary to produce a crude nuclear fraction first. After the whole homogenate is adjusted to 25% (w/v) iodixanol, this is loaded directly on to a discontinuous iodixanol gradient; therefore, the method is quicker and simpler to execute. The protocol below is adapted from Graham et al. (1994). Ford et al. (1998) reported that a comparable iodixanol gradient is also able to separate the nuclei and starch granules from wheat germ (see alternative steps). This is not possible with sucrose gradients, since both pellet through the 2.3 M sucrose.

Additional Materials (also see Basic Protocol 1)

- Filtered homogenate (animal cells or tissue; see Basic Protocol 1) or wheat germ
- 50% (w/v) iodixanol (see recipe; animal cells or tissue)
- 30% and 35% (w/v) iodixanol gradient solutions (see recipe; animal cells or tissue)
- 25% and 40% (w/v) iodixanol gradient solutions (see recipe; wheat germ)
- Wheat germ medium (WGM; see recipe)
- High-speed centrifuge with a swinging-bucket rotor and appropriate 50-ml tubes

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C, and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

NOTE: Protease inhibitors (UNIT 3.4) may be added to any or all solutions.

For animal cells or tissue

- 1a. Prepare the filtered homogenate as described (see Basic Protocol 1, steps 1 to 8).
- 2a. Mix the homogenate with an equal volume of 50% iodixanol.
- 3a. In a 50-ml centrifuge tube, layer 15 ml each of 35% and 30% iodixanol solutions, and sample.

If it is necessary to reduce the number of tubes used for this centrifugation, the volume of 35% iodixanol per tube may be reduced to 7.5 ml while increasing the volume of homogenate by a corresponding amount.

- 4a. Centrifuge in a high-speed centrifuge with a swinging-bucket rotor 20 min at $10,000 \times g$, 4°C .

The nuclei from some tissues or cells may be smaller and/or less dense than those from mammalian liver; therefore, it may be necessary to increase the RCF to $20,000 \times g$ and/or modulate the concentration (w/v) of iodixanol in the three layers (e.g., 20%, 25%, and 30%).

- 5a. Harvest the nuclei from above the 35% iodixanol and dilute with 2 vol of NIM.

For wheat germ

- 1b. Make a crude suspension of nuclei by vigorously shaking ~5 g of wheat germ in ~50 ml WGM.
- 2b. Allow the debris to settle for 10 min. Carefully decant the supernatant.
- 3b. Layer 20 ml crude nuclear suspension over 5 ml each 25% and 40% iodixanol gradient solutions.
- 4b. Centrifuge in a high-speed centrifuge with a swinging-bucket rotor 30 min at $5600 \times g$, 4°C .
- 5b. Harvest the nuclei, which band above the 40% iodixanol.

The starch granules form a pellet.

Concentrate nuclei

6. Centrifuge 10 min at $2000 \times g$, 4°C in a low-speed centrifuge with a swinging-bucket rotor. Resuspend the pellet in NIM (animal cells or tissue) or WGM (wheat germ), or other suitable medium compatible with the next process, to ~5 mg protein/ml.

Metabolic assays should be carried out as soon as possible upon preparation; however, some enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20° or -80°C . Always check that the measurements made are relatively unaffected by the storage conditions by comparing with those made on fresh material. Thawing a frozen suspension of nuclei may cause release of DNA, and consequently almost irreversible aggregation of the particles.

ISOLATION OF NUCLEI FROM RAT-LIVER HOMOGENATE IN A NONAQUEOUS MEDIUM

**ALTERNATE
PROTOCOL 2**

It may be beneficial to use homogenization and gradient media that are nonaqueous in order to eliminate, as far as possible, any loss of proteins from the external surface of the nuclei, or the leaching out of proteins from the matrix. This approach, in particular, eliminates loss of DNA polymerase from the nuclei. The method, developed by Rickwood et al. (1997), in which an aqueous sucrose medium is replaced by ethylene glycol, permits the use of standard Potter-Elvehjem homogenization techniques. Moreover, Nycodenz (Axis-Shield) is freely soluble in the same solvent so purification in a density gradient is also possible.

Additional Materials (also see Basic Protocol 1)

- Ethylene glycol homogenization medium (EGHM; see recipe)
10% and 40% (w/v) Nycodenz (Axis-Shield; available from Accurate Chemicals)
in EGHM
Two-chamber gradient maker or Gradient Master (BioComp Instruments)

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NOTE: All solutions, glassware, centrifuge tubes, and equipment should be precooled to 0° to 4°C, and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

NOTE: Protease inhibitors (*UNIT 3.4*) may be used in any or all solutions.

1. Prepare a filtered homogenate as described (see Basic Protocol 1, steps 1 to 8), except omit the use of NIM for washing the liver (step 3), replacing the ~40 ml NIM (step 5) with ~50 ml EGHM, and increasing the number of strokes of the pestle from 4 to 5 to 6 to 9 (step 6).
2. Centrifuge in a low-speed centrifuge with a swinging-bucket rotor 10 min at $1000 \times g$, 4°C.
3. Discard the supernatant and resuspend the pellet in 10% (w/v) Nycodenz in EGHM (~2 ml/g liver), using a Dounce homogenizer.
4. Prepare 12-ml continuous gradients from equal volumes 10% and 40% Nycodenz in EGHM in a 14-ml ultracentrifuge tube using a two-chamber gradient maker or Gradient Master, and layer 2 ml resuspended nuclear pellet on top.
5. Ultracentrifuge in a swinging-bucket rotor 90 min at $80,000 \times g$, 4°C.
6. Remove the white band of nuclei from the middle of the gradient (i.e., ~22% Nycodenz) using a syringe or pipet.
7. Dilute the suspension with 2 vol EGHM. Centrifuge in a low-speed centrifuge with a swinging-bucket rotor 10 min at $1000 \times g$, 4°C.
8. Resuspend in EGHM or some other medium appropriate to further analysis.

Metabolic assays should be carried out as soon as possible upon preparation; however, some enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20°C or -80°C. Always check that the measurements made are relatively unaffected by the storage conditions, by comparing with those made on fresh material. Note that thawing a frozen suspension of nuclei may cause release of DNA, and consequently almost irreversible aggregation of the particles.

BASIC PROTOCOL 2

ISOLATION OF NUCLEAR MEMBRANES: HIGH-IONIC-STRENGTH METHOD

High-ionic-strength methods for removing chromatin from the nucleus were first developed by Monneron et al. (1972), and more recently, high concentrations (i.e., 1.6 M) of NaCl were used by Kaufmann et al. (1983). In these methods, nucleus-sized hollow spheres are produced that are good for permeability studies. DNase I and RNase are also commonly included to digest the nucleic acids, and phenylmethylsulfonyl fluoride (PMSF) is included as a protease inhibitor.

As with the preparation of nuclei, most nuclear membrane methods are also based on rat liver and application to nuclei from other sources will need to be approached empirically to determine which, if any, of the current methods are suitable. This protocol is adapted from Kaufmann et al. (1983).

Materials

200 mM PMSF (*APPENDIX 2A*)
Nuclei suspension medium (NSM; see recipe)
DNase I
RNase
Incubation buffer (IB; see recipe)
High-NaCl buffer (HNB; see recipe)
2-mercaptoethanol
Nuclear membrane storage medium (NMSM; see recipe)
~20-ml Dounce homogenizer with loose-fitting Wheaton Type B pestle
Refrigerated low-speed centrifuge with swinging-bucket rotor

1. Prepare the nuclei (see Basic Protocol 1 or Alternate Protocol 1), using 1 mM PMSF in all media, diluted from a 200 mM stock.
2. Suspend the nuclei in nuclear suspension medium (NSM) at a concentration of 5 to 8 mg DNA/ml (see Support Protocols 1 and 3), using 3 to 4 gentle strokes of the ~20-ml Dounce homogenizer.
3. Add DNase I and RNase to a final concentration of 250 $\mu\text{g/ml}$ and stir gently for 1 hr at 4°C.
4. Sediment the nuclei in a low-speed centrifuge with a swinging-bucket rotor for 10 min at $1000 \times g$, 4°C. Resuspend the pellet in the same volume incubation buffer (IB) to a concentration of 5 to 8 mg DNA/ml.
5. While stirring at 4°C, add 4 vol high-NaCl buffer (HNB) dropwise. Add 2-mercaptoethanol to a final concentration of 1% (v/v).

Make sure each drop of HNB is well mixed before adding the next.

6. Stir for a further 15 min, then pellet the nuclear envelopes in a low-speed centrifuge with swinging-bucket rotor for 30 min at $1600 \times g$, 4°C.
7. Resuspend the pellet in IB and repeat steps 5 and 6, but omit the 2-mercaptoethanol.
8. Harvest the membranes by centrifugation in a low-speed centrifuge with swinging-bucket rotor for 30 min at $1600 \times g$, 4°C.
9. If the membranes are not to be analyzed immediately, suspend in nuclear membrane storage medium (NMSM) and maintain at -20°C .

Metabolic assays should be carried out as soon as possible upon preparation; however, some enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20°C or -80°C . Always check that the measurements made are relatively unaffected by the storage conditions by comparing with those made on fresh material.

ISOLATION OF NUCLEAR MEMBRANES: LOW-IONIC-STRENGTH METHOD

The low-ionic-strength method described in this protocol uses 0.1 mM MgCl_2 and is relatively rapid. This method produces fragments that range from incomplete “ghosts” to much smaller pore-sized fragments that retain the lamina and pores and are good for compositional studies (Kay et al., 1972; Harris and Milne, 1974; Rickwood et al., 1997). The protocol is adapted from Kay et al. (1972).

The application of this and other methods to nuclei from sources other than mammalian liver will need to be approached empirically to determine which of these protocols are suitable.

BASIC PROTOCOL 3

Subcellular Fractionation and Isolation of Organelles

3.10.7

Materials

200 mM PMSF (*APPENDIX 2A*)
Low-ionic-strength suspension medium (LISM; see recipe)
1.0 mg/ml DNase I
Sucrose buffer, pH 7.4 and 8.5 (SB7.4 and SB8.5; see recipe)
NMSM (see recipe)
~10-ml Dounce homogenizer with loose-fitting Wheaton Type B pestle
High-speed centrifuge with fixed-angle rotor for 15-ml tubes

NOTE: All centrifugations are carried out at 4°C and all incubations are at 22°C.

1. Prepare the nuclei (see Basic Protocol 1, Alternate Protocol 1, or Alternate Protocol 2), using 1 mM PMSF in all media, diluted from a 200 mM stock.
2. Suspend the nuclei in low-ionic-strength suspension medium (LISM) to a concentration of 3 to 4 mg DNA/ml (see Support Protocols 1 and 3), using 4 to 5 gentle strokes of the pestle of the ~10-ml Dounce homogenizer.
3. Add DNase I to a final concentration of 5 µg/ml from the 1 mg/ml stock and add 4 vol sucrose buffer, pH 8.5 (SB8.5).
4. Incubate with gentle stirring for 15 min at 22°C and then add an equal volume ice-cold distilled water.
5. Transfer to an appropriate centrifuge tube and centrifuge in a high-speed centrifuge with fixed-angle rotor 15 min at 38,000 × g, 4°C.
6. Resuspend the pellet in the same volume of sucrose buffer, pH 7.4 (SB7.4), and add DNase I to a final concentration of 1 µg/ml.
7. Incubate 15 min with gentle stirring at 22°C and then add an equal volume ice-cold distilled water.
8. Centrifuge 15 min at 38,000 × g, 4°C. If the membranes are not to be analyzed immediately, resuspend in NMSM and store at -20°C.

Metabolic assays should be carried out as soon as possible upon preparation; however, some enzyme assays can be carried out after storage at 4°C for up to 16 hr. Many assays can be carried out on material frozen to -20°C or -80°C. Always check that the measurements that are made are relatively unaffected by the storage conditions by comparing with those made on fresh material.

SUPPORT PROTOCOL 1

DIPHENYLAMINE ASSAY FOR DNA

This well-established chemical method for quantifying DNA, devised by Schneider (1957), is still probably one of the best, simplest, and most reliable assays. It measures the deoxyribose released by acid hydrolysis; however, because of interference by other carbohydrates, the DNA of samples in sucrose or any other gradient medium containing a sugar residue (e.g., metrizamide), must be first precipitated with trichloroacetic acid (TCA). Although a later method based on the use of the dye methyl green (Peters and Dahmus, 1979) does not require hydrolysis of the nucleic acid and is compatible with most gradient media, it is necessary to extract the methyl green with chloroform to remove contaminating crystal violet, and some sources of methyl green do not give consistent results (Graham and Ford, 1983).

Materials

500 µg/ml DNA standard solution
5%, 10%, and 20% (w/v) trichloroacetic acid (TCA), ice-cold
Samples
Diphenylamine reagent (see recipe)

1. Dilute the 500 $\mu\text{g/ml}$ DNA standard to give 0 to 200 μg DNA in 1.0 ml of 5% (w/v) TCA, diluted from a 20% (w/v) stock.
2. *Optional:* For samples in sucrose, precipitate the DNA by adding an equal volume ice-cold 20% (w/v) TCA.
Although this is not strictly necessary for iodixanol-containing fractions, it is a useful way of concentrating dilute fractions. Omit steps 2 to 4 if precipitating DNA is not necessary.
3. After 10 min on ice, sediment the DNA by centrifuging in a low-speed centrifuge with a swinging-bucket rotor 20 min at $1000 \times g$, 4°C .
4. Wash the pellet twice in ice-cold 10% TCA.
5. Hydrolyze the DNA in the standards, membrane suspensions, or pellets in 5% (w/v) TCA (1 ml final volume), 20 min at 90°C .
6. Centrifuge 2 min at full speed in a microcentrifuge.
7. To 0.5 ml of each supernatant (and a blank containing 0.5 ml 5% TCA), add 1 ml diphenylamine reagent and heat 10 min at 100°C .
8. Cool to room temperature and measure the absorbance at 595 nm.

ORCINOL ASSAY FOR RNA

Like the diphenylamine assay for DNA, this simple method was devised by Schneider (1957) and measures the ribose released from RNA by acid hydrolysis. Gradient media (e.g., sucrose, metrizamide) containing carbohydrate residues must be removed by precipitation of the nucleic acid with trichloroacetic acid (TCA) prior to hydrolysis. As the deoxyribose from DNA also interferes, standard curves for both DNA and RNA should be constructed.

Materials

500 $\mu\text{g/ml}$ DNA and RNA standard solutions
 5%, 10%, and 20% (w/v) TCA
 Samples
 Orcinol reagent (see recipe)

1. Dilute the 500 $\mu\text{g/ml}$ RNA and DNA standards to give 0 to 200 μg nucleic acid in 0.5 ml water.
2. *Optional:* For samples in sucrose, precipitate the nucleic acids by adding an equal volume ice-cold 20% TCA.
Although this is not strictly necessary for iodixanol-containing fractions, it is a useful way of concentrating dilute fractions. Omit steps 2 to 5 if precipitating the DNA is not necessary.
3. After 10 min on ice, sediment the DNA by centrifuging in a low-speed centrifuge with a swinging-bucket rotor 20 min at $1000 \times g$, 4°C .
4. Wash the pellet twice in ice-cold 10% TCA.
5. Resuspend the pellet in 0.5 ml water.
6. To 0.5 ml standards, membrane suspensions, or resuspended pellets, add 0.1 ml water and 0.2 ml 20% (w/v) TCA.
7. Hydrolyze the nucleic acids 20 min at 90°C .

SUPPORT PROTOCOL 2

Subcellular Fractionation and Isolation of Organelles

3.10.9

8. Microcentrifuge for 2 min at full speed.
9. To 0.6 ml of all supernatants and a blank containing 0.6 ml of 5% TCA, add 0.6 ml orcinol reagent and heat 20 min at 100°C.
10. Cool to room temperature and measure the absorbance at 660 nm.

To obtain an accurate value of the RNA content of a gradient fraction the contribution of the deoxyribose to the total absorbance must be subtracted. Determine the amount of DNA in the fraction using the diphenylamine method (see Support Protocol 1) and using the orcinol DNA standard curve convert this to an A_{660} value. Deduct this value from the A_{660} of the sample.

**SUPPORT
PROTOCOL 3**

ETHIDIUM BROMIDE ASSAY FOR DNA AND RNA

This very sensitive fluorometric method devised by Karsten and Wollenberger (1972, 1977), although very simple to execute, does require a large number of controls. Although the method avoids the use of the strong acids needed for the chemical method (see Support Protocols 1 and 2), ethidium bromide is extremely toxic. The characteristic fluorescence is produced by the ethidium bromide intercalating the DNA, which therefore indicates that ethidium bromide is a strong mutagen. The alternative fluorescent method (Brunk et al., 1979) using diamidinophenylindole (DAPI) is less potentially harmful, but the binding of the DAPI is highly specific for A-T base pairs, and it is therefore essential that the standard and sample have the same base composition (Graham and Ford, 1983). Both methods are compatible with any gradient medium.

NOTE: Use phosphate-buffered saline with Ca^{2+} and Mg^{2+} (PBSCM) to make all solutions and dilutions in this protocol.

Materials

- Phosphate-buffered saline with Ca^{2+} and Mg^{2+} (PBSCM; see recipe)
- 25 $\mu\text{g}/\text{ml}$ DNA standard solution: store at -20°C for 2 to 3 months
- 25 $\mu\text{g}/\text{ml}$ heparin
- Sample
- 50 $\mu\text{g}/\text{ml}$ RNase: heat at 100°C for 10 min to denature DNase
- 25 $\mu\text{g}/\text{ml}$ ethidium bromide
- 3-ml cuvettes
- Spectrophotofluorometer with an emission wavelength of 580 nm and an excitation wavelength of 360 nm

CAUTION: Ethidium bromide is a mutagen and should be handled, stored, and disposed of with appropriate care.

1. Just before use, dilute 25 $\mu\text{g}/\text{ml}$ DNA standard with an additional 4 vol PBSCM.
2. In 3-ml cuvettes, labeled a to f, make up the following mixtures:
 - a. standard: 0.5 ml of 5 $\mu\text{g}/\text{ml}$ DNA standard, 0.5 ml heparin solution and 1.0 ml PBSCM
 - b. blank I: 0.5 ml heparin solution and 1.5 ml PBSCM
 - c. blank II: 2.5 ml PBSCM
 - d. sample (DNA + RNA): 0.5 ml sample, 0.5 ml heparin solution and 1.0 ml PBSCM
 - e. sample (DNA): 0.5 ml sample, 0.5 ml heparin solution, 0.5 ml 50 $\mu\text{g}/\text{ml}$ RNase, 0.5 ml PBSCM
 - f. sample blank: 0.5 ml sample, 2.0 ml PBSCM

Dilute samples as required with PBSCM, in order to dilute the DNA to a level compatible with the assay.

3. Incubate all cuvettes at 37°C for 20 min.
4. Add 0.5 ml 25 µg/ml ethidium bromide to all cuvettes except c and f.
5. Stir the contents before measuring the fluorescence at an emission wavelength of 580 nm and an excitation wavelength of 360 nm.

The fluorescence of the standard (a) should be set at 100 and the temperature should remain constant ($\pm 0.5^\circ\text{C}$) during the measurements. Measurements should be made at least 1 min after adding the ethidium bromide, but within an hour. Check the RNase solution for fluorescence and subtract if necessary.

6. Calculate the mass of DNA in the sample using the following equation:

$$A_{\text{DNA}} = \frac{A_{\text{std}} (F_e - F_b - F_f + F_c)}{F_a - F_b}$$

Calculate the mass of RNA according to the following equation:

$$A_{\text{RNA}} = \frac{A_{\text{std}} (F_d - F_e)}{0.46 \times (F_a - F_b)}$$

where A_{DNA} is the mass of DNA in sample mixture, A_{RNA} is the mass of RNA in sample mixture, A_{std} is the mass of DNA in standard mixture, and F is the fluorescence intensity.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Diphenylamine reagent

To 100 ml glacial acetic acid add 1 g diphenylamine (1% w/v final) and 2.75 ml concentrated sulfuric acid (H_2SO_4). Store up to 1 month at room temperature.

CAUTION: *Take care when adding the H_2SO_4 and use safety goggles.*

Ethylene glycol homogenization medium (EGHM)

To 100 ml ethylene glycol add 20 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (1 mM final). Store up to 1 month at 4°C.

High-NaCl buffer (HNB)

To 100 ml H_2O add:

23.4 g NaCl (2.0 M final)

40 µl 1 M MgCl_2 (APPENDIX 2A; 0.2 mM final)

2 ml 1 M Tris·Cl (APPENDIX 2A; 10 mM final)

Adjust to pH 7.4 if necessary

Add H_2O to 200 ml

Store 2 to 3 days at 4°C

Immediately before use, add 1 ml of 200 mM PMSF (APPENDIX 2A)

continued

Incubation buffer (IB)

To 100 ml H₂O add:
40 µl 1 M MgCl₂ (*APPENDIX 2A*; 0.2 mM final)
2 ml 1 M Tris·Cl (*APPENDIX 2A*; 10 mM final)
Adjust to pH 7.4 if necessary
Add H₂O to 200 ml
Store 2 to 3 days at 4°C
Immediately before use, add 1 ml of 200 mM PMSF (*APPENDIX 2A*)

Iodixanol, 50% (w/v)

Dilute 5 vol OptiPrep (60% iodixanol; Axis-Shield; available from Accurate Chemical) with 1 vol OptiPrep diluent for nuclei (ODN; see recipe). Store 2 to 3 days at 4°C.

Iodixanol gradient solution, 30% and 35% (animal cells or tissues)

For 30% and 35% iodixanol, dilute 50% (w/v) iodixanol (see recipe) with NIM (see recipe) at a ratio of 6:4 and 7:3 by volume, respectively. Store 2 to 3 days at 4°C.

Iodixanol gradient solutions, 25% and 40% (wheat germ)

For 25% and 40% iodixanol, dilute OptiPrep (60% iodixanol; Axis-Shield; available from Accurate Chemical) with WGM (see recipe) at a ratio of 2.5:3.5 and 4:2 by volume, respectively. Store 2 to 3 days at 4°C.

Low-ionic-strength suspension medium (LISM)

To 200 ml H₂O, add 20 µl 1 M MgCl₂ (*APPENDIX 2A*; 0.1 mM final). Store up to 2 weeks at 4°C.

Nuclear isolation medium (NIM)

To 100 ml H₂O add:
17.1 g sucrose (0.25 M final)
0.37 g KCl (25 mM final)
1 ml 1 M MgCl₂ (*APPENDIX 2A*; 5 mM final)
2 ml 1 M Tris·Cl (*APPENDIX 2A*; 10 mM final)
Adjust to pH 7.4 if necessary
Add H₂O to 200 ml
Store 2 to 3 days at 4°C

Nuclear membrane storage medium (NMSM)

To 100 ml H₂O add:
40 ml glycerol (20% v/v final)
2 ml 100 mM disodium EDTA (*APPENDIX 2A*; 1 mM final)
2 ml 1 M Tris·Cl (*APPENDIX 2A*; 10 mM final)
Adjust to pH 7.5 if necessary
Add H₂O to 200 ml
Store 2 to 3 days at 4°C

Nuclei suspension medium (NSM)

To 100 ml H₂O add:
17.1 g sucrose (0.25 M final)
1 ml 1 M MgCl₂ (*APPENDIX 2A*; 5 mM final)
10 ml 1 M Tris·Cl (*APPENDIX 2A*; 50 mM final)
Adjust to pH 7.4 if necessary
Add H₂O to 200 ml
Store 2 to 3 days at 4°C
Immediately before use add 1 ml of 200 mM PMSF (*APPENDIX 2A*)

OptiPrep diluent for nuclei (ODN)

To 100 ml H₂O add:
17.1 g sucrose (0.25 M final)
2.22 g KCl (150 mM final)
6 ml 1 M MgCl₂ (APPENDIX 2A; 30 mM final)
12 ml 1 M Tris·Cl (APPENDIX 2A; 60 mM final)
Adjust to pH 7.4 if necessary
Add H₂O to 200 ml
Store 2 to 3 days at 4°C

Orcinol reagent

To 50 ml concentrated HCl, add 0.5 g orcinol (1% w/v final) and 0.25 g FeCl₃·6H₂O (0.5% w/v final). Make up fresh and keep on ice until required.

Phosphate-buffered saline with Ca/Mg (PBSCM)

To 100 ml H₂O add:
0.026 g CaCl₂·2H₂O (0.9 mM final)
0.04 g KCl (2.68 mM final)
0.04 g KH₂PO₄ (1.47 mM final)
100 μl 1 M MgCl₂ (APPENDIX 2A; 0.5 mM final)
0.16 g NaCl (0.137 M final)
0.23 g Na₂HPO₄ (8.1 mM final)
Adjust to pH 7.5 with NaOH or HCl
H₂O to 200 ml
Store up to 1 week at 4°C

Sucrose buffer, pH 7.4 (SB7.4), pH 8.5 (SB8.5)

Sucrose buffer, pH 8.5 (SB8.5)
To 100 ml H₂O add:
20.5 g sucrose (0.3 M final)
20 μl 1 M MgCl₂ (APPENDIX 2A; 0.1 mM final)
78 mg 2-mercaptoethanol (5 mM final)
0.37 g triethanolamine (10 mM final)
Adjust to pH 8.5 with HCl
Add H₂O to 200 ml
Store 2 to 3 days at 4°C
Sucrose buffer, pH 7.4 (SB7.4)
Prepare as SB8.5, but adjust to pH 7.4

Sucrose density barrier (SDB)

To 95 ml H₂O add:
157 g sucrose (2.3 M final)
0.37 g KCl (25 mM final)
1 ml 1 M MgCl₂ (APPENDIX 2A; 5 mM final)
2 ml 1 M Tris·Cl (APPENDIX 2A; 10 mM final)
Adjust to pH 7.4 if necessary
Add H₂O to 200 ml
Store 2 to 3 days at 4°C

(continued)

Wheat germ medium (WGM)

To 100 ml H₂O add:
27.4 g sucrose (0.4 M final)
0.37 g KCl (25 mM final)
1 ml 1 M MgCl₂ (APPENDIX 2A; 5 mM final)
0.39 g 2-(*N*-morpholino)sulfonic acid (MES; 10 mM final)
Adjust pH to 6.2 with NaOH
Add H₂O to 200 ml
Store 2 to 3 days at 4°C

COMMENTARY

Background Information

Isolation of nuclei from a mammalian tissue homogenate has proved to be one of the easier tasks of sucrose density gradient separation technology. In a homogenate the nuclei are generally regarded as the largest organelle (4 to 12 μm in diameter), although some plasma membrane sheets, often present in a tissue homogenate but not in homogenate from cultured cells, may be up to 20 μm in length. Consequently a “low-speed” or “nuclear” pellet, produced by centrifugation of a homogenate at 500 to 1000 × *g* for 10 min, will contain these two components, plus some of the largest mitochondria. Unbroken tissue, cells, and connective tissue also sediment under these conditions, and this is the reason why nuclear isolation methods invariably preface the low-speed centrifugation with a filtration step to remove these elements which would otherwise contaminate the nuclear pellet and compromise the subsequent gradient fractionation. Smaller subcellular particles will also contaminate the pellet because of entrapment and the inefficiency of differential centrifugation (UNIT 3.3). These can be partially removed by washing the pellet, but this might be regarded as superfluous, as any subsequent gradient will resolve these contaminants, since the density of nuclei (>1.32 g/ml in sucrose) is also greater than that of any other major subcellular component.

It is also possible to purify the nuclei from a low-speed pellet by washing the crude nuclei in a medium containing 0.5% Triton X-100 in order to solubilize contaminating membranes (Hoffmann and Chalkley, 1978); however, the method tends to make the nuclear membrane rather more fragile, the nuclei prone to aggregation, and endogenous nuclease activity can be activated (Rickwood et al., 1997). Thus, density barrier centrifugation is the most widely used technique. As the nuclei are the largest and densest of all the subcellular organelles, simple density barriers or discontinuous

gradients are usually effective for their purification.

Sucrose solutions are not dense enough to band nuclei ($\rho > 1.32$ g/ml in sucrose) within a gradient; thus, pelleting through a 2.2 to 2.3 M sucrose density barrier is the only option. Because of the high viscosity of the sucrose density barrier, the sedimentation of the particles is very slow and the nuclei lose much of the water from their internal space due to the osmotic gradient across the membrane. This process may disrupt the macromolecular structures, which are normally highly hydrated. Nevertheless, the nuclei do rehydrate when they are transferred back to an isoosmotic medium, and the method is still widely used. After centrifugation of sucrose barriers at 100,000 to 200,000 × *g* (for up to 1 hr), the nuclei are recovered as a pellet. Because resuspension of the pellet is sometimes difficult (aggregation of the nuclei is often a problem), banding the organelles at an interface offers some significant benefits.

Although both CsCl and sodium diatrizoate can provide solutions of sufficient density to band nuclei at an interface, their ionic strength can cause disruption of the nucleoprotein structure, unless the chromatin is first fixed using a cross-linking agent such as formaldehyde to prevent dissociation of the protein from DNA (Gollin and Wray, 1984). However, the non-ionic iodinated density gradient media can be used in this mode. With iodixanol, the gradient is isoosmotic, so the nuclei do not become condensed. Consequently, they have a much lower density (i.e., 1.20 to 1.22 g/ml), and the lower viscosity of the density gradient allows the separation to be carried out at much lower RCFs (i.e., 10,000 × *g* for 20 min) than with sucrose. It is also considered unnecessary to produce a crude nuclear fraction first. The recommended strategy is to adjust the homogenate with OptiPrep, or a high-density working solution produced from it, to a density that is higher than most organelles ($\rho \sim 1.16$ g/ml), and layer

it over a double iodixanol barrier ($\rho = 1.175$ and 1.20 g/ml, respectively). In mammalian cells, the only other organelles that have a density approaching that of nuclei are peroxisomes (Graham et al., 1994), but, as they are much smaller, only the nuclei have the size and density to band above the densest layer at the relatively-low g forces (i.e., $10,000 \times g$) and short times (i.e., 20 min) used in this method (Graham et al., 1994; Provost et al., 1996).

Early methods that attempted to use nonaqueous media for the isolation of organelles relied on organic solvents such as benzene and carbon tetrachloride and the preparation of lyophilized cells and tissues, so it is hardly surprising that they never attained any degree of popularity whatsoever (see Rickwood et al., 1997 for more information). Nevertheless, a method such as homogenization of lyophilized cells in pure glycerol (Gurney and Foster, 1977) may be useful for analysis of inorganic ions and soluble proteins which are normally leached out by the normal aqueous media. However, the method developed by Rickwood et al. (1997), in which the liver is homogenized in ethylene glycol and the nuclei are purified in gradients of Nycodenz in the same solvent, probably provides a much more acceptable manner for avoiding the use of aqueous media (see Alternate Protocol 2).

Critical Parameters and Troubleshooting

Homogenization

For mammalian tissues, inclusion of divalent cations in the homogenization medium, usually in the form of $MgCl_2$ ($CaCl_2$ is generally avoided because of the problem of Ca^{2+} -stimulated phospholipases), as well as KCl in buffered 0.25 M sucrose, helps to stabilize and preserve nuclear structure (e.g., the 0.25 M sucrose/25 mM KCl/5 mM $MgCl_2$ /10 mM Tris-Cl, pH 7.4 medium; see Basic Protocol 1). This buffer was devised by Blobel and Potter (1966). While KCl is sometimes omitted, inclusion of $MgCl_2$ is invariant; Widnell and Tata (1964), for example, used 0.25 M sucrose/5 mM $MgCl_2$ /10 mM Tris-Cl, pH 7.4. For nuclei from nonmammalian sources, inclusion of Mg^{2+} is also common, although the details of the homogenization medium are generally specific for the organism. Yeast spheroplasts have been homogenized in 8% polyvinylpyrrolidone (PVP; M_r 40,000)/1 mM $MgCl_2$ /0.02 M KH_2PO_4 /0.02% Triton X-100, while for intact yeast cells an Mg^{2+} -free medium has been used

(i.e., 1 M sorbitol/20% glycerol/5% PVP), and for plant cells the media are even more diverse. For a review of these media see Rickwood et al. (1997).

With mammalian liver, KCl and $MgCl_2$ can be present at all stages of the procedure, including homogenization. On the other hand, homogenization media for hard tissues (e.g., skeletal muscle) may contain KCl, but they may also contain EDTA rather than Mg^{2+} . In such circumstances, isolation procedures need to be developed empirically. If adequate homogenization does not occur in the absence of EDTA, or in the presence of Mg^{2+} , then a compromise must be reached in which the tissue or cells are homogenized in a suitable medium and then an isoosmotic medium containing KCl and/or $MgCl_2$ might be added to the homogenate. Alternatively the nuclei can be pelleted and then resuspended in a KCl/ $MgCl_2$ medium.

The same problem applies to cultured cells in which it is common to use media that are not ideally suited to nuclei (e.g., 0.25 M sucrose/1 mM EDTA/10 mM triethanolamine-acetic acid, pH 7.4 medium; Marsh et al., 1987). If the cells do not disrupt readily in an isoosmotic Mg^{2+} -containing medium, then it may be better to use a hypoosmotic medium such as 15 mM KCl/1.5 mM magnesium acetate/1 mM dithiothreitol/10 mM HEPES-KOH, pH 7.5 (Goldberg and Kornfeld, 1983; Dunphy and Rothman, 1985). Birnie (1978) recommended a swelling medium of 10 mM NaCl/1.5 mM $MgCl_2$ /10 mM Tris-Cl, pH 7.4. After homogenization, the medium is returned to isoosmoticity by adjusting to 0.25 M sucrose. With cultured-cell nuclei, it is also common to include a number of washing steps prior to centrifugation through 2.2 to 2.3 M sucrose. Moreover, Birnie (1978) used a Ca^{2+} -containing buffer (0.25 M sucrose/3 mM $CaCl_2$ /10 mM Tris-Cl, pH 7.4) as a wash medium; apparently Ca^{2+} -stimulated phospholipases are less of a problem with cultured cells (i.e., as compared to liver).

For cells and tissues that do not homogenize satisfactorily in any of these media, the method adopted by Wray et al. (1977) may be useful. Homogenization of liver in 0.5 M hexylene glycol (2-methyl-2,4-pentanediol)/1.0 mM $CaCl_2$ /50 mM PIPES, pH 7.5, allowed the isolation of nuclei with good functional integrity. Although this method only produced a yield of 40% to 50%, it was applicable to a very wide range of tissues, including those which do not respond well to the normal sucrose-based meth-

Table 3.10.1 Enzyme Content of Nuclear Membranes^a

	Glucose-6-phosphatase activity (μmol phosphate/hr/mg/protein)	NADHCR ^b activity (μmol cytochrome <i>c</i> reduced/hr/mg/protein)	DNA polymerase activity (pmol TMP incorporated/min/mg protein)
Nuclei	1.32	6.48	68.3
Nuclear membranes	9.6	16.0	9.0
Microsomes	10.56	45.5	Not applicable

^aData adapted from Kay et al. (1972) with kind permission.

^bNADHCR = NADH cytochrome *c* reductase.

ods (e.g., Novikoff hepatoma cells and mouse brain).

Selection of a suitable nonaqueous system is a difficult problem, and the approach adopted by Rickwood et al. (1997) is something of a compromise in that, although the homogenization medium is nonaqueous, water will be present in the tissue since lyophilization is not used. Although such an approach cannot remove cytosolic water, the water in the liver vasculature can be eliminated by perfusion with ethylene glycol prior to homogenization, if this is regarded as important. Use of a nonaqueous medium generally makes homogenization more difficult, and the liver should be minced very finely before using the Potter-Elvehjem homogenizer; indeed it may be advantageous to use a small mincer rather than scissors.

Isolation of mammalian nuclei by sucrose density gradient centrifugation

At the high concentrations of sucrose used in the first protocol of this unit (see Basic Protocol 1), the extreme viscosity is a major problem, not only in preparing the solutions, but also in accurate dispensing of aliquots into centrifuge tubes. It may be easier to make up the solutions on a weight/weight basis, rather than to try and dissolve the sucrose in a smaller volume than the final, and then make up to the volume (e.g., 2.2 and 2.3 M sucrose are equivalent to 59% and 61% w/w, respectively). However, it should be noted that the liquid used to dissolve the sucrose should contain approximately twice the required concentrations of buffer and salts to allow for "dilution of the water by the sucrose." If the crude nuclear pellet is resuspended directly in 2.3 M sucrose rather than in 0.25 M sucrose first (see Basic Protocol 1, step 14), great care has to be taken to ensure that the pellet is properly resuspended; using such a viscous medium in an homogenizer is technically difficult.

Isolation of mammalian nuclei by iodixanol density gradient centrifugation

For material other than mammalian liver it may be necessary to modulate the density of the three layers of iodixanol and/or increase the time of centrifugation (see Alternate Protocol 1). This separation is based to some extent on the sedimentation rate of the nuclei as well as their density. In dense hyperosmotic sucrose, all nuclei shrink and attain the same limiting density irrespective of their real size and density. In isoosmotic iodixanol gradients, the organelles retain their native size and density; therefore, the rate at which they sediment and their banding density may vary with the material source.

Gradient purification of nonmammalian nuclei

Nuclei from plant cells pose additional problems in that other subcellular particles such as chloroplasts and starch granules sediment at low RCFs and are as dense or denser than nuclei in sucrose gradients. Consequently, sucrose gradients are ineffective for resolving and purifying plant cell nuclei; however, since nuclei are much less dense in iodixanol than in sucrose, the opportunity exists for resolution in these gradients. Ford et al. (1998) reported that starch granules are able to pellet through an iodixanol solution of density 1.234 g/ml, and that centrifugation of a crude nuclear preparation over a discontinuous gradient of iodixanol (i.e., 1.167 and 1.234 g/ml) produces nuclei free of starch granules at the lower interface. It is not known at what density chloroplasts band in this system. Rickwood et al. (1997) also noted that the melanin granules of *Xenopus* liver also pellet with the nuclei in sucrose and that only a 58% (w/v) metrizamide barrier (i.e., 10,000 × *g* for 20 min) was effective in separating these particles (the melanin granules pellet). At this concentration, metrizamide is hyperosmotic

and it may be possible to use a lower concentration of iodixanol.

Nuclear membranes

In the 1970s, two quite different strategies were evolved for the isolation of nuclear envelopes from mammalian liver nuclei. One used high concentrations of $MgCl_2$ (i.e., 0.5 to 0.7 M) to disrupt the nuclear chromatin (Monneron et al., 1972) while the other used low-ionic-strength media (Kay et al., 1972; Harris and Milne, 1974). Since that time, these two basic strategies have been developed almost in parallel, with some workers favoring high ionic strength and others low ionic strength.

There are no particular technical problems associated with either method of nuclear membrane preparation (see Basic Protocols 2 and 3). Either method works well for rat liver; only experimentation will reveal whether this is true for other tissues and cells. Choice of methodology depends to some extent on the subsequent studies to be carried out on the envelopes. The high-ionic-strength method produces nucleus-sized hollow spheres, which are good for permeability studies. The yield of envelopes is very high and they are intact, retaining the lamina and pores. Low-ionic-strength procedures tend to produce high-purity fragments of the nuclear membrane, with intact pores and lamina, and are particularly good for compositional studies. The fragments of membrane have a variety of sizes ranging from incomplete “ghosts” to much smaller pore-sized fragments; therefore, a high-speed centrifuge is required to harvest the membranes in high yield. The high-ionic-strength procedure, on the other hand, in which the whole envelope is retained, requires only a low-speed centrifuge. All the modern methods incorporate the use of enzymes to digest the released nucleic acids.

Assays

Determination of the purity of a nuclear preparation can be assessed by phase-contrast microscopy (i.e., absence of any particles other than nuclei) and measurement of DNA and RNA. The chemical methods that measure the content of either deoxyribose or ribose (Schneider, 1957) are probably the most widely used. Neither Nycodenz nor iodixanol interferes significantly, but sucrose, metrizamide, and other sugar-containing molecules do. The newer fluorometric methods (Karsten and Wollenberger, 1972, 1977) are certainly more sensitive and are unaffected by most gradient

media. There are no easily measurable enzyme markers for the nuclear membrane, but enzymes that are characteristic of the endoplasmic reticulum (ER) are found in nuclear and nuclear-membrane preparations. Since there is evidence for some continuity between the outer nuclear membrane and the ER, this is probably not surprising, but the levels of enzymes such as glucose-6-phosphatase, NADH cytochrome *c* reductase, and NADPH cytochrome *c* reductase (*UNIT 3.5*) observed in nuclear membrane preparations suggest that they are true components, rather than merely a reflection of the presence of some “attached” ER. It is more common to express the purity of nuclei in terms of the DNA and RNA content and in addition, protein and phospholipid, for nuclear membrane fractions (see Anticipated Results).

Anticipated Results

Nuclei

The yield of nuclei is measured in terms of percentage recovery of DNA from the homogenate, and for both the sucrose and iodixanol methods a figure of at least 90% is expected. Approximately 4% of the total RNA is recovered in the nuclear pellet, and the DNA/RNA ratio should be ~0.11. By phase-contrast microscopy, the nuclei should appear as characteristic dark-gray structures, 4 to 12 μm in diameter, and although smaller organelles cannot be discerned at the levels of magnification achievable by this technique, there should be a complete absence of any “stippled” background. In neither method can any marker enzymes of any other membrane be detected, except those of the ER (e.g., glucose-6-phosphatase and NADH cytochrome *c* reductase), which may be present at specific activities that are characteristically 10% to 15% of the levels in microsomes.

Nuclear membranes

Because the preparation of nuclear membranes is always made from previously purified nuclei, it is common to express efficacy of the membrane separation procedure in terms of the percentage recovery of protein, DNA, RNA, and phospholipid from the nuclei. Typical values are 8% to 12%, 0.6% to 2%, 6% to 8%, and 55% to 60%, respectively (Kay et al., 1972; Monneron et al., 1972). Phospholipid is only present in the membrane, so 55% to 60% is also a measure of the overall recovery of nuclear membranes. The relative amounts of each of these four components in the membrane is

typically 73%, 0.6%, 3%, and 20%, respectively, by weight.

Table 3.10.1 gives the specific activity of some typical enzyme markers in the nuclear membranes, and, for comparison, levels in the nuclei and in the microsomes (Kay et al., 1972). There is a clear enrichment in the nuclear membrane (i.e., compared to the nuclei) of the two enzyme markers, which are generally regarded as characteristic of the endoplasmic reticulum (glucose-6-phosphatase and NADH cytochrome *c* reductase). The specific activity of the glucose-6-phosphatase, in particular, is close to that of the microsomes, and thus this enzyme would appear to be a true component of the nuclear membrane rather than a result of contamination by the ER. It should be noted that in some preparations the levels of glucose-6-phosphatase are much lower (for a review of this data, see Harris and Agutter, 1976). The situation with the NADH cytochrome *c* reductase is rather less clear, in as much as the membranes contained <10% of the protein present in the nuclei, but the specific activity of this enzyme only increased approximately two-fold. Again the reported levels for this enzyme vary considerably (Harris and Agutter, 1976).

Time Considerations

There are no points at which any of the basic or alternate protocols can be interrupted. The crude nuclear fraction (see Basic Protocol 1) must be used immediately for any subsequent density barrier or gradient separation. Discontinuous (see Alternate Protocol 1) and continuous gradients (see Alternate Protocol 2) should be made up immediately prior to use, although time can be saved by preparing these gradients during the immediately previous step or steps. Basic Protocol 1 and Alternate Protocol 2 will take about 2.5 hr, while the shorter centrifugation time and avoidance of use of an ultracentrifuge means that Alternate Protocol 1 can be accomplished in less time (i.e., ~1.75 hr).

Nuclear membrane preparations should be executed as soon as possible upon purification of the nuclei. Basic Protocol 2 will take about 3.15 hr, while Basic Protocol 3 is more rapid (i.e., ~1.75 hr).

Functional assays should be carried out as soon as possible after the preparation, but for simple marker-enzyme assay, material can be stored at 0° to 4°C for up to 16 hr without much loss of activity. Enzyme assays may be carried out on nuclear membrane preparations frozen to -20° or -80°C, but when frozen nuclei are thawed, the tendency to leak DNA may cause

serious aggregation. There are no general rules regarding the suitability of a particular storage regime, and it is strongly recommended that the validity of results on stored material be checked by comparison with those made on fresh organelles. Chemical assays for DNA and RNA, which may take up to 3 hr if the DNA is precipitated first, can be carried out on frozen material, although again the aggregation that can occur when frozen suspensions of nuclei are thawed may cause sampling errors. The ethidium bromide assay for DNA and RNA is very rapid and will take no more than 1 hr, and this is also best carried out on unfrozen material. Any concentration of the membrane fractions and/or removal of gradient medium must be carried out prior to freezing.

All the solutions can be made ahead of time and stored at 4°C for 1 to 2 days or frozen to -20°C for longer periods; however, note that any enzymes, PMSF, or other protease inhibitors, should be added immediately prior to use.

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Free-Flow Electrophoretic Analysis of Endosome Subpopulations of Rat Hepatocytes

UNIT 3.11

Endosomes constitute a functionally, morphologically, and biochemically heterogeneous population of intracellular organelles that play a major role in sorting of incoming ligands, receptors, membrane, and content. To characterize their composition and properties, highly purified endosomes are required. Given the heterogeneity of endosomes and the lack of a general and suitable marker, endosome subpopulations have to be specifically labeled by pulse-chase internalization of ligands routed to the pathway of interest. However, due to their heterogeneity and similarity in buoyant density to other intracellular organelles, isolation procedures using standard cell fractionation techniques usually do not result in sufficient purification and adequate resolution of the endosome population of interest. Thus, alternative protocols have to be used for endosome purification. These involve the selective modification of the density of endosomes, immunoisolation, and free-flow electrophoresis (FFE). The latter technique has been applied to isolate highly purified and, most importantly, functional endosomes from cultured cells and tissues (Marsh et al., 1987). Furthermore, endosome subpopulations can be resolved by FFE, allowing for the kinetic analysis of ligand transport through distinct endosome compartments (Schmid et al., 1988).

This unit describes the application of FFE for analysis of endosome subpopulations of polarized rat hepatocytes (see Basic Protocol). As a prerequisite, endosomes involved in transport to lysosomes and transcytosis have to be labeled. In rat liver, asialoorosomucoid (ASOR) and polymeric immunoglobulin A (pIgA) can be used as ligands to selectively label the lysosomal and transcytotic route in hepatocytes. The respective receptors—i.e., the asialoglycoprotein receptor (ASGPR) and the pIgA receptor (pIgR)—are expressed in parenchymal but not in other cells in the liver. Procedures for preparation of ASOR, fluorescein isothiocyanate (FITC)-ASOR, and [¹²⁵I]pIgA are detailed in Support Protocols 4, 5, and 6, respectively. In vivo labeling of rat liver endosomes is achieved by intravenous injection of the ligands. As starting material for the FFE separation, endosome-enriched fractions have to be prepared from rat liver homogenates. The FFE protocol (see Basic Protocol) can be extended for the preparative isolation of endosomes from rat liver (see Alternate Protocol). The characterization of the endosome-containing FFE-fractions with respect to contamination by other intracellular organelles and the analysis of the protein composition are outlined in Support Protocols 1 and 2, respectively. Highly purified endosomes obtained by FFE are fully functional with respect to in vitro endosome acidification as described in Support Protocol 3.

FREE-FLOW ELECTROPHORESIS OF HEPATOCYTE ENDOSOMES

Endosome subpopulations of rat hepatocytes can be most accurately labeled in the isolated perfused liver using single-pass perfusion and a pulse-chase protocol (Mueller and Hubbard, 1986; Perez et al., 1988). This method, however, requires a skillful person trained in this specific operating technique and the instruments (e.g., pumps, drop counter, bile flow recorder) for the isolated perfusion of the liver. For a detailed description of the method see Exton (1975). Alternatively, markers can be injected into the rat for in vivo endosome labeling (Hoppe et al., 1985). However, using in vivo marker injection, a detailed pulse-chase protocol for two or more endocytic markers is not applicable and consequently the labeling of distinct endosome subpopulations is less precise in comparison to the isolated perfused liver. Nevertheless, the endosomes are well resolved from the

**BASIC
PROTOCOL**

**Subcellular
Fractionation and
Isolation of
Organelles**

3.11.1

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Supplement 14

main protein peak and the kinetics of marker transport through distinct subcompartments can also be analyzed.

For FFE separation of rat liver endosomes, the sample injected into the FFE apparatus must already be considerably enriched in endosomes with respect to the original homogenate. This can be achieved by differential centrifugation of the initial homogenate, preparation of microsomes, and flotation of microsomes in discontinuous sucrose density gradients. The protocol for preparation of endosome-enriched fractions described below (also see Fig. 3.11.1) is a modification of the method used by Khan et al. (1986) and has been successfully used as starting material for FFE isolation of rat liver endosomes (Fuchs et al., 1989; Stefaner et al., 1997).

Endosome-enriched fractions prepared from rat liver are used as the starting material for the separation of endosome subpopulations from each other—and from contaminating organelles such as plasma membrane, endoplasmatic reticulum, and lysosomes—by free-flow zone electrophoresis, also known as continuous flow electrophoresis (the

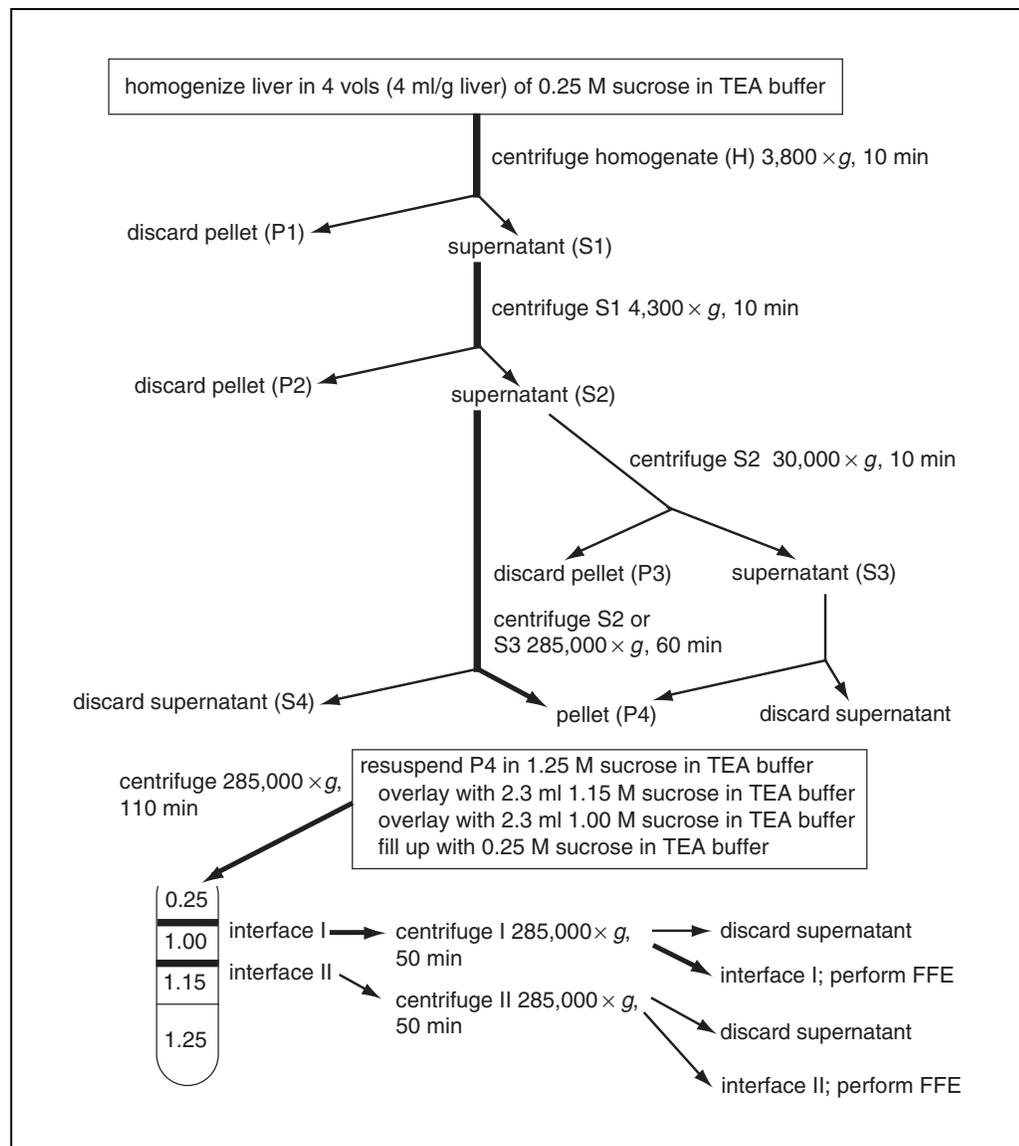


Figure 3.11.1 Flow chart of the fractionation protocol applied to prepare endosome-enriched fractions (interface I and interface II) from rat liver. Interface I and II are subsequently subjected to separation by free-flow electrophoresis (FFE).

abbreviation FFE is used in this protocol for this specific type of free-flow electrophoresis). The starting membrane material is treated with trypsin, a procedure which alters the electrophoretic mobility of endosomes and lysosomes (Marsh et al., 1987). Samples are then injected as a fine jet into the chamber of the FFE apparatus, where they are subjected to separation by the laminar flow of the chamber buffer and an high-voltage electrical field. Endosomes are deflected more towards the anode from the flow direction of the medium and contaminating organelles such as plasma membranes and endoplasmic reticulum. Among the endosome subpopulations, late endosomes are more deflected towards the anode than transcytotic or early endosomes, respectively (Schmid et al., 1988; Stefaner et al., 1997). Once the samples have reached the end of the separation chamber at the top, they can be fractionated continuously and collected by means of a 96-fold splitting device. The presence of endosomes and other organelles in these fractions is then identified by determination of endosomal markers and organelle-specific marker enzyme activities.

Carrier-free high voltage electrophoresis involves high-tech instruments. The user must have well-founded knowledge with regard to the theory and practice of the method and operation of the instrument. The protocol described below has been established for the Elphor VaP 22, Bender and Hobein, FFE apparatus. Application of the protocol to other free-flow electrophoresis instruments (e.g., Octopus) might require modifications with respect to buffer volumes, chamber buffer flow, sample flow rate, and voltage/current.

CAUTION: Wear gloves and carry out experiments in radioactivity-certified areas. This procedure must be performed in a laboratory certified for work with ^{125}I -labeled material. All personnel using radioactive material must be properly trained. Make sure that all radioactive waste (including the rat corpse and perfusion buffer) is disposed appropriately (also see *APPENDIX 1D*).

NOTE: All solutions, rotors, centrifuge tubes and glassware should be prechilled to 4°C and kept on ice throughout the isolation procedure. All manipulations and centrifugations following step 17 are carried out at 4°C .

NOTE: All protocols using live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to government regulations regarding the care and use of laboratory animals. Anesthesia, surgery, and killing of the rats should be supervised or carried out by an experienced animal technician.

Materials

- 150- to 200-g male Sprague-Dawley rats (e.g., Taconic Farms, The Jackson Laboratory)
- Urethane anesthetic solution: 10 g urethane ethyl carbamate/10 ml 0.9% (w/v) NaCl (prepare immediately before use)
- 0.25 M, 1 M, 1.15 M, and 2.5 M sucrose in TEA buffer, ice cold (see recipes)
- Endocytic tracers: 220 μg FITC-ASOR (see Support Protocols 4 and 5) and 1.4×10^6 cpm [^{125}I]pIgA (see Support Protocol 6) per 500 μl phosphate-buffered saline (PBS; *APPENDIX 2A*)
- Protease inhibitor solutions (*UNIT 3.4*)
- 1 \times TEA buffer (see recipe)
- FFE chamber buffer (see recipe)
- FFE electrode buffer (see recipe)
- 1 mg/ml trypsin solution (trypsin-TPCK treated; Worthington), freshly prepared
- 2 mg/ml trypsin inhibitor solution (trypsin inhibitor, Type I-S, from soybean; Sigma-Aldrich), freshly prepared
- 2-ml syringes and 25-G needles

Plastic dissecting trays (~15 cm width × 30 cm length) with legs (~8 cm height)
 Plastic basins (~35 cm width × 35 cm height × 15 cm depth)
 500-ml buffer reservoir with outlet at bottom and connecting tube to fit the 18-G cannula (e.g., 500 ml squibb, separatory pear-shaped funnel with stopcock; Cole Parmer No. P-34506-04) or 500 ml aspirator bottle)
 Cannula (e.g., 18-G plastic cannula)
 Dissecting instruments including: microscissors, scissors, and curved forceps
 4-0 surgical silk
 15-ml and 40-ml Dounce homogenizers (Kontes Glass) with loose- and tight-fitting pestles (size A and B, respectively)
 50-ml thick-walled polycarbonate centrifuge tubes for Beckman J6-B centrifuge
 Low-speed centrifuge with swinging bucket-rotor for 50-ml tubes (e.g., Beckman J6-B)
 30-ml thick-walled polycarbonate centrifuge tubes for Sorvall SS-34 rotor
 High-speed centrifuge (e.g., Sorvall RC-5B) with fixed-angle rotor (e.g., Sorvall SS-34 rotor) for 30-ml tubes
 14-ml Ultraclear ultracentrifuge tubes
 Ultracentrifuge (e.g., Beckman L8-55) with swinging-bucket rotor (e.g SW 40 or equivalent)
 1-ml and 2-ml syringes with blunt-ended 1.8 × 18-mm needles (for underlaying gradients)
 Zeiss Refractometer
 5-ml syringe with blunt-ended 1.8 × 10-mm needle (for collecting fractions from gradients)
 2-ml syringe with 0.6 × 30-mm blunt-ended needle (for resuspending pellets)
 Conductivity meter
 FFE apparatus (e.g., Elphor VaP 22, Bender and Hobein)
 Additional reagents and equipment for determining the protein concentration according to Bradford (*APPENDIX 3B*)

Label hepatocellular endosomes in situ

1. Starve a 150-to 200-g male Sprague-Dawley rat overnight.

For analytical separation, one 7- to 10-g liver provides sufficient material. For preparative work (see Alternate Protocol), use up to 30 g of liver at a time (three to four rats and scale up volumes proportionally to number of livers used).

2. Anesthetize rats by an intraperitoneal injection of urethane anesthetic solution (1 ml per 100 g body-weight), using an 25-G needle.
3. Shave abdomen of rat.
4. Fasten animal on a plastic tray (~15 cm × 30 cm with legs ~8 cm in height) using adhesive tape and place the tray in a plastic basin (~35 cm × 35 cm × 15 cm) to collect blood and perfusion buffer contaminated with ¹²⁵I.

Make sure that the dimensions of the plastic basin are large enough to collect the entire fluid dropping from the plastic tray.

Perfuse the liver

5. Place a buffer reservoir with an outlet at the bottom ~50 cm above the animal. Fix a tube to the outlet that is long enough to allow for connection to a cannula (e.g., an 18-G plastic cannula) to be placed later into the portal vein of the rat (step 9). Fill the reservoir and the tube with ~500 ml ice-cold 0.25 M sucrose in TEA buffer.

6. Open the abdomen widely by a midline incision without injuring liver or diaphragm. Expose the liver by gently moving intestines to the left side of the rat to gain easy access to the liver, the portal vein, and the inferior vena cava.
7. Prepare two ligatures of 4-0 silk in loose knots around the portal vein 1 to 2 cm and 4 to 5 cm, respectively, before the vessel enters the liver. Leave bile duct exterior to the ligatures.
8. Inject endocytic tracers (e.g., 220 μg FITC-ASOR and 1.4×10^6 cpm ^{125}I -pIgA in 500 μl PBS) into the saphenous vein using a 2-ml disposable syringe with a 25-G needle.

The endocytic tracers FITC-ASOR and [^{125}I]-pIgA can be injected simultaneously in 500 μl PBS or successively (e.g., each tracer in 500 μl PBS) to label the endosome subpopulation of interest. Due to variability of the animals, the injection procedure and the resulting labeling of the individual endosome subpopulation, injection of two or more tracers in the same animal is required.

If labeling is carried out for more than 5 min, the body temperature of the animal should be stabilized by e.g., covering the body with paper towels and placing the animal under a 60 W light bulb.

9. At the end of the labeling time (1 to 20 min), hold the portal vein with forceps and incise on the ventral surface with a fine pair of scissors between the two prepared ligations. Lift and steady the vein and insert the cannula into the hepatic portal vein. Immediately remove the needle and place the tip of the cannula ~ 3 mm before the first bifurcation of the portal vein to ensure perfusion of all lobes. Fix the cannula with the ligature proximal to the liver and then also tie the distal ligature.
10. Remove any air bubble at the end of the cannula by adding ice-cold 0.25 M sucrose in TEA buffer from a syringe with a 25-G needle immediately before it is connected to the tube. Then rapidly connect the cannula air bubble-free to the tube and start to perfuse the liver with ice-cold 0.25 M sucrose in TEA buffer by gravity.

When labeling endosomes, a rapid subsequent perfusion with ice-cold 0.25 M sucrose in TEA buffer is important to halt endocytic processes. In addition, this perfusion will remove any contaminating blood and extracellular marker trapped within the liver. Since binding of ASOR to the ASGPR is calcium dependent, plasma membrane bound FITC-ASOR is also removed using this calcium-free and EDTA-containing TEA buffer.

11. Cut the inferior vena cava soon after connecting the cannula to the tube.

If this is not done, back-pressure will damage the liver. The perfusion fluid is allowed to drain from the vena cava into the body and then drain into the basin. The fluid remaining in the corpse after removing the liver should be drained into the basin before disposal of the corpse.

12. After perfusion, cut the diaphragm and immediately proceed to prepare endosome-enriched fractions, beginning with step 13.

All subsequent steps are performed at 4°C.

Isolate and mince liver

13. After in vivo labeling of hepatocyte endosomes and perfusion of the liver with ~ 500 ml ice-cold 0.25 M sucrose in TEA buffer, carefully excise the liver and cut away connective tissue.
14. Determine the liver weight and place the liver in a prechilled beaker containing 4 vol (4.0 ml/g liver) of 0.25 M sucrose in TEA buffer (i.e., 28 ml) with protease inhibitors on ice.

For the case of 150- to 200-g male Sprague-Dawley rats, the range of liver weight is 7 to 10 g. The conditions described below are based on a liver weight of 7 g.

15. Use scissors to mince the liver into $\sim 0.5\text{-cm}^3$ pieces. Pour the mixture into a 40-ml Dounce homogenizer.

If the liver weighs more than 10 g, divide it into two equal portions and homogenize separately.

Homogenize the liver

16. Homogenize the liver with eight up-and-down strokes with loose-fitting pestle (A), followed by three up-and-down strokes with a tight-fitting pestle (B). Measure total volume of the homogenate (H) and remove an aliquot ($\sim 100\ \mu\text{l}$) for determination of radioactivity, fluorescence, protein, and enzymes.

To avoid producing bubbles during homogenization, move the pestle slowly and steadily. Wipe off any connective tissue that sticks to the pestle.

Prepare microsomes

17. Pour homogenate into 50-ml thick-walled polycarbonate tubes (1 to 2 tubes/liver). Centrifuge 10 min at $3800 \times g$, 4°C , in a low-speed centrifuge to pellet nuclei and unbroken cells. Remove supernatant (S1) and discard pellet (P1).
18. Transfer supernatant (S1) into a another 50-ml thick-walled polycarbonate tube, and centrifuge for 10 min at $4300 \times g$, 4°C , in a low-speed centrifuge. Remove supernatant (S2) and discard pellet (P2).

These two centrifugation steps remove nuclei and unbroken cells. Furthermore, the resulting supernatant (S2) is depleted in marker for mitochondria and plasma membranes.

19. Measure volume of supernatant S2 and remove an aliquot ($\sim 100\ \mu\text{l}$) for analysis. Continue endosome preparation either at step 20a or step 20b.
- 20a. *To prepare "microsomes"*: Transfer S2 to a 30-ml thick walled polycarbonate tube (one tube per 10 g liver, maximum) and centrifuge 10 min at $30,000 \times g$, 4°C , in a high-speed centrifuge to pellet lysosomes and mitochondria. Discard the pellet and transfer the supernatant (S3) to 14-ml ultraclear ultracentrifuge tubes. Proceed directly to step 21.

This additional centrifugation step results in a higher enrichment of endosomal markers in the microsomal pellet (P4; see step 24) and consequently in endosome-enriched fractions due to removal of the majority of lysosomes and mitochondria. As a further consequence, the amount of trypsin required to achieve separation of endosomes by free-flow electrophoresis can be reduced from 3% to 0.3% (see steps 39b and 40b). This is of major importance when the acidification properties of the isolated endosomes are analyzed (see Support Protocol 3).

- 20b. *To prepare "total microsomes"*: Transfer S2 to 14-ml Ultraclear ultracentrifuge tubes and proceed directly to step 21.

If this alternative is chosen, centrifugation of S2 at $30,000 \times g$ is omitted. For quantitative analysis of endosome subpopulations total microsomes are used.

21. Place a 1.3-ml cushion of 2.5 M sucrose in TEA buffer at the bottom of each of the tubes (containing either S2 or S3) using a 2-ml syringe equipped with a $1.8 \times 18\text{-mm}$ needle with a blunted end.

The high-density cushion prevents the breakage of organelles, and therefore the loss of endocytic markers or vesicular content, by preventing the organelles from being pelleted against the walls of the tube. In addition, gentle resuspension of the pellet is facilitated.

22. To prepare total microsomes (i.e., from S2, where step 20a has not been performed) or microsomes (i.e., from S3, where step 20a has been performed), respectively, centrifuge either supernatant S2 or S3 for 50 min at $285,000 \times g$ (40,000 rpm in an SW 40 rotor), 4°C .
23. Discard supernatant (S4).
24. Transfer the pellet (P4) and the cushion to a 15-ml Dounce homogenizer. Rinse tube with 2 to 3 ml of 0.25 M sucrose in TEA buffer.
25. Resuspend the membranous material by using 10 up-and-down strokes with a tight-fitting pestle (B). Monitoring with a Zeiss refractometer, adjust the sucrose concentration with 2.5 M sucrose (in TEA buffer) to 1.25 M (refractive index = 1.3934) and to a final volume of 1.3 to 1.5 ml per gram liver.

If assaying all fractions for yield and enrichment, save them on ice and use on the same day.

Prepare endosome-enriched fractions

26. Transfer 5 ml of resuspended total microsomes (or microsomes) to a 14-ml ultraclear ultracentrifuge tube (for 10 g liver, three tubes are required). Overlay with 2.3 ml of 1.15 M sucrose in TEA buffer, then overlay that with 2.3 ml of 1.0 M sucrose in TEA buffer. Finally, fill up tube to 3 mm below the top with 0.25 M sucrose in TEA buffer.

Figure 3.11.1 contains a diagram of the preparation of the sucrose gradient.

27. Centrifuge gradients for 110 min at $285,000 \times g$, 4°C .
28. Using a 5-ml syringe with a blunt-ended 1.8×10 -mm needle, collect and pool the endosome-enriched fractions at the 0.25/1.0 M sucrose interface (interface I) and the fractions at the 1.0/1.15 M interface (interface II), from each tube. Transfer the pooled interface I fractions and the pooled interface II fractions into separate 14-ml Ultraclear centrifuge tubes.

For a 7-g liver, one tube for the pooled interface I fraction and another tube for the pooled interface II fraction are sufficient.

29. Determine refractive index of the pooled interface I fraction and of the pooled interface II fraction and add TEA buffer to bring the sucrose concentration to 0.25 M in each fraction.
30. Fill up tubes to ~14 ml with 0.25 M sucrose in TEA buffer and underlay each tube with 100 μl 2.5 M sucrose in TEA buffer using a 1-ml syringe with a 1.8×18 -mm needle with a blunted end.

Concentrate endosome-enriched fractions by centrifugation

31. Ultracentrifuge tubes for 50 min at $285,000 \times g$, 4°C , to concentrate material in the sucrose cushion.
32. Carefully remove the supernatants, add 500 μl of 0.25 M sucrose in TEA buffer, and resuspend the pellets and the sucrose cushion using a 2-ml syringe with a 0.6×30 -mm needle.
33. Determine the sucrose concentration of the resuspended material using a refractometer and adjust the sucrose concentration with TEA buffer to 0.25 M (refractive index = 1.3453).

Adjust protein concentrations of enriched fractions

34. Determine the protein concentration of each fraction using the Bradford method (APPENDIX 3B).

From a 7 g liver, one may expect to obtain ~2.5 mg protein in the final interface I fraction and 7 mg in the final interface II fraction. To quantitate yield and enrichment, remove aliquots of all supernatants and pellets (~100 μ l to 500 μ l) and also of the fractions applied to the free-flow electrophoresis apparatus (designated interface I and interface II). Furthermore, determine and record the volumes of the respective fractions. Either keep fractions at 4°C and assay immediately or store at -20°C.

35. Adjust the protein concentration to 1 mg/ml by adding 0.25 M sucrose in TEA buffer.

High protein concentrations (>1 mg/ml) result in reduced separation of endosomes from other subcellular organelles by FFE. Low protein concentrations (<1 mg/ml) complicate detection of markers in the FFE fractions.

Set up instrument

36. Make sure that the FFE instrument is assembled correctly, and the conductivities of the chamber and electrode buffers are correct. Fill chamber with FFE chamber buffer without inclusion of air bubbles, and add FFE electrode buffer to the electrodes.

Degass chamber buffer using a vacuum pump immediately before use. Fill the chamber slowly to prevent generation of air bubbles during filling. Carefully read the manufacturer's manual on how to avoid air bubbles during filling of the chamber.

37. Before turning on the high voltage (and injecting sample), make sure that all 96 collecting tubes are not clogged.

For the separation profile, a continuous dropping of buffer from all collecting tubes is of great importance. Due to the presence of sucrose in the chamber buffer, the tubes tend to clog as a result of limited washing after the run.

38. Follow the manufacturer's instructions for starting the instrument. Set the following parameters and maintain constantly:

Chamber buffer flow: 2.0 to 2.5 ml/hr/fraction

Chamber temperature: 6°C

Voltage: ~1400 to 1550 V

Current: ~120 to 135 mA.

Values indicated have been established for the Elphor VaP 22 (Bender and Hobein) FFE apparatus, and might need to be retested in other systems.

Stabilization of voltage and current requires that the FFE instrument be run with the settings listed above for 1 to 2 hr before sample injection. This should be considered in the time schedule. For the stability of the field, the conductivities of electrode buffer (~7.2 mS) and chamber buffer (~720 μ S) are of great importance and need to be verified.

Treat endosome-enriched fractions with trypsin

For analysis of "total microsomes" (i.e., where step 20a has not been performed)

- 39a. Immediately before injection of samples into the FFE apparatus, treat interfaces I and II obtained from total microsomes with 3% trypsin (0.03 mg trypsin/mg protein; added from 1 mg/ml trypsin solution) for 5 min at 37°C.

The amount of trypsin is "standardized as percent trypsin/mg protein; 0.03 mg trypsin/mg protein is therefore equal to 3% trypsin.

- 40a. Stop the reaction by adding 6% soybean trypsin inhibitor (0.06 mg soybean trypsin inhibitor/mg protein; added from 2 mg/ml soybean trypsin inhibitor solution), and cooling immediately to 4°C.

For analysis of “microsomes” (i.e. where step 20a has been performed)

- 39b. Immediately before injection of samples into the FFE apparatus, treat interfaces I and II derived from microsomes with 0.3% trypsin (0.003 mg trypsin/mg protein) for 5 min at 37°C.
- 40b. Stop the reaction by adding 0.6% soybean trypsin inhibitor (0.006 mg soybean trypsin inhibitor/mg protein; added from 2 mg/ml soybean trypsin inhibitor solution), and cooling immediately to 4°C.

Subject samples to separation by FFE

41. Inject trypsin-treated sample into the FFE apparatus at a rate of 1 to 1.5 ml/hr and 4°C.

Avoid introducing an air bubble into the chamber. Wash sample inlet with FFE chamber buffer after injection of sample.

Turn off the room light and try to visualize the sample jet in the FFE- chamber using a flashlight. Splitting of the jet into two unequal beams will give a first indication for separation of endosomes (thin beam, deflected more towards the anode) from the majority of other organelles (thick beam, majority of protein). By watching the sample in the chamber one knows when the fraction collection has to be started and stopped, to avoid unnecessary dilution of the fractions.

42. Begin collecting the fractions when the sample reaches the top of the separation chamber. Collect FFE fractions on ice during the entire run.

Keep an eye on the volume in the tubes collecting the 96 fractions. Try to avoid clogged tubes. Usually, ~2 to 3 ml/fraction are obtained from the FFE separation of interface I or II from one rat liver.

Analyze samples

43. At the end of the run, immediately analyze the collected fractions for protein concentration (e.g., Bradford assay; *APPENDIX 3B*), endosomal marker, and marker enzymes (see Support Protocol 1).

Turn off the FFE instrument by exactly following the manufacturer’s instructions. Wash extensively with water to prevent deposition of sucrose or salt in the apparatus.

PREPARATIVE FFE

Usually, one rat liver yields enough material to analyze FFE fractions for marker enzymes, for kinetic analysis of transport through endosomal compartments, or for functional studies such as in vitro acidification of endosomes. However, to characterize the composition of endosomes and identify endosomal proteins or lipids—by, e.g., SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*) or by immunoprecipitation (*UNIT 7.2*), larger quantities of purified fractions are required than what are obtained from one rat liver. To avoid the labeling of endosomes in all livers subjected to fractionation, endosomes from one liver are labeled with the appropriate marker (see the Basic Protocol), and two to four livers that have been flushed with buffer by in situ perfusion without prior endosome labeling are processed in parallel.

For materials, see Basic Protocol.

ALTERNATE PROTOCOL

Subcellular Fractionation and Isolation of Organelles

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Table 3.11.1 Suggested Dilutions and Volumes Used for Protein, Endocytic Marker, and Marker Enzyme Determination

Fraction ^a	Dilution/volume used			
	Protein (Bradford)	[¹²⁵ I]IgA	FITC-ASOR	Enzymes
H	1:20/5 µl	No dilution/50 µl	-	1:20/10 to 100 µl
S2	1:10/5 µl	No dilution/50 µl	-	1:10/10 to 100 µl
P4	1:10/5 µl	No dilution/5 µl	No dilution/5 µl	1:10/10 to 100 µl
Interface I	No dilution/5 µl	No dilution/10 µl	No dilution/5 µl	No dilution - 1:5/10 to 60 µl
Interface II	No dilution/5 µl	No dilution/10 µl	No dilution/5 µl	No dilution - 1:5/10 to 60 µl
FFE fractions	No dilution/300 µl	No dilution/500 to 1000 µl	No dilution/500 µl	No dilution/60 to 200 µl

^aSee Basic Protocol and Figure 3.11.1 for explanations of the terms used for the various fractions. Abbreviations: H, homogenate; P4, pellet 4; S2, supernatant 2.

1. Label endosomes of one rat liver and halt endocytosis by ice-cold perfusion with 0.25 M sucrose in TEA buffer (see Basic Protocol, steps 1 to 12).
2. Flush one to four livers by in situ perfusion with ice-cold 0.25 M sucrose in TEA buffer (see the Basic Protocol, steps 1 to 12, but omit pulsing with endocytic tracers at step 8)
3. Prepare endosome-enriched fractions from labeled and unlabeled livers, respectively (see Basic Protocol, steps 13 to 35). Prepare the FFE apparatus (see Basic Protocol, steps 36 to 38).
4. Pool interfaces derived from unlabeled livers.
5. Trypsinize interface I or II that contain labeled endosomes (see Basic Protocol, steps 39 to 40).
6. Subject the interfaces containing labeled endosomes (e.g., FITC-ASOR and [¹²⁵I]pIgA) to FFE separation, and collect the 96 fractions (see Basic Protocol, steps 41 to 42).
7. Trypsinize pooled interface I or II from unlabeled livers (see Basic Protocol, steps 39 to 40).
8. Immediately after the FFE separation of the labeled samples (step 6 of this Alternate Protocol), inject the pooled fractions of the unlabeled samples (step 7 of this Alternate Protocol) and subject to FFE. Collect the 96 fractions.
According to the amount of livers used, the duration of separation as well as the fraction volumes will be increased.
9. During FFE of unlabeled samples, analyze labeled fractions for protein (APPENDIX 3B) and endosome marker distribution (see Support Protocol 1).
10. Analyze FFE fractions of unlabeled samples for the distribution of proteins (see APPENDIX 3B and Support Protocol 1).
The localization of the major protein peak and the endosome marker proteins should be identical in FFE-separations carried out under the same condition.
Fractions containing endosomes derived from the unlabeled livers can be identified by comparison to the separation profile of labeled fractions.
11. Pool fractions of interest and continue with analysis (see, e.g., Support Protocol 2).

DETERMINATION OF PROTEIN, ENDOSOMAL MARKER, AND MARKER ENZYMES

The amount and dilution used for analysis of individual fractions is listed in Table 3.11.1. Protein concentration is determined using the Bradford assay (*APPENDIX 3B*). The enrichment of and purity of [¹²⁵I]pIgA-labeled endosomes is determined by γ counting of individual fractions. Since the liver homogenate and most fractions obtained from it are colored and have a high level of autofluorescence, an accurate determination of the FITC-ASOR concentration and the enrichment of FITC-ASOR labeled endosomes is difficult and requires the parallel analysis of rat liver fractionation where endosome labeling was omitted. Nevertheless, the distribution of FITC-ASOR in interface I and II and in free-flow fractions can be carried out following this protocol. Furthermore, ATP-dependent acidification can be used to determine the enrichment of FITC-ASOR labeled compartments in free-flow fractions in comparison to P3 or P4 (see Support Protocol 3). In order to determine the enrichment of ASOR-labeled endosomes with respect to the initial homogenate it is preferable to use ¹²⁵I-labeled ASOR.

The following organelle-specific markers can be used to determine the degree of contamination of the endosomal fraction after free-flow electrophoresis. For the apical and basolateral plasma membrane, alkaline phosphodiesterase I (APDE I), which is localized at both plasma membrane domains, is determined (see Support Protocol 1, *UNIT 3.2*). NADPH cytochrome *c* reductase is an oxidoreductase present in the endoplasmic reticulum and is determined according to the Support Protocol in *UNIT 3.5*. As a marker enzyme for lysosomes, β -*N*-acetylglucosaminidase can be assayed using the less sensitive *p*-nitrophenyl derivative as substrate instead of 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide (see Support Protocol 2 in *UNIT 3.6*; also see *UNIT 3.4*). It has to be pointed out that lysosomal enzymes are also found in endosomes en route to lysosomes. Furthermore, lysosomal enzymes are also found in the biosynthetic pathway (e.g., Golgi) where they can be partially active. As the *cis*, *medial*, and *trans* Golgi are involved in glycoprotein and proteoglycan modification carrying out distinct modifications at distinct sites in the Golgi stack, the respective enzymes involved in these modifications exhibit a site-specific localization. Most commonly, UDP-galactose galactosyltransferase, a marker of the *trans*-Golgi, is used to determine contamination by Golgi membranes (see Support Protocol in *UNIT 3.9*). Since most of the enzymes are facing the interior of the organelles and many substrates for the enzymes are membrane impermeable, it is important to include detergents in the reaction mixture. For practical considerations of the assay procedure see *UNIT 3.4-3.9*.

Materials

- FFE fractions enriched in endosomes or other organelles (see Basic Protocol or Alternate Protocol)
- Phosphate-buffered saline (PBS; *APPENDIX 2A*), pH 7.4
- PBS, pH 7.4 (*APPENDIX 2A*) containing 0.2% (v/v) Triton X-100
- 0.25 M sucrose in TEA buffer (see recipe)
- Disposable plastic cuvettes for spectrofluorometer
- Spectrofluorometer (e.g., Jasco FP 777)
- 2.0 ml scintillation vials
- γ -counter

Determine FITC-ASOR fluorescence

1. When required (Table 3.11.1), dilute fractions with PBS, pH 7.4.
2. Use 300 μ l to 500 μ l of each free-flow fraction and add PBS containing 0.2% (v/v) Triton X-100 up to 2 ml.

3. Use 300 μ l to 500 μ l of 0.25 M sucrose in TEA buffer as blank.
4. Determine fluorescence of the fractions using a spectrofluorimeter at 485 nm excitation (excitation slit, 10 nm) and at 515 nm emission wavelength (emission slit, 10 nm).
5. Subtract blank.

Calculate and present data

6. To determine the distribution of marker enzymes and endosome subpopulations (radioactivity of [¹²⁵I]pIgA and FITC-ASOR fluorescence) in free-flow fractions, set the total amount of the respective marker recovered in all free-flow fractions (in arbitrary units) to 100% and express the activity in individual fractions, counted in 2-ml scintillation vials in a γ -counter in percent of the total amount.
7. Plot the percent of marker in individual fractions versus fraction number.

**SUPPORT
PROTOCOL 2**

CHARACTERIZATION OF PROTEINS IN ENDOSOME SUBPOPULATIONS

The distribution of proteins of interest associated with endosome subpopulations (or other organelles) separated by FFE can be determined by processing the FFE fractions obtained for SDS-PAGE (UNIT 6.1) and immunoblotting with specific antibodies (UNIT 6.2). FFE fractions are pooled and concentrated, and proteins are dissolved in SDS sample buffer. In order to detect a protein of interest, the effect of trypsin on its antigenicity needs to be tested, as the cytoplasmic portions of proteins can be cleaved by trypsin during preparation for FFE.

Materials

- 1 mg/ml trypsin
- 2 mg/ml soybean trypsin inhibitor
- FFE fractions enriched in endosomes or other organelles (Interfaces I and II; see Basic Protocol or Alternate Protocol)
- 0.25 M sucrose in TEA buffer, ice cold (see recipe)
- 1 \times SDS sample buffer (UNIT 6.1)
- Ultracentrifuge with swinging-bucket rotor (e.g., Beckman SW 40 or equivalent) and 14-ml Ultraclear tubes
- Additional reagents and equipment for protein determination (APPENDIX 3B), SDS-PAGE (UNIT 6.1), and immunoblotting (UNIT 6.2)

Determine effect of trypsin on the integrity of proteins by immunoblotting

The incubation of endosome-enriched fractions with trypsin is an absolute requirement for separation of endosomes from other organelles; however, when dealing with a membrane-associated or membrane-spanning protein, the trypsin incubation and the resulting degradation of the protein of interest might result in a loss of antigenicity. Test endosome-enriched fractions (i.e., interfaces I, II) for the effect of trypsin on the protein of interest before subjecting them to FFE separation. When starting the protocol, the conditions for SDS-PAGE and immunoblotting required to detect the protein of interest with the antibody should be known.

1. Prepare endosome-enriched fractions (interfaces I, II; see Basic Protocol, step 29, including step 20a).
2. Determine protein concentration in the interfaces (e.g., Bradford assay, APPENDIX 3B).

3. If necessary, adjust protein concentrations of interfaces as described (see Basic Protocol, steps 34 to 35).
4. Digest, e.g., 100 μ g protein from interfaces with increasing concentrations of trypsin (e.g., 0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%; see Basic Protocol, step 39) from 1 mg/ml trypsin stock.

The amount of trypsin is “standardized as % trypsin/mg protein; 0.003 mg trypsin/mg protein is therefore equal to 0.1% trypsin.

5. Stop incubation as described in Basic Protocol, step 40 by addition of 0%, 0.2%, 0.4%, 0.6%, 0.8%, and 1% soybean trypsin inhibitor from 2 mg/ml.

The results obtained from immunoblotting the samples treated with various concentrations of trypsin should indicate whether trypsin treatment does or does not interfere with detection of the protein of interest by immunoblotting.

Process FFE fractions for SDS-PAGE

6. Determine the protein concentration of pooled FFE fractions of interest (e.g., Bradford assay, *APPENDIX 3B*) and measure the total volume. Calculate total amount of protein in the pooled fractions.
7. Load 14-ml Ultraclear ultracentrifuge tubes with the pooled fractions. Fill up tubes with 0.25 M sucrose in TEA buffer.
8. Ultracentrifuge 50 min at 285,000 $\times g$ (e.g., 40,000 rpm in Beckman SW 40 rotor), 4°C, to concentrate material.
9. Carefully remove the supernatants and add a small volume (e.g., 50 μ l) of 1 \times SDS sample buffer to the pellet.
10. Perform SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*).

IN VITRO ACIDIFICATION OF ISOLATED ENDOSOMES

Endosomes purified by free-flow electrophoresis maintain their functional integrity. In vivo, endosomes are acidified by a proton ATPase (so called vacuolar or V-ATPase) resulting in the generation of a pH gradient across the endosomal membrane (Mellman et al., 1986). Due to passive proton efflux, this pH gradient can only be sustained by constant proton influx energized by the proton ATPase. This functional property of endosomes is also found in vitro. Thus, a cell-free acidification assay can be applied to characterize proton transport and ion permeabilities of the isolated endosomes, taking advantage of the pH-dependent fluorescence of FITC-labeled ligands (such as FITC-ASOR), whereby lowering the pH from 7.4 to 5.0 results in a pH-dependent quenching of the fluorescence intensity of FITC. In the absence of permeable ions (such as in 0.25 M sucrose in TEA buffer) the in vivo acidic pH of the endosomes is not dissipated during the isolation procedure and purified endosomes are therefore still acidic. Consequently, this pH gradient has to be dissipated by incubation with permeable cations and anions (e.g., in KCl-containing buffers). Subsequently, ATP is added, and a rapid decrease in the initial fluorescence is indicative of ATP-dependent intravesicular acidification.

Due to trypsinization of the interface I fraction (with 0.03 mg trypsin/mg interface I protein) prior to free-flow electrophoresis, the proton permeability of the endosomes is increased, and, as a consequence, ATP-dependent acidification is decreased. Thus, for acidification experiments, endosome-enriched fractions are prepared that have been depleted in mitochondria and lysosomes (by centrifugation of S2 at 30,000 $\times g$; see Fig. 3.11.1). For such endosome fractions (interface I) the amount of trypsin required can be

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reduced to 0.003 mg trypsin/mg protein to achieve separation by free-flow electrophoresis. 0.003 mg trypsin/mg protein does not affect ion permeabilities and acidification properties of free-flow purified endosomes (Fuchs et al., 1989).

Materials

FFE fractions from microsomes (Interface I, see Basic Protocol, step 20a)
containing FITC-ASOR labeled endosomes (see Basic Protocol), treated with
reduced quantity of trypsin (see Basic Protocol, steps 39b and 40b)
Acidification buffer (see recipe)
pH calibration buffers (see recipe)
ATP stock (see recipe)
500 μ M nigericin stock (see recipe)
1 mg/ml rabbit anti-fluorescein antibody: IgG(H+L)-fraction (Molecular Probes)
Spectrofluorometer (e.g., Jasco FP 777)
Disposable plastic cuvettes for spectrofluorometer (e.g., Sarstedt)

Generate pH calibration curve of internalized FITC-ASOR

1. Add 0.2 to 10 μ l interface I endosome fraction (0.2 to 10 μ g protein) to 2 ml of pH calibration buffer (pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.4) in disposable plastic cuvettes.

FITC-ASOR labeled endosomes are mainly enriched at interface I, regardless of whether endosomes were labeled by injection of markers for 1 min or 10 min.

2. Add 1 μ M nigericin (from 500 μ M stock) and 1 μ l anti-fluorescein antibody.

The K^+/H^+ ionophore nigericin results in pH equilibration between the pH calibration buffer and the interior of the vesicle. Any FITC-ASOR that leaks out during incubation is quenched by the anti-fluorescein antibody and therefore does not contribute to the fluorescence measurements.

3. Incubate for 2 to 3 hr at room temperature in the dark to equilibrate extra- and intravesicular pH.
4. Transfer cuvettes into spectrofluorometer and determine the fluorescence intensities at 485 nm excitation (excitation slit, 10 nm) and at 515 nm emission wavelength (emission slit, 10 nm).
5. Generate a pH calibration curve by setting fluorescence intensity at pH 7.4 to 100% and plotting pH versus fluorescence intensities (in percent), to obtain a calibration curve of internalized FITC-ASOR.

Dissipate initial acidic endosomal pH

6. Add endosome fraction (0.1 to 10 μ l of interface I; 100 to 500 μ l of free-flow fraction) to 2 ml acidification buffer in a plastic cuvette.
7. Add 1 μ l anti-fluorescein antibody.
8. Place cuvette into spectrofluorometer and continuously record the fluorescence until it reaches a steady-state level.

Dissipation of the initial pH to neutrality requires 30 min to 1 hr.

Acidify endosomes

9. Add 10 μ l ATP stock (final concentration 2.5 mM) to the cuvette and mix rapidly by carefully pipetting up and down.
10. Record fluorescence decrease until steady-state level is obtained.

Usually, ATP-dependent acidification results in a fluorescence decrease at least to the initial fluorescence prior to pH dissipation. Steady-state low pH is obtained after ~15 min. If a fluorescence increase is then observed, the amount of ATP added was insufficient and must be increased. Due to other ATPases present in the fraction, the ATP concentration decreases, and this may result in insufficient levels to provide the driving force for the V-ATPase.

11. Add 4 μ l of 500 μ l nigericin stock (final concentration, 1 μ M), mix rapidly by carefully pipetting up and down, and record the rapid fluorescence increase.

Nigericin leads to an immediate dissipation of the intravesicular pH. The fluorescence value upon nigericin addition should be identical to the value prior to ATP addition (i.e., neutral pH inside the endosomes).

Calculate initial endosomal pH and the pH obtained after ATP addition

12. Express initial fluorescence in percent of the value obtained after dissipation of the pH gradient (i.e., after the intravesicular pH has reached 7.4).
13. Express ATP-dependent fluorescence-decrease in percent of the value prior to ATP addition (i.e., at intravesicular pH of 7.4).
14. Use pH calibration curve to calculate the initial (acidic) endosomal pH and the pH obtained after ATP-dependent acidification.

The pH obtained after in vitro acidification amounts to 6.5 to 6.0 for early endosomes and to 5.0 to 5.5 for late FITC-ASOR labeled endosomes, and is comparable to the initial pH. Peak fractions from free-flow fractions have the highest capacity for ATP-dependent acidification/ μ g protein, demonstrating a high level of enrichment of these fractions.

PREPARATION OF ASIALOOROSOMUCOID (ASOR)

The unique and high expression of a galactose-specific receptor, i.e., asialoglycoprotein receptor (ASGPR) at the sinusoidal (basolateral) plasma membrane of rat hepatocytes leads to uptake and removal of desialylated glycoproteins from the circulation (Wall et al., 1980). Many desialylated proteins with terminal galactose residues bind to this receptor. High binding affinities are exhibited by proteins such as ASOR that expose at least three terminal galactose residues. ASOR can be obtained by enzymatic desialylation of α 1-acid glycoprotein (orosomucoid; Wall et al., 1980), as described in this protocol. Subsequent conjugation of ASOR to fluorescein isothiocyanate (FITC) is described in Support Protocol 5.

Materials

Insoluble neuraminidase (type X-A, attached to beaded agarose; Sigma-Aldrich)
0.1 M sodium acetate buffer, pH 5.5 (APPENDIX 2A)
2% (w/v) sodium azide in H₂O, prepare before use
Human α 1-acid glycoprotein (Sigma-Aldrich)
0.1 M sodium borate buffer, pH 9.3
Sephadex G-25 M column (prepacked; Pharmacia Biotech PD-10; bed volume 9.1 ml)
Low-speed centrifuge with swinging bucket rotor for 50-ml tubes (e.g., Beckman JA-20 rotor or equivalent)
50-ml conical centrifuge tubes

Prepare neuraminidase

1. Resuspend 0.25 U insoluble neuraminidase in 1.5 ml of 0.1 M sodium acetate buffer, pH 5.5, in a 2.0-ml microcentrifuge tube at room temperature.

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2. Microcentrifuge for 10 min at maximum speed, (16,000 × g) room temperature.
3. Remove and discard supernatant.
4. Resuspend the pellet of insoluble neuraminidase in 1.5 ml of 0.1 M sodium acetate buffer, pH 5.5.
5. Pellet insoluble neuraminidase by centrifugation as in step 2.
6. Repeat steps 4 and 5 once more.

Desialylate α 1-acid glycoprotein with insoluble neuraminidase

7. Dissolve 100 mg α 1-acid glycoprotein in 2 ml of 0.1 M sodium acetate buffer, pH 5.5, containing 0.02% (w/v) sodium azide (added from a 2% w/v stock solution).
8. Add this α 1-acid glycoprotein solution to the pellet of neuraminidase and incubate 48 hr under rotation at 37°C.
9. Pellet neuraminidase by microcentrifuging 10 min at maximum speed, at room temperature.
10. Remove supernatant containing ASOR and free sialic acid in 0.1 M sodium acetate buffer, pH 5.5 for subsequent spin desalting. Use this solution in 1-ml aliquots for spin desalting (steps 11 – 15).

Spin desalt ASOR

11. Equilibrate prepacked Sephadex G-25 M column with two bed volumes (20 ml) of 0.1 M sodium borate buffer, pH 9.3.

One column is needed to exchange the buffer and remove free sialic acid from 1 ml of ASOR solution.

12. Centrifuge column for 5 min at 2500 × g (in JA-20 rotor) at room temperature to remove excess liquid.

Centrifugation can be done by cutting a hole in the screw cap of a 50-ml conical plastic tube to fix the column at the top of the tube. Excess liquid is then collected at the bottom of the tube and discarded. It is important to allow liquid to be removed by centrifugation without back-flow into the column, otherwise the protein solution subsequently applied cannot be quantitatively removed by the centrifugation step below.

13. Insert the column into a new 50 ml conical plastic tube and place a 2.0-ml microcentrifuge tube under the outlet of the Sephadex G 25 column at the bottom of the 50-ml centrifuge tube.
14. Apply 1 ml of ASOR solution (in sodium acetate buffer, pH 5.5; see step 10) to the column.
15. Centrifuge column for 5 min at 2500 × g, at room temperature, to collect the ASOR in 1 ml 0.1 M sodium borate buffer, 9.3 in the 2.0-ml microcentrifuge tube. Cool ASOR solution to 4°C.

Since excess liquid has been removed from the column by centrifugation (step 12) the 1 ml ASOR in 0.1 M sodium acetate buffer, pH 5.5, applied should be recovered as ASOR in 1 ml of 0.1 M sodium borate buffer, pH 9.3.

16. Use an aliquot (20 mg) of this solution for FITC-labeling (see Support Protocol 5) and store remaining aliquots (20 mg) at –20°C.

COUPLING OF FLUORESCHEIN ISOTHIOCYANATE (FITC) TO ASOR

To follow endocytosis and to determine the endosomal pH, FITC conjugates of various ligands or fluid-phase markers have been used (van Renswoude et al., 1982). The fluorescence intensity of FITC is pH dependent and the fluorophore can be easily conjugated to the protein of interest. Specific receptor binding of FITC-ligands has to be verified by competing internalization using a 10- to 100-fold excess of unconjugated ligand.

Materials

20 mg ASOR (see Support Protocol 4) in 1 ml of 0.1 M sodium borate buffer, pH 9.3

40 mg FITC on celite (Isomer I on celite; ~10% FITC; Sigma-Aldrich): use 2 mg FITC on celite/mg protein for coupling

TBS, pH 7.38 (see recipe)

PBS, pH 7.38 (APPENDIX 2A)

Thermomixer for microcentrifuge tubes (e.g., Eppendorf thermomixer)

Dialysis tubing (Spectra/Por 2; 0.32 ml/cm; MWCO, 12,000 to 14,000 Da)

Sephadex G-25 M column (prepacked; Pharmacia Biotech PD-10; bed volume 9.1 ml)

Spectrofluorometer (e.g., Jasco FP 777)

Microcuvettes for spectrofluorometer

Additional reagents and equipment for dialysis (APPENDIX 3C)

Label ASOR with FITC

1. Add 40 mg FITC on celite to 20 mg ASOR in 1 ml 0.1 M sodium borate buffer, pH 9.3, at 4°C, in a 1.5-ml microcentrifuge tube.
2. Incubate under constant mixing at maximum mixing frequency in an Eppendorf Thermomixer in the dark for 40 min at 4°C.
3. Pellet celite by microcentrifuging 10 min at maximum speed, (16,000 × g), 4°C.
4. Remove supernatant containing FITC-ASOR and free unconjugated FITC with a plastic transfer pipet and transfer this solution into a prewetted (APPENDIX 3C) dialysis tubing.

Avoid aspiration of any celite, which is not removed during dialysis and which would otherwise interfere with the endocytosis of FITC-ASOR.

5. Dialyze supernatant (APPENDIX 3C) against eight 2-hr changes of 1 liter TBS at 4°C in the dark.
6. Finally, dialyze against 1 liter PBS at 4°C in the dark.
7. Remove FITC-ASOR (~1 ml of 40 mg/ml in PBS) from the dialysis tubing, determine the coupling ratio (steps 8 to 10), and store aliquots (e.g., 100 µg) at -20°C.

Determine the FITC/ASOR ratio (coupling ratio)

8. Determine the protein concentration of the conjugate using the Bradford method (APPENDIX 3B).
9. Dilute the FITC-ASOR solution (~1:40) with PBS, pH 7.38, and measure the absorbance of the conjugate at 496 nm (maximal FITC absorbance, A_{496}).

It is important to adjust the pH of the PBS to exactly 7.38, as the fluorescence of FITC is strongly pH dependent.

10. Calculate the amount (μg) FITC bound to ASOR (mg) using the following equations (coupling ratio; Jobbágy and Király, 1966):

$$\mu\text{g FITC}/\text{mg ASOR} = \text{cFITC}/\text{cASOR}$$

$$\text{cFITC (FITC content of the conjugate; } \mu\text{g/ml)} = 6.09 \times A_{496} \times \text{dilution}$$

$$\text{cASOR} = \text{concentration of ASOR in the conjugate (mg/ml)}$$

Following this protocol, the authors found that ~18 μg FITC bound to 1 mg ASOR; this corresponds to a molar ratio of FITC:ASOR of 2:1. The FITC-to-ASOR ratio should not exceed 8 mol FITC per mol ASOR to ensure specific binding to the ASGP receptor.

Analyze the purity of the FITC-ASOR conjugate by chromatography on Sephadex G-25

11. Equilibrate Sephadex G-25 column with two bed volumes (20 ml) of PBS.
12. Apply 100 μl of diluted (1:100 in PBS) FITC-ASOR solution to the column.
13. Elute column with PBS and collect 2-ml fractions of the eluate.
14. Determine fluorescence of individual fractions in a spectrofluorometer at excitation and emission wavelengths of 485 nm and 515 nm, respectively (slit, 10 nm).

The FITC-ASOR conjugate elutes at fraction 2, whereas the peak of free FITC, if present, is found in fractions 10 to 11. In the case of free FITC, the conjugate can be further purified by chromatography on Sephadex G-25 (follow steps 11 to 14 of this protocol using undiluted FITC-ASOR as starting material).

Specific binding of FITC-ASOR can be demonstrated by competing binding and internalization by a 10-fold excess of unlabeled ASOR; under this condition no fluorescence signal in isolated endosomes is obtained (Fuchs et al., 1989).

SUPPORT PROTOCOL 6

PREPARATION OF ^{125}I -LABELED HUMAN POLYMERIC IMMUNOGLOBULIN A (^{125}I]pIgA)

The pIgA-receptor (pIgR) on rat hepatocytes efficiently transports pIgA from the circulation into bile via vesicular transcellular transport. To label transcytotic endosomes in rat liver, rat, human or mouse pIgA can be used as unmodified or radiolabeled ligand (Hoppe et al., 1985; Giffroy et al., 1998).

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

CAUTION: This procedure must be performed in a hood certified for radioiodination. All personnel performing iodinations or using radioactive materials must be properly trained.

Materials

Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing 0.1% (w/v) bovine serum albumin (BSA)

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Iodo-Beads (iodination reagent; Pierce)

$\text{Na}[^{125}\text{I}]$ (17 mCi/ml; NEN Life Sciences)

Human polymeric immunoglobulin A (pIgA), purified from human myeloma serum (Vaerman and Lemaitre-Coelho, 1979) and kindly provided by Jean-Pierre Vaerman and Pierre J. Courtoy (Catholic University of Louvain, Brussels, Belgium).

TBS (APPENDIX 2A)

10% (w/v) trichloroacetic acid (TCA)

Pre-packed anion-exchange cartridges (e.g., Bio-Rad Econo-Pac Q; bed volume 5 ml)

5-ml conical-bottom screw-cap glass reaction vials

2.0-ml scintillation vials

γ counter (e.g., Beckman Instruments)

Prepare the anion exchange column

1. Equilibrate the prepacked anion-exchange column with three bed volumes (15 ml) of PBS/0.1% (w/v) BSA to reduce nonspecific protein absorption.
2. Remove excess BSA with 1 bed volume (5 ml) PBS.

Radiolabel pIgA

3. Wash one Iodo-Bead with 500 μ l PBS and dry bead on filter paper.
4. Put 100 μ l PBS containing 1 mCi (2.2×10^9 cpm) Na[¹²⁵I] in a 5-ml glass reaction vial.
5. Add Iodo-Bead to the vial, cap vial, and incubate in the hood for 10 min at room temperature.
6. Add 50 μ g pIgA (in 5 μ l PBS) to the vial and incubate for 30 min at room temperature in the hood.

Alternatively, mouse pIgA can be used. The polymer forms of mouse IgA can be purified from clarified ascites (Sigma-Aldrich) or from the purified myeloma immunoglobulin (Sigma-Aldrich) of a plasmacytoma cell line (TEPC) as described by Hoppe et al., 1985.

7. Stop the iodination reaction by applying the mixture to the anion-exchange column.
8. Rinse the vial with 100 μ l PBS and transfer the rinse to the anion-exchange column.
9. Elute iodinated pIgA with 100 μ l portions of PBS at room temperature and collect ~100- μ l fractions in pre-chilled microcentrifuge tubes on ice.

Analyze labeling

10. Quantify radioactivity by counting 3- μ l aliquots of each fraction in a γ -counter.
11. To determine protein-bound radioactivity, place a 5- μ l sample of each fraction in a microcentrifuge tube and add 1 ml ice cold 10% (w/v) TCA, mix, and incubate on ice for 30 min.
12. Pellet precipitated proteins by microcentrifuging 10 min at maximum speed, 4°C.
13. Remove and count 500 μ l of the supernatant (i.e., TCA-soluble radioactivity) as well as the remaining 500 μ l containing soluble and precipitable (¹²⁵I-labeled pIgA) radioactivity.
14. Determine cpm in a γ counter and calculate free (soluble) and precipitable cpm.

This procedure should yield pIgA with a specific activity of ~1.5 μ Ci/ μ g pIgA (3.3×10^6 cpm/ μ g pIgA).

15. Use the fractions with at least 95% to 98% TCA-precipitable counts for in vivo endosome labeling. Store ¹²⁵I-labeled pIgA in 50- μ l aliquots for up to 1 month at 4°C in a lead container.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Acidification buffer

5.6 g KCl (150 mM final)
3.4 g HEPES (*N*-[2-Hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; 20 mM final)
616.2 mg MgSO₄·7H₂O (5 mM final)
H₂O to 500 ml (final volume)
Adjust pH with tetramethylammonium hydroxide to pH 7.4
Filter through a qualitative filter paper (e.g., Whatman no. 4)
Store up to 1 month at 4°C

ATP stock

2917 mg adenosine 5'-triphosphate (dipotassium salt; Sigma-Aldrich; 500 mM final)
H₂O to 10 ml (final volume)
Adjust pH with 1 M KOH (prepared freshly) to 7.4
Store aliquots (100 µl) indefinitely at -20°C

FFE chamber buffer

Prepare 5 liters of 1× TEA buffer from 10× stock solution (see recipe). Adjust pH to 7.4. Add 427.9 g sucrose (250 mM final) to a final volume of 5 liters of 1× TEA buffer, stir overnight, filter through a qualitative filter paper (e.g., Whatman no. 4) before use, and determine conductivity (should be 720 µS). Prepare fresh and use at 4°C

With a chamber buffer flow rate of 2.0 to 2.5 ml/h/fraction, this volume is sufficient to run the FFE for at least 20 hr nonstop. The FFE separation of one interface by the Elphor Vap 22 takes ~45 to 60 min.

FFE electrode buffer

10× TEA buffer (see recipe), room temperature
Determine conductivity before use (conductivity: 7.2 mS)
Prepare 5 liters for one FFE run

Nigericin stock, 500 µM

5 mg nigericin, sodium salt (Sigma-Aldrich; 500 µM final)
Ethanol to 13.4 ml
Store 1-ml aliquots up to 1 year at -20°C

pH calibration buffers

Mix solution A (see recipe) with solution B (see recipe) until the desired pH (5.0, 5.5, 6.0, 6.5, 7.0, and 7.4) is obtained. Prepare 100 to 200 ml of each buffer. Store up to 1 month at 4°C

Solution A for pH calibration buffer

11.2 g KCl (150 mM final)
9.8 g MES (2-[*N*-Morpholino]ethanesulfonic acid; 50 mM final)
H₂O to 1000 ml (final volume)
Adjust pH with KOH to 5.0
Filter through a qualitative filter paper (e.g., Whatman no. 4)
Store up to 1 month at 4°C

Solution B for pH calibration buffer

11.2 g KCl (150 mM final)
16.9 g HEPES (*N*-[2-Hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]); 50 mM final)
H₂O to 1000 ml (final volume)
Adjust pH with KOH to 7.4
Filter through a qualitative filter paper (e.g., Whatman no. 4)
Store up to 1 month at 4°C

Sucrose in TEA buffer, 0.25 M

171.2 g sucrose (250 mM final)
1× TEA buffer (prepare from 10×; see recipe) to 2000 ml (final volume)
Adjust to pH 7.4
To control the molarity of the solution, check the refractive index (refractive index = 1.3453 ± 0.0005) at room temperature. Filter through a qualitative filter paper (e.g., Whatman no. 4) and store up to 48 hr at 4°C.

Sucrose in TEA buffer, 1.00 M

85.6 g sucrose (1.0 M final)
1× TEA buffer (prepare from 10×; see recipe) to 250 ml (final volume)
Adjust to pH 7.4
To control the molarity of the solution, check the refractive index (refractive index = 1.3816 ± 0.0005) at room temperature. Filter through a qualitative filter paper (e.g., Whatman no. 4) and store up to 48 hr at 4°C.

Sucrose in TEA buffer, 1.15 M

98.4 g sucrose (1.15 M final)
1× TEA buffer (prepare from 10×; see recipe) to 250 ml (final volume)
Adjust to pH 7.4
To control the molarity of the solution, check the refractive index (refractive index = 1.3888 ± 0.0005) at room temperature. Filter through a qualitative filter paper (e.g., Whatman no. 4) and store up to 48 hr at 4°C.

Sucrose in TEA buffer, 2.5 M

214.0 g sucrose (2.5 M final)
1× TEA buffer (prepare from 10×; see recipe) to 250 ml (final volume)
Adjust to pH 7.4
To control the molarity of the solution, check the refractive index (refractive index = 1.4528 ± 0.0005) at room temperature. Filter through a qualitative filter paper (e.g., Whatman no. 4) and store up to 1 month at 4°C.

TEA buffer, 10×

66.6 ml triethanolamine, free base (100 mM final)
28.6 ml acetic acid (glacial; 100 mM final)
100 ml 500 mM EDTA (*APPENDIX 2A*; 10 mM final)
H₂O to 5000 ml (final volume)
Adjust pH to 7.4 with NaOH
Store at room temperature

Using the FFE instruments available from Elphor VaP 22, Bender and Hobein, this volume is sufficient for the FFE electrode buffer volume required for one FFE run. An additional 500 ml to 1000 ml will be needed to prepare the FFE chamber buffer and the solutions containing sucrose in TEA buffer used.

continued

Tris-buffered saline (TBS)

87.7 g NaCl (150 mM final)
24.2 g Tris base (20 mM final)
H₂O to 10,000 ml (final volume)
Adjust pH with HCl to 7.38
Store indefinitely at 4°C

This volume is sufficient to dialyze 20 mg FITC-ASOR.

COMMENTARY

Background Information

Free-flow electrophoresis (FFE) is an efficient method providing both analytical capabilities and the opportunity for continuous operation on a preparative scale. The range of substances to be separated is wide, including

small ions, proteins, membrane particles, and even viable cells, and the recoveries of membrane and proteins are high (~90%). FFE separates membranous cell components and organelles (e.g., endosomes) based on surface charge (Morré et al., 1994). As the procedure is non-

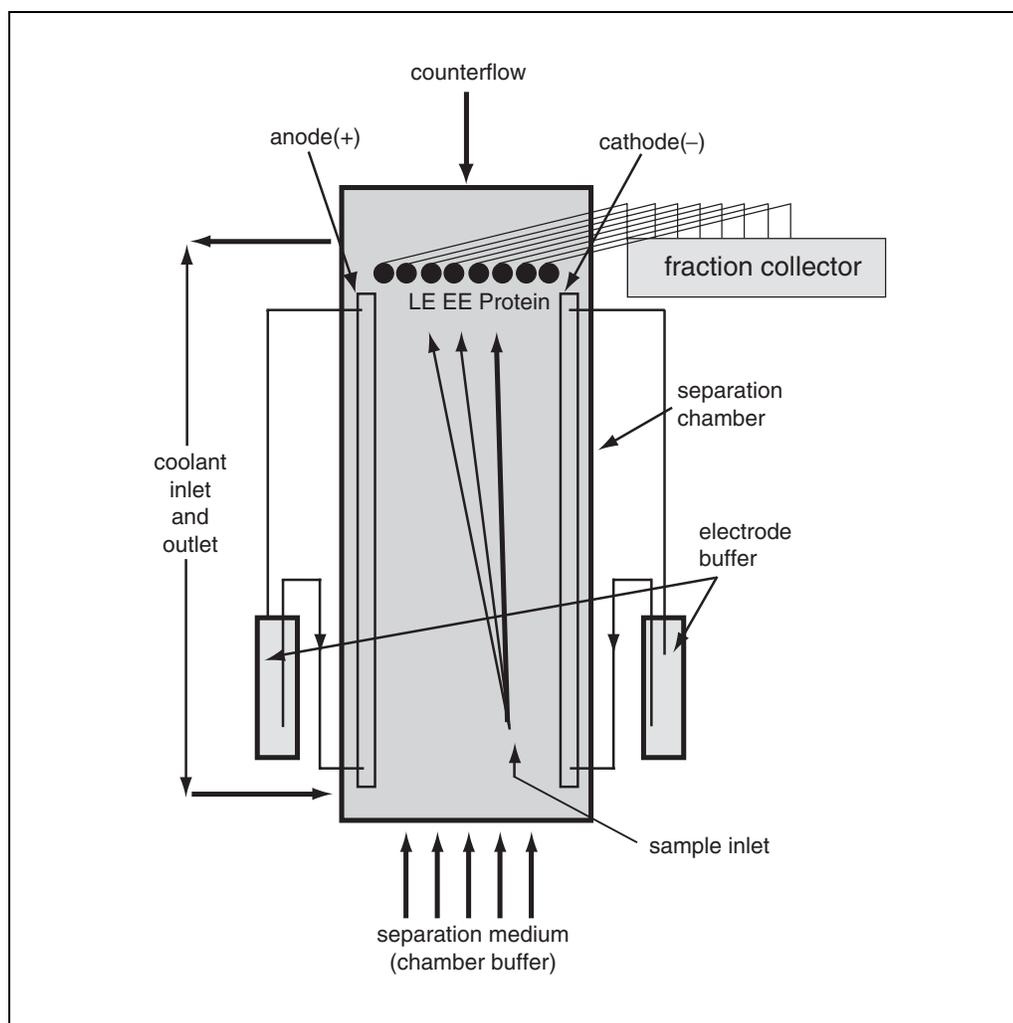


Figure 3.11.2 Basic principle of endosome separation by free-flow zone electrophoresis, referred to as free-flow electrophoresis (FFE), represented in a schematic view. A basic electrolyte with defined pH (chamber buffer) is continuously pumped through the chamber. A fine stream of sample is injected into the laminar flow of the chamber buffer. Due to their different electrophoretic mobilities the different organelles exhibit different angles of deflection and can be collected in different fractions. Early endosomes (EE) are less deflected towards the anode than late endosomes (LE) and the main protein peak elutes nearly undeflected.

destructive and fractions obtained retain full *in vitro* function, organelles such as endosomes isolated by FFE can be subsequently analyzed for their characteristic functions (e.g., acidification).

A mixture of components to be separated is injected as a fine jet into a separation buffer moving perpendicular to the field lines of an electric field (see Fig. 3.11.2). The electric field leads to deflection of membranes bearing different electrical charge densities according to their mobility or isoelectric point. The sample and the separation buffer enter the separation chamber at one end. On the opposite side, the fractionated sample and the separation buffer are collected. Though FFE can be performed in various modes depending on the electrolyte

system applied (e.g., isotachopheric mode, isoelectric focusing mode), the simplest and most widely used free-flow electrophoresis technique is free-flow zone electrophoresis, also known as continuous flow electrophoresis. In this mode, the chamber buffer has a constant composition, pH, and conductivity, and the electrically charged components of the samples (e.g., organelles) are deflected from the flow direction of the medium to a defined angle, which is determined by flow velocity and by electrophoretic migration speed (separation according to the size-to-charge ratio).

With regard to the isolation of intracellular organelles from rat liver, FFE has been applied (Krivankova and Bocek, 1998) for the isolation of lysosomes (Stahn et al., 1970), peroxisomes

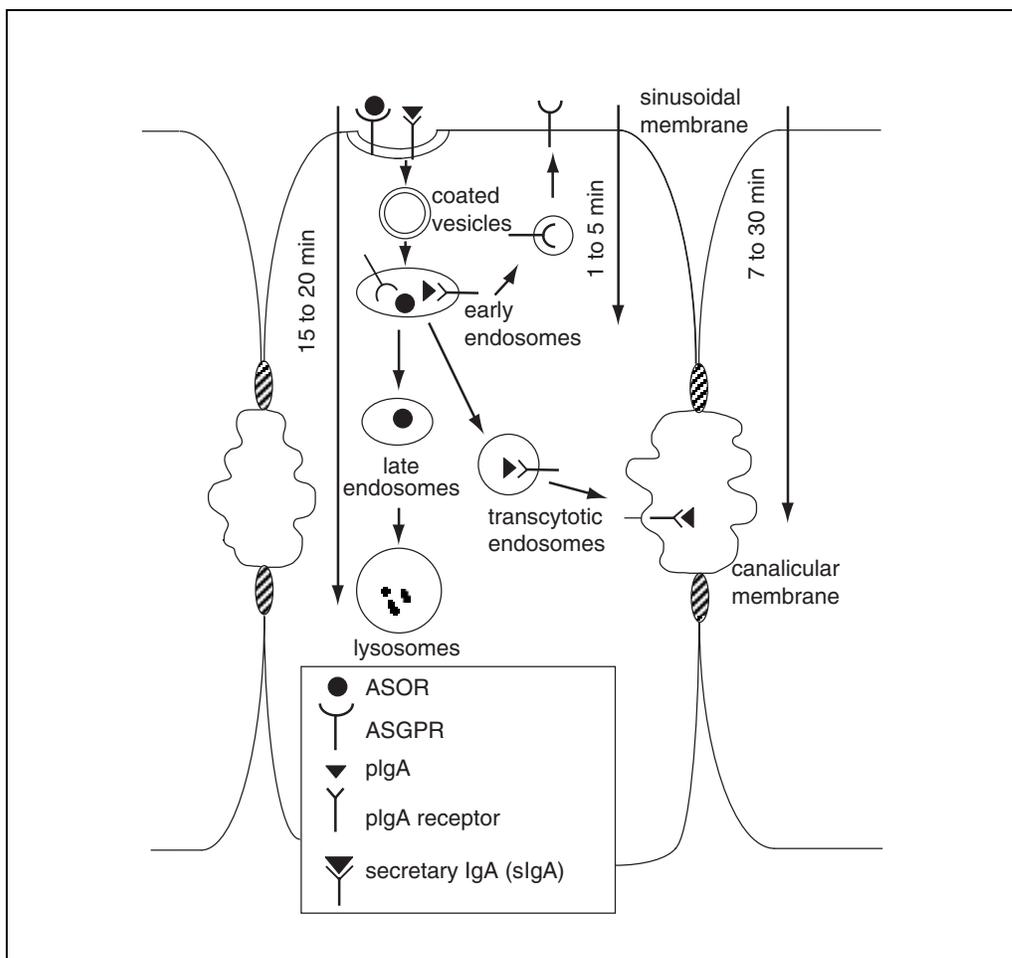


Figure 3.11.3 Kinetics of ASOR and pIgA transport through endocytic compartments in rat hepatocytes. Endocytic subcompartments of rat hepatocytes can be selectively labeled by cointernalization of FITC-ASOR and [¹²⁵I]pIgA. Within 1 to 5 min after *in vivo* injection, both ligands can be localized in a common early endosomal compartment. Thereafter, pIgA is sorted into the transcytotic pathway and is secreted into bile (as secretory IgA) 7 to 30 min after injection. In contrast, ASOR is dissociated from its receptor in acidic early endosomes and transported via late endosomes to lysosomes. Lysoosomal degradation of ASOR is observed 15 to 20 min after uptake. The authors therefore labeled (common) early endosomes by injection of both markers for 1 min. Late endosomes and transcytotic endosomes are preferentially labeled when the markers are injected for 10 min.

(Volkl et al., 1997) and for subfractionation of the Golgi apparatus (Morré et al., 1983). The buffer system and FFE conditions described in this protocol can also be used for the purification of lysosomes, and with some modifications for peroxisome isolation, and for subfractionation of the Golgi apparatus. In any case, an organelle-enriched fraction is subjected to separation by FFE. Although trypsinization is omitted, the starting material is, for example, treated with amylase (Morré, 1998) or reacted with antibodies (Volkl et al., 1997). As marker enzymes β -*N*-acetylglucosaminidase (lysosomes), urate oxidase (peroxisomes), thiamine pyrophosphatase and UDP-galactose galactosyltransferase (trans-Golgi) can be used to follow the organelle purification and enrichment.

An important application of FFE is the isolation and analysis of endosomes and endosome subpopulations. Although endosomes per se are not separated from contaminating material (e.g., endoplasmic reticulum, plasma membranes, mitochondria) when subjected to FFE, separation can be achieved by mild trypsin treatment of the sample, which results in a dose-dependent increase in separation (Marsh et al., 1987). Although the effect of trypsin is still obscure, the electrophoretic mobility of endosomes and lysosomes is enhanced after trypsinization. Applying this technique, it was demonstrated that late endocytic compartments are more shifted to the anode than early compartments (Schmid et al., 1988). Migration of late endosomes to the anode appear to correlate with the internal lower (more acidic) pH of late compartments as compared to the higher (less acidic) pH of early compartments. This led Morré et al. (1994) to suggest that ATP-dependent vesicle (endosome) acidification generates an outside negative membrane potential and as a consequence results in a more anodal migration of vesicles with a lower internal pH, as compared to those maintaining a higher pH. ATP-dependent acidification was then applied to achieve endosome separation by FFE without the need for trypsinization (Morré et al., 1994). In the authors' experience, rat liver endosomes maintain their initial low internal pH during the isolation procedure; nevertheless no separation is achieved without trypsinization (Fuchs et al., 1989).

FFE has been widely used to characterize endocytic pathways and compartments in non-polarized cells, primarily in tissue culture cells. The isolation of endosomes from polarized tissue culture cells requires the culture of the

cells on permeable filters in sufficient quantities to permit subcellular fractionation. This is time-consuming and costly. Alternatively, the rat liver provides a tissue where trafficking in polarized hepatocytes can be studied and where sufficient material is provided from one liver to isolate intracellular organelles. Therefore, the authors describe a protocol for the labeling of endosomal subcompartments of rat hepatocytes and the subsequent FFE isolation and analysis of endosomal fractions.

Polarized hepatocytes express many receptors also commonly found in other cell types (e.g., transferrin receptor, LDL receptor, insulin receptor) and in addition exhibit unique receptor expression (e.g., ASGPR) at their basolateral surface (Courtroy, 1991). Furthermore, hepatocytes are involved in selective transcytosis and secretion of serum proteins (e.g., pIgA) into bile. After internalization via clathrin-coated vesicles, internalized ASGPs are first delivered to kinetically early endosomes that maintain a mildly acidic intravesicular pH. In these compartments, ligands and receptors are sorted into distinct pathways: ASGPs dissociate from their receptors in the acidic endosomal milieu and are transported via kinetically late endosomal compartments to lysosomes, where they are rapidly degraded. Similar to the transferrin receptor, the ASGPR is recycled to the sinusoidal membrane. Although the pIgA-receptor complex is initially delivered via clathrin-coated vesicles to the same early endosomal compartment as ASGPs, the complex is subsequently sorted into transcytotic endosomes and pIgA/pIgR is transported to the canalicular (apical) plasma membrane. Due to proteolytic cleavage near or at the canalicular membrane, the extracellular (ligand-binding) domain of the pIgR (now called secretory component) still bound to pIgA is secreted into bile (hence the term secretory IgA, or sIgA). Although hepatocytes internalize membrane and extracellular material at the canalicular surface (Tuma et al., 1999), this pathway is less characterized due to experimental inaccessibility of the canalicular domain.

Based on the kinetics of ASOR transport through early (receptor-positive) and late (receptor-negative) compartments and its arrival and immediate degradation in lysosomes, early or late endosomes en route to lysosomes can be labeled in a time-dependent manner (see Fig. 3.11.3). Similarly, early and late compartments involved in transcytosis of pIgA from the sinusoidal to the canalicular domain can be marked by short or prolonged internalization

of [125 I]pIgA. The labeling procedure applied in the Basic Protocol is based on *in vivo* studies and cell fractionation experiments reported by various laboratories (Courtoy, 1991; Hoppe et al., 1985; Mueller and Hubbard, 1986; Perez et al., 1988; Sztul et al., 1991; Stefaner et al., 1997). Early endosomes were labeled by coinjection of FITC-ASOR and [125 I]pIgA for 1 min, whereas late compartments of the transcytotic and lysosomal pathway, respectively, were labeled by coinjection of the markers for 10 min.

Critical Parameters and Troubleshooting

Due to variability of the animals, the injection procedure and the resulting labeling of the individual endosome subpopulation, injection of two or more tracers in the same animal is required. To obtain reproducible endosome labeling by *in vivo* marker injection, rapid cooling of the liver by *in situ* perfusion is very important, especially when early compartments have to be labeled, since markers are rapidly sorted (within 1 to 5 min) from these compartments. Furthermore, the livers have to be processed (minced, homogenized) immediately after cooling. The entire isolation procedure

(including homogenization) is carried out in isotonic buffers to maintain the integrity of the organelles and to prevent loss of endocytic markers. Thus, care has to be taken during homogenization, and protease inhibitors are included in the buffer to inhibit damage due to release of proteases. It is also critical for subsequent FFE separation to use the 0.25 M sucrose in TEA buffer, which is also used as FFE chamber buffer, throughout the entire isolation procedure. Most tissue culture cells, and also liver cells, are effectively broken up in this buffer.

To obtain good separation of samples by free-flow zone electrophoresis in general, an accurate preparation of FFE electrode and chamber buffer is of major importance. The composition, the pH, and the conductivity of these buffers are critical parameters; therefore, pH and conductivity should be tested prior to every run. In general, all parameters (buffer composition, coolant volume, filter membranes) directly or indirectly influencing the high-voltage field must be regularly checked. To avoid loss of essential samples, a constant electrical field should be maintained for at least 1 hr before the sample is injected. This might allow one to observe and find a reason for any

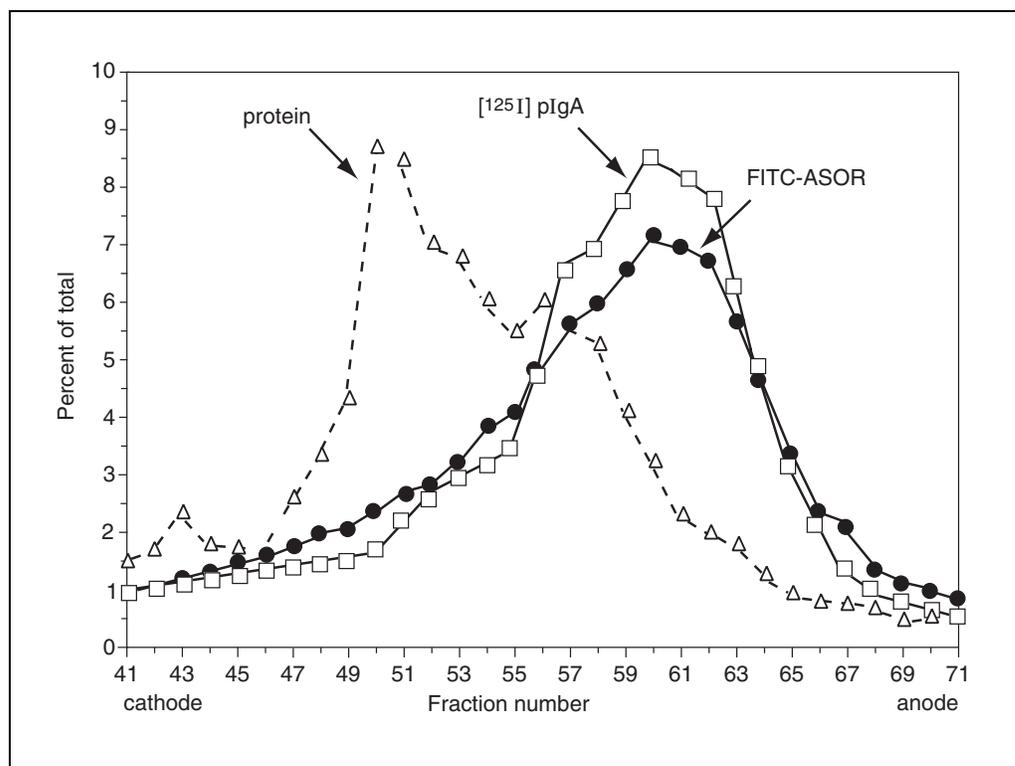


Figure 3.11.4 Distribution of protein and endocytic marker after free-flow electrophoresis of interface I after *in vivo* injection of FITC-ASOR and [125 I]pIgA for 1 min. Endosome-enriched fractions were prepared. Material at the 0.25/1.0 M sucrose interface was collected, trypsin-treated, and subjected to FFE.

problem before the sample is lost. Filtering of chamber buffer is essential, too, as small particles introduced into the thin chamber can disturb the separation by, e.g., deflecting the jets of separated substances. For the same reason, it is essential to accurately clean the chamber before it is filled and to avoid the introduction of small air bubbles either during filling of the chamber with chamber buffer or during injection of the sample. Separated samples are collected via 96 tubes. Clogged tubes (especially several clogged tubes in series) severely alter the separation profile. It is therefore critical to extensively wash the chamber and all connecting tubes with water after every run to remove the sucrose. Tubes blocked at the beginning of a run might be forced to flow again by applying a vacuum (e.g., sucking with a large syringe). Also wash the sample inlet immediately after sample injection with TEA/0.25 M sucrose buffer to prevent clogging. Separation of endosomes from other organelles requires brief trypsinization. As demonstrated by Marsh et al. (1987), even a small increase or decrease in the trypsin concentration can alter the separation profile. Therefore, the amount of trypsin added, the incubation time of samples with trypsin at 37°C, and an exact determination of the protein

concentration in the interfaces are critical parameters. The amount of trypsin is “standardized” as percent trypsin per milligram protein. Since the amount of trypsin required to separate endosomes depends on the enrichment of endosomes in the sample, the optimal trypsin concentration has to be experimentally determined, whenever the fractionation protocol is modified. The adjustment of a certain protein concentration (1 mg/ml) in the loading sample has important implications: lower concentrations make it difficult to detect marker proteins in fractions; high concentrations cause broadening of jets and reduced separation of organelles. When the expected separation (~10 fractions) of endosomal markers from the major protein peak is not obtained (e.g., with a new instrument), alter the relevant conditions (sample flow rate, chamber buffer flow rate, trypsin concentration, voltage/current) one by one.

When two different endosome-labeling conditions (e.g., 1 min versus 10 min labeling) are analyzed, these two livers should be processed in parallel and subjected to FFE on the same day, one after another, to have comparable isolation and FFE conditions. When individual FFE runs at different days and liver preparations are compared, the actual fraction num-

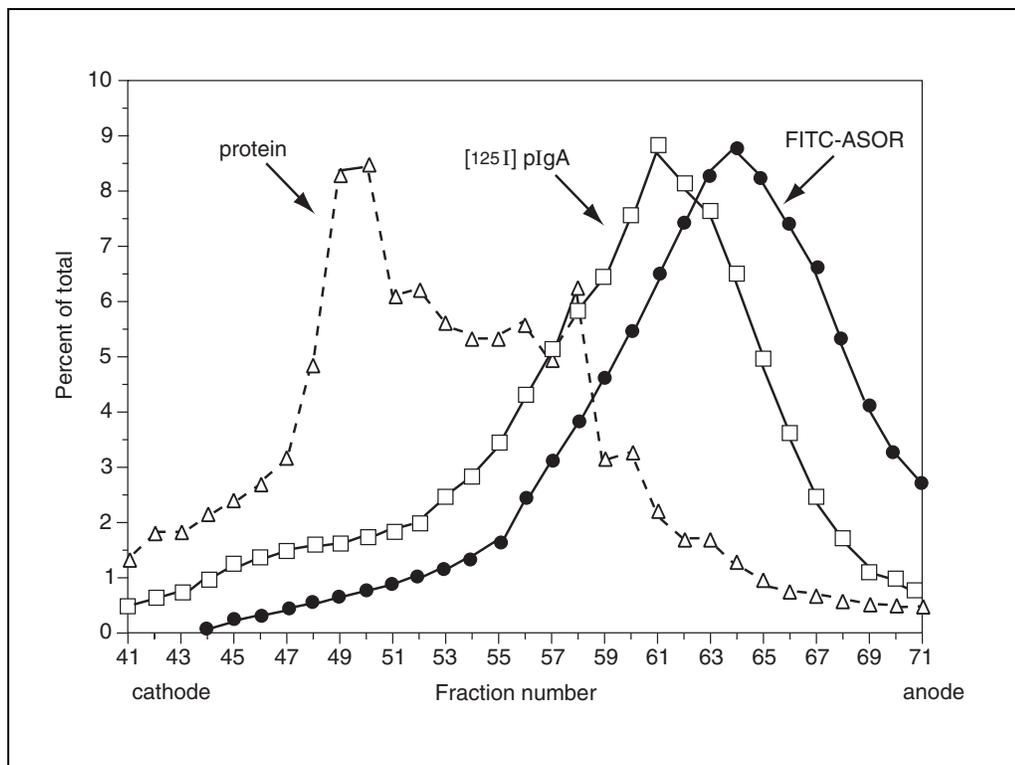


Figure 3.11.5 Distribution of protein and endocytic marker after free-flow electrophoresis of interface I. FITC-ASOR and [¹²⁵I]pIgA were injected for 10 min. Endosome-enriched fractions were prepared. Material at the 0.25/1.0 M sucrose interface was collected, trypsin-treated, and subjected to FFE.

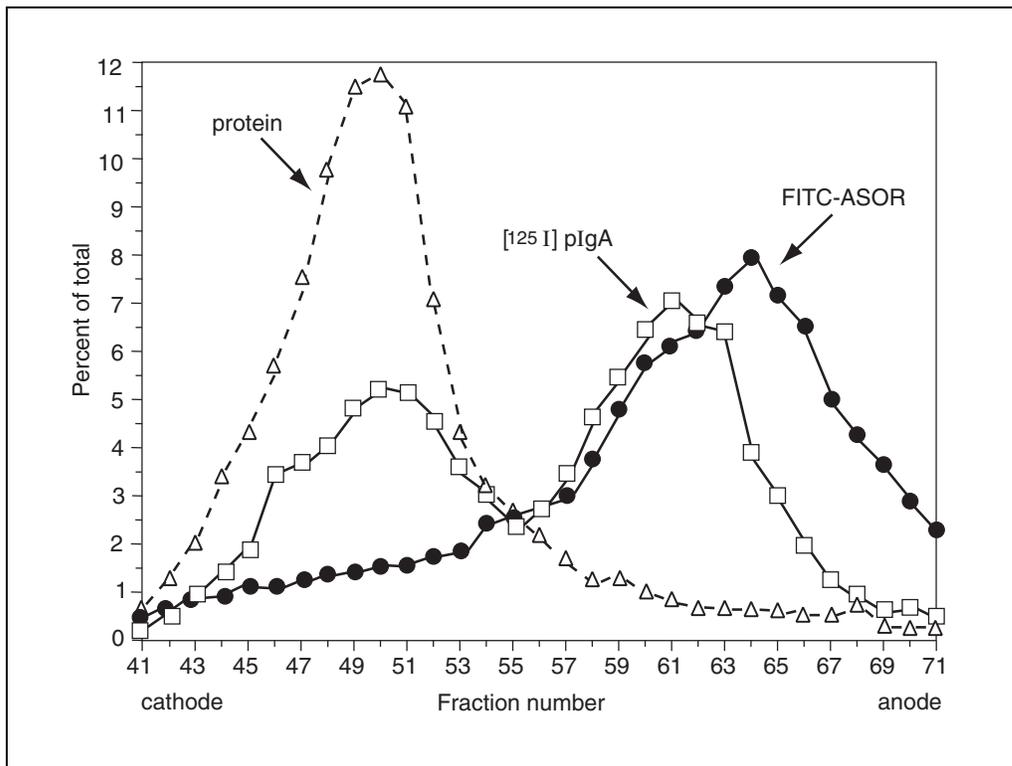


Figure 3.11.6 Distribution of protein and endocytic marker after free flow electrophoresis of interface II. FITC-ASOR and [^{125}I]pIgA were injected for 10 min. Endosome-enriched fractions were prepared. Material at the 1.0/1.15 M sucrose interface was collected, trypsin-treated, and subjected to FFE.

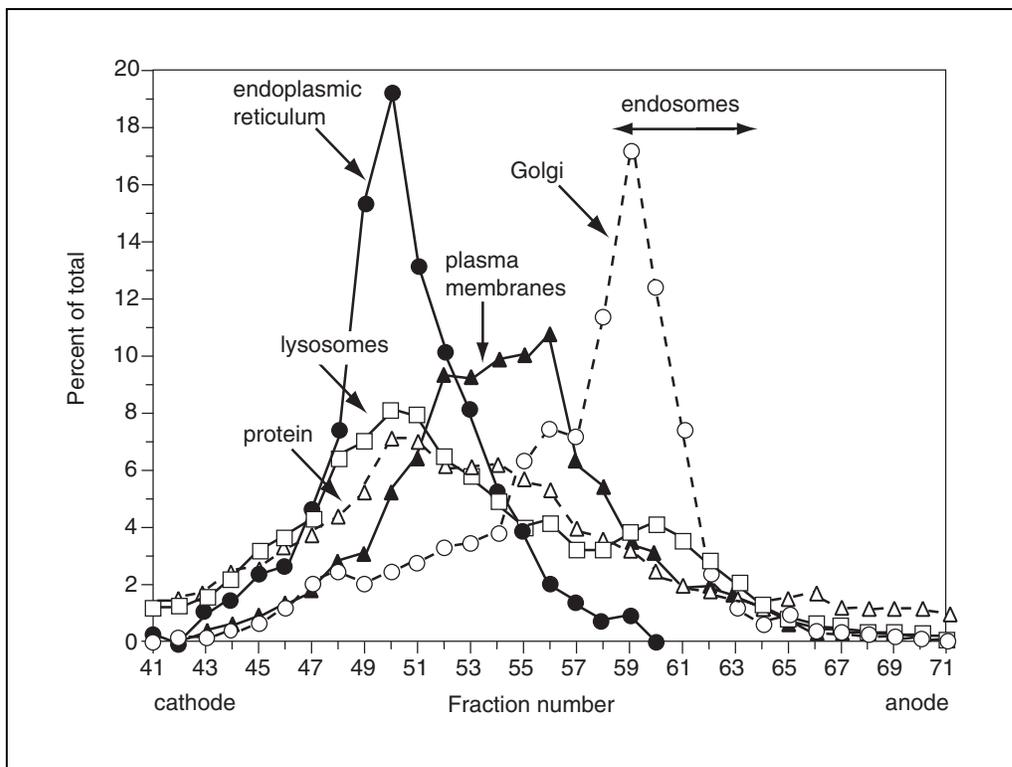


Figure 3.11.7 Distribution of protein and marker enzymes after free-flow electrophoresis of interface I. The following marker enzymes were determined: NADPH cytochrome *c* reductase (endoplasmic reticulum); alkaline phosphodiesterase I (plasma membranes); β -*N*-acetylglucosaminidase (lysosomes); UDP-galactose galactosyltransferase (*trans*-Golgi).

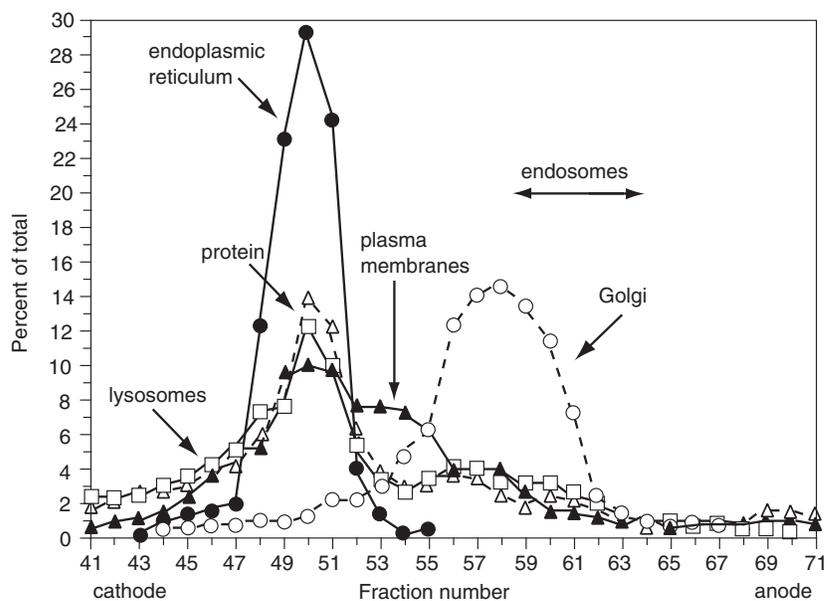


Figure 3.11.8 Distribution of protein and marker enzymes after free flow electrophoresis of interface II. The following marker enzymes were determined: NADPH cytochrome *c* reductase (endoplasmic reticulum); alkaline phosphodiesterase I (plasma membranes); β -*N*-acetylglucosaminidase (lysosomes); UDP-galactose galactosyltransferase (*trans*-Golgi).

ber—i.e., the position at which protein and endosomal peaks elute—varies, but separation (number of fractions between endosomal and protein peaks, and between endosome subcompartments) is fairly constant.

Anticipated Results

Basic Protocol

Preparation of endosome-enriched fractions. Rat liver endosomes are mainly enriched in so-called Golgi fractions that can be obtained by flotation of microsomes or total microsomes (see Fig. 3.11.1) in discontinuous sucrose density gradients. According to the Basic Protocol, a “Golgi fraction” is prepared that corresponds to a combined Golgi light, intermediate, and heavy fraction as described by Khan et al. (1986). The combined Golgi fraction, here called interface I (interface 0.25/1.0 M sucrose), is primarily enriched in endosomes labeled with ASOR. In contrast, pIgA is equally distributed between interfaces I and II (1.0/1.15 M interface) and also within the 1.15 M sucrose layer. Nevertheless, due to the higher amount of protein-contaminating material (primarily endoplasmic reticulum and plasma membranes) present in interface II, pIgA is less enriched in interface II as compared to interface

I. Both interface I and interface II have been analyzed by FFE to demonstrate that the distribution and separation of endosome subcompartments is very similar in these interfaces and therefore independent of their density properties. For further functional studies, endosomes derived from interface I are preferentially used. These endosome-enriched fractions are mainly contaminated by the Golgi apparatus, which is highly enriched at interface I. Although the majority of plasma membranes are removed by the initial low-speed centrifugation steps, interfaces I and II are slightly enriched in APDE I with a higher enrichment found in interface II as compared to interface I. This becomes apparent when FFE fractions of interface I and II are compared (see below) and plasma membrane localization of pIgA is evident in interface II but not in interface I. For a detailed characterization (yield, enrichment, purity) of endosome-enriched gradient fractions and FFE fractions see previous reports (Fuchs et al., 1989; Khan et al., 1986; Marsh et al., 1987; Schmid et al., 1988; Stzul et al., 1991; Stefaner et al., 1997).

FFE separation of endosome-enriched fractions: To analyze the kinetics and potential subcompartments involved in endocytic transport, analytical FFE can be applied. For these separations, the distribution of marker enzymes

is first determined to ensure the resolution of the technique. For subsequent analysis, the distribution of protein and endosomal markers is determined. The quality of the separation is usually estimated from the number of fractions between the main protein peak and the endosome peak fractions. Since early endosomes are less deflected to the anode than transcytotic and late endosomes, a separation is considered as “good” if the protein and early endosome peaks are 9 to 10 fractions apart. Late endosomes are separated by 13 to 15 fractions from the protein peak, at best. In general, the separation of rat liver endosomes is usually less when compared to the separation of endosomes from tissue culture cells.

Early endosomes were labeled by coinjection of FITC-ASOR and [¹²⁵I]pIgA for 1 min. Since ~1 to 2 min are required to cool the liver and halt endocytosis, both ligands are internalized for ~2 to 3 min. The FFE separation of the endosome-enriched fraction at interface I is shown in Figure 3.11.4. The major protein peak elutes at fraction 50, whereas endosomes are deflected towards the anode and are well resolved from the protein peak. As expected, the major peak of ASOR and pIgA-labeled endosomes are localized in the same fraction (60).

Internalization of FITC-ASOR and [¹²⁵I]pIgA for 10 min and FFE separation of interface I and interface II is shown in Figures 3.11.5 and 3.11.6. In comparison to early endosomes labeled with ASOR, late ASOR-labeled compartments are more deflected to the anode, and the peak fraction of the late ASOR-labeled endosomes now elutes at fraction 64. In contrast, labeling of late transcytotic compartments with pIgA leads to a small but reproducible shift of the peak fraction from 60 (see Fig. 3.11.4) to 61 (see Fig. 3.11.5 and 3.11.6). Similar results were obtained by labeling early, late, and transcytotic endosomes in the isolated perfused liver (Stefaner et al., 1997). The second peak of [¹²⁵I]pIgA at fraction 50 after FFE separation of interface II (Fig. 3.11.6) is due to pIgA still present in the plasma membrane. This also demonstrates the higher contamination of interface II fractions by plasma membranes, since this peak is largely absent in interface I fractions (see Fig. 3.11.5). In contrast to ASOR, binding of pIgA to its receptor is neither calcium- nor pH-dependent and plasma membrane-associated pIgA is not displaced from the plasma membrane during isolation.

The distribution of marker enzymes after FFE separation of interface I and II (see Fig. 3.11.7 and 3.11.8) is very similar with respect

to elution of peak fractions. The major protein peak consists of endoplasmic reticulum and plasma membranes. Lysosomes are a minor contaminant and the lysosomal enzyme activity found in the FFE fractions from rat liver does not colocalize with endosomal fractions, in contrast to FFE of endosomes from tissue culture cells (Marsh et al., 1987). Similar to endosomes, marker for the *trans*-Golgi is also shifted to the anode, eluting at fraction 59. Thus, it is primarily early endosomes that are contaminated by Golgi membranes. Due to removal of the majority of the protein (i.e., endoplasmic reticulum, plasma membranes), upon FFE the enrichment of the endosomal peak fractions is further increased 2- to 4-fold as compared to the starting material (interface I and II).

In conclusion, FFE separation of endosome-enriched fractions from rat liver results in the identification of three distinct endosomal sub-compartments: (common) early, late, and transcytotic endosomes. When the acidification properties of these three peak fractions is analyzed using Support Protocol 3, these populations are functional with respect to ATP-dependent proton transport and differ in their internal pH.

Time Considerations

In contrast to the FFE purification of endosomes from tissue culture cells, the isolation procedure for rat liver endosomes (see Basic Protocol) is time-consuming and cannot be interrupted at any time. The following time requirements have to be calculated for individual steps (preparation of buffers, ligands, and solutions is not included in this timetable):

In vivo endosome labeling, and liver perfusion: 1 hr;

Preparation of endosome-enriched fractions ready for FFE separation: ~8 to 9 hr;

FFE-set up before separation: 2 to 3 hr (this can be done in parallel during the centrifugation procedure);

Endosome separation: calculate ~1 to 1.5 hr/sample to obtain material for analytical purpose; at least two samples are analyzed, i.e., 2 to 3 hr; For preparative endosome isolation: 3 to 5 hr;

Analysis of protein and endosomal marker in FFE fractions: ~1 to 2 hr; For analysis of marker enzymes the fractions can be frozen and assayed when appropriate;

Enzyme assays: 1 to 2 days.

It has to be pointed out that the result of the separation and the reproducibility largely depend on how fast endosome-enriched fractions ready to be used for the FFE are prepared. Since the entire protocol, starting at marker injection into the rat in the morning and ending after FFE fractions are obtained, has to be carried without interruption, at least 13 to 14 hr must be allotted. When cell-free acidification assays are carried out for functional analysis, these assays have to be performed immediately after analysis of the FFE fractions for endosomal markers and for protein. This functional assay is similarly time-consuming. To dissipate the initial pH gradient and to subsequently acidify endosomes, ~1 hr is required. In most cases, dissipation of the initial gradient does not have to be determined in each fraction, and fractions can be pre-equilibrated consecutively while determining ATP-dependent acidification of the preceding samples. Nevertheless, ~5 hr are required to carry out these assays.

For preparative isolations, at least 15 hr must be allotted. In the case of analysis of endosomes by SDS-PAGE and immunoblotting, protein and endosomal markers have to be analyzed immediately after FFE separation in order to pool the fractions of interest and to concentrate the membranes by centrifugation (2 to 3 hr). Thereafter, the endosomal (membrane) pellets can be stored frozen for further analysis (e.g., SDS-PAGE and immunoblotting: 1 to 2 days).

Disclaimer

This article was written by Renate Fuchs and Isabella Ellinger in their private capacity and without any official support.

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CHAPTER 4

Microscopy

INTRODUCTION

Light microscopy historically has been an important technique of scientific discovery because it provides enlarged images of small objects. Recent advances in optical design and the development of probes has made light microscopy an essential tool for both research and diagnosis, enabling analysis of structure, physiology, and function. This chapter deals with methods for using the microscope, including the principles underlying different applications of light microscopy—e.g., phase-contrast, differential interference contrast (DIC), and fluorescence microscopy—as well as how to align and adjust the microscope for these different applications, how to prepare and label specimens, and how to image them accurately.

Although the structural detail in images resolved by the microscope is continually being improved by video and digital cameras and electronic imaging, the quality and accuracy of the image is primarily dependent on proper microscope alignment and adjustment. *UNIT 4.1* details methods for aligning a typical research light microscope for bright-field, phase-contrast, DIC, and epifluorescence microscopy. The unit emphasizes how proper alignment and adjustment are essential for optimal image quality and accurate quantitative measurements. The principles underlying Köhler illumination are explained, providing the background for discussions of image formation and microscope alignment for bright-field, phase-contrast, and DIC imaging. *UNIT 4.1* also describes methods for camera attachment, calibration of image formation, care and cleaning of the microscope, and assessment of microscope performance.

UNIT 4.2 describes fluorescence microscopy and its use as a tool for imaging biological specimens. Fluorescence microscopy has long been used to characterize cellular organization and is based on the property of fluorescent molecules whereby they absorb light of a particular wavelength and emit light at longer wavelengths. This unit includes methods for aligning the arc lamp and provides information on how to choose wavelength selection devices and objectives (also see *APPENDIX 1E*). *UNIT 4.2* also explains how to image fluorescent specimens and optimize image brightness and resolution. The availability in recent years of economical camera and software systems for capturing, storing, and analyzing digital images obtained from fluorescence microscopes has led to the replacement of photography by digital analysis as the preferred method for documenting images. This is described in the section of *UNIT 4.2* entitled “The Digital Darkroom,” in which the advantages of digital images over conventional photography are described. These include convenience of image storage, retrieval, and manipulation. In addition, digital imaging allows quantitative analysis of fluorescent data and greatly facilitates the analysis of large numbers of images.

UNIT 4.3 provides methods for what is perhaps the most widely used application of fluorescence microscopy, immunofluorescent staining. In this technique fluorophore-conjugated antibodies are used as sensitive and specific probes for staining antigens in fixed and/or permeabilized cells, and the location of specific proteins can be determined. When two or more antibodies labeled with different fluorophores are used to stain cells, multiple protein distributions can be visualized simultaneously. This unit provides the

basic methodology for fixation and optimal staining of cells and provides a variety of tips for troubleshooting potential problems.

The advent of fluorescent dyes that selectively label intracellular organelles provides an additional application of light microscopy whereby the spatial distribution of specific cellular components may be examined *in vivo*. *UNIT 4.4* describes protocols for fluorescently labeling three subcellular organelles—endoplasmic reticulum (ER), Golgi complex, and mitochondria—using selective fluorescent probes, including DiOC₆(3), C₆NBD-ceramide, and BODIPY-ceramide. These lipid-based dyes have been shown to incorporate into the membranes of the ER, Golgi complex, and mitochondria in living cells, so they can be used to follow the dynamics of these organelles in real time. This unit includes an extensive troubleshooting guide for staining, as well as detailed descriptions of anticipated results.

Another powerful system for imaging specimens is the confocal microscope, which produces sharp images of structures by selectively collecting light from a thin focal plane within the specimen. *UNIT 4.5* provides important background information for the use of such microscopes in cell biological applications. It details how confocal optical sectioning is accomplished and how the technique can be used to reconstruct a three-dimensional view of a specimen. The unit also describes the different types of confocal microscope systems (including laser-scanning, rapid-scan, and two-photon) and their advantages and disadvantages, and provides practical advice for using these instruments.

UNIT 4.6 describes immunoperoxidase methods for localizing intracellular antigens. With these methods the distribution of proteins can be characterized at both the light and electron microscope levels, providing insight into cellular organization at different size scales. Included in the description are tips for assuring that antibodies have access to the intracellular environment where antigen is localized, preserving antigenicity, and maintaining good cellular morphology during fixation and staining. Also described are the advantages and disadvantages of immunoperoxidase staining and practical tips for ensuring its success.

Cryo-immunogold electron microscopy (*UNIT 4.7*) has become the method of choice for high-resolution detection of antigens at the subcellular level. This technique involves preparation of ultrathin cryosections from cells or tissues followed by immunogold labeling to localize antigens. Because harsh organic solvents such as those used for plastic embedding are not required, preservation of protein antigenicity and ultrastructural details is better than with conventional electron microscopic embedding. In cryo-immunogold labeling, gold particles of different sizes conjugated to *Staphylococcus* protein A can be used to label different populations of antibodies that have been bound to antigens. This allows several antigens to be localized simultaneously, which is not possible with other immuno-electron microscopy labeling techniques. *UNIT 4.7* describes how to perform cryo-immunogold electron microscopy labeling of samples, including fixation, embedding, cryosectioning, and labeling. The unit also provides useful discussions on methods to improve the structural integrity of cryosections, the common problems and artifacts that arise during section preparation and the choice of antibodies and gold particle sizes for optimal immunolabeling results.

UNIT 4.8 describes a method known as correlative video light electron microscopy (CVLEM) that combines fluorescent microscopy and electron microscopy for analyzing dynamic structures with complex morphology within cells. In this technique, cells expressing a fluorescent reporter protein such as a GFP protein chimera are first monitored to identify and record a structure of interest. The cells are fixed and the same structure of interest is then analyzed by electron microscopy using three-dimensional reconstruction

methods. This technique requires considerable skill at the various steps involved in specimen preparation, but it can yield tremendous insight into the high-resolution organization and morphology of complex structures within the cell, such as the Golgi complex, membrane transport intermediates, and cytoskeletal elements.

UNIT 4.9 describes polarization microscopy, a technique that allows the organization and dynamics of cells and organelles to be studied based on their anisotropic properties, such as birefringence and dichroism. These properties derive from differences in the fine structural detail of an object that makes it reflect light in a polarized manner (i.e., differently in different directions). The unit starts out with a brief explanation of the phenomenon of polarized light and birefringence. It then describes how to optimally use a polarizing microscope, which is an ordinary microscope equipped with a polarizer underneath a condenser, an analyzer above the objective, and a compensator in between. Several examples of applications of the polarizing microscope are discussed, including study of the assembly and disassembly dynamics of spindle microtubules and the packing arrangement of DNA in sperm chromosomes.

UNIT 4.10 characterizes a fluorescent imaging technique for visualizing the dynamics, movement, and turnover of intracellular protein assemblies like actin and tubulin—fluorescent speckle microscopy (FSM). The technique is based on the incorporation of low concentrations of microinjected fluorescent subunits into a macromolecular structure through co-assembly with endogenous unlabeled subunits. This results in the structure of interest acquiring a fluorescent speckled pattern that can be observed in resolution-limited fluorescent images. Changes in the fluorescent speckle pattern can then be used to quantitatively analyze the assembly, disassembly, and movement of the structure. The virtue of this technique compared to conventional fluorescent imaging is that it significantly reduces out-of-focus fluorescence so the visibility of the speckles of a fluorescently labeled structure is greatly improved. Moreover, FSM can provide information about protein dynamics throughout the cell in contrast to methods like photoactivation and photobleaching, which follow a small, marked region of a structure. Use of FSM to study microtubules and actin cytoskeleton dynamics in living cells is presented as an example of the usefulness of this technique, so methods for fluorescently labeling tubulin and actin and time-lapse imaging are also detailed.

UNIT 4.11 describes the basic principles and uses of two-photon excitation microscopy. This technique allows three-dimensional imaging of a specimen without absorption above and below the plane of focus. The result is that thick samples, including brain slices, whole organs, and embryos, can be readily imaged since there is no out-of-focus light. This unit discusses the advantages and limitations of its use relative to confocal and deconvolution microscopy as well as selected applications of two-photon microscopy to illustrate the unique features of this technique, particularly its depth penetration in a specimen, its avoidance of phototoxic effects from ultraviolet irradiation, and its use in fluorescence lifetime imaging.

UNIT 4.12 discusses total internal reflection microscopy (TIR-FM), also known as evanescent wave microscopy. The fundamental principle of TIR-FM is that light will be totally internally reflected back into a medium with higher refractive index when the incident angle reaches a critical angle. A standing field is then generated into the medium of lower refractive index on the side opposite the incident wave. This field, called the evanescent field, has a short characteristic distance for decay, so it can be used to limit the depth of penetration of excitation light into the specimen. This permits imaging of the specimen within 50 nm of the cell surface. Recent technical advances in fast imaging and image analysis, together with the availability of TIR fluorescence microscopes has popularized this technique. It is now used in a variety of live-cell applications, including the study of

cell-surface contact zones, exocytosis and endocytosis of vesicles, and submembrane actin dynamics. This unit describes the theory behind TIR-FM; it provides tips for optimizing this method; and it describes the necessary steps for setting up a TIR-FM system. Finally, it describes how TIR-FM can be used for addressing simple cell biological questions.

UNIT 4.13 describes a range of labeling techniques for fluorescent labeling of yeast cells, which because they are quite small and difficult to visualize by fluorescence microscopy. Among the techniques described in this unit are fluorescent dye labeling of membranes and visualization of expressed GFP fusion proteins in both fixed and living yeast cells. With these approaches combined with the use of newer confocal imaging instruments, yeast organelle structure and protein localization can be characterized. Specific applications that are described include labeling of yeast vacuoles, filamentous actin patterns, and yeast budding morphologies.

Chapter 4 also contains the Organelle Atlas, which is a collection of figures that shows organelles of the cell visualized using a variety of reagents and techniques.

Jennifer Lippincott-Schwartz

Proper Alignment and Adjustment of the Light Microscope

Optical microscopes can be powerful tools in biomedical research and diagnosis if properly aligned and adjusted. This is essential for optimal image quality and accurate quantitative measurements. Video cameras, digital cameras, and electronic image processing can improve visibility of structural detail resolved by the microscope optics in comparison to viewing by eye. However, the quality and accuracy of the image still depends critically on proper microscope alignment and adjustment.

This unit presents protocols for alignment and adjustment of a typical research compound light microscope for transillumination and epi-illumination imaging modes typically used today in biomedical research. The transillumination light modes include bright-field, phase-contrast, and differential interference contrast (DIC). The primary epi-illumination mode is fluorescence microscopy.

The described procedures are for alignment of a research upright microscope (Fig. 4.1.1). The procedures are applicable to inverted microscopes that have similar imaging and illumination light paths to the upright microscope. In either case, the specimen image is produced by an objective lens and the image is projected either to the eye with an eyepiece or to a camera with (and sometimes without) a projection lens. One lamp attached to the back of the microscope provides light for transillumination of the specimen through a condenser lens (Fig. 4.1.2 and Fig. 4.1.3). Another lamp attached to the back of the microscope provides light for epi-illumination of the specimen through the objective using a mirror in a filter cube to bring the illuminating light into the objective light path (Fig. 4.1.4). For both the transillumination and epi-illumination paths, there are field diaphragms for controlling the specimen region illuminated and condenser diaphragms for controlling illumination from the condenser. The lamps, the field diaphragm, and the condenser diaphragm, as well as the condenser and the objective, must be properly focused and centered for the best image formation. In addition, each different mode of image formation has special optical components that also require alignment and adjustment for optimal performance.

The first section in this unit (see discussion of Major Components of the Light Microscope) describes the location and basic functions of the most important features of the upright compound microscope. The second section (see discussion of Basic Imaging and Köhler Illumination Light Paths For Bright-Field and Fluorescence Microscopy) introduces the imaging and illumination light paths of the microscope and describes the principles of Köhler illumination, which is the alignment used typically for both transillumination and epi-illumination in the light microscope. Basic Protocol 1 lists steps in microscope alignment for transmitted-light Köhler illumination. Basic Protocol 2 lists steps in microscope alignment for epifluorescence Köhler illumination. Basic Protocols 3 and 4 provide brief descriptions of the principles of image formation and microscope alignment for phase-contrast and differential interference contrast (DIC) microscopy; these optical modes produce contrast of transparent specimens using transmitted-light illumination. Support Protocol 1 deals with mating cameras to the microscope and Support Protocol 2 deals with calibrating image magnification. Several procedures (see Support Protocols 3, 4, and 5) are given for testing the optical performance of the microscope; these also describe test specimens for microscope performance and their sources. Finally, Support Protocol 6 deals with the care and cleaning of microscope optics. The Commentary refers the reader to references that provide more comprehensive

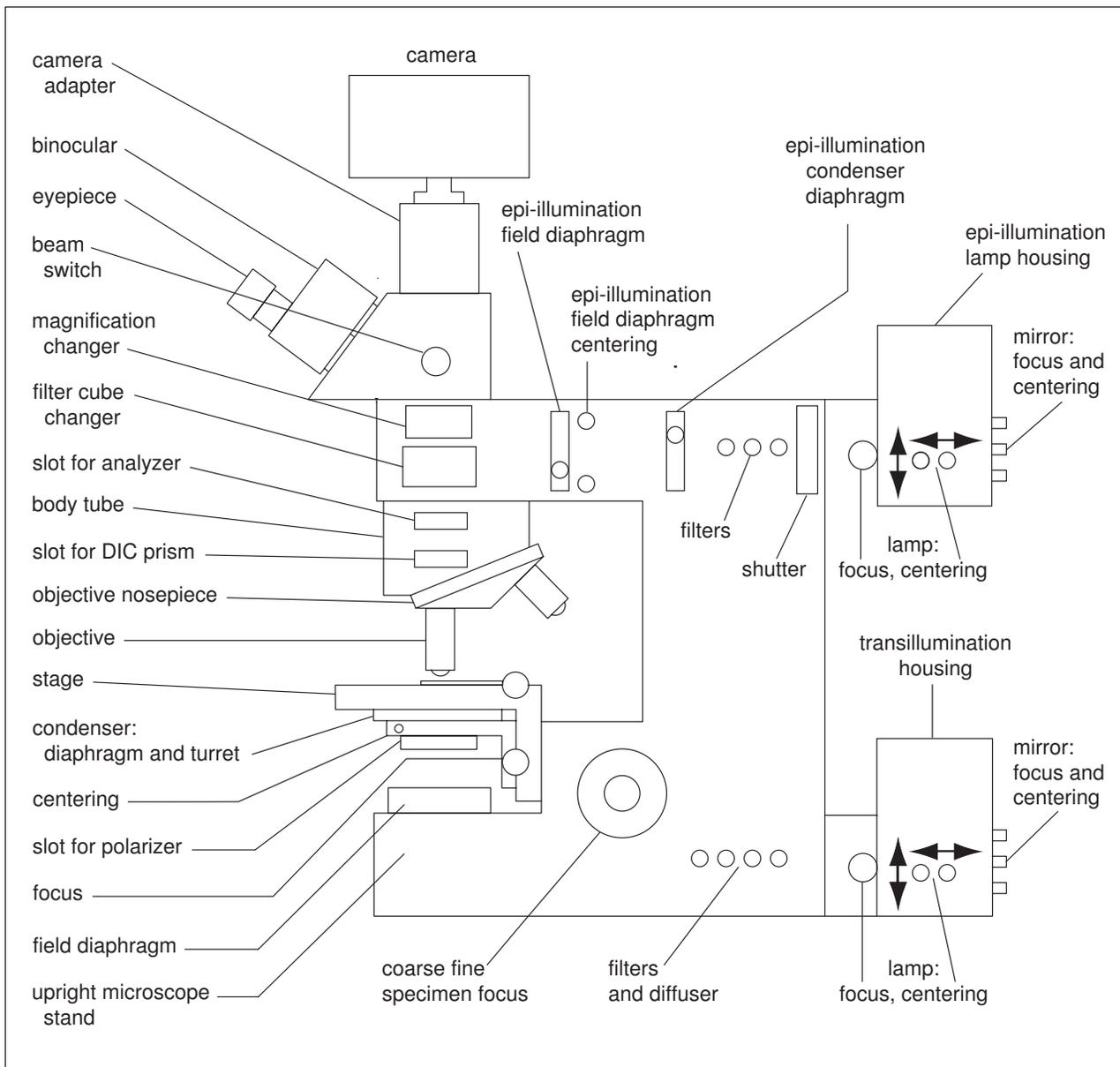


Figure 4.1.1 Diagram of the major component parts and centering screws for a research upright light microscope.

treatments of the basic modes and methods of light microscopy as well as advanced microscope imaging methods like multiwavelength, confocal, or multiphoton imaging, and electronic imaging techniques, including video and digital microscopy.

MAJOR COMPONENTS OF THE LIGHT MICROSCOPE

Familiarizing oneself with the components of the light microscope is best done using the manual for the microscope as reference. Figure 4.1.1 provides a diagram of an upright research compound light microscope equipped for both transmitted light and epifluorescence microscopy. Locate the following components on the microscope, identify their adjustment screws (if applicable) and movements (e.g., condenser focus), and ascertain that they appear correctly mounted. In this and the following sections, z is a direction along the microscope axis; x and y are perpendicular directions.

Image-Forming Components

Specimen stage and focus

A mechanical carrier on the stage holds the specimen slide. Knobs control movement in the x - y direction. Look for the vernier scales that mark the x and y positions. Rotatable stages are typically used for DIC microscopy. The objective is usually fixed and the specimen is focused by moving the stage along the z axis using coarse- and fine-focus knobs on the microscope body. Check the scale on the fine focus. On research microscopes, this is usually 1 μm /unit.

Objectives and revolving nosepiece

Examine the different objectives on the nosepiece. Each objective is usually labeled with the following designations: magnification (e.g., 60 \times); the degree of optical correction (Apochromat is better than Fluor which is better than an Achromat); the label Plan (if both the center and edges of the field are in focus); numerical aperture (NA, which measures the half-angle of the cone of light from the specimen accepted by the objective; Fig. 4.1.3); immersion medium to be used (e.g., air, oil, water, or glycerin between the objective front lens and the specimen); coverslip thickness; optical tube length (previously 160 mm, currently infinity); and other special features like phase contrast, DIC, or long working distance. Working distance refers to the distance from the front element of the objective to the specimen. Working distance usually decreases with increasing objective magnification and NA. Check that the objectives are mounted in series from the objective of the lowest magnification to the objective of the highest magnification. It is usually best to find the specimen or region of a specimen with a low-magnification objective, and then swing in objectives with higher magnifications and shorter working distances. The high-NA, short-working distance oil-immersion objectives often have the ability to lock up the nosepiece when changing objectives. This prevents running the front element of the objective into the mounting medium on the edges of the coverslip. Check that the nosepiece is down for imaging.

Specimen slides and coverslips

Microscope condenser lenses are usually corrected optically for 1-mm-thick glass slides. The objectives are corrected optically for coverslips with a thickness of 0.17 mm; no. 1.5 coverslips are on average this thick. The image quality decreases for thinner (e.g., no. 1) or thicker (e.g., no. 2) coverslips, particularly for non-oil immersion (“dry”) objectives with high NA. For oil-immersion objectives, this problem is not critical when the refractive index of the oil and coverslip (~1.515 to 1.52) match. Coverslips thinner than no. 1.5 are often used with oil immersion, to allow greater range of focus.

Body tube

In the modern research light microscope, the imaging light leaving the objective and nosepiece is unfocused and the image is projected to “infinity” (Fig. 4.1.2, left). This infinity space above the objective allows insertion of filters without changing the focal position of the image at the intermediate image plane. A positive focusing lens, the tube lens, above the infinity space, is used to bring the imaging light into focus for the eyepieces or camera.

Check for the following possible inserts in the infinity space of the microscope.

1. DIC prisms for the DIC objectives, which are usually right above the objective.
2. Analyzer; used for DIC microscopy (which should be removed from the light path for bright field, phase-contrast, and fluorescence microscopy).

3. Epi-illuminator filter-cube changer. These devices usually hold 2 to 4 filter cubes for fluorescence microscopy, each of which contains an excitation filter, an emission filter, and a dichroic mirror (Fig. 4.1.4) designed specifically for different fluorophores. Check the numbers on the filter cubes and mark the corresponding positions on the outside of the filter changer to identify the proper cube position for a given fluorophore.
4. Magnification changer and Bertrand lens. Body tube magnifications are selected from a turret, and possible values are 1.0×, 1.25×, 1.5×, and 2.0×. Often one position of the turret contains a Bertrand lens. This lens is used in combination with the eyepieces to produce a telescope view of the objective back focal plane (Fig. 4.1.2, far right). This is an important device for checking that the lamp image is centered and in focus during alignment for Köhler illumination, and for adjustment of the condenser diaphragm (see discussion of Transillumination Components).

Beam switch

This device switches the light between the binocular and the camera port. Check for the percentage of light in each direction. For fluorescence microscopy, it is important to be able to send 100% of the imaging light either to the eye or to the camera.

Eyepieces

Eyepiece magnification to the eye is marked on the barrel (e.g., 10×). Note that there are two and maybe three adjustments. The interpupillary distance for the eyes is adjusted by grasping the bases of the eyepiece tubes and moving them closer or further apart. At least one of the eyepieces is adjustable so that the eyes are parfocal.

Camera adapters

Check the type of camera adapter and whether it matches the detector. There are several different types of camera adapters. One type uses an eyepiece tube (not the binocular tube) and a projection eyepiece combined with a camera lens to project an image onto the camera detector. This method is common in photographic film cameras (which use ≥ 35 -mm film) and for some video cameras with large-size detectors (e.g., 1-in., equivalent to 2.54-cm). The recent video and cooled slow and progressive scan charge-coupled device (CCD) cameras have small detectors ($\leq \frac{2}{3}$ in., equivalent to 1.7 cm). These cameras require smaller projection magnifications or none—in the latter case the detector is mounted at the intermediate image plane, the focal plane of the objective (Fig. 4.1.2), and no eyepiece or projection lens is used.

Transillumination Components

Lamp and housing

The lamp is typically a low-voltage 100-W quartz halogen bulb with a tungsten-filament light source and variable control. Some lamp housings have no adjustments for centering the bulb; the socket is prefixed. Most lamp housings have x - y adjustments for the bulb socket, while an advanced lamp housing also has a mirror in the back for reflecting an image of the lamp back along the microscope axis. This mirror usually has adjustments for x , y , and z positions of the mirror image of the lamp. There is usually a knob on the side of the lamp housing for moving the collector lens back and forth along the z axis to focus the tungsten-filament image onto the condenser diaphragm plane.

Diffuser and filters

A diffuser and other filters are often inserted in slots in the base of the microscope. The diffuser helps spread the image of the source at the condenser diaphragm plane in order to uniformly fill the condenser aperture. This is important for achieving high resolution.

A heat-reflecting filter (e.g., BG 58) blocks infrared light. Video cameras, but not the eye, are often sensitive to this wavelength of light. A green filter is frequently preferred for illuminating living cells. High-efficiency interference filters with a 40-nm bandwidth around 540 nm are often best. Various neutral-density (ND) filters, which are not wavelength selective, are useful for attenuating light to cameras.

Field diaphragm

This is usually located just above or beneath the mirror that deflects the light up to the condenser lens. It controls the specimen area illuminated by the condenser (Fig. 4.1.2, left).

Polarizer

This is inserted below the condenser diaphragm for polarization and DIC microscopy. Otherwise, it is removed from the light path.

Condenser focus knob

This translator moves the condenser along the microscope z axis to focus an image of the field diaphragm on the specimen.

Condenser centering screws

There are usually two screws on the condenser carrier which move the condenser in an x - y plane. These screws are used to center the image of the field diaphragm on the z axis.

Condenser diaphragm

The condenser diaphragm is located on the bottom of the condenser. It controls the angle (NA) of the condenser cone of illumination of the specimen (Fig. 4.1.3).

Condenser turret

Condensers have turrets with inserts for special image-contrast techniques such as phase-contrast and DIC microscopy. Each insert matches certain objectives. In phase-contrast microscopy, the inserts are annuli of different diameters designed to match the phase ring in phase-contrast objectives (Fig. 4.1.5). For DIC, the inserts are typically DIC prisms designed for certain objectives (Fig. 4.1.7). To see these inserts, as well as the opening and closing of the condenser diaphragm, remove the condenser from the condenser carrier by loosening the locking screen and turning it upside down.

Condenser lens

The condenser's main function is to provide bright, uniform illumination of both the specimen field (Fig. 4.1.2, right) and objective aperture (Fig. 4.1.3) for objectives with different NAs. Check the markings on the condenser to see if it is designed for air (dry) or oil immersion with the glass slide. Dry (no oil-immersion) condensers have NA values of ≤ 0.9 and should not be used with immersion oil. Oil-immersion condensers usually have NA values of 1.0 to 1.4 when using immersion oil.

Epi-illumination Components

Lamp and housing

The light source for the epifluorescence illumination is typically an HBO 50-W or HBO 100-W mercury arc lamp or a xenon lamp of similar wattage. These lamps need to be handled with care because they can blow up if mistreated. Their glass envelopes should be carefully cleaned with 70% ethanol before installation to prevent fingerprints or other materials from inducing hot points that may result in fracturing of the bulbs. Look for the adjustment screws to adjust the x - y position of the lamp. Look also for the rear mirror

and its x , y , and z adjustment screws. Identify the focusing knob for the lamp collector lens. Do not turn the lamp on until the bulb has been installed according to the manufacturer's instructions, the collector lens has been inserted properly, and the lamp housing has been installed on the back of the microscope. These lamps produce intense light, and the mercury bulbs have large peaks in the UV range. Therefore, UV-protective glasses should always be worn when handling and installing these lamps. The light intensity decreases and the probability of explosion increases with the number of hours of operation and lamp starts. Check the timer on the lamp power supply to see that the recommended limits are not exceeded.

Shutter

Photobleaching is a big problem in fluorescence microscopy. A shutter is used to block the light from the specimen when not taking exposures on a camera or viewing by eye.

Filters

A heat-reflecting filter is used to prevent infrared illumination of the specimen and to keep infrared scattered light from reaching the camera. Neutral-density (ND) filters are used to reduce the light intensity of fluorescence illumination by the amount indicated on each filter.

Epi-illumination condenser diaphragm

This diaphragm provides variable adjustment of the illumination intensity. Some microscopes lack this diaphragm.

Epi-illumination field diaphragm

This diaphragm controls the area of the specimen illuminated. Look for the centering screws that control the x - y position of the field diaphragm.

Filter cubes

See discussion of Image-Forming Components.

BASIC IMAGING AND KÖHLER ILLUMINATION LIGHT PATHS FOR BRIGHT-FIELD AND FLUORESCENCE MICROSCOPY

The second major step in learning proper alignment of the light microscope is to understand the basic image-forming and Köhler illumination light paths and the functions of the key optical components and diaphragms. These are outlined in Figure 4.1.2, Figure 4.1.3, and Figure 4.1.4.

Imaging-Ray Paths

The upper left section of Figure 4.1.2 shows ray paths for the image-forming light from the objective. The objective, in combination with the tube lens, produces a real, magnified image of the specimen at the intermediate image plane. The eyepiece provides a second stage of magnification to the eye. The magnification to the eye is the product of objective magnification and eyepiece magnification multiplied by any magnification provided by additional lenses in the body tube of the microscope between the objective and the eyepieces.

When a camera detector—film, video, or charge coupled device (CCD)—is mounted at the intermediate image plane, the magnification to the camera is the magnification produced by the objective and body tube. When a camera is mounted above an eyepiece, then the unfocused light leaving the eyepiece must be focused onto the camera detector by a camera lens, which modifies the total magnification to the detector. Special adapters (see Image-Forming Components, Camera adapters) are also available for projecting the

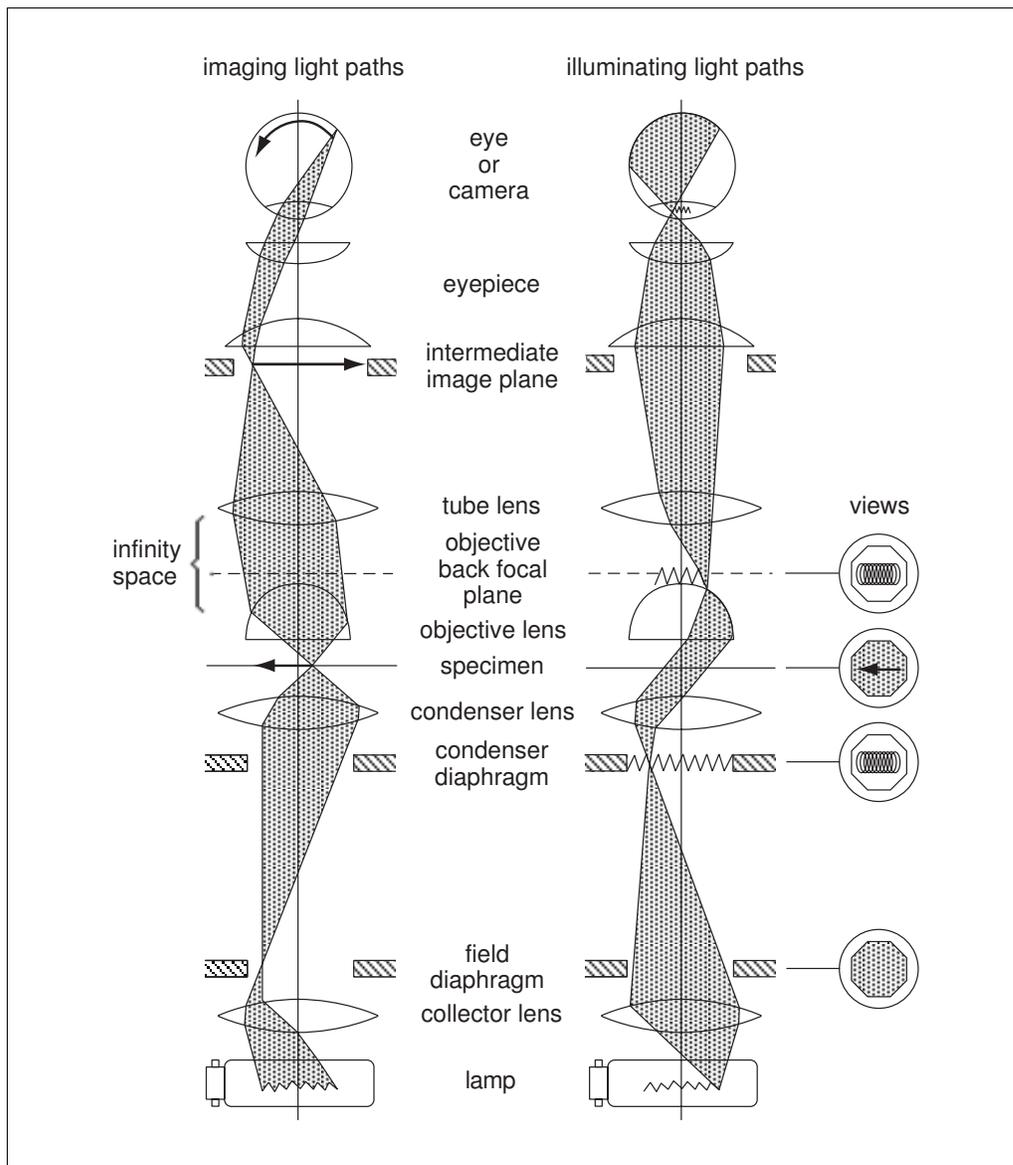


Figure 4.1.2 The imaging and illuminating light paths for a bright-field microscope aligned for transmitted light Köhler illumination. Modified from Keller (1998).

objective image onto a camera detector with magnification, but without the need for an eyepiece.

Transillumination Ray Paths

In the illustration of the standard Köhler method (Fig. 4.1.2), trace the illumination ray paths for a bright-field microscope aligned for Köhler transillumination (right side). A point on the light source is focused by the lamp collector lens onto the front focal plane of the condenser lens, where the condenser diaphragm is located. This light is projected, out of focus, through the specimen by the condenser lens, producing uniform illumination of the specimen. The objective collects the unfocused illumination light and brings an image of the light source into focus at its back focal plane, which is at the back aperture of the objective (Fig. 4.1.3). The light source is again out of focus at the intermediate image plane and at the retina of the eye or the detector of the camera. In between these two points, the light source is in focus at the exit pupil of the eyepiece, at a position ~15

mm above the eyepiece. This position is also called the eyepoint, the position of eye placement above the eyepiece (Fig. 4.1.2, right).

The condenser diaphragm controls the NA (cone angle) of specimen illumination by the condenser lens (Fig. 4.1.3). Opening the diaphragm increases the aperture of illumination, which increases both the light intensity and resolution in bright-field light microscopy (see Support Protocol 3). Note on the right side of Figure 4.1.2 that the image of the condenser diaphragm is in focus where the lamp image is in focus along the microscope axis, at the objective back focal plane and the exit pupil of the eyepiece.

Trace the imaging light rays from the field diaphragm through the microscope (Fig 4.1.2, left). Note that the field diaphragm controls the specimen area illuminated by the condenser. Note also that the field diaphragm is placed between the lamp collector lens and the condenser lens in a plane where the lamp image is out of focus (compare Fig. 4.1.2, left and right). When the condenser lens has focused the image of the field diaphragm onto the specimen, then the field diaphragm will be in focus with the specimen at the intermediate image plane and at the eye or detector.

Epi-illumination Ray Paths

Figure 4.1.4 shows the optical alignment for an epi-illuminator and Köhler illumination through the objective. As in transillumination (Fig. 4.1.2), the light source is focused by a collector lens onto the condenser diaphragm of a condenser lens in the light path. The condenser lens illuminates a field diaphragm. Another lens collects this light and projects it off a reflective mirror into the objective. The image of the light source is focused at the back focal plane of the objective so that it is out of focus at the specimen plane. In epifluorescence microscopy, filter cubes containing dichroic mirrors are used in combination with excitation and emission filters to efficiently reflect the excitation light into the objective and transmit to the eyepiece or camera only the longer-wavelength fluorescent light emitted from the specimen (see *UNIT 4.2* and Taylor and Salmon, 1989).

BASIC PROTOCOL 1

ALIGNMENT FOR KÖHLER ILLUMINATION IN BRIGHT-FIELD, TRANSMITTED LIGHT MICROSCOPY

The following procedures assume that the positions of the quartz-halogen tungsten filament and collector lens are adjustable. Inexpensive, non-research compound microscopes may have the illuminator built into the stand, with no adjustment for the lamp. Such microscopes depend on a ground-glass filter for even illumination. For these microscope stands, skip steps 2, 3, 4, and 8.

Focus the light source

1. If possible, remove the diffusion filter in the transillumination pathway during alignment so that a crisp image of the light source can be viewed at the condenser diaphragm plane and at the objective back focal plane. When alignment is complete, reinsert the diffusion filter.
2. Center and focus the lamp filament near the condenser diaphragm plane. Remove the condenser lens. Place a sheet of lens paper on the microscope stage. Close down the field diaphragm and adjust the intensity of the lamp so that the lens paper is moderately illuminated. Use the lamp-focusing knob to move the condenser lens (or lamp) along the z axis until an image of the filament is in focus on the lens paper. Roughly center the lamp on the microscope axis with the adjustment screws on the lamp housing, then adjust the mirror image (if there is a mirror) using its adjustment screws on the lamp housing.

Table 4.1.1 Suppliers for Items Used In Light Microscopy

Test specimen	Supplier
Hematoxylin/eosin-stained skeletal muscle	Carolina Biological Supply
Diatom test plate	Carolina Biological Supply
Squamous cheek cells	Freshly prepared (see Support Protocol 4)
Stage micrometer	Fisher
Red, green, and blue fluorescent tissue culture cells	Molecular Probes

3. Place the lens paper on the condenser carrier and raise the condenser carrier until it is ~20 mm from the top of the stage.

This is the approximate position of the condenser diaphragm when a condenser is installed and in focus.

4. Refocus the lamp and mirror images at this position of the lens paper.

If there is no mirror image, center the lamp image. If there is a mirror image, then position the images so that they sit side by side to fill the condenser aperture. Remove the lens paper and replace the condenser.

Focus for low-power viewing

5. Obtain an in-focus image of a specimen with the low-power objective (10× or 16×) by placing a test specimen that absorbs light on the stage (e.g., stained muscle section; see Table 4.1.1).

The focus position can be estimated from the working distance of the objective lens. For a standard 10× lens, this is ~4 mm. So, use the coarse focus to bring the specimen to ~16 mm from the coverslip by viewing the objective position from the side of the microscope.

6. Move the condenser lens up close to the lower surface of the slide and open the field and condenser diaphragms all the way. Look down the eyepieces, and use the coarse- and fine-focus knobs to bring the specimen into sharp focus.
7. Focus the image of the field diaphragm centered on the specimen by adjusting the condenser *x*-*y* screws and the condenser focus. Initially close down the field diaphragm until an edge of the image can be focused by the condenser, then close the field diaphragm further as the image is centered, using the *x*-*y* translation screws.

Usually, the field diaphragm is opened just enough to match the field of view in the eyepiece or the camera. However, the best image contrast is obtained if the field diaphragm is opened just enough to illuminate the region of interest. This eliminates the presence of scattered light from outside the region of interest.

8. Center an in-focus image of the lamp and mirror images at the objective back focal plane using the focus and adjustment screws on the lamp housing. View the objective back aperture (the position of the back focal plane; Fig. 4.1.3) by using the Bertrand lens in the magnification changer in combination with the eyepieces, by replacing one eyepiece with a telescope, or by simply removing one eyepiece and peering down the body tube. Open the condenser diaphragm all the way. Readjust the collector lens and the mirror so that the lamp images are in focus and sit side by side to fill the objective aperture.

This is a rough adjustment with the low-power objective and will need to be fine-tuned using the objective with the highest NA.

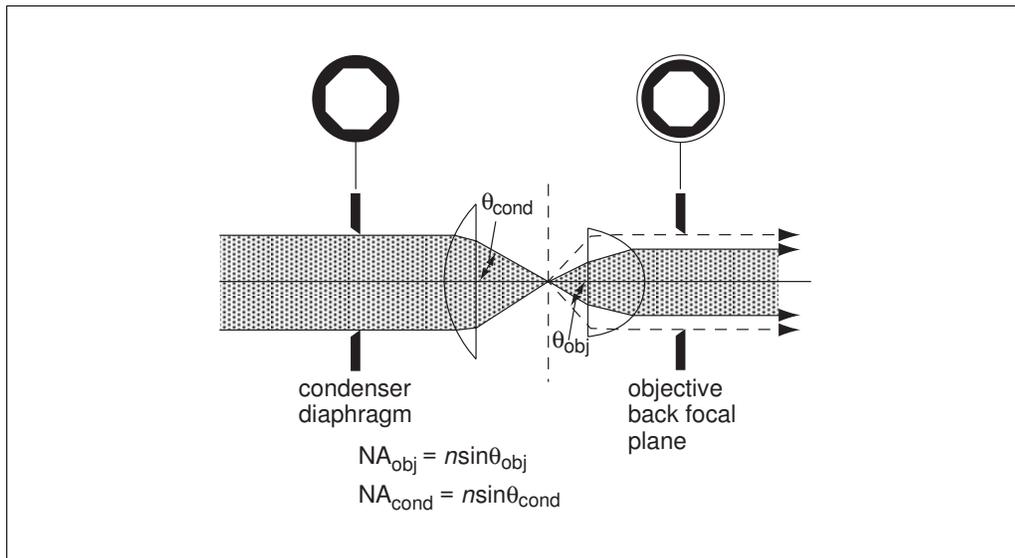


Figure 4.1.3 Numerical aperture (NA) of objective light collection and condenser illumination. The objective numerical aperture (NA_{obj}) depends on the angle of the cone of light from the specimen, which is accepted by the objective aperture while the numerical aperture of condenser illumination (NA_{cond}) is controlled by the condenser diaphragm and limited by the maximum NA of the condenser when the condenser diaphragm is wide open.

9. Adjust the opening of the condenser diaphragm so that the diameter of its image at the objective back focal plane (see far right of Fig. 4.1.2; also see Fig. 4.1.3) is slightly less than the diameter of the objective back aperture.

Some research condensers also have centering adjustments for the condenser diaphragms. The image of the condenser diaphragm should be centered in the objective back aperture.

10. Adjust the light intensity for comfortable viewing of the specimen by adjustment of the light-source intensity, either using the power-supply rheostat or by inserting neutral-density filters in the illumination light path.

The microscope is now adjusted properly for the low-power objective.

Adjust for 40× viewing

11. Switch the turret to the 40× high-dry objective.

Note that because of parfocal objective design the specimen is almost in focus. Focus on the specimen with the fine focus.

12. Focus and center the field diaphragm with the condenser focus and the condenser-carrier *x*–*y* adjustment screws.

13. View the objective back aperture (Fig. 4.1.3).

Notice that the image of the condenser diaphragm adjusted for the low-power objective is only about half the diameter of the 40× back aperture. The ratio of the diameter of this image to the diameter of the objective back aperture is equal to the ratio of the NA of the condenser illumination to the NA of the objective (Fig. 4.1.3). When the condenser illumination NA equals the objective NA, the aperture of the objective is filled with light and maximum resolution will be achieved for the objective NA (see Commentary).

14. Adjust the condenser diaphragm so that the condenser illumination NA just about matches the objective NA.

Each time objectives are changed, these procedures should be repeated.

Adjust for high-power viewing

15. Rotate the nosepiece so that the high power (60× to 100×, NA 1.25 to 1.4) oil-immersion lens is coming into place, but stop before it clicks into place. Place a small drop (~2 mm diameter) of immersion oil directly onto the coverslip above the point upon which the condenser beam is focused.

This oil drop must be free of air bubbles and dirt. The refractive index of the immersion oil should be close to that of the glass coverslip.

16. Complete the rotation of the nosepiece so that the oil-immersion objective clicks into place. Lower the objective nosepiece if appropriate.

The space between its front lens and the coverslip is now filled with immersion oil.

IMPORTANT NOTE: *Use only the fine adjustment knob when working with the oil-immersion lenses.*

17. Remove the eyepiece (or use the telescope or Bertrand lens) and inspect the back aperture of the objective. Open the condenser diaphragm as much as possible to try and match its image with the objective aperture (Fig. 4.1.3).

This will be impossible if the condenser is dry and not designed for oil immersion, since dry condensers have $NA \leq 0.9$ and the relative size of the image of the condenser aperture or condenser diaphragm seen in the objective back focal plane is given by the ratio of NA_{cond}/NA_{obj} (Fig. 4.1.3).

18. View the objective back aperture and tune up the focus and position of the lamp images so that they fill the objective aperture with light as uniformly as possible.

19. Replace the eyepiece (or remove the Bertrand lens) and examine the specimen. Adjust the field diaphragm until its margins just match the field of view.

With inexpensive condensers, the image of the edge of the field diaphragm will not be in good focus, even at the optimum position. If a condenser designed for oil immersion is being used, enormous improvement in the field iris image can be achieved by oil immersion.

20. Oil the condenser (if possible) to achieve the highest resolution and image quality. To oil a condenser, rotate the objective out of the way, remove the slide, oil the condenser (it takes much more air-bubble-free oil than the objective), then replace the slide and refocus the objective.

Before oiling the condenser, make sure it is not designed to be a “dry” condenser by checking that it is marked with an $NA > 0.9$.

The image of the field diaphragm should now be much sharper when in focus and centered.

21. Look at the objective back aperture. Again, tune up the focus and position of the lamp images so that they fill the objective aperture with light as uniformly as possible.

After oiling, the aperture should be filled by the condenser illumination when the condenser diaphragm is wide open.

Notice also that viewing the objective back aperture is the best way to see if air bubbles have become trapped in the oil. If so, carefully wipe off the oil with lens paper and repeat steps 15 through 21.

22. Reinsert the diffusion glass in the illumination light path.

This will reduce light intensity, but make the illumination of the objective aperture more uniform.

23. When finished for the day, use lens paper to remove excess oil from the objective and condenser lens surfaces to prevent dripping on specimens.

It is not necessary, however, to completely clean oil from the optics after every use (see Support Protocol 6).

ALIGNMENT OF THE EYEPIECES

The binocular usually has adjustments for the inter-eyepiece distance and visual acuity in each eye. The goal is for the image to be in focus for each eye without any eyestrain or discomfort. Only the left eyepiece tube or eyepiece is adjustable on a typical microscope. However, if the microscope has a target reticle in the microscope stand that can be rotated into view, then usually both eyepieces are adjustable.

1. With a low-power objective (10× or 16×), focus on a stained specimen placed on the stage of the microscope (e.g., stained muscle section, see Table 4.1.1) and align the microscope for Köhler illumination.
2. Move the bases of the eyepiece tubes together or apart to set the proper interpupillary distance for your eyes.
- 3a. *If the microscope has a target reticle:* Rotate the reticle into the field of view. Close the left eye and use the diopter-adjustment ring on the right eyepiece until the target is in sharp focus for the right eye. Then close the right eye and use the diopter-adjustment ring on the left eyepiece until the target is in sharp focus for the left eye. Remove the target to view the specimen.
- 3b. *If the microscope has no target reticle:* Set the right eyepiece tube to the inter-eyepiece setting (if possible). Close the left eye and bring into sharp focus a structural detail near the center of the field of view with the right eye. Close the right eye and use the diopter-adjustment ring on the left eyepiece tube to bring the specimen detail into sharp focus for the left eye. With both eyes open, fine tune the focus of the left eyepiece tube.

ALIGNMENT FOR KÖHLER ILLUMINATION IN EPIFLUORESCENCE MICROSCOPY

The eye is most sensitive to green light. Hence, the following protocol is best done using a filter cube that produces green excitation light (e.g., a filter cube for rhodamine).

Focus the lamp

1. Remove one objective, and rotate the nosepiece so the open position is centered on the microscope axis. Place a white card on the microscope stage, on top of supports that position the card at ~2 to 3 cm from the nosepiece.

This is approximately the position of the objective back focal plane, where the images of the light source should be in focus and centered after alignment (Fig. 4.1.4).

Some microscopes have a special device for lamp alignment. It screws into the objective nosepiece and projects an image of the epi-illumination arc and electrodes onto a small diffusion screen within the barrel of the device.

2. Install the mercury bulb in the lamp housing and attach the lamp housing on the back of the microscope according to the manufacturer's instructions.
3. Turn on the power source and ignite the lamp.

It takes ~10 min for the arc to brighten.

CAUTION: It is usually a good idea to make sure computers in the vicinity are turned off before starting the lamp because the high voltage pulse used to ignite the arc plasma may damage the electronics. Laboratory personnel should protect their eyes against UV light from the lamp.

4. Close down the field diaphragm and open the condenser diaphragm (if there is one) all the way.

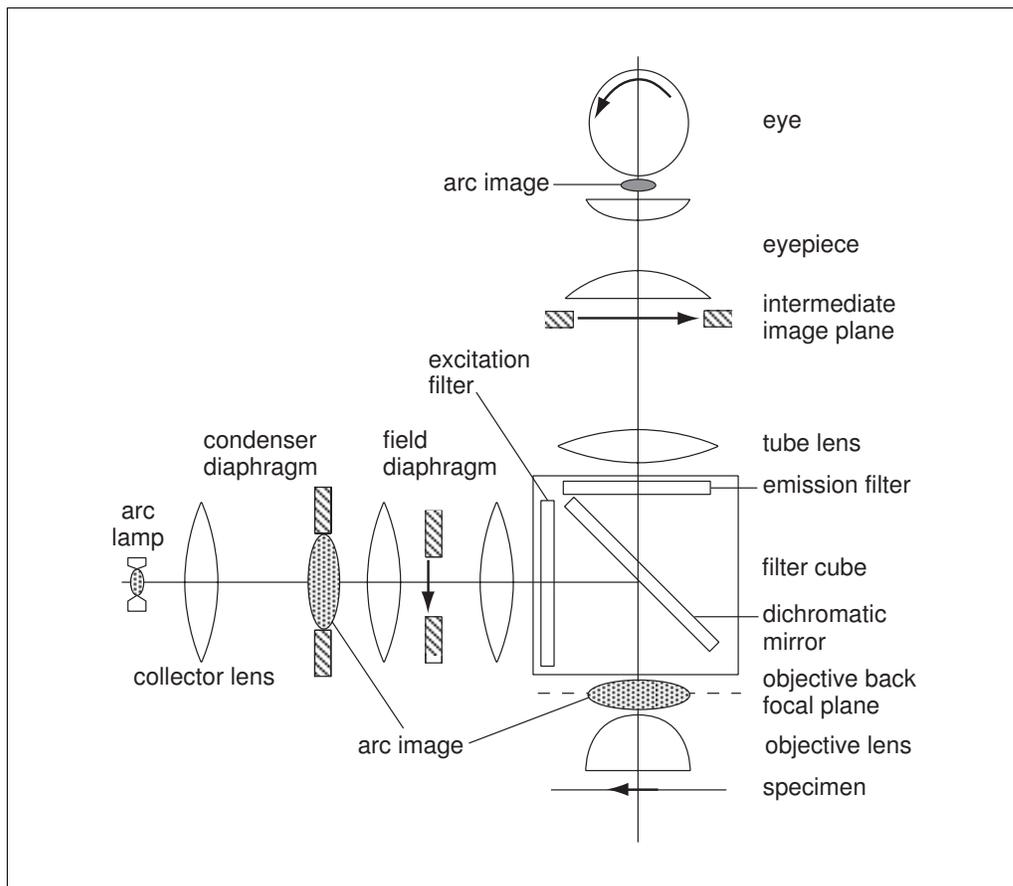


Figure 4.1.4 Microscope alignment for epifluorescence Köhler illumination.

5. On the diffusion glass screen or on the white card on the stage, the image of the arc between the tips of the two electrodes should be visible. Use the lamp focusing knob to obtain an in-focus image of the lamp arc and electrodes. Use the lamp *x-y* adjustment screws to approximately center the image of the arc and electrode tips (see Figure 4.1.2).
6. If there is a lamp mirror, use its adjustment screws to focus the mirror image of the arc and electrode tips, centered on the target.

Often the primary image and the mirror image of the arc are adjusted side-by-side and slightly overlapping, but centered on the microscope axis.

Focus the field diaphragm

7. Place a test fluorescent specimen (see Table 4.1.1) on the stage and rotate the objective nosepiece to a low-power objective. Select the appropriate filter cube for the fluorescent specimen. Open up the epi-illuminator field diaphragm all the way. Open the shutter and view the specimen.
8. Focus the specimen, then close down the field diaphragm until an edge comes into the field of view. Use the *x-y* adjustment screws for the field diaphragm to center the field diaphragm image within the field of view.
9. Close down the field diaphragm until only the region of interest is illuminated.

This prevents photobleaching of areas outside the region of interest and reduces the amount of scattered fluorescent light in the image from outside the region of interest.

10. Switch to a high-power objective. For an oil-immersion objective, place a small drop (~2 mm diameter) of immersion oil directly onto the coverslip above the region of interest. Carefully swing in the objective. Open the shutter and focus on the specimen. Readjust the size and centration of the field diaphragm.

This oil drop must be free of air bubbles and dirt.

11. To prevent photobleaching, be sure to close the epi-illumination shutter when not viewing or taking camera exposures.

ALIGNMENT FOR PHASE-CONTRAST MICROSCOPY

Phase-contrast microscopy is often used to produce contrast for transparent, non-light-absorbing, biological specimens. The technique was discovered by Zernike, in 1942, who received the Nobel prize for his achievement (Zernike, 1942, 1955, 1958). The last of these references describes the principles of phase contrast and provides an excellent introduction to the wave optics of image formation, resolution, and contrast in the microscope.

The phase-contrast microscope is a bright-field light microscope with the addition of special phase-contrast objectives (Fig. 4.1.5) containing a phase plate or ring and a condenser annulus instead of a diaphragm; the annulus is usually located on a condenser turret because it has to be selected for different objectives. The microscope optics are usually aligned for bright-field specimen illumination by the standard Köhler method. However, there is no condenser diaphragm to adjust. Instead, the phase annulus must be selected and adjusted properly. Modern phase-contrast objectives have a phase plate containing a ring in the back focal plane within the barrel of the objective. This ring

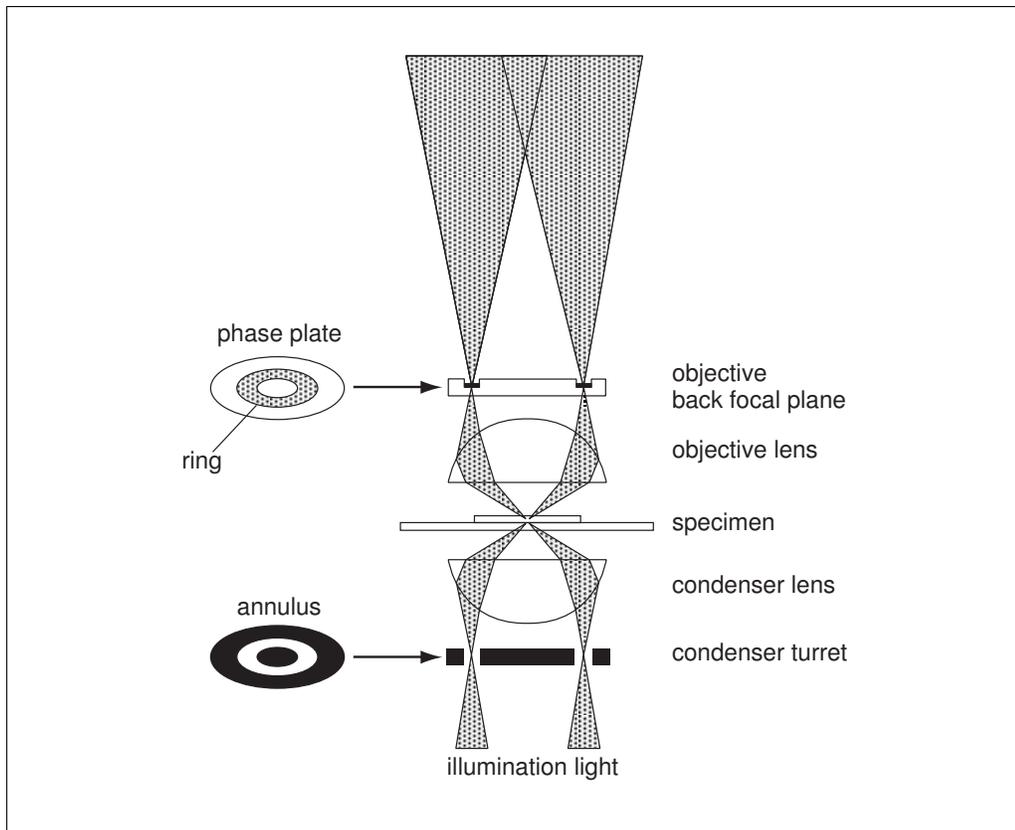


Figure 4.1.5 Illumination light path through the condenser annulus and objective phase ring in a microscope aligned for phase-contrast microscopy.

absorbs and advances the phase of the light passing through it by $\frac{1}{4}$ wavelength in comparison to light passing through the rest of the objective aperture. For each phase objective, there is a corresponding annulus in the condenser turret that has about the same relative size as the phase ring in the objective. Light passing through this annulus passes through the phase ring in the objective when the annulus is selected and aligned properly. Light scattered by the specimen mainly passes through the objective aperture outside the phase ring. Light scattered from a thin transparent specimen is $\sim\frac{1}{4}$ wavelength retarded from the unscattered illumination light. The additional $\frac{1}{4}$ wavelength retardation between the scattered and illumination light produced by the objective phase ring makes the scattered and illumination light 180° out of phase. They destructively interfere with each other at the image plane to produce the “dark” contrast of structural detail typical of phase contrast images of biological specimens.

Because the phase annulus and the phase ring reduce the intensity of the background light, a bright illuminator—e.g., a 100-W quartz-halogen illuminator—is necessary at high magnifications (because image intensity decreases as $1/\text{magnification}^2$). For living cells, heat reflection and green illumination filters should be used.

To examine the objective phase ring and matching condenser phase annulus, remove the low- and medium-power phase objectives and the phase condenser from the microscope. View the phase ring (phase plate) within the objective by looking in the back end. The phase ring is located at the position of the objective back focal plane; its diameter is usually $\sim\frac{2}{3}$ that of the objective aperture. Note that it is situated within the objective body and is visible because it absorbs light. Locate the annulus for each objective in the condenser turret. The annulus is located at the condenser diaphragm plane, which is situated at the condenser front focal plane. Notice that as the objective NA increases, the diameter of the corresponding annulus in the condenser turret increases. The NA of condenser illumination from an annulus is designed to match that of the phase ring in the corresponding objective.

Replace the condenser on the microscope and illuminate with white light. Hold a piece of tissue paper near the top of the condenser and examine the illumination cone. Notice that it is an annular cone of illumination. Change the annulus from the setting for the low-power objective to the $40\times$ setting. Note that the NA or angle of illumination increases. Points of illumination further from the central axis in the condenser diaphragm plane produce higher-aperture rays passing through the specimen.

Perform alignment using the following steps.

1. Align the microscope for bright-field Köhler illumination using the low-power phase objective ($10\times$ or $16\times$) and a cheek cell preparation for the specimen (see Support Protocol 4).

The cheek cell specimen is an excellent transparent test specimen for alignment of the microscope for phase contrast and DIC. The cheek cells are transparent and only barely visible by bright-field illumination. To find the plane of focus in bright field, initially close down the condenser diaphragm and look for the edges of air bubbles in the preparation. These edges scatter much light and appear dark in the image.

2. Align the condenser annulus with the phase ring by rotating the condenser turret to the position where an annulus matches the phase ring in the objective; use the telescope, your eye, or the Bertrand lens to view the objective back aperture.

As the condenser turret is rotated, notice in the objective back focal plane the images of the different-diameter annuli designed for the different numerical aperture objectives. The correct annulus may not be properly aligned with the objective phase ring.

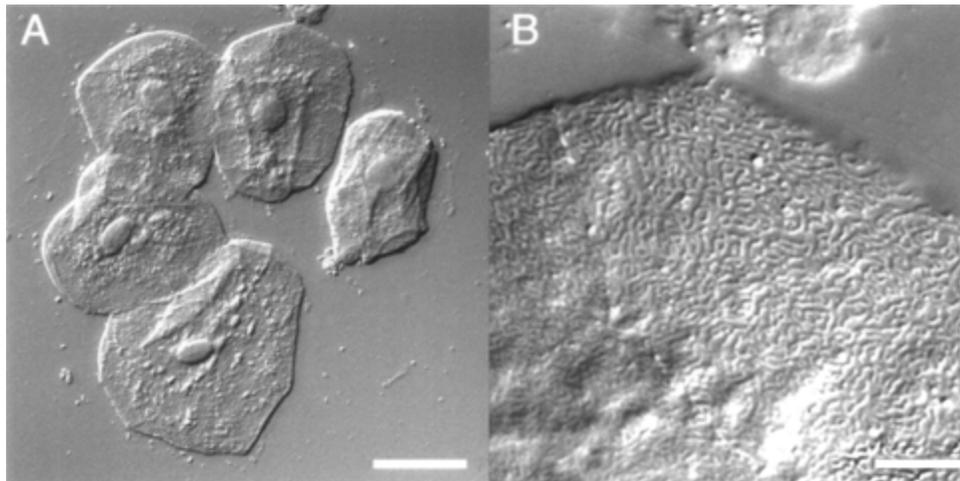


Figure 4.1.6 DIC images of a human cheek cell test specimen. **(A)** Low magnification of cheek cell preparation with a 20 \times objective. Bar = 20 μm . **(B)** High-resolution image of the surface of the cell at the top of (A) using a 60 \times /(NA = 1.4) Plan Apochromat objective and matching condenser illumination. The ridges on the cell surface are often diffraction limited in width. Bar = 5 μm . From Salmon and Tran (1998), reprinted with permission from Academic Press.

- Use the adjustment screws in the condenser (and the special tools if necessary) to move the annulus in the x - y plane to achieve alignment with the phase ring in the objective.

Note that the phase ring is slightly wider than the image of the correct annulus. It is critical that the image of the annulus be within the phase ring, but it is not critical if it is very slightly off center.

- When the objective phase ring and condenser annulus are aligned, view the specimen and properly adjust the focus and centration of the field diaphragm.

Notice that if the phase ring and annulus are slightly misaligned (rotate the turret slightly), the background light intensity goes up. This is because the phase ring is designed to absorb much of the illumination light. The unscattered illumination light becomes closer to the intensity of the light scattered by the specimen, which passes through the objective aperture outside of the phase ring. Minimizing the background light intensity while viewing the specimen image can also be used to align the annulus with its phase ring or to touch up the alignment done by viewing down the body tube.

- Switch to the 40 \times phase objective and then the high-power 60 \times or 100 \times phase objective (NA 1.25 to 1.4) and repeat steps 1 to 4.

It is necessary to increase light intensity at the higher magnifications. It should be possible to see the ridges on the cell surface (see Fig. 4.1.6 for a DIC image of the ridges). Notice the “phase halos” at discrete edges, a problem that limits conventional phase contrast in high-resolution imaging.

BASIC PROTOCOL 5

Proper Alignment and Adjustment of the Light Microscope

4.1.16

ALIGNMENT FOR DIC MICROSCOPY

Since its introduction in the late 1960s (e.g., Allen et al., 1969), DIC microscopy has been popular in biomedical research because it highlights edges of specimen structural detail, provides high-resolution optical sections of thick specimens—including tissue cells, eggs, and embryos—and does not suffer from the “phase halos” typical of phase-contrast images. See Salmon and Tran (1998) for details of image formation and video-contrast enhancement.

The DIC microscope is a bright-field light microscope with the addition of the following elements (Fig. 4.1.7, middle): a polarizer beneath the condenser; a DIC beam-splitting

prism (Nomarski or Wollaston) in the condenser turret; a DIC beam-combining prism (Nomarski) just above the objective; an analyzer above the objective prism in the infinity body tube space; a compensator after the polarizer or before the analyzer in some microscopes (not shown in Fig. 4.1.7); and a rotatable stage. The microscope optics are usually aligned for bright-field specimen illumination by the standard Köhler method.

The polarizer, which produces plane-polarized light (Fig. 4.1.7, left), is typically oriented with its transmission azimuth in an east-west direction facing the front of the microscope. Polarizers with high transmission efficiency are preferred. Polarizers are usually high-quality polaroid material held between thin optical glass flats. Another polarizer is used as an analyzer. The transmission azimuth of the analyzer is oriented north-south at 90° with respect to the polarizer azimuth to produce extinction of the illumination light in the absence of the DIC prisms.

The specimen is held on a rotating stage. Contrast depends on orientation, and frequently specimens must be reoriented to achieve maximum contrast of the structures of interest. Either the stage or the objective and condenser must be centerable.

For the highest sensitivity, the objective and condenser lenses should be selected free of birefringence (Inoué and Spring, 1997). Rectifiers can be used to correct for the rotation of the plane of polarization of light which occurs at the periphery of lens surfaces (Inoué and Spring, 1997).

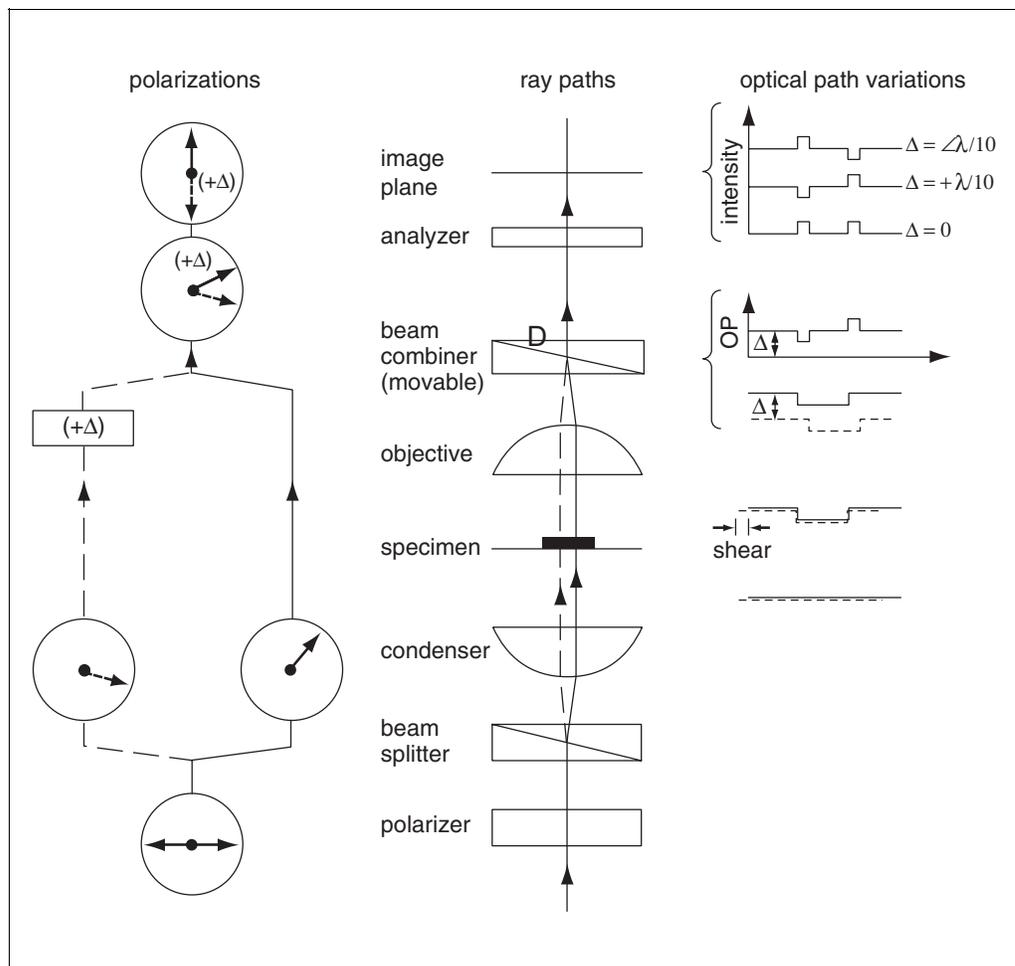


Figure 4.1.7 The optical system for DIC microscopy. From Salmon and Tran (1998), reprinted with permission from Academic Press.

Figure 4.1.7 outlines the principles of DIC image formation and contrast. The DIC beam-splitting prisms are located at the condenser diaphragm plane, just above the polarizer in the condenser turret. Objectives of different magnification and/or NA usually require condenser prisms of different optical characteristics.

The condenser prism splits the light coming from the polarizer into divergent polarized light wavefronts whose planes of polarization are orthogonal to each other and at 45° with respect to the initial plane of polarization (Fig. 4.1.7, left). The divergent beams are converted by the condenser into two wavefronts, which pass through the specimen separated laterally from each other in the direction of the prism wedge (the shear direction, Fig. 4.1.7, middle) by a tiny amount that is usually less than the resolution limit of the condenser-objective lens combination (Fig. 4.1.7, right). These two wavefronts are recombined just above the objective by a beam-combining prism. Often, each objective has its own prism so that it accurately matches the action of the condenser prism. In some microscopes, there is one beam-combining prism for all objectives and a different condenser prism for each objective. Check which is the case for your microscope.

DIC image contrast depends on the “compensation” or “bias retardation” (Δ) between the two wavefronts along the microscope axis (Fig. 4.1.7, right). When the objective beam-combining prism is perfectly aligned with the condenser beam-splitting prism and there is no compensation ($\Delta = 0$), the background light is extinguished and the edges of objects are bright against a black background (Fig. 4.1.7, upper right). When one wavefront is retarded relative to the other by Δ , this increases the optical path (OP) between the wavefronts (Fig. 4.1.7, middle right) and brightens the background light. One edge of an object becomes brighter than the background while the opposite edge becomes darker (Fig. 4.1.7, upper right). This produces the “shadow cast” appearance of DIC images. Reversing the sign of retardation, reverses the contrast of the specimen edges.

In some microscopes, the objective beam-combining prism (Fig. 4.1.7, middle) is used as a compensator by translating the prism in the direction of prism wedge away from the position of background light extinction. One direction produces positive while the other produces negative retardation (Δ). In other microscopes, both the objective and condenser prisms are fixed at positions that produce background light extinction, and typically a deSenarmont compensator (a birefringent quarter-wave retarder in combination with a rotatable polarizer or analyzer; Inoué and Spring, 1997) is inserted just above the polarizer or beneath the analyzer.

Bright illumination sources are required for high magnification because of the crossed polarizer and analyzer; at least the 100-W quartz-halogen illuminator is usually needed.

To examine the DIC prisms, remove the objective prisms and the DIC condenser from the microscope. Examine the objective prism. Notice that it is very thin and wide enough to cover the objective aperture. Also notice if there is a screw that can be used to translate the prism back and forth in one direction across the objective aperture. Examine the prisms in the condenser turret. Notice that there are different prisms for low- and high-NA objectives. The highest-resolution (NA) objectives often have special prisms designed only for the optical properties of that objective. In aligning the microscope for DIC, be careful to use the condenser Wollaston prism that matches the objective in use. Reinstall the condenser.

Steps 1 to 6 in the following procedure are for the initial alignment of the microscope. Once this is done, then begin at step 7 for routine use.

Perform initial alignment

1. Align the microscope initially for Köhler illumination using the low-power objective and the cheek cell preparation. Again look for the bright air-bubbles in the preparation to find the specimen plane.
2. Check that the polarizer is oriented with its transmission azimuth in an east-west direction as determined facing the front of the microscope (look for the line or double-headed arrow on the polarizer mount). Rotate the deSenarmount compensator (if there is one) so that it is at its zero position (no compensation).
3. Remove the objective prism and rotate the condenser turret to an open position. Make sure that the analyzer transmission azimuth is crossed to the polarizer by checking that the background light is at extinction.

This exercise is best done with the brightest light position of a 100-W quartz-halogen illuminator.

4. Insert the objective DIC prism (observe caution as the image will be very bright). Observe the extinction fringe in the middle of the prism (you must use the telescope, remove an eyepiece, or insert the Bertrand lens to focus on the objective focal plane). Make a drawing of your view of the fringe in the back aperture.

The fringe should be at 45° with respect to the analyzer-polarizer transmission azimuths.

5. Remove the objective DIC prism and rotate into place the condenser prism that matches the objective. Observe the objective back focal plane.

The orientation of the fringe should match the orientation of the extinction fringe for the objective prism in your drawing.

6. Insert the objective prism and observe the objective back focal plane. Rotate in the other condenser prisms and notice that the extinction fringe is no longer spread across the objective aperture. Rotate in the correct condenser prism.

If the condenser and objective prisms are properly matched and oriented in the same direction, the fringe should become spread across the objective aperture (a dark cross will still occur in high-power, unrectified objectives; Inoué and Spring, 1997).

Align for specimen viewing

7. View the specimen with the correct objective and condenser prisms in place. Translate the objective prism (or rotate the deSenarmount compensator if the objective prism is fixed) to induce a retardation (Δ) between the two wavefronts to brighten the background light and make the edges of the cell appear shaded. If possible, rotate the specimen and observe that contrast is directional—45° with respect to the analyzer-polarizer orientations.

As the upper prism is translated (or the deSenarmount compensator is rotated) to compensation of opposite sign, the initially bright edges become darker and the initially dark edges become brighter than the background.

8. Adjust for proper bright-field, transmitted-light Köhler illumination.

The quality of the DIC image depends critically on the field diaphragm being sharply focused on the specimen (Fig. 4.1.2, left), since this puts the condenser prism in the proper place. The highest possible resolution is achieved when the condenser diaphragm is adjusted to match the diameter of the objective aperture (view the objective back focal plane during this adjustment).

Optimum edge contrast is produced when the retardation between the wavefronts is adjusted to extinguish the light coming from one set of edges in the specimen. Further retardation increases light intensity, but not contrast to the eye. When using video cameras,

it is often important to view the specimen by eye and adjust the compensation for best contrast, then change illumination intensity to provide the camera with enough light for good image quality.

9. Repeat steps 2 to 8 for the other objectives.

Once you are sure of the correct prisms and their orientation, then only steps 7 and 8 are necessary for each objective.

SUPPORT PROTOCOL 1

MATCHING MICROSCOPE MAGNIFICATION TO DETECTOR RESOLUTION

This procedure uses the diatom test slide (see Table 4.1.1 for supplier information). For more details on matching a camera to the microscope see Hinsch (1998) or Inoué and Spring (1997).

1. Look down the eyepieces and obtain an in-focus image of the frustule pores of the diatom *Pleurosigma angulatum* (Fig. 4.1.8, number 6 in panel A) using 40 \times /(NA \sim 0.7) phase-contrast or DIC and proper Köhler illumination.

The pores should be clearly visible by eye.

2. Project the diatom image onto the faceplate of a video or CCD camera.

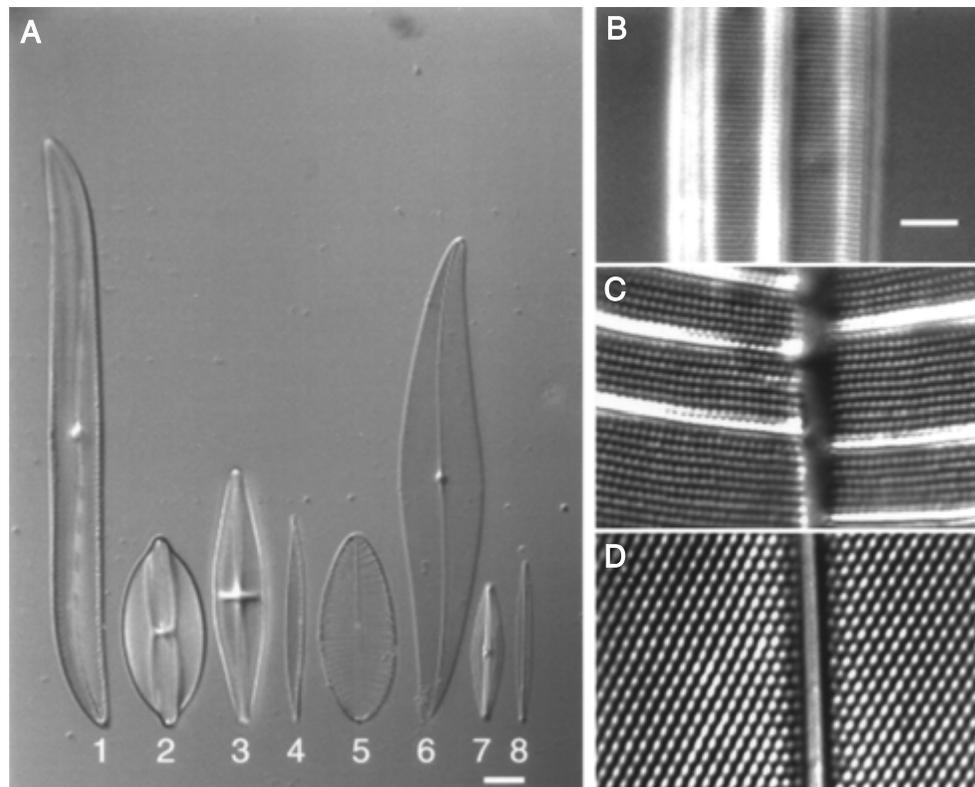


Figure 4.1.8 (A) The diatom test plate. The rows of pores are spaced in the silica shell by $\sim 0.25 \mu\text{m}$ in *Amphipleura pellucida* (panel B; also number 8 on panel A), $0.41 \mu\text{m}$ in *Surrella gemma* (panel C; also number 5 on panel A), and $0.62 \mu\text{m}$ in *Pleurosigma angulatum* (panel D; also number 6 on panel A). Bar: panel A, $10 \mu\text{m}$; panels B, C, and D, $2.5 \mu\text{m}$. From Salmon and Tran (1998), reprinted with permission from Academic Press.

3. Adjust the gain and contrast camera controls for optimum image brightness and contrast.

As you increase contrast, you will need either more camera gain or brighter illumination. Usually, a better signal-to-noise ratio is achieved by leaving the gain control in the middle of its adjustment and increasing illumination intensity.

If the projection magnification to the detector is too small, the frustrule pore lattice will be invisible in the video image, although it will be clearly visible when viewing the image by eye.

4. Increase the magnification to the camera as much as possible.

As magnification increases, resolution in the image becomes less limited by the resolution of the camera. However, the size of the field of view decreases inversely with magnification. In addition, the intensity of light in the image decreases as $1/\text{magnification}^2$. The image will become noisy at low light intensities. It will be necessary to increase illumination intensity as much as possible at high magnification or increase the integration period for the camera exposure.

CALIBRATING IMAGE MAGNIFICATION WITH A STAGE MICROMETER

1. Insert the stage micrometer (see Table 4.1.1) on the microscope stage.
2. With the low-power objective, find the region of the micrometer with 10- μm scale intervals.

The larger intervals are 100 μm .

3. Use the 100- or 10- μm scales to calibrate distance in images taken with your objectives.

The magnifications on the objective barrel and projection lenses are only approximations; accurate measurements of distances in images require a calibration scale.

4. Acquire images of the scale in both the horizontal and vertical directions to check if the camera has square pixels.

TESTS FOR THE OPTICAL PERFORMANCE OF THE MICROSCOPE

Test slides are used to evaluate the performance of the microscope under different conditions.

Testing Phase-Contrast and DIC Using Diatom Testing Slide

Diatoms have silica shells shaped like pillboxes. There are pores in the shell arranged in a lattice pattern specific for each diatom species. Figure 4.1.8 shows a low-magnification view of the eight diatoms on the test slide (panel A) plus higher-magnification views of the lattices of three diatoms most useful in testing the resolution performance of microscope optics using phase-contrast or DIC. Number 6 in panel A of Figure 4.1.8, *Pleurosigma angulatum*, has a triangular pore lattice with spacing of $\sim 0.61 \mu\text{m}$ between rows (illustrated in Fig. 4.1.8, panel D). Number 5, *Surrella gemma*, has rows of pores where the rows are separated by $\sim 0.41 \mu\text{m}$ (illustrated in Fig. 4.1.8, panel C). Number 8 is *Amphipleura pellucida*, which has horizontal rows of pores separated by $\sim 0.25 \mu\text{m}$ (illustrated in Fig. 4.1.8, panel B). In transmitted light, the diffraction limit to lateral resolution, r , is given by (Inoué, 1989):

$$r = \lambda / (\text{NA}_{\text{obj}} + \text{NA}_{\text{cond}})$$

where λ is the wavelength of light, NA_{obj} is the objective NA, and NA_{cond} is the condenser NA (see Fig. 4.1.3). The lateral resolution, r , is equal to $0.195 \mu\text{m}$ for the highest objective

**SUPPORT
PROTOCOL 2**

**SUPPORT
PROTOCOL 3**

Microscopy

4.1.21

NA, which is equal to 1.4, with $NA_{\text{cond}} = NA_{\text{obj}}$ and 546 nm green light. As seen in Figure 4.1.8B, this objective is capable of resolving the rows of pores in the shell of *Amphipleura*, but not the individual pores, which are slightly $<0.19 \mu\text{m}$ apart.

Use the above equation to calculate the diffraction limit of resolution for your other objectives and use the diatoms to test whether they achieve their theoretical limits.

**SUPPORT
PROTOCOL 4**

Testing Phase-Contrast and DIC Using Squamous Cheek Cell Test Slide

Cheek cells are a convenient specimen for testing the performance of phase-contrast or DIC microscopes. As seen in the low-magnification view (Fig. 4.1.6A) they are large and flat, $\leq 3 \mu\text{m}$ thick except near the cell center which contains the nucleus. The upper and lower surfaces have fine ridges which swirl around much like fingerprints. Many of the ridges are $<0.2 \mu\text{m}$ in width and separated by <0.5 to $1.0 \mu\text{m}$.

To prepare the cheek cells, gently scrape the inside of your mouth with the tip of a plastic pipet or similar soft tool and spread the cells and saliva on the center of a clean no. 1.5 coverslip. Invert the coverslip quickly onto a clean slide and press down to spread the cell preparation into a thin layer. Seal the edges with a thin layer of nail polish.

**SUPPORT
PROTOCOL 5**

Testing Fluorescence Using Red, Green, and Blue Fluorescent Tissue Culture Cell Test Slide

The cells in Figure 4.1.9 are triple labeled: DAPI stained nuclei and chromosomes (blue fluorescence; Fig. 4.1.9A); Alexa 488-phalloidin labeled actin filaments (green fluorescence; Fig 4.1.9B); and X-Rhodamine immunofluorescently labeled microtubules (red fluorescence; Fig. 4.1.9C). The microtubules and fine actin filamentous arrays are $<100 \text{ nm}$ in width, but they should appear sharp and in high contrast in the microscope. There should be no “bleed-through” of fluorescence from one fluorescence channel to another if the filters are chosen properly (Taylor and Salmon, 1989).

In fluorescence microscopy, the diffraction-limited lateral resolution is given by (Inoué 1989; Inoué and Spring, 1997):

$$r = 0.61\lambda/NA_{\text{obj}}$$

and the intensity of the image, I_{image} , is given approximately by:

$$I_{\text{image}} \approx I_{\text{ex}} NA_{\text{obj}}^4 / M_{\text{p}}^2$$

where I_{ex} is the excitation light intensity entering the objective and M_{p} is the projection magnification from the objective to the eye or camera. The rate of photobleaching, R_{p} , of a specimen depends on:

$$R_{\text{p}} \approx I_{\text{ex}} NA_{\text{obj}}^2 M_{\text{obj}}^2$$

where M_{obj}^2 is the magnification of the objective.

Note from these equations that resolution depends inversely on NA_{obj} ($r = 0.24 \mu\text{m}$ for $NA_{\text{obj}} = 1.4$ and 546 nm green light), image intensity depends on the fourth power of NA_{obj} , while loss of intensity by photobleaching of the fluorophore increases with the square of objective NA (a measure of the excitation light collected by the objective) and magnification (which concentrates the excitation light on the specimen).

Verify these equations by imaging the fluorescent specimen (the microtubules in the spread cells are a good choice) for objectives with different magnifications and numerical apertures. It will quickly be seen why in fluorescence one wants to use the minimum total

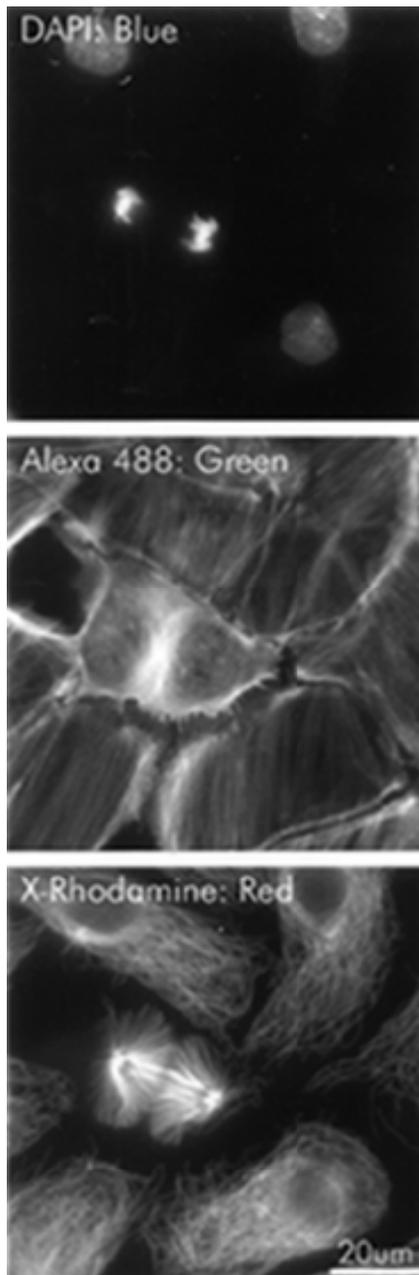


Figure 4.1.9 Epifluorescent images of fixed tissue culture cells stained with (A) DAPI, making DNA fluorescent blue; (B) Alexa 488 bound to phalloidin to label actin filaments fluorescent green; and (C) X-rhodamine labeled antibodies against tubulin to label microtubules fluorescent red. Bar = 20 μm . Images recorded with a 40 \times (NA = 1.4) Plan Fluor objective, 1.5 magnification, to a cooled CCD camera and the multi-modem multiwavelength microscope described by Salmon et al. (1998).

magnification necessary to resolve the structures of interest in the detector, as well as use the maximum NA_{obj} in order to maximize light intensity for the least amount of excitation light (and the least amount of photobleaching).

CARE AND CLEANING OF MICROSCOPE OPTICS

Keeping the microscope optics clean is important for high-quality imaging. Dust, fingerprints, excess immersion oil, or mounting medium on or in a microscope causes reduction in contrast and resolution. DIC is especially sensitive to contamination and scratches on the lens surfaces. Below are steps for keeping the microscope clean.

1. Always keep microscopes covered when not in use.
2. Make sure that all ports, tubes, and unoccupied positions on the lens turrets are plugged.

Plastic plugs are usually supplied with the microscope.

**SUPPORT
PROTOCOL 6**

Microscopy

4.1.23

3. Store objectives in screw-top containers when not on the microscope. Keep accessories—e.g., condensers and compensators—in plastic bags or boxes. Keep slides and coverslips covered.
4. Be careful with salt water, corrosive liquids, and all solvents.
5. When cleaning lens surfaces, avoid touching the lens surface with anything (even lens paper if possible).

IMPORTANT NOTE: Never use Kimwipes or commercial facial tissue, because they may contain a filler that is part diatomaceous material (glass). One pass of a Kleenex could ruin an objective.

6. Remove dust by gently brushing with an oil-free (ether-washed) camel's hair brush or by using a low-velocity stream of purified air.
7. Remove water-soluble contamination using distilled water with a small amount of detergent, such as Kodak Photoflow solution.

Much (modern) immersion oil can also be removed by washing with detergent.

8. Remove most immersion oil by passing a high-quality lens tissue over the objective or condenser front element.

IMPORTANT NOTE: Do not rub. No area on the tissue should come in contact with the lens twice. This prevents dust and dirt removed from the lens from coming back and possibly scratching it. This is easily accomplished by passing the tissue over the lens in a "Z" pattern or by making parallel passes.

9. Clean objective lenses by holding a piece of doubled lens paper over the objective and placing a few drops of solvent on the paper. Draw the paper across the lens surface so that the solvent flows rapidly in a circular pattern over the recessed lens surface (see Inoué and Spring, 1997). Finish the stroke with a dry portion of the paper. Repeat as necessary.

In this way, the solvent contacts the lens but the paper does not, because the lens is recessed. For solvent, first use a 1% solution of Kodak Photoflow in distilled water to remove much of the oil and water-soluble material. Then use a small amount of oil solvent like ethyl ether or xylene to clean all the oil from the surface. Avoid soaking a lens with solvent, to prevent damage to lens cements.

10. To clean recessed front elements of dry objective lenses or to remove stubborn dirt, use a cotton-tipped applicator that has been soaked in cleaning solution and then shaken to remove excess fluid. Rotate the cotton tip over the lens surface to clean. Again, first use a 1% solution of Kodak Photoflow in distilled water to remove much of the oil and water-soluble material. Then use a small amount of oil solvent like ethyl ether or xylene to clean all the oil from the surface.

11. Use a detergent solution or ethanol to clean the surfaces of the eyepiece lenses.

Do not use xylene as it may solubilize enamel surfaces.

COMMENTARY

The development of lasers, electronic cameras, digital image analysis, and specific fluorescent molecular probes have recently made light microscopy an enormously powerful tool in the biomedical sciences. There are a number of excellent books and review articles about these applications, as well as the optical principles of light microscope design and image

formation. Listing of all these references is beyond the scope of this unit; only a few of these books and reviews are listed, and these can be used to find other useful references.

Spencer (1982), Zernike (1958), and Keller (1995, 1997, 1998) provide excellent introductions to the principles of image formation and contrast in the light microscope for beginners,

while Pluta (1988, 1989) provides a more comprehensive treatment. Inoué and Oldenbourg (1995) also review the basic concepts and principles of microscope design and different methods of image formation. The abovementioned references describe the many modes of transmitted-light imaging in the microscope, including bright-field, phase-contrast, and DIC, which are described in this unit, as well as dark-field, polarization, interference contrast, Hoffman modulation, and reflection interference contrast methods. Taylor and Salmon (1989) and *UNIT 4.2* give clear introductions to fluorescence microscopy. The recent edition of *Video Microscopy* by Inoué and Spring (1997) is a comprehensive introduction to the principles and practical aspects of light microscopy, video, and digital imaging. The *Video Microscopy* volume of *Methods in Cell Biology* edited by Sluder and Wolf (1998) also has contributions that cover many basic concepts and practical aspects of light microscopy in the biomedical sciences; it also includes chapters on multiwavelength, multimode digital imaging methods, camera selection, ion ratio imaging, and specimen chambers. Salmon and Tran (1998) review the principles of video-enhanced DIC methods used to image macromolecular complexes invisible in the microscope when viewed by eye. Taylor et al. (1992), Salmon et al. (1998), and Rizzuto et al. (1998) review microscope design for multiwavelength, multimode digital imaging of fluorescent specimens, and four-dimensional microscopy. Advanced quantitative fluorescence methods like ratio imaging, photobleaching, photoactivation, resonance energy transfer, chromophore-assisted laser ablation, and fluorescence lifetime imaging are also reviewed in the *Methods in Cell Biology* volumes edited by Taylor and Wang (1989), and Sluder and Wolf (1998) as well as in Herman and Jacobson (1990) and Inoué and Spring (1997). Reviews on imaging cells containing expressed protein coupled to green fluorescent protein (GFP) include Heim and Tsien (1996), and Sullivan and Kay (1998). Agard et al. (1989), Carrington et al. (1995), and Rizzuto et al. (1998) review how deconvolution of three-dimensional image stacks can produce super resolution in the light microscope. The recent edition of *Handbook of Biological Confocal Microscopy* edited by Pawley (1995) is an excellent reference on this important method for imaging fluorescent structures in thick specimens (Smith, 1997). The new technique of multiphoton laser scanning microscopy is described by Denk et al. (1994),

while biological applications of optical traps to manipulate organelles and measure molecular forces is reviewed by Svoboda and Block (1994).

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Fluorescence is a property exhibited by some molecules whereby energy of a particular wavelength (λ) is absorbed, and, after a certain period of time (termed the fluorescence lifetime), a portion of this absorbed energy is emitted. The amount of energy emitted as fluorescence is always less than the amount of energy absorbed. Absorption of energy by molecules in the form of light and emission of energy in the form of fluorescence can occur only at certain wavelengths, which are characteristic for a given molecule. The difference in the maximal wavelengths (corresponding to energy levels) of absorption and emission is known as the Stokes shift. Each fluorescent molecule is characterized by its own distinct absorption and emission energy levels (i.e., excitation and emission spectrum). This fact—combined with the fact that a number of processes can affect the emission of fluorescence from a fluorophore during its excited-state lifetime—allow the specificity, quantitative and environmental sensitivity, and high temporal resolution provided by fluorescence to be brought to bear in the examination of microscopic structures. To accomplish this, the fluorescence microscope is constructed in such a way as to permit the delivery of excitation energy to the specimen in the form of light of specific energy levels corresponding to wavelengths that match the maximal absorbance band of the fluorescing species. The microscope must also have the capacity to separate the weaker emitted fluorescence light from the brighter excitation light for observation.

FLUORESCENCE MICROSCOPE OPTICS

Fluorescence microscopes must accomplish the following four functions: (1) deliver excitation light of the appropriate wavelengths to the specimen; (2) separate the excitation light from the emitted fluorescence; (3) collect as much of the emitted fluorescence given off by the emitted fluorophores as is possible; and (4) allow observation of fine detail in the specimen.

Fluorescence microscopes have been designed with these functions in mind. Excitation light sources and wavelength-selection devices allow selection of appropriate excitation wavelengths. Excitation light is delivered to the specimen through the microscope objective. A special mirror, known as a dichroic beam-splitting mirror, allows separation of the excitation light from the emitted fluorescence. The dichroic beam-splitting mirror has the special property of being able to reflect light below a specific wavelength, yet allow light above this specific wavelength to pass through the mirror unobstructed. Objective lenses have been developed that allow maximal collection of a portion of the emitted fluorescence from the sample, as well as magnification and observation of fine detail (resolution) of the specimen.

The most commonly employed illumination system is that invented by Ploem, termed Ploem illumination, incident-light illumination, or epiillumination. In epiillumination, a dichroic beam-splitting mirror reflects the excitation light into the back aperture of the objective. The objective acts as a condenser and focuses the excitation light onto the specimen. A portion of the emitted fluorescence (the amount being determined by the numerical aperture, or NA, of the objective) is collected by the objective and passes through the dichromatic beam-splitting mirror either to eyepieces or a detector. The existence of Stokes shift and the special property of the dichroic beam-splitting mirror described above allow the separation of the excitation light from the emitted fluorescence.

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COMPONENTS OF THE FLUORESCENCE MICROSCOPE

Excitation Light Sources

There is a variety of excitation light sources that can be used for fluorescence microscopy. The choice of which light source to employ will depend on the fluorescent probes being used. Because many of the available fluorophores (see Table A.1E.1) require excitation in the blue/green portion of the visible light spectrum, mercury (Hg), xenon (Xe), or Hg/Xe combination lamps are generally employed. Lasers can also be used. Each light source has benefits, depending on the wavelength and strength of excitation energy required in a given experimental situation. For example, Hg light sources emit energy at discrete wavelengths (e.g., 365, 400, 440, 546, and 580 nm) making this light source useful for caged compound release—i.e., the release of chemically caged (inert) molecules such as Ca^{2+} and inositol 1,4,5-triphosphate (IP_3) inside living cells with UV excitation—as well as for Hoescht-, 4',6-di-

amidino-2-phenylindole (DAPI)–, fluorescein- and rhodamine-based fluorophores. Conversely, Xe lamps have a spectrally uniform intensity profile from the ultraviolet to the far red; this light source is particularly suited for use of Fura-2, a calcium-sensitive dye. Metal halide lamp spectral output is the same as that of the Hg arc lamp, but the energy output per unit area for metal halide is less than that of the Hg arc lamp. However, the 150-W metal halide bulb has an average lifespan five times that of an Hg lamp (1000 versus 200 hr). A Xe lamp (75-W) lasts ~400 hr. These times are estimates, as the lifetime of a bulb is determined by the total hours that it is on and the number of times it is turned on and off. When first used or when first ignited, the spark of these lamps can flicker or wander. This can be minimized by an initial 2-hr “burn in” or 10-min warm-up period. If the lamp has been turned off and is to be used again, ≥20 min must pass before turning on the lamp again.

Lasers (light amplification by stimulated emission of radiation) offer monochromatic light of very high energies and can be used in continuous-wave (CW) or pulsed modes of operation. Laser light is coherent, monochromatic (although a single laser can output multiple wavelengths of light), and polarized (propagates in one plane). Pulsed lasers are finding more and more applications in fluorescence microscopy, especially in two-photon excitation microscopy (TPEM) and in the two- and three-dimensional measurement of fluorescence lifetimes in intact cells and tissues. An important concern with the use of lasers in

microscopy is the potential for scattering and diffraction due to dirt and interference on optical surfaces in the beam path. This can be overcome by using a multimode fiber-optic or liquid light pipe.

Köhler illumination

When using a fluorescence microscope, it is necessary to provide bright and even illumination of the whole field of view, while at the same time not illuminating any areas outside of the field of view. This reduces scattered light and photodamage. The optical design that can accomplish this, named after its inventor, is known as Köhler illumination (Fig. 4.2.1). In Köhler illumination, a collector lens in front of the excitation light source focuses an image of the excitation light onto a condenser field diaphragm. The condenser lens then focuses an image of the field diaphragm into the plane of the specimen (for a step-by-step procedure see *UNIT 4.1*). When this is done, the microscope is aligned in such a way that the planes carrying the illumination information are separate from the set of planes carrying the specimen information. The advantages of Köhler illumination are that the back focal plane of the objective is fully illuminated, providing a microscope field that is homogeneously bright. In addition, this system makes possible maximal lateral and axial resolution, optimal contrast, and reduction of flare from the microscope optics.

Arc lamp alignment

Alignment of the arc lamp is required each time a new lamp is inserted into the lamp

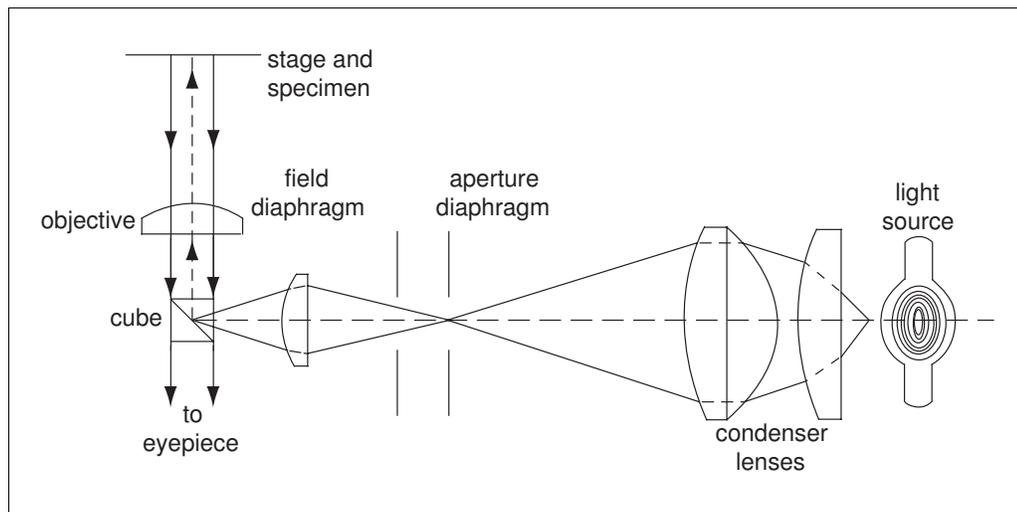


Figure 4.2.1 Köhler illumination. Condenser lenses are placed in front of the light source to make sure that the image of the centerable aperture diaphragm is focused on the back aperture of the objective, which is then focused on the specimen.

housing, and occasionally during routine usage. It is important to remember, when replacing an arc lamp, that the bulb should never be touched with one's hands (lens tissue or lint-free gloves should be used) or when it is hot (because it might explode). Also, whenever an arc lamp is replaced, the lamp power supply should be unplugged, protective eye gear should be worn, the lamp should never be used outside of its housing, and one should never look directly at or expose one's skin to the lamp (because it could cause burns and/or skin cancer). After placing a new lamp bulb in its housing, perform the following steps.

1. Reattach the lamp to the microscope, turn it on, and remove one of the objective lenses. Put a white piece of paper (a business card works well), on the microscope stage to visualize and align the lamp arc.

2. To align the arc (e.g., Hg or Xe) lamp, first focus the real and mirror images of the lamp arc to their smallest size using the collector lens in the lamp housing (Fig. 4.2.2).

3. Next, adjust the real and mirror images so that they are visible in the same field and are at the same focus or size. Adjust the real image until it is centered in the vertical direction in the field and is just to the side of the center.

Move the mirror image so that it is also centered vertically in the image, but is on the opposite side of center from the real arc image.

4. Lastly, using the collector lens focusing knob on the lamp housing, defocus the arc image to provide an evenly illuminated field.

Photobleaching

Photobleaching or "fading" is the loss of fluorescence intensity following illumination of the specimen. Photobleaching decreases the fluorescence signal and hence the signal-to-noise (S/N) ratio of the microscope image. Photodynamic photobleaching is the most common type of photobleaching that occurs in fluorescence microscopy; it involves the interaction of the fluorophore with light and oxygen. In their excited state, fluorescent molecules can interact with nearby oxygen (O_2) molecules, resulting in oxidation of the fluorophore and loss of fluorescence. Fluorophores in their excited state can collide with molecular oxygen and "sensitize" singlet oxygen production. Singlet oxygen is highly reactive and can interact with the fluorophore to bleach it. The amount of photobleaching or fading that occurs in a given situation will depend on the concentration of molecular oxygen, the fluorescence life-

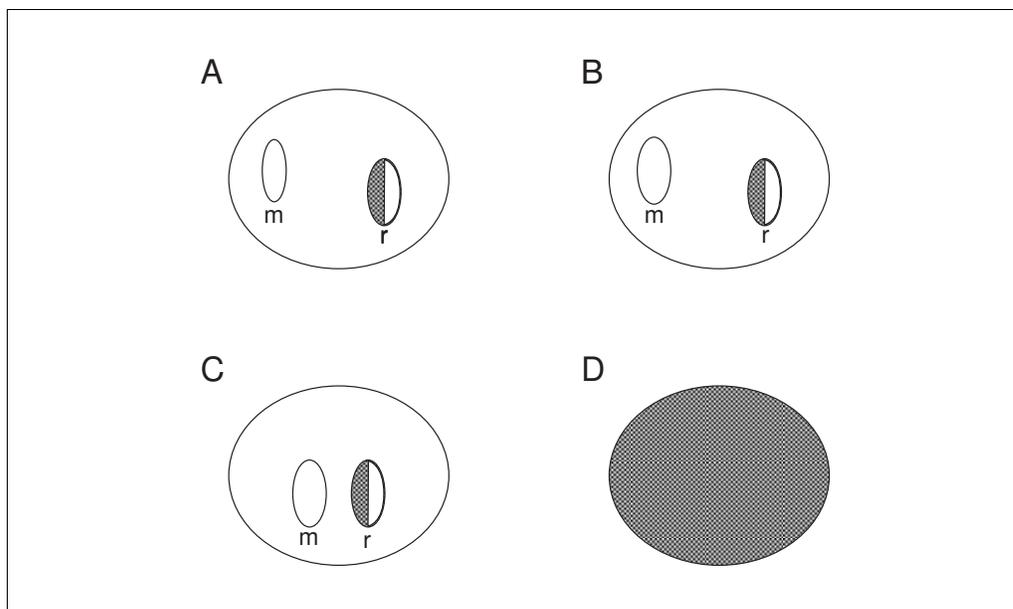


Figure 4.2.2 Alignment of arc lamp. **(A)** After placing a new lamp bulb in the lamp housing, use the collector lens focusing knob on the lamp housing so that the real (r) and mirror (m) images of the lamp bulb are at their smallest size (i.e., most focused). **(B)** Next, using a socket wrench and the ports on the lamp housing, adjust the real and mirror images so that they are visible in the same field and are at the same focus or size. **(C)** Adjust the real image until it is centered in the vertical direction in the field and is just to the side of the center. Move the mirror image so that it is also centered vertically in the field, but is on the opposite side of center from the real arc image. **(D)** Using the collector lens-focusing knob on the lamp housing, defocus the arc image to provide an evenly illuminated field. Once the lamp is focused, replace the objective.

time of the excited state (the longer the lifetime the more chance for interaction with molecular oxygen), and the distance between the fluorophore, molecular oxygen, and any other cellular constituents.

From a theoretical standpoint, whether the excitation energy is delivered in a pulsed versus continuous fashion should not affect the photochemical lifetime (rate of photobleaching). Therefore, at low excitation energy levels, photobleaching is not prevented, but the rate of photobleaching is merely reduced. However, anecdotal reports suggest that the delivery of excitation light in a pulsed versus continuous fashion may have experimental advantages in certain situations. For example, it may be advantageous to get several high signal-to-noise digital video images before photobleaching has occurred, using intense short-duration pulses. Alternatively, for certain cell physiological events, one may need to illuminate for much longer times to study the time evolution of a phenomenon, but at considerably lower S/N ratios in the individual images.

Protection against photobleaching involves reducing exposure time or excitation energy. However, these actions will also reduce the measurable signal. Solutions of molecules or suspensions of cells could also be deoxygenated, although this is not feasible for living cells and tissues. Antifade reagents such as *n*-propyl gallate, or others that are commercially available, can be added to fixed specimens. Singlet oxygen quenchers such as histidine, diphenylisobenzofuran, or crocetin (a water-soluble ca-

rotenoid) can also be employed. The use of a computer-controlled electronic shutter that is open only when experimental data is being collected will prolong filter life as well as keep exposure of the sample to harmful radiation at a minimum.

While photobleaching is usually to be avoided at all costs, there is one experimental technique that uses photobleaching to examine the lateral diffusional mobility of fluorescent molecules. This technique, termed fluorescence recovery after photobleaching (FRAP), involves irreversible photobleaching of a small area of fluorescent molecules with a short, intense burst of a laser, and observation of the reappearance of fluorescence in the bleached area (Fig. 4.2.3). The reappearance of fluorescence is due to the diffusion of unbleached fluorescent molecules into the bleached region. The kinetics of this process are related to the lateral diffusional mobility of the fluorescent material.

Wavelength Selection Devices

In the fluorescence microscope, excitation light of a specific energy level (wavelength) from Hg or Xe arc lamps or a laser is isolated using an exciter filter and reflected onto the specimen by a dichroic beam-splitting mirror. The emitted fluorescence is viewed through a cutoff or barrier filter that blocks all wavelengths below a set value from reaching the observer.

Filters can consist of a variety of substances such as absorption glass (including gelatin) or

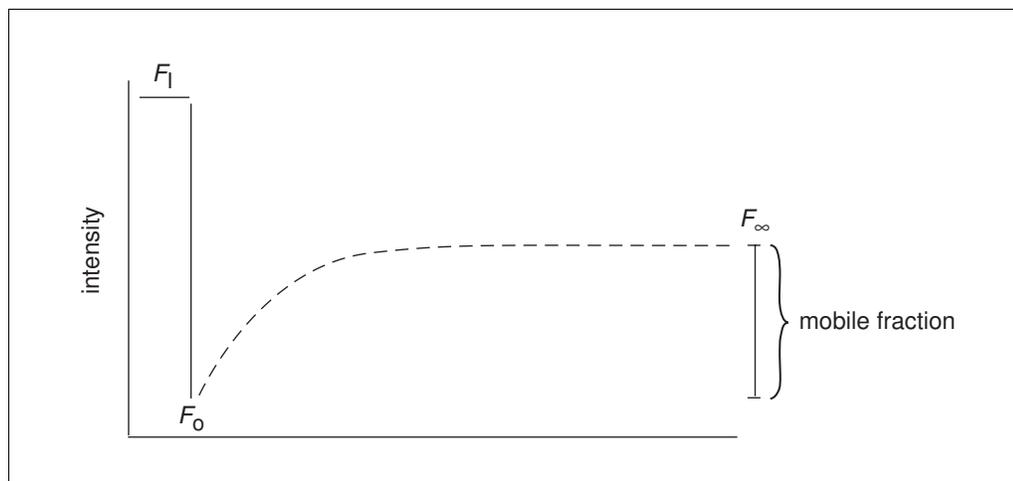


Figure 4.2.3 Fluorescence recovery after photobleaching (FRAP). A small region of the sample containing a fluorophore of some intensity value (F_i) is irreversibly bleached by a strong laser beam resulting in a drop in the observed fluorescence intensity (F_o). A much weaker laser beam then monitors the recovery of fluorescence into the bleached area as a function of time, until there is no further increase in fluorescence. The mobile fraction is defined as the difference between F_∞ and F_o .

thin-film coatings. Thin-film coatings may be either of a metallic nature (for fully reflective mirrors and neutral-density filters) or interference coatings (for interference filters). Several standard exciter filter/dichroic beam-splitting mirror/barrier filter combinations are available from each microscope manufacturer. The choice of which combination to use will depend on the fluorochrome(s) employed.

Filters are described according to their transmission properties. A short-pass filter will pass light up to a certain wavelength, but will not allow light of higher wavelengths to pass through the filter. A long-pass filter will pass only wavelengths above a certain value, and will prevent light of lower wavelengths from passing through the filter. Both short-pass and long-pass filters are named by the wavelength at which the transmission of light is 50% of maximum.

Filters also exist that can transmit only a specific range of wavelengths (interference or band-pass filters). Both wide band-pass and narrow band-pass filters exist. The narrower the range of wavelengths that can pass through the filter, the more specific is its ability to discriminate individual fluorophores in a mixture (i.e., the higher its contrast is). However, higher specificity means less intensity. Broad-band filters provide more signal but less contrast (i.e., where contrast is defined as the ability to distinguish specific versus nonspecific fluorescence or the ability to distinguish individual fluorophores when more than one fluorophore is present). These filters are named by their center wavelength (the arithmetic means of the wavelengths at 50% of peak transmission) and by the range of wavelengths (bandwidth) of light they transmit at 50% peak transmittance (full width half maximum, or FWHM). For example, BP 490/30 is an interference filter with maximal transmission at 490 nm (for fluorescein excitation) and which transmits light from 475 to 505 nm. A short-pass and long-pass filter can be sandwiched together to generate specific band-pass filters.

Dichroic beam-splitting mirrors reflect the shorter wavelengths of excitation light to the specimen and allow the long wavelength emitted fluorescence to pass to the detector, while at the same time preventing the passage of the shorter excitation wavelengths to the detector. Optimally, >90% reflectance of the excitation light and >90% transmission of the emission light is desired. Dichroic beam-splitting mirrors are identified by the highest wavelength of light that will be reflected onto the specimen.

Often more than one fluorophore will be visualized in a single specimen. In order to visualize each fluorophore individually without any contamination from the other fluorophores, microscopes have been designed to accommodate multiple exciter/dichroic/emission filter units, each optimally matched for its respective fluorophore. Of course this type of arrangement does not allow observation of all of the fluorophores simultaneously. To accomplish this, single filters with multiple band passes have been designed that can be used to image more than one fluorophore simultaneously. In addition to filters, monochromators and electro-optic instruments can be employed as wavelength-selection devices.

Fluorescein and rhodamine represent two of the most common fluorophores utilized in fluorescence microscopy. Typical fluorescein and rhodamine filter sets might be constructed according to the following considerations. Fluorescein absorbs maximally at 490 nm and emits maximally at 525 nm. Rhodamine absorbs maximally at 550 nm and emits maximally at 580 nm. Therefore, the two filter sets have to be constructed in such a way that the appropriate wavelengths of excitation (490 nm and 550 nm) can be delivered to the specimen, and the emitted fluorescence (525 nm and 580 nm) can be separated from the excitation light and any other scattered light before reaching the detector. Using an Hg lamp, an excitation interference filter is placed in the light path—comprised of a 450 to 490 nm filter for fluorescein and a 546/12 nm filter for rhodamine—to select the appropriate wavelengths of excitation for the two probes. After passing through the excitation filter, the excitation light encounters a dichroic beam-splitting mirror (DM)—comprised of a DM 510 nm mirror for fluorescein and a DM 580 nm mirror for rhodamine. These dichroic mirrors will reflect light below 510 nm and 580 nm, respectively, into the back focal plane of the objective and then to the sample. The emissions of fluorescein and rhodamine are then isolated using an emission filter—comprised of a long-pass (LP) 520 nm and an LP 590 nm filter. While these filter units work to enable observation of fluorescein and rhodamine independently in the same sample, they do not correct for the following possibility (which actually occurs). Some excitation of the rhodamine can occur at the wavelengths of excitation of fluorescein, and a small portion of the emission spectrum of fluorescein overlaps a portion of the emission spectrum of rhodamine. To overcome this problem, emission

interference filters—515 to 565 nm for fluorescein and LP 610 nm for rhodamine—can be employed to visually isolate, specifically, the emission of fluorescein from rhodamine.

There are other types of filters that are employed in fluorescence microscopy—i.e., heat and neutral-density filters. Heat filters are used to protect the excitation filters and detectors from infrared radiation. They are placed in front of the light source to reduce heat transfer to the excitation interference filter and can also be used to reduce the UV excitation intensity. Neutral-density filters reduce the intensity of all wavelengths of the exciting light equally, and are commonly used for reducing the intensity of the exciting light to prevent photobleaching of fluorophores and photodamage of living cells and tissue. The neutral-density filter should be interposed between the heat filter and excitation filter.

Objectives

In fluorescence microscopy, the microscope objective serves the functions of a condenser, a magnifier, and a collector of the emitted fluorescence. In epifluorescence microscopy, reflected-light objectives have been developed, which feature specially coated glass surfaces to avoid reflection in the optics (“antireflection coating”). Most (if not all) objectives in use today are infinity-focused objectives. In infinity-focused objectives, light rays emanating from any point in the specimen are parallel between the objective and the eyepiece. The

advantage of infinity-designed objectives is their insensitivity to other components introduced in the beam path (e.g., filters).

When selecting an objective for use in fluorescence microscopy, it is important to remember that objectives differ in magnification, light-gathering ability (numerical aperture or NA), wavelengths of light that they transmit, immersion medium required, and the specific applications for which they were developed. Table 4.2.1 lists some current types of objectives and their special properties.

Numerical aperture

In most biological applications involving living cells, it is important to collect as much of the emitted fluorescence as possible. Employing the largest NA possible will allow maximal collection of emitted fluorescence. NA (where $NA = \eta \sin \alpha$, η being the refractive index of the medium between the specimen and the objective) is defined as the angle, α , of the emitted fluorescence given off from a fluorophore relative to the original direction of the exciting light (Fig. 4.2.4). Objectives that are capable of collecting light over a large angle α will provide brighter images. Such objectives are said to possess a large NA. However, there is a limit to the size of the NA, resulting from a mismatch between the refractive indices of the medium (air) between the front surface of the objective and the coverslip. To increase the NA of the objective, a better match between the refractive indices of the front surface of the

Table 4.2.1 Properties of Objective Lenses^a

Lens	Properties	Uses
Plan achromat	Flat field; color-corrected; short working distance	Routine fluorescence
Ultrafluor ^b	Broad spectral transmission (200-700 nm)	Calcium measurements with Fura-2
Quartz	UV transmission	UV fluorescence
Multi-immersion	For use with or without coverslip	Low-magnification, high-NA applications
Water-immersion	High NA	Live cell and tissue physiology
Achromat	Low-cost; partially color-corrected	Phase-contrast microscopy
Plan/Epiplan ^b	Good image contrast; flat field; long working distance	Photomicroscopy
Plan/Epiplan Neofluor ^b	Flat field; corrected for spherical and chromatic errors over broad range of visible spectrum	Calcium measurements with Fura-2

^aAbbreviation: NA, numerical aperture.

^bUltrafluor, Neofluor, and Epiplan are trademarks of Carl Zeiss, Inc.

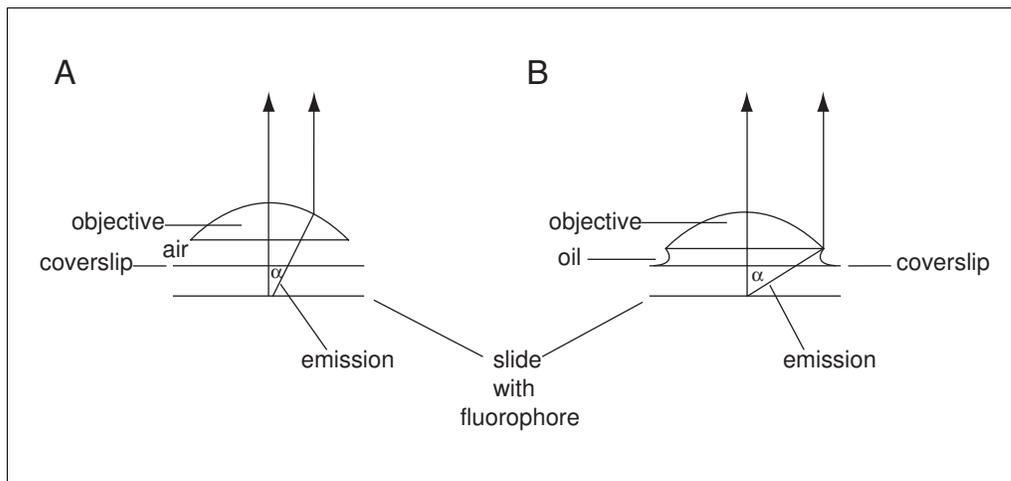


Figure 4.2.4 The numerical aperture (NA) is a measure of the light-collecting ability of an objective lens. The larger the NA, the greater the quantity of light collected by the objective lens. Larger NA is associated with shorter working distance of the objective lens (distance between the front of the objective lens and the sample). Since NA is proportional to the refractive index (n) of the medium between the specimen and the objective, matching the refractive indices of the objective lens and the sample results in a larger NA and collection of more of the emitted fluorescence. Compare (A), representing an objective without oil immersion and (B), representing an objective with oil immersion to match the refractive indices of lens and sample. Note the shorter working distance and larger angle (α) between the excitation and emission beams in B.

objective and cover glass must be made. This is done by placing immersion oil between the front lens of the objective and the coverslip. The n of both glass and immersion oil is 1.515, thereby eliminating all reflections on the path from the object to the objective and increasing the NA of the objective as well as the amount of light that can be captured by the objective.

Resolution

Resolution (R) is defined as the smallest distance (d) between two small objects that still can be discerned as two separate objects. $R = 0.61(\lambda/NA)$, where λ is the wavelength of light and NA is the numerical aperture of the objective lens. Because R is determined by the wavelength of the light and the objective NA, objectives with higher NA values result in smaller d (i.e., greater resolving power). Shorter wavelengths are also beneficial for increasing objective resolving power.

Magnification

Magnification is defined as the power to cause objects to appear larger than they are. The useful range of overall magnification of a microscope should be >500 times but <1000 times the objective aperture. The magnification and NA of an objective are related to each other in such a way that the brightness of the image

decreases as the magnification increases. In the typical fluorescence microscope employing epiillumination, the objective lens serves both as a condenser and an objective. This results in the light intensity (brightness) being proportional to $(NA)^4$. Brightness is also related to the magnification but in the opposite direction—i.e., brightness is proportional to $1/(\text{Mag})^2$. Therefore, the overall brightness is proportional to $(NA)^4/(\text{Mag})^2$. Thus, when performing fluorescence microscopy, it is important to use the highest NA and the lowest overall magnification objective possible.

Optimizing image brightness and resolution

Optimizing image brightness involves the following considerations.

1. The sample should be supplied with sufficient light energy for excitation (but not photobleaching) at the appropriate (fluorophore-dependent) wavelength.
2. The emitted fluorescence should be observed without any contamination from the excitation light.
3. The light source must provide a large amount of excitation energy in very narrow ranges of the spectrum.
4. Appropriate filters that transmit the required wavelengths but block the unrequired ones should be employed.

5. The objectives should have high transmission from the UV through a large part of the visible spectrum.

6. Objectives with the highest NA should be employed. A good rule of thumb is that if the objective aperture is doubled in size, approximately four times more fluorescence light can be gathered.

7. Nonautofluorescent, PCB-free immersion oil with the proper refractive index ($n = 1.51$), to eliminate light loss caused by light reflection on surfaces, should be used.

8. None of the optical components of the microscope should be autofluorescent. Autofluorescence will increase the brightness of the background and reduce the contrast in the image.

9. The objective lenses must be clean.

10. Coverslips of the appropriate thickness (0.17 mm) should be used. For objectives with $NA > 0.7$, the thickness of the coverslip can vary ± 0.01 mm (from 0.17 mm) and still provide a high-resolution image. When the NA of the objective is between 0.3 and 0.7, coverslip thickness can vary by ± 0.03 mm and still provide high-resolution images.

11. Air bubbles must be avoided in the immersion-oil layer.

12. To improve contrast, the field diaphragm should be closed down.

Cleaning the microscope optics

The following are some general rules that should always be followed when working with any optical microscope to maintain optimal performance.

1. Always work in as clean an environment as possible.

2. Avoid dust at all costs.

3. Keep the microscope covered at all times when not in use.

4. Store all microscope components in appropriate containers and avoid salt water, corrosive solvents, and all solvents if possible.

5. Clean the microscope thoroughly after each use.

6. Do not smoke.

7. Before cleaning an objective lens, directly inspect the lens using an inverted ocular from the microscope as a magnifier in room light. It is also possible to inspect the objective lens by placing the lens under a dissecting microscope and focusing on the lens to observe any imperfections.

8. Use oil only on lenses designed to be used with oil. It is a tedious process to clean immer-

sion oil off a dry lens so as not to damage the lens.

9. Properly store objectives that are not in use in the container that they came in.

The first rule of cleaning microscope optics is do not touch the lens surface if at all possible, not even with lens paper. Dust particles in the air and even components of cleaning tissues can contain abrasive materials that will scratch the lens surface. The best way to clean an objective lens is as follows.

1. Soak a previously unused Q-tip in any of the following solvents: a mixture of 55% acetone/30% ether/15% ethanol; pure alcohol; the glass cleaner Sparkle; distilled H₂O; lighter fluid; or a well-shaken 1:1:1 mixture of chloroform/alcohol/water. Avoid benzene, toluene and xylene. The organic solvents are usually more effective, but bear in mind that some of these solvents are carcinogens and flammable and need to be used in a well-ventilated environment with no nearby flames (i.e., no smoking).

2. Very gently roll the solvent-laden Q-tip over the surface of the lens once.

3. Take another fresh Q-tip dipped in solvent and pass it over the lens very gently, barely touching the lens surface. Another option is to place a drop of solvent onto the objective lens (without directly touching the lens), and then gently blot the drop of solvent up using lens paper, but not touching the lens directly.

If you are using oil-immersion objectives and scanning a number of slides, it is not necessary to clean the objective after each slide. Rather, wipe off excess oil with lens paper and place the new slide (with a drop of oil on it) onto the objective so that the oil on the slide and the residual oil on the objective meet without producing air bubbles.

THE DIGITAL DARKROOM

Photographs of images viewed through the microscope have long been the preferred means of disseminating microscopic information. This has historically meant using film as the medium for recording images. However, the use of computers for acquisition and display of images, along with the explosion of the World Wide Web (WWW), has led to the rapid expansion of the use of computer-based image analysis and presentation programs for production of permanent hard-copy microscopic images. Digital imaging provides the user with great latitude with respect to speed of image acquisition and data presentation. While it is a great improvement over film, the use of computer-

based image-analysis and presentation software provides a ready means for altering image information, and care must be taken to maintain the original data in the final image. There are also other problems with electronic image presentation—e.g., there often exists incompatibility between the data formats of the software used for image acquisition and presentation, and the electronic components themselves can induce distortion of the original image information.

The overall design of a typical digital darkroom for fluorescence microscopy is as follows. A one- or two-dimensional detector (e.g., various cameras, photomultiplier tubes, or photodiode arrays) is attached to the microscope port at the in-focus plane of image formation. The image formed by the microscope is recorded by the detector, whose output is sent to a computer. The computer may require specific hardware to receive the image from the camera as well as a substantial amount of fast access RAM and hard disk space to store the image(s). Since images contain a large amount of information (and therefore require huge amounts of storage space), a number of storage media can be employed to store microscopic images for subsequent display and processing in digital format. These include optical memory disk recorder (OMDR), computer floppy disks, Zip drives, Jazz drives, and CD-ROM disks, as well as hard drives, laser disks, and optical disks.

Once in computer memory or on the computer hard disk, a number of software programs (e.g., Image-1, Photoshop, Adobe Illustrator, Designer, and Corel Draw) can be used to create publication-quality images. These programs allow a number of operations, some of which include sharpening or blurring the image, removal of background and/or nonspecific noise, pseudocoloring, creating montages, and labeling of the figure. A word of caution is required regarding these programs. It may be the case that the input/output file formats used by these programs are not compatible with other image presentation-programs. As a general consideration, image-processing software is most efficient and useful if it contains the flexibility of user-defined parameters and multiple data and file formats. It is very advantageous to be able to generate digital data sets for computer-based processing in a uniform format that is easily understood by the variety of image analysis/processing software currently available. The tagged image file format (TIFF) is one such universal file format. Other important needs for

software used for presentation of microscopic images include bitmap manipulation capabilities to allow adjustment of the printed color image so that it matches the image seen on the computer monitor. For printing color images, another important feature is the ability to print in both CMYK and RGB format. It is also necessary to make sure that, if the software uses PostScript drivers for color printing, the PostScript color interpreter exploits the full ability of the printing objects.

Once completed, a publication-quality hard copy of the computer image can then be produced using a number of techniques. These include photographic printing and color copiers as well as a variety of types of computer printers—e.g. color or black-and-white laser, ink-jet (which are becoming quite good and cost-effective), and dye-sublimation (which require special paper but have higher resolution and better color rendition). LCD projectors, which allow display of computer acquired/processed images directly from the computer, are also available.

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Immunofluorescence Staining

UNIT 4.3

This unit describes the widely used and powerful technique of localization of proteins in cells by immunofluorescence (see Basic Protocol). The location can be determined by double labeling with an antibody directed against a protein of known location. The technique can be used as a supplement to immunolocalization by electron microscopy and subcellular fractionation (Chapter 3). It allows not only identification of the antigen distribution in the cell, but also a survey of the dynamic aspects of protein movements in the cell—on and off membranes, into and out of the nucleus, and through membrane traffic pathways.

IMMUNOFLUORESCENCE LABELING OF CULTURED CELLS

**BASIC
PROTOCOL**

The following is a basic “generic” method for localizing proteins and other antigens by indirect immunofluorescence. The method relies on proper fixation of cells to retain cellular distribution of antigen and to preserve cellular morphology. After fixation, the cells are exposed to the primary antibody directed against the protein of interest, in the presence of permeabilizing reagents to ensure antibody access to the epitope. Following incubation with the primary antibody, the unbound antibody is removed and the bound primary antibody is then labeled by incubation with a fluorescently tagged secondary antibody directed against the primary antibody host species. For example, incubation with a mouse IgG primary antibody might be followed by incubation with a RITC (rhodamine isothiocyanate)-labeled goat anti-mouse IgG secondary antibody. After removal of the secondary antibody, the specimen is ready for viewing on the fluorescence microscope. Once the conditions for observing specific immunolocalization have been identified for a given antibody and cell type, double labeling with two antibodies can be employed to compare localizations. To do this, primary antibody incubation can contain two antibodies generated in two species (e.g., mouse and rabbit), followed by incubation with two secondary antibodies coupled to different fluorophores. Care should be taken, however, that the two antibody combinations, especially the secondary antibodies, do not cross-react.

Materials

- Cells of interest, growing in tissue culture
- 2% formaldehyde (see recipe)
- Phosphate-buffered saline (PBS; see recipe), pH 7.4
- PBS/FBS: PBS, pH 7.4, containing 10% fetal bovine serum (FBS)
- 0.1% (w/v) saponin in PBS/FBS: prepare fresh from 10% (w/v) saponin stock solution (*APPENDIX 2A*; store stock up to 2 months at 4°C or in aliquots up to 1 to 2 years at -20°C)
- Primary antibody
- Controls: preimmune serum (if using rabbit polyclonal antibody) or antigen added in excess to primary antibody
- Secondary antibodies (against Ig of species from which primary antibody was obtained) coupled to fluorophore: e.g., RITC (rhodamine isothiocyanate), FITC (fluorescence isothiocyanate) Cy3, or Texas Red
- Mounting medium (see recipe)
- 10-cm diameter tissue culture dishes
- 12-mm no. 1 round glass coverslips, sterilized by autoclaving or soaking in 70% ethanol
- 12-well tissue culture plates
- 150-mm petri dishes

Microscopy

Contributed by Julie G. Donaldson

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4.3.1

Watchmaker's forceps
Microscope slides
Nail polish
Fluorescence microscope with 63× oil-immersion lens

Additional reagents and equipment for trypsinization of cells (*UNIT 1.1*)

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

1. For adherent cells, 1 to 2 days prior to experiment trypsinize cells and seed onto 10-cm culture dishes, each containing 15 to 20 sterilized coverslips, so that on day of experiment cells are 20% to 50% confluent.

Nonadherent cells can be coaxed into adhering to the coverslips by precoating the coverslip with poly-L-lysine (see recipe). Apply 10 to 20 μl of suspended cells to each coverslip, let sit 10 min, then proceed with fixation (step 3).

Alternatively, cells can be attached to coverslips using a cytocentrifuge by following the manufacturer's instructions.

2. On day of experiment, transfer each coverslip individually to a well of a 12-well tissue culture dish containing 1 ml culture medium. Subject cells to the desired experimental conditions (e.g., treat with various drugs, inhibitors, or temperatures prior to fixation and immunostaining).
3. Aspirate medium and add 1 ml of 2% formaldehyde to each well. Allow cells to fix at room temperature for 10 min.
4. Aspirate the formaldehyde fixative and wash coverslips twice, each time by adding 1 ml PBS, pH 7.4, letting stand 5 min, then aspirating the PBS. Add 1 ml PBS/FBS to the fixed coverslips and let stand 10 to 20 min to block nonspecific sites of antibody adsorption.

NOTE: Throughout the procedure, do not let cells dry out.

5. In 1.5-ml microcentrifuge tubes dilute primary antibodies in 0.1% saponin/PBS/FBS.

Typically affinity-purified antibodies are diluted in the range of 1 to 10 μg/ml, and rabbit antisera are diluted between 1:100 and 1:1000. If using a commercial antibody, follow suggested dilutions from manufacturer.

During initial characterization, it is wise to try a range of dilutions of antibody.

6. Prepare controls containing only 0.1% saponin/PBS/FBS or (if available) containing preimmune antiserum (if rabbit polyclonal antibody is being used) or specific (primary) antibody with the antigen added in excess.

Controls are often the most important part of an immunofluorescence experiment.

7. Microcentrifuge antibody dilutions and control solutions 5 min at maximum speed, room temperature, to bring down aggregates in pellet.

Pipet the antibody solution from above the aggregate pellet.

8. Place a 10 × 10-cm piece of Parafilm in the bottom of a 150-mm petri dish. In a grid pattern that replicates the 12 wells used to incubate the coverslips, label the appropriate place on the Parafilm for each coverslip with a marker.

9. Apply a 25- μ l drop of appropriate primary antibody solution to each numbered section. Carefully remove each coverslip from the 12-well plate with watchmaker's forceps, blot the excess fluid by touching the edge to a Kimwipe, then invert the coverslip over the appropriate 25- μ l drop, making sure that the side with the cells is down. Place the top on the petri dish and incubate 1 hr at room temperature.

Sometimes proper labeling will require a longer incubation time or the petri dish will be incubated >1 hr for convenience. For longer incubations, add some wetted Kimwipes to the dish to maintain a humid atmosphere. The incubation can be extended overnight at 4°C, if necessary.

NOTE: It is important to always be aware of which side of the coverslip the cells are on. Cells should be facing up when in the 12-well plate, but facing down when placed on the antibody. Picking up coverslips with the forceps is awkward at first but becomes easier with practice.

10. Carefully pick up each inverted coverslip and flip it over so that it is cell-side-up, then place in a well of a 12-well plate. Wash each coverslip three times to remove unbound antibody, each time by adding 1 ml PBS/FBS, letting stand 5 min, then aspirating the solution.

11. Dilute fluorophore-conjugated secondary antibodies in 0.1% saponin/PBS/FBS. Mix, then microcentrifuge as in step 7 to remove aggregates.

Typically, commercial preparations are diluted between 1:100 and 1:500.

12. Prepare an incubation chamber as in step 8. Apply 25 μ l of appropriate secondary antibody solution to each numbered section and invert coverslip over drop as in step 8. Cover petri dish and protect from light with aluminum foil or place chamber in drawer. Incubate 1 hr at room temperature.

13. Wash coverslips as in step 10. After removal of last PBS/FBS wash, add 1 ml PBS.

14. Label slides and place 1 drop of mounting medium onto slide. Pick up coverslip from well, gently blot off excess PBS by touching the edge to a Kimwipe, then invert coverslip, cell-side-down, onto drop. Gently blot mounted coverslip with paper towel, then seal edge of coverslip onto slide by painting the edge with a rim of nail polish. Let dry.

The fixed, mounted, and nail polish-sealed coverslips can be stored in the dark for 6 months to 1 year at 4°C.

15. View specimen on fluorescence microscope using an 63 \times oil immersion objective.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Formaldehyde, 2%

In a chemical fume hood, dilute 2 ml 37% reagent-grade formaldehyde into 35 ml PBS (see recipe), pH 7.4.

As reagent-grade formaldehyde contains 11% methanol, as an alternative (or if necessary) make up formaldehyde from paraformaldehyde by dissolving 0.4 g paraformaldehyde powder in 10 ml H₂O that has been heated to 60°C, then diluting 1:1 with 2 \times PBS, pH 7.4. It might be desirable to try both procedures for preparing the formaldehyde solution and see which gives better results.

Mounting medium

Use Fluormount G (Southern Biotechnology) or prepare 50% (w/v) glycerol and 0.1% (w/v) *p*-phenylenediamine in PBS (see recipe), pH 8.0.

Phosphate-buffered saline (PBS)

0.144 g KH_2PO_4

9.0 g NaCl

0.795 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$

H_2O to 1 liter

Adjust to desired pH with 1 M NaOH or 1 M HCl

Store indefinitely at room temperature

Poly-L-lysine-coated coverslips

Apply 25 μl of 1 mg/ml poly-L-lysine to each sterilized no. 1 coverslip in a hood and allow to stand \sim 10 min. Carefully rinse coverslips three times with water, then allow to air dry.

COMMENTARY

Background Information

The first use of fluorescently labeled antibodies to localize a protein in cells occurred over 50 years ago (see Coons, 1961, for a reminiscence). Since that time, the wide availability of numerous antibodies and improvements in indirect labeling methods and fluorophores has made immunofluorescent localization of proteins in cells both a routine and a vital component in any study. Immunofluorescence labeling is quite effective when combined with biochemical or ultrastructural studies because the technique is rapid and so many parameters can be assessed. Furthermore, in contrast to biochemical studies, which assume uniformity of the sample, immunofluorescence technique allows analysis of individual cell differences.

Immunofluorescence labeling has been employed in a variety of cell-biological studies, including the first description of peptide targeting sequences specifying retention of endoplasmic reticulum (ER) luminal proteins (Munro and Pelham, 1987) and initial studies describing the dynamic membrane trafficking between the ER and Golgi complex (Lippincott-Schwartz et al., 1990). The first study describing the family of Rab GTPases used immunofluorescence to demonstrate distinct localization of the different Rabs to different organelles in the cell (Chavrier et al., 1990). Finally, immunofluorescent localization of proteins encoded by novel genes, including those associated with human diseases and cancer (Nathke et al., 1996), will provide critical information for determining the cellular function of these proteins.

Critical Parameters

To ensure success with immunofluorescence, three parameters are critical—fixation, permeabilization, and determination of the specificity of labeling. The fixation and permeabi-

lization conditions must be assessed individually for each antibody and each cell type investigated. Refer to Griffiths (1993) for an excellent discussion on fixation and issues related to specificity of antibody labeling.

Different fixatives might be investigated to optimize preservation of the antigen, its distribution, and the morphology of other cellular constituents. For example, some epitopes are lost upon aldehyde fixation but preserved with alcohol fixation, and vice versa. Alternative fixatives to try include methanol at -20°C (5 min exposure) or formaldehyde fixation followed by a brief (1-min) exposure to methanol at 0°C . Methanol fixation is often quite effective for localizing cytoskeletal elements. Alcohols work by extracting lipids and precipitating remaining proteins, whereas aldehydes are cross-linking reagents that generally preserve membranous structures better (McCaffery and Farquhar, 1995).

Sometimes, even when material is appropriately fixed, the epitope is obscured and not accessible to antibody binding. Permeabilizing reagents are typically detergents that partially denature fixed proteins, exposing the epitope. In addition to saponin, which the authors include throughout the staining procedure, treatments after fixation with 0.2% to 0.5% Triton X-100 or SDS can also be tried. Often a short treatment with these reagents prior to antibody incubations is sufficient to expose the epitope. Special attention should be directed toward ensuring that the access to the epitope is the same even if the protein has undergone a translocation; for example proteins that shuttle between the cytoplasm and nucleus are not always equally accessible to antibody labeling (Pines, 1997).

The primary antibody should be purified to the extent that it recognizes only the protein of interest on immunoblots. Although affinity pu-

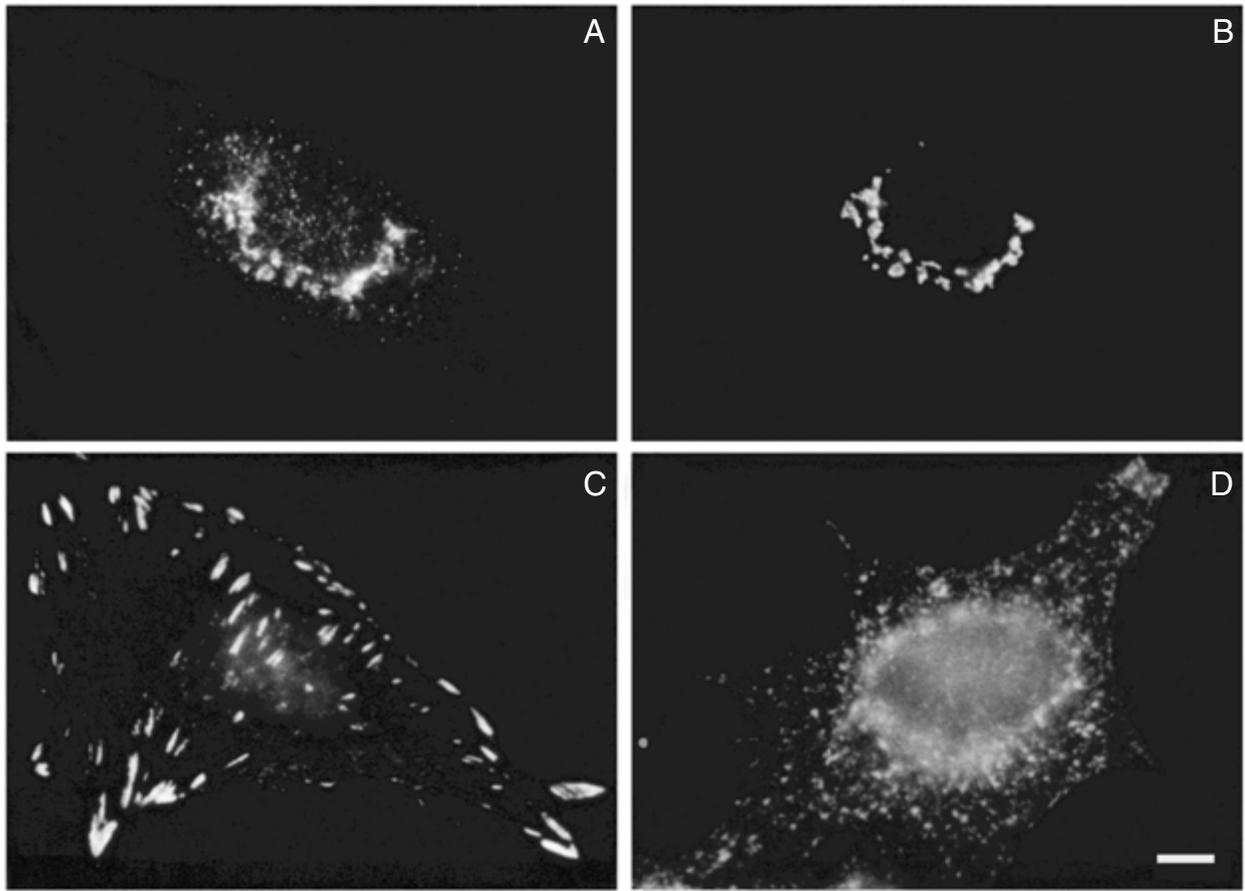


Figure 4.3.1 Examples of immunofluorescence labeling of formaldehyde-fixed cells. **(A)** and **(B)** Double labeling of a normal rat kidney cell with a mouse monoclonal antibody to **(A)** the β -COP component of coatamer and **(B)** a rabbit polyclonal antibody to mannosidase II. **(C)** Distribution of vinculin in a formaldehyde-fixed normal rat kidney cell using a mouse monoclonal antibody. **(D)** Distribution of transferrin receptor in formaldehyde-fixed HeLa cells using a mouse monoclonal antibody. Bar is equal to 10 μ m.

rification often resolves this, be aware that “affinity-purified” antibodies do not always result in antibodies that recognize only a single protein. Especially in the case of anti-peptide antibodies, multiple bands are sometimes labeled even after affinity purification. By immunoblotting on a gel (UNIT 6.2), it is easy to determine on the basis of molecular weight whether it is the protein of interest that is recognized. However, not all antibodies that can be used to detect a specific protein by immunoblotting work for immunofluorescence detection. Likewise, there are antibodies that recognize the protein by immunofluorescence that do not recognize the separated, denatured proteins transferred to nitrocellulose.

Next, it is important to be able to determine what constitutes “specific labeling” with the antibody. To assess this, control-stained slides (incubated with secondary antibody only or

with preimmune serum) should first be viewed to determine what constitutes “nonspecific staining.” Ideally this is negligible, and, by contrast, the staining pattern obtained with the specific antibody is much brighter and more distinct. When initially characterizing immunolocalization using a particular antibody, a range of dilutions of the primary and secondary antibodies should be examined to determine optimal concentrations that minimize nonspecific staining and maximize specific staining. Minimal background staining from secondary antibodies can often be achieved by dilution or trying different secondary antibodies made in different host species or obtained from different commercial suppliers. Then, a dilution of the primary antibody is selected to optimally label the specific protein. At this point, further controls should be analyzed to ensure that the staining observed is due to the presence of the protein of

interest. If a rabbit antiserum is used, preimmune serum should yield a pattern of staining similar to that of the secondary antibody alone. If the immunizing antigen is available, it can be added to the antibody dilution to compete for the specific staining; and it should yield “background” levels of fluorescence. Lastly, support for specific labeling can be confirmed by lack of staining in a cell type that does not express the protein of interest and increased labeling in cells overexpressing the protein by transient transfection. Finally, the use of different antibodies to the protein, if available, can independently confirm the localization pattern.

Troubleshooting

If background staining with secondary antibodies is too high, there are several possible remedies. The secondary antibodies may be diluted further, or different secondary antibodies, different hosts, or different fluorophores may be tried. Also, it is possible to try other “sorbing” reagents. PBS/FBS is generally a good sorbing reagent that effectively blocks out nonspecific sites, but other agents can be used—e.g., 1% (w/v) BSA (immunoglobulin-free) or gelatin.

If no specific staining is observed, it is possible to increase the concentration of or the time of exposure to the primary antibody. One can also try alternative fixation and permeabilization regimens to visualize specific staining.

If specific staining is observed, but it is very dim, it is possible to increase the concentration of the primary and/or secondary antibodies. As long as background staining is low, the signal can be enhanced by using a fluorescence double-sandwich technique. To do this, following incubation with primary antibody (e.g., a mouse monoclonal antibody) and washing, incubate first in FITC-conjugated goat anti-mouse IgG and then in FITC-conjugated donkey anti-goat IgG. Caution should be observed that secondary antibodies do not alter the pattern of single antibody labeling.

Anticipated Results

Once conditions for specific localization have been optimized, the distribution of the protein in a variety of cells under a variety of conditions can be observed (see Fig. 4.3.1). Double labeling of two antigens allows the distribution of one protein to be compared with that of another protein in the same sample using two secondary antibodies attached to fluorophores that can be

monitored in two different channels by flow cytometry. Thus, the location of a known protein, say an antibody to a Golgi-resident protein, can be used as a marker for the Golgi complex to see whether the antigen under study colocalizes with it (Fig. 4.3.1, panels A and B).

Time Considerations

The entire procedure can be performed in ~3 hr. Fixed, washed coverslips can be stored at 4°C for several days prior to immunolabeling. Incubations with primary antibodies can be extended to overnight at 4°C if necessary (or if it is desirable to increase staining).

Disclaimer

This article was written by Julie Donaldson in her private capacity. No official support or endorsement by the NHLBI or NIH is intended and none should be inferred.

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Fluorescent Staining of Subcellular Organelles: ER, Golgi Complex, and Mitochondria

The ability to distinguish and identify different subcellular compartments is crucial for understanding organelle function, biogenesis, and maintenance within cells, as well as for defining protein trafficking pathways. This unit describes protocols for labeling three subcellular organelles—endoplasmic reticulum (ER), Golgi complex, and mitochondria—using fluorescent dyes and fluorescently labeled lipid derivatives. Many fluorescent lipid derivatives have been shown to preferentially partition into membrane compartments of living cells. This finding has been used to generate protocols for fluorescently labeling specific intracellular organelles *in vivo*. Such protocols have been enormously valuable for enabling researchers to identify different organelles and to study their dynamics and spatial organization within cells (Terasaki et al., 1984; Cooper et al., 1990; Pagano et al., 1991).

The unit first describes how to stain the endoplasmic reticulum (ER) by using the fluorescent dye DiOC₆(3). This dye can be used to stain fixed cells (see Basic Protocol 1) or living cells (see Alternate Protocol), and the protocols are very simple. Since the dye stains many other organelles in addition to the ER, DiOC₆(3) is particularly useful for staining the thin peripheral regions of cultured cells, where it is usually easy to distinguish the ER on the basis of morphology.

Fluorescent analogs of ceramide, including *N*-[7-(4-nitrobenzo-2-oxa-1,3-dizole)]-6-aminocaproyl *D*-erythrosphingosine (C₆-NBD-Cer) and *N*-[5-(5,7-dimethyl BODIPY)-1-pentanoyl]-*D*-erythrosphingosine (BODIPY-Cer), have proved to be particularly valuable for labeling the Golgi complex (reviewed in Pagano, 1989), which receives, processes, and sorts newly synthesized proteins exported from the ER. These dyes readily accumulate in the Golgi complex of most cell types by a preferential membrane partitioning process (Lipsky and Pagano, 1983, 1985a; Pagano et al., 1989). They can be used to image the Golgi complex in fixed or living cells (see Basic Protocol 2).

Mitochondria are unique among intracellular organelles in that they have a large membrane potential, which is negative inside. This characteristic makes it relatively easy to specifically stain mitochondria in living cells. Many fluorescent lipophilic cationic molecules can be used. These molecules pass through the plasma membrane by their lipophilicity and then accumulate in the mitochondria as a result of the attraction of the plus charge on the molecule to the high negative mitochondrial membrane potential. This unit describes the use of TMRE (tetramethylrhodamine, ethyl ester) to stain mitochondria in mammalian cells in culture (see Basic Protocol 3). Accumulation of this dye follows the Nernst equation and is therefore independent of other factors such as binding to mitochondrial components (Ehrenberg et al., 1988; Farkas et al., 1989). This establishes TMRE as a good monitor for the mitochondrial membrane potential (Loew et al., 1993). TMRE has an additional advantage in that live cell staining is accomplished simply by equilibrating cells with the dye, then mounting them for observation in the presence of the dye. Other fluorescent dyes are available for investigating mitochondrial properties in interesting ways and are discussed briefly in the Commentary.

STAINING THE ENDOPLASMIC RETICULUM IN FIXED CELLS

In this protocol, cells are fixed in glutaraldehyde for 3 min, stained with the fluorescent dye DiOC₆(3) for 10 sec, then mounted in dye-free buffer. Live cell staining is accomplished by mounting cells directly in the fluorescent dye (see Alternate Protocol).

Cells growing on coverslips are handled in a similar way in this protocol as for immunofluorescence, with two major differences. One is that the cells must be kept away from detergents, organic solvents, and nail polish, since these will extract DiOC₆(3). The second is that the time scale is much shorter.

Materials

Fixative: prepare 0.25% glutaraldehyde in PBS (*APPENDIX 2A*) from 7% to 70% commercial stock solution (store up to 1 week at 4°C)

Cells of interest, growing on coverslips

2.5 µg/ml DiOC₆(3) working solution (see recipe)

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Silicone high-vacuum grease

Small petri dish

Watchmaker's forceps

Microscope slides

Silicon rubber chamber (see recipe)

Fluorescence microscope with fluorescein filters and either 63× or 100× oil-immersion objective (*UNIT 4.2*)

Additional reagents and equipment for fluorescence microscopy (*UNIT 4.2*)

1. Put some fixative in a small empty petri dish. Using watchmaker's forceps, remove coverslip containing cells from culture dish and put it into the fixative. Let cells fix 3 to 5 min.
2. Remove coverslip from fixative and place cell-side-up on a piece of Parafilm. Remove excess fixative using a Pasteur pipet, but do not let the coverslip dry out.
3. Apply enough 2.5 µg/ml DiOC₆(3) working solution to cover the coverslip.
A volume of 100 µl should be sufficient for most coverslip sizes.
4. After ~10 sec, remove the DiOC₆(3) solution with a Pasteur pipet, then apply sufficient PBS to cover the coverslip.
5. Grease both sides of a silicon rubber chamber sparingly with silicone high-vacuum grease. Press the greased chamber firmly on a microscope slide, avoiding air bubbles. Fill the well with DiOC₆(3) working solution, then place the coverslip, cell-side-down, on the chamber. Press down lightly on the coverslip to make contact between the glass and the silicon rubber and make a seal that keeps the coverslip adherent to the chamber (Fig. 4.4.1). Dry off excess fluid with a Kimwipe.

One way to do this is to hold the Pasteur pipet used for filling the chamber in the left hand and hold the coverslip with watchmaker's forceps in the right hand.

Use a minimal amount of grease, since this can be very difficult to remove if it gets onto the microscope objective lens. For short-term observations it is possible to mount coverslips without the silicone grease.

An alternative method (not involving a silicon rubber chamber) is to gently place the coverslip cell-side-down directly on a microscope slide with a minimum of bubbles. Carefully dry off excess fluid at the sides and especially on top of the coverslip. Sealing the sides is not required for short-term observations. Do not let the microscope immersion oil

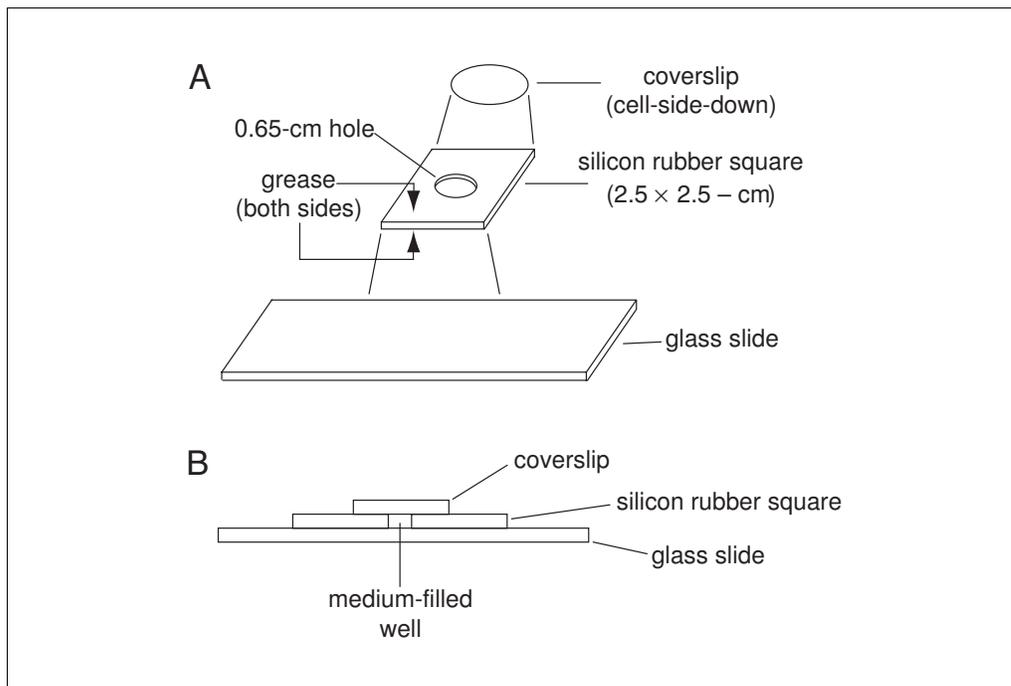


Figure 4.4.1 Microscope slide with silicon rubber chamber. **(A)** Assembly; **(B)** side view in cross-section.

leak underneath the coverslip. Also, be aware that the coverslip will start to dry out from the edges, and that dried cells are not suitable for viewing. If sealing of the coverslip is desired, use paraffin or Valap (UNIT 13.1), not nail polish, because the organic solvents in the nail polish will extract the stain.

6. Observe cells using fluorescence microscope with fluorescein filters and oil-immersion 63× or 100× objective lens.

The results are best observed during the first 10 to 20 min after staining. Afterwards, the dye starts to accumulate in lysosomes. After longer periods of time, autofluorescence from glutaraldehyde begins to obscure the staining.

STAINING THE ENDOPLASMIC RETICULUM IN LIVING CELLS

The ER of cells that have not been fixed can also be labeled with DiOC₆(3).

Additional Materials (also see Basic Protocol 1)

0.5 μg/ml DiOC₆(3) working solution in growth medium appropriate for cells (see recipe)

1. Grease both sides of silicon rubber chamber sparingly with silicone high-vacuum grease and press onto microscope slide.

The silicon rubber chamber does not need to be sterilized for short-term observations of a few hours.

2. Using watchmaker's forceps, remove coverslip containing cells from culture dish. Fill chamber with 0.5 μg/ml DiOC₆(6) working solution, then put coverslip cell-side-down on chamber. Press down carefully on the coverslip to make a seal that keeps the coverslip adherent to the chamber, and dry off excess fluid with a Kimwipe.

If a large bubble is seen in the chamber, remount the coverslip. If a chamber with temperature control and ability to perfuse is desired, see Basic Protocol 3, introduction, for information on live cell chamber.

ALTERNATE PROTOCOL

Microscopy

4.4.3

3. Observe cells using fluorescence microscope with fluorescein filters and oil-immersion 63× or 100× objective lens.

It should take 5 to 10 min for ER staining to develop. The mitochondria are usually swollen at dye concentrations that stain the ER. After 10 min, if only the mitochondria are stained, try 1.0 µg/ml DiOC₆(3) staining solution. If the cells look sick and are starting to detach from the coverslip, use a lower concentration of dye. To stain only the mitochondria with DiOC₆(3), use 0.1 µg/ml.

If photodynamic damage or bleaching is hindering the experiments, scavenge the oxygen in the medium with Oxyrase, a bacterial enzyme system. The enzyme is used at 0.3 U/ml (1:100 dilution of enzyme as purchased from Oxyrase, Inc.; Waterman-Storer et al., 1993).

BASIC PROTOCOL 2

STAINING THE GOLGI COMPLEX IN LIVING CELLS

Vital staining of the Golgi complex with C₆-NBD-Cer or BODIPY-Cer involves incubation of cells with either of these fluorescent lipids at low temperature, followed by washing. The ER, nuclear envelope, and mitochondria are initially fluorescent, but over time the Golgi complex begins to fluoresce as a result of preferential insertion of the fluorescent lipids into its membranes. Upon warming the cells to 37°C, intense labeling of the Golgi complex occurs, but only for a finite period of time. This is because the ceramide derivatives within the Golgi complex are metabolized at 37°C to the corresponding fluorescent analogs of sphingomyelin and glucosylceramide. These traffic to the cell surface, causing fluorescent labeling of the plasma membrane over time. Fluorescent labeling of the cell surface can be reduced by incubating cells in medium containing nonfluorescent liposomes or defatted BSA. This results in “back-exchange” of fluorescent sphingomyelin and glucosylceramide into the medium (Lipsky and Pagano, 1985b; van Meer et al., 1987). Labeled cells can be fixed and further processed for double labeling with fluorescently tagged antibodies (see *UNIT 4.3*), to compare the localization of the Golgi complex with other markers within the cell.

Golgi membranes labeled with the fluorescent ceramide derivatives are viewed on a fluorescent microscope equipped with filter combinations appropriate for fluorescein. BODIPY-Cer can also be visualized with filter combinations appropriate for rhodamine optics, since at high concentrations within Golgi membranes BODIPY-Cer forms excimers, which emit >590-nm light when excited with blue light.

Materials

- Ethanol
- 0.05% trypsin in HBSS (*APPENDIX 2A*) without calcium and magnesium
- 1 mM ceramide fluorescent derivative working solution in ethanol (see recipe)
- Serum-free medium appropriate to cells
- Cells of interest
- Phosphate-buffered saline (PBS; *APPENDIX 2A*), 4°C
- 10% (w/v) defatted BSA (Sigma) in serum-free medium appropriate to cells
- Fluoromount G (Southern Biotechnology Associates or Electron Microscopy Sciences) *or* mounting medium (see recipe)
- HEPES-buffered culture medium appropriate to cells, pH 7.0, with 10% serum and without phenol red
- 12-mm diameter no. 1 round glass coverslips
- Watchmaker's forceps
- 10-cm sterile tissue culture dishes
- Hamilton syringe
- Silicon rubber chambers (see recipe)
- Microscope slides

Conventional fluorescence microscope with standard fluorescein and rhodamine filter cubes *or* confocal microscope with Kr/Ar laser (UNIT 4.2)
63× (1.4 NA) or 100× (1.3 NA) oil-immersion objective
Microscope air-stream incubator (Nevtek)

Additional reagents and equipment for growing cells in tissue culture and trypsinization of cells (UNIT 1.1) and fluorescence microscopy (UNIT 4.2)

Prepare cells on coverslips

1. Sterilize 12-mm diameter no. 1 round glass coverslips by soaking in 95% ethanol and flaming. Handling the coverslips with watchmaker's forceps, place in a 10-cm sterile tissue culture dish (which will accommodate up to eight coverslips).

Be sure the coverslips are no. 1 thickness for viewing at high magnification on the microscope. Thicker coverslips will not match the numerical aperture of the objective and can give significant spherical aberration, resulting in poor microscopic images.

2. One to two days prior to start of experiment, trypsinize cells (UNIT 1.1) in 0.05% trypsin and seed onto the culture dishes containing the coverslips.

For optimal viewing, the cells should have grown to only ~50% confluency by the day of the experiment.

If cells fail to grow well, the coverslips can be treated prior to seeding with 1 mg/ml poly-D-lysine or poly-L-lysine for 15 min.

Label cells

3. Take a small aliquot of the 1 mM ceramide derivative in ethanol and disperse it into serum-free medium at room temperature by injecting it into the medium with a Hamilton syringe while rapidly vortexing. Use quantities of Cer-derivative solution and medium such that the final working concentration of Cer-derivative in the medium will be 5 to 10 μ M and the final concentration of ethanol will be <0.5% (in order to prevent damage to the cells).

This step causes the Cer-derivatives to form small vesicles which are more easily delivered to the cell.

4. Place coverslips in a tissue culture dish containing ice-cold PBS for 2 min. Remove PBS and replace with fresh ice-cold PBS, then repeat one more time.

This step removes serum from outside the cells. Cer-derivatives have a very high affinity for serum, so any serum present on the cells will lower the effective concentration of the Cer-derivative.

5. Add medium containing the Cer-derivative to cells so that the bottom of the dish, along with the surface of the coverslip, is completely covered with medium and incubate 30 min at 4°C.

6. Wash cells three times with cold PBS to remove unbound lipid, then incubate with medium containing 10% defatted BSA for 30 min at 4°C.

This removes or "back exchanges" the excess NBD- or BODIPY-Cer that is not incorporated into intracellular membranes.

7. Warm cells to 37°C, and continue incubating at that temperature, examining cells under a fluorescence microscope to determine when optimal labeling of the Golgi complex and minimal labeling of other intracellular membranes occurs.

Golgi labeling should be prominent after 15 to 40 min.

View cells by fluorescence microscopy

8. Place a small drop of Fluoromount G or mounting medium on the cell side of the coverslip. Invert coverslip onto a silicon rubber chamber attached to a microscope slide (see Basic Protocol 1, step 5) that contains HEPES-buffered medium with serum, pH 7.0, without phenol red.

Phenol red is autofluorescent and therefore increases the background level of fluorescence within cells.

Alternatively, the cells can be grown in commercially supplied coverslip–bottom open dishes (Lab-Tek chamber coverglass system, item nos. 136307, 136420, Nunc). These dishes allow drugs or other reagents to be easily added while the cells are being imaged. Such chambers, however, can only be used with an inverted microscope.

9. Place a drop of immersion oil on the non-cell side of the coverslip and view cells with a fluorescent microscope using a 63× or 100× oil immersion objective.
 - a. View C₆-NBD-Cer–labeled cells using a filter combination on the microscope appropriate for fluorescein optics (i.e., one that excites the fluorophore at 450 to 490 nm and collects its emission at 520 to 560 nm).
 - b. View BODIPY-Cer–labeled cells using the above filter combination or a filter combination that excites the fluorophore at 450 to 490 nm and collects its emission at >590 nm. For confocal microscopes, image with the 488-nm line of the Kr/Ar laser.

The filter set collecting emission at >590 nm allows the distribution of BODIPY-Cer excimers to be visualized. These usually only form in Golgi membranes, where the lipid has become greatly concentrated. Because BODIPY-Cer excimer formation usually only occurs in the Golgi complex, imaging the red-shifted BODIPY-Cer signal rather than the green BODIPY-Cer signal provides a convenient way to avoid imaging the green signal from non-Golgi membranes (including the ER and plasma membrane) containing BODIPY-Cer.

To keep cells at 37°C while viewing under the microscope, warm microscope stage by blowing warm air upon it with an air-stream incubator.

STAINING MITOCHONDRIA

This staining protocol (which is for living cells only, not fixed cells) is simple, since it merely involves incubating the cells in the presence of the dye. An additional consideration, however, is how to maintain normal cell behavior on the microscope stage. For physiological experiments on mammalian cells, where mitochondrial potential is to be measured or where the motility or distribution of mitochondria is being studied, it is necessary to regulate the temperature. The ability to perfuse different solutions or media may also be necessary. Several temperature-regulated chambers are commercially available. For experiments on an inverted microscope, the authors have used a thermoregulated microscope chamber from Bioptechs equipped for perfusion and solution exchange. Coverslips (no. 1, 31-mm diameter) were also obtained from Bioptechs. For experiments in which mitochondria are to be observed at room temperature, simpler mounting strategies can be used (e.g., see Chen, 1989).

Materials

100 nM TMRE working solution (see recipe) in NB
Normal buffer (NB; see recipe)

No. 1, 31-mm diameter coverslips (Bioptechs)
60-mm petri dishes
Forceps

Q tips or Kimwipes

Temperature-regulated microscope chamber (Bioptechs)

Conventional fluorescence microscope with conventional rhodamine filter *or*
confocal microscope (UNIT 4.2)

40× or greater oil-immersion objective

1. Grow cells on no. 1, 31-mm glass coverslips.

Culturing procedures will depend on the application. For example, to clearly observe the endoplasmic reticulum network of fibroblasts, trypsinize the cells, plate them on the coverslip at a relatively low density so that most cells are not in contact with another cell, and observe them after allowing them to spread overnight.

2. Fill three 60-mm petri dishes with NB and one tissue culture dish with 100 nM TMRE working solution in NB. Handling the coverslip with forceps, gently wash by serially dipping in the three NB dishes and finally in the TMRE solution. Return the coverslip to a new dish and incubate 10 min in 100 nM TMRE working solution in NB.
3. Remove the coverslip from the culture dish, dry the bottom of the coverslip with a Q-tip or Kimwipe, and mount in a microscope chamber. Fill the chamber with 100 nM TMRE in NB.
4. Observe cells using either a conventional rhodamine filter on a conventional fluorescence microscope or the 540- or 568-nm laser excitation line on a confocal microscope, and a 40× or greater oil-immersion objective lens.

The staining should be sufficient for observing mitochondrial distribution, motility, or relative levels of mitochondrial membrane potential. Quantitative measurements of the membrane potential requires low-light-level imaging and digitization of images. These topics are covered in Loew et al. (1993) and Loew (1998).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Ceramide fluorescent derivatives

Stock solution: For long-term storage, dissolve an appropriate amount of powdered Cer-derivative (C₆NBD-Cer or BODIPY-Cer; Molecular Probes) in a glass scintillation vial with a Teflon-lined screw cap containing 19:1 (v/v) chloroform/methanol. Blow argon gas on top of the liquid before sealing to prevent oxidation. Keep the tubes sealed and store up to 2 years at –20°C.

Working solution (1 mM): When ready to label cells, dry an aliquot of the chloroform stock under nitrogen and redissolve in absolute ethanol to make a solution of 1 mM.

For short-term storage (i.e., 1 to 2 weeks), dissolve the Cer-derivatives in 100% ethanol to a concentration of 1 mM, seal, and keep at –20°C.

DiOC₆(3) stock and working solutions

Stock solution (0.5 mg/ml): Prepare ~10 ml of 0.5 mg/ml DiOC₆(3) (D-273 from Molecular Probes) in ethanol. Store in a scintillation vial wrapped in aluminum foil at room temperature (probably good for several years).

Working solution for Basic Protocol 1 (2.5 μg/ml): Make a 0.5% (v/v) dilution in PBS (APPENDIX 2A) of the 0.5 mg/ml DiOC₆(3) stock solution [e.g., 5 μl DiOC₆(3) stock in 1 ml PBS].

Working solution for Alternate Protocol (0.5 μg/ml): Make a 1:1000 dilution of the 0.5 mg/ml stock solution in the growth medium appropriate to the cells of interest.

This working solution can be used for a few hours, but do not store overnight.

Mounting medium

PBS (APPENDIX 2A) containing:
50% (w/v) glycerol
0.1% (w/v) *p*-phenylenediamine
Adjust pH to pH 8.0 using NaOH
Store up to 1 to 2 weeks at 4°C

Normal buffer (NB)

7.6 g NaCl (130 mM final)
0.41 g KCl (5.5 mM final)
0.2 g CaCl₂ (1.8 mM final)
0.2 g MgCl₂·7H₂O (1.0 mM final)
4.5 g glucose (25 mM final)
4.8 g HEPES (20 mM final)
H₂O to 1 liter
Adjust pH to pH 7.4 with 1 N NaOH
Store up to 1 month at 4°C

Silicon rubber chamber

Using a razor blade, cut a 2.5 × 2.5-cm piece of Ronsil calendared sheet, 0.3 in. (0.76 mm) thick (purchase from North American Reiss; 100 feet minimum order). Using a hole puncher, cut a hole ~0.65 cm in diameter in the middle of the square over which the coverslip will be placed.

After the experiment, the silicon rubber chamber can be peeled off the slide, wiped clean with a Kimwipe, and reused.

TMRE

Stock solution (0.5 mM): To make 10 ml, add 2.5 mg TMRE (T-669 from Molecular Probes) to 10 ml of 95% ethanol. Store up to 1 year at –20°C.

Working solution (100 nM): Add 20 μl of 0.5 mM TMRE stock to 100 ml normal buffer (NB; see recipe). Use only on day of preparation.

One may consider using growth medium instead of NB to prepare the working solution, but this often produces higher background fluorescence, probably from phenol red or from serum binding of dyes.

COMMENTARY

Background Information

Endoplasmic reticulum

The endoplasmic reticulum (ER) was discovered by electron microscopy in the peripheral regions of osmium-fixed cells grown on formvar-coated grids (Porter et al., 1945; Porter, 1953). The peripheral regions of cells are thin enough that the organization can be imaged without sectioning. The name ER was given to a network (reticulum) that was more abundant in the central (endoplasmic) portion as opposed to the motile periphery (ectoplasm). Therefore, the ER was first defined purely in terms of morphology. With the advent of thin-section electron microscopy and differential centrifugation, the functions of the membranes became established.

The use of fluorescent dyes to stain the ER grew out of the use of dyes for monitoring membrane potential (Cohen et al., 1974). Dicarboxyanine dyes are of the “slow” class of potential-sensitive dyes (Sims et al., 1974). Rhodamine 123, like many of the dicarboxyanine dyes, was found to label mitochondria in living cells (Johnson et al., 1980). Michael Weiss found that one of the dicarboxyanines labeled a network when viewed at high magnification (Terasaki et al., 1984). Rhodamines and dicarboxyanines were screened, and DiOC₆(3) was chosen as the brightest and most photostable stain for the network. The network was identified as ER, based on its morphology. In addition, conditions for staining fixed cells were described. It was initially stated that the dye did not stain Golgi or endosomes, but it was

later concluded that it did stain these organelles (Terasaki et al., 1986; Terasaki and Reese, 1992). There was some uncertainty among cell biologists whether the network stained was actually the ER, even though this network had been originally defined as the ER. Double imaging of BiP, a protein agreed to be in the ER, proved that the peripheral network stained by DiOC₆(3) was identical to the BiP immunofluorescence pattern (Terasaki and Reese, 1992).

This two-dimensional network of ER in the periphery of many cultured cell types has some noteworthy characteristics. All of the ER network in this region is close to the plasma membrane. This is in contrast to the bulk of the ER of most cells, which is in the interior and not close to the plasma membrane. The ER network is usually composed of tubules that are connected by three-way junctions; sometimes, large sheets that correspond to ER cisternae are also seen (Terasaki et al., 1986). The tubules near the leading edge of the cell are often aligned with microtubules that are responsible for extending the ER tubules outward (Terasaki et al., 1986; Dailey and Bridgman, 1989). It is also noteworthy that the ER network in the periphery is relatively sparse. First of all, due to the limitations in light microscopy, the fluorescence image of the ER tubules is larger than its actual width, which is on the order of 100 nm. When the ER is seen in whole-mount electron micrographs, it looks surprisingly thin (Dailey and Bridgman, 1991). Secondly, it is easy to see that the total amount of ER membrane in the peripheral regions is less than the amount of plasma membrane in those regions. This is in contrast to the situation in most cells, where the area of the ER is many times as large as that of the plasma membrane.

Another dicarbocyanine dye, DiI [DiIC₁₈(3)] has been used to label the ER (Terasaki and Jaffe, 1991). This method results in more specific labeling of the ER and is well suited for three-dimensionally arranged ER, but requires microinjection (Terasaki and Dailey, 1995).

Golgi complex

The Golgi complex plays a central role in secretory protein processing and trafficking within cells. It receives membrane-enclosed packets of protein cargo from the ER, modifies and processes cargo proteins, and then repackages them into transport intermediates for delivery to the plasma membrane and other intracellular destinations (Mellman and Simons, 1992). To perform these tasks, the Golgi complex is enriched in a variety of resident enzymes

involved in carbohydrate processing. It also has a unique lipid composition that includes high concentrations of cholesterol and other lipids relative to the concentrations found in the ER. This property is the basis for vital staining of the Golgi with fluorescent lipids. The C₆-NBD- and BODIPY-fluorescent analogs of ceramide have proved to be particularly valuable for this purpose. C₆-NBD-Cer is formed by *N*-acylation of the long-chain base, sphingosine, with the short-chain fluorescent fatty acid, *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproic acid (C₆-NBD-FA). C₆-NBD-Cer can be synthesized in the laboratory (Kishimoto, 1975; Schwarzmann and Sandhoff, 1987) or purchased from Molecular Probes. The newer fluorescent ceramide analog, BODIPY-Cer, is a conjugate of *D-erythro*-sphingosine and a fluorescent fatty acid containing the fluorophore boron dipyrromethene difluoride (BODIPY). It has ~2- to 3-fold higher fluorescent yield and greater photostability than NBD-ceramide (Pagano et al., 1991). An additional advantage of the BODIPY-Cer analog is that its fluorescence emission spectrum is dramatically red shifted as the probe concentrates in membranes. This is due to excimer formation of the BODIPY-Cer molecules. The Golgi complex is usually the only membrane system where BODIPY-Cer accumulates to high enough levels to form excimers and hence is the only organelle where the red shift occurs (Pagano et al., 1991). By using microscope filters appropriate for visualizing the red-shifted BODIPY-Cer that has formed excimers in Golgi membranes, non-Golgi membranes containing low concentrations of the dye will not be visualized.

The mechanism whereby C₆-NBD-Cer and BODIPY-Cer initially accumulate in the Golgi complex is not understood. It has been suggested that Golgi labeling by C₆-NBD-Cer and BODIPY-Cer is due to preferential partitioning into Golgi membranes as a result of the specific physical properties of these membranes. This could explain why NBD-Cer labeling of the Golgi complex occurs at low temperature or in the presence of metabolic inhibitors, and can even occur in cells that have been fixed before incubation.

The finding that C₆-NBD-ceramide labels the Golgi complex after cells have been fixed (Pagano et al., 1989) is the basis for a novel methodology for visualizing the Golgi complex at the electron microscope (EM) level (Pagano and Martin, 1998). In this protocol, fixed cells labeled with C₆-NBD-ceramide are photobleached in the presence of diaminoben-

zadine (DAB). The photooxidation products catalyze the polymerization of DAB to yield a high-molecular-weight osmiophilic compound that can be visualized at the EM level. Previous work has shown that under these conditions, the C₆-NBD-ceramide product labels the trans-Golgi elements (Pagano et al., 1989, 1991).

In addition to being useful markers for Golgi membranes in localization studies (Lipsky and Pagano, 1985a; van Meer et al., 1987; Kobayashi and Pagano, 1989; Rosenwald and Pagano, 1993; van't Hof and van Meer, 1990), C₆-NBD-Cer and BODIPY-Cer have been used in fluorescent time-lapse imaging studies to analyze the dynamics of the Golgi complex in cultured cells (Cooper et al., 1990; Sciaky et al., 1997; see Fig. 4.4.2). These studies have shown that while Golgi elements have a large-scale stability in organization, they are remarkably dynamic. Long tubule processes were often seen extending off the rims of Golgi elements during time-lapse imaging. The Golgi tubule processes appeared to serve both to interconnect adjacent Golgi elements and to carry Golgi membrane out to the cell periphery.

Mitochondria

Mitochondria in tissue culture cells can be seen by phase-contrast or bright-field microscopy. Johnson et al. (1980) described the use of rhodamine 123 for viewing mitochondria by fluorescence microscopy. Many fluorescent dyes that had been developed for monitoring plasma-membrane potential have turned out to stain mitochondria similarly. Fluorescence staining of mitochondria produced images of much higher contrast than available with transmitted light microscopy, and it was also realized that they could be used for monitoring mitochondrial membrane potential. Some mitochondrial dyes have been reported to selectively kill cultured cancer cells that retain them longer (Bernal et al., 1982). Molecular Probes has generated the greatest number of new mitochondrial probes in recent years.

Critical Parameters

Endoplasmic reticulum

The best way to fluorescently label the ER is probably to express a GFP chimera targeted to the ER (Hampton et al., 1996; Terasaki et al., 1996). However, it may be difficult to express GFP constructs in a given cell, or the fluorescence may not be bright enough. DiOC₆(3) is an older method for staining the ER, but is still useful because of the ease of use and brightness

of staining. Its great weakness is the lack of specificity of staining; DiOC₆(3) will stain all of the ER but will stain other organelles as well.

It must be stressed that DiOC₆(3) does not stain the ER specifically. DiOC₆(3) is a positively charged lipophilic molecule that permeates through the plasma membrane. At low concentrations, it accumulates in mitochondria due to the effect of the large negative mitochondrial membrane potential on the positively charged dye. At higher concentrations, the dye stains other membranes, including the ER. The mitochondria are swollen at these higher concentrations, suggesting that they are no longer concentrating dye, and that the dye is free to associate with other membranes. In fixed cells, the dye stains ER, mitochondria, and large vesicles even at the lowest concentrations. The simplest interpretation is that the dyes are associating with all intracellular membranes. Some of the membranes may be so small that the amount of dye contained in them is not visible. Since DiOC₆(3) probably stains all membranes, it is only useful as an ER stain when the ER pattern can be distinguished from the other membranes. The thin peripheral region of many cultured cells has often been used, because in that region the ER is a single-layered network that is easily identified.

Other dyes with the same staining properties as DiOC₆(3) include rhodamine 6G, DiOC₆(5), DiIC₆(3), and DiSC₆(3) (all screened in Terasaki et al., 1984), as well as hexyl ester rhodamine B (Terasaki and Reese, 1992). All of these are lipophilic cationic dyes that stain only mitochondria at low doses in living cells. If it is desired to observe ER staining with a rhodamine filter set, hexyl ester rhodamine B (Molecular Probes R-648, also known as "R6") may be the best dye to try first.

The quality of the images obtained with DiOC₆(3) is generally much better than that obtained with immunofluorescence staining. One reason is that the glutaraldehyde used for DiOC₆(3) staining is a superior fixative that is usually not used for immunofluorescence because of the autofluorescence that develops with time. DiOC₆(3) methods use lower glutaraldehyde concentrations and images are observed before autofluorescence develops. Another reason is that immunofluorescence requires permeabilization of the cells with detergents or organic solvents. These treatments generally disrupt the delicate membranes of the ER, so that they are no longer continuous, or are so disfigured that the network pattern is no longer discernible. Immuno-

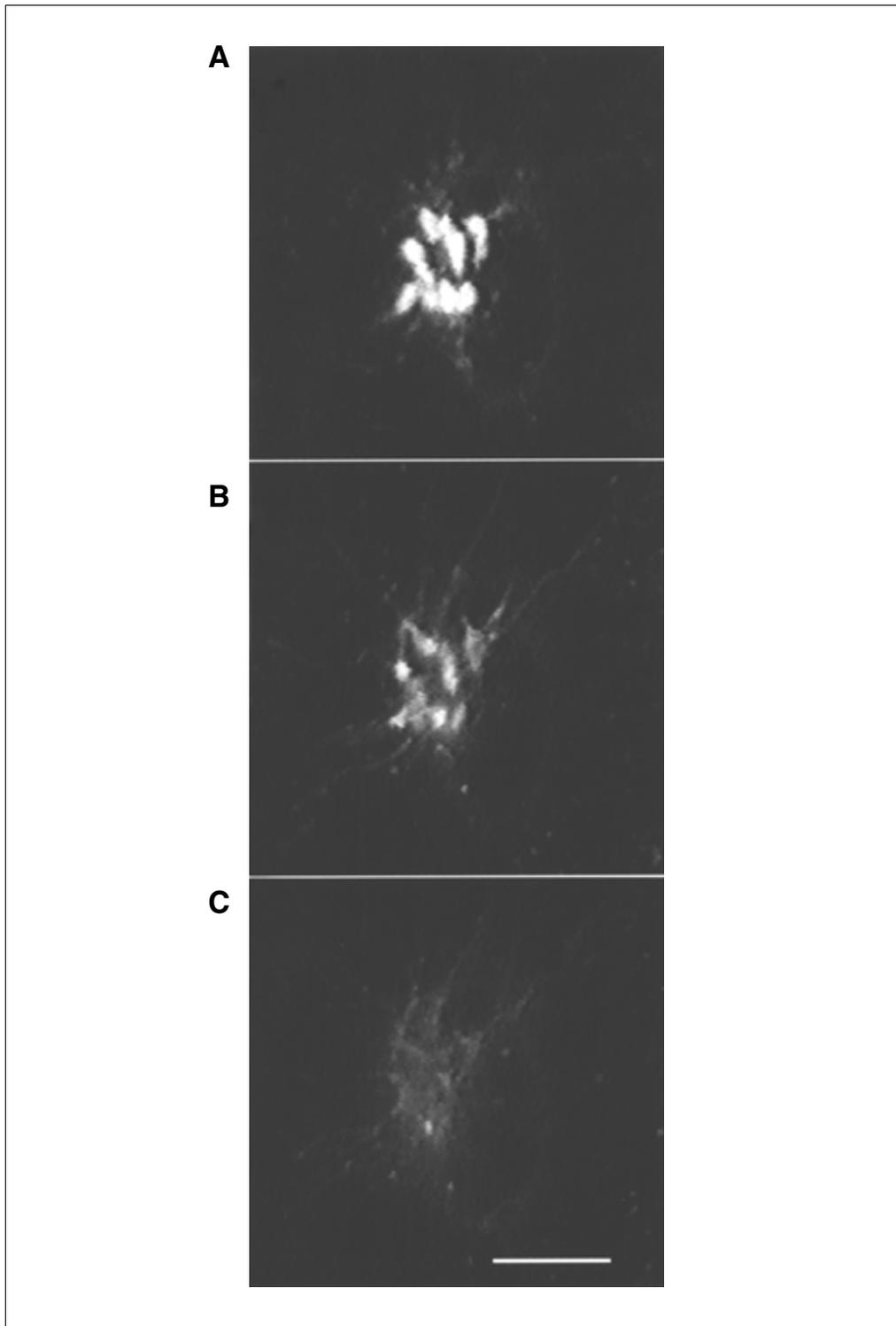


Figure 4.4.2 BODIPY-Cer labeling of Golgi membranes to study the effects of brefeldin A (BFA). Golgi membranes were labeled with BODIPY-Cer and then treated with brefeldin A before imaging at 37°C on a cooled CCD microscope system. Note that Golgi membranes tubulated in response to the drug and then dispersed (Sciaky et al., 1997). **(A)** Image collected immediately after addition of BFA; **(B)** image collected 5 min later; and **(C)** image collected 10 min after addition of drug. Bar is equal to 10 μm .

fluorescence has the advantage, however, of labeling specific molecules in the ER. Immunofluorescence can also show compartmentalization in the ER, and is also much better suited than DiOC₆(3) for screening the results of multiple experimental variations, because of the short period during which the dye staining is optimal.

DiOC₆(3) is well suited for observations in living cells. One drawback is that the mitochondria are swollen at dye concentrations required to stain the ER, so that there is a possibility that the cells are not behaving normally. Nevertheless, DiOC₆(3) has been used successfully for observing ER dynamics at video rates (Lee and Chen, 1988), in time-lapse (Dailey and Bridgman, 1989; Terasaki and Reese, 1994), and during mitosis (Waterman-Storer et al., 1993). It is particularly good for observing ER movements during cytoplasmic streaming in plant cells (Allen and Brown, 1988; Knebel et al., 1990; McCauley and Hepler, 1990). When using the fluorescent dyes on a new organism, it is important to optimize the staining conditions. Koning et al. (1993) give a good description of how the cell chamber and optical conditions were optimized for yeast. For some discussion of other cell preparations and of other aspects of this method, see earlier reviews (Terasaki, 1989, 1993, 1998). For live-cell imaging by confocal microscopy, see Terasaki and Dailey (1995).

Golgi complex

Attention to several critical parameters is important for ensuring the success of C₆-NBD- and BODIPY-Cer labeling of cells. These parameters include the amount of fluorescent lipid added to cells, the length of time for incorporation of the lipid into cells, and the method of imaging cells. Each of these parameters needs to be optimized to ensure that Golgi labeling is bright and specific, and that cells are not damaged during the labeling or imaging process.

Although incubation of cells with medium containing 5 to 10 μ M fluorescent Cer-derivative for 30 min at 4°C, washing, and warming to 37°C for 30 min is recommended, the optimal conditions for Golgi labeling vary depending on cell type and therefore need to be tested. The level of brightness of Golgi labeling also depends on the purpose of the experiment. If cells are to be used for time-lapse imaging of the Golgi complex, for example, a brighter signal than normal is recommended since the fluorescent lipid is quickly photobleached dur-

ing repetitive imaging. To increase levels of Golgi labeling, simply incubate cells with a higher concentration of fluorescent-ceramide solution, and vice-versa for lower levels of Golgi labeling.

Higher levels of Golgi labeling are not always desirable. Labeling of non-Golgi membranes also usually increases under conditions where Golgi labeling increases. In addition, if the Golgi complex is too bright, images will be saturated (with low signal-to-noise ratios) and the risk of photobleaching damage upon imaging is greater.

For time-lapse imaging studies, BODIPY-Cer is preferable to C₆-NBD-Cer because it has a slower rate of photobleaching. Even BODIPY-Cer photobleaches quickly relative to other fluorophores (e.g., rhodamine) so imaging with a low-light detection system is essential. To reduce photobleaching of fluorescent ceramide derivatives in time-lapse studies, minimal exposure of the specimen to bleaching radiation is essential. Control of the exposure time during imaging is critical in these types of experiments. This can be done by using a computer-controlled shutter to control exposure time and by using neutral-density filters during illumination to reduce the light reaching the specimen. Imaging with low light intensity through the use of neutral-density filters will lead to significantly less photobleaching. This is essential for time-lapse studies of C₆NBD- or BODIPY-Cer labeled cells.

Photobleaching results in permanent inactivation of the fluorophore due to its interaction with neighboring molecules. This frequently involves the generation of free radicals that can damage the cell. In time-lapse imaging studies where the specimen becomes photobleached due to repetitive imaging, it is essential to monitor the overall appearance of the cell to assess potential light-induced damage to the cell. A typical response by cells that are being damaged by photobleaching radiation is for them to round up and then eventually die.

The process of finding a labeled cell and focusing it can cause extensive photobleaching if performed without neutral-density filters. A useful approach is to find and focus cells in phase optics before imaging the specimen with fluorescent light. Cells that are fixed after staining for double-label studies should be immediately processed using as little light exposure as possible. The fixed cells should be imaged immediately when they are ready, since they do not store for very long periods. If double labeling with an antibody is being performed after fixa-

Table 4.4.1 Troubleshooting Guide for Fluorescent Staining of Organelles

Problem	Possible causes and/or solution(s)
<i>Endoplasmic reticulum</i>	
Staining pattern is too dense to see a network	This means that the the staining of the ER and/or other organelles is too dense relative to the resolution of light microscopy. If ER membranes are separated from each other or from other organelles by <math><0.5\ \mu\text{m}</math> or so, it will be difficult to resolve them by light microscopy. If the ER has a three-dimensional organization, the overlapping membranes compound the problem. Sometimes, using a 100 \times lens or a confocal microscope can help. It may be necessary to use another cell type or to resort to other means to localize the ER, such as electron microscopy.
Vesiculation of ER network	One possible cause is poor fixation. Fixation with formaldehyde often causes vesiculation of the ER network. If glutaraldehyde is being used, it may help to try a fresh stock solution. Another cause of vesiculation is the fixation buffer. Make sure that the tonicity and pH are physiological. For fixed cells, it may help to replace PBS with another buffer (100 mM sucrose/100 mM sodium cacodylate, pH 7.4). Another cause of vesiculation is drying of the cells during the processing. It is important to keep the cells hydrated at all times.
Smear-out network	This can be caused by organic solvents or detergents that extract the membranes and the dye staining. Do not use nail polish to seal coverslips to microscope slides since the organic solvents in the nail polish will have this same effect.
Out-of-focus, distorted network	This is usually a problem of the microscope optics. Make sure that there is no water in the immersion oil on the coverslip. Also check for dried material, broken pieces of coverslip, and other debris on the objective lens.
No network visible	In one cell type, fish chromatophores, DiOC ₆ (3) did not stain the ER and gave a diffuse background staining (M. McNiven, pers. comm.). This was surprising, since preparations from many higher vertebrates, as well as many invertebrates and unicellular organisms, have been successfully stained with DiOC ₆ (3). Perhaps the lipid composition of the ER in the fish chromatophores somehow prevents the partitioning of DiOC ₆ (3) into the membrane bilayer.
<i>Golgi complex</i>	
Background labeling with fluorescent lipid is too high	Use lower concentrations of fluorescent lipid (<math><5\ \mu\text{M}</math>) during initial labeling of cells.
There is no specific labeling	Check stock solutions of fluorescent lipids to see if they were properly prepared and are not too old. Check to see if incubation with fluorescent lipid solution for longer times at 4 $^{\circ}\text{C}$ and/or 37 $^{\circ}\text{C}$ result in specific labeling.
Nonspecific labeling is too high	Reduce concentration of fluorescent lipid added to cells. Wait for longer times after shift to 37 $^{\circ}\text{C}$ before viewing cells. Check to make sure cells are not autofluorescent (i.e., whether unlabeled cells show the same non-specific labeling). Consider labeling with BODIPY-Cer and viewing cells in rhodamine optics so that fluorescence from the BODIPY-Cer excimers is only being imaged.
Specific staining is too dim	Add higher concentrations of fluorescent lipid to cells and incubate at 4 $^{\circ}\text{C}$ for longer periods.

*continued***Microscopy****4.4.13**

Table 4.4.1 Troubleshooting Guide for Fluorescent Staining of Organelles, continued

Problem	Possible causes and/or solution(s)
Cells round up and appear unhealthy during imaging	Use attenuated light to image cells. Image for only short time periods. Label cells with lower concentrations of fluorescent lipid.
Fluorescence is lost too quickly because of photobleaching	Find cells and focus them using phase optics to reduce the extent of photobleaching prior to imaging. Use attenuated light to image cells and image for as short a time as possible. Imaging systems that detect low light levels are useful since photobleaching is reduced when specimens are imaged with less light. Use BODIPY-Cer instead of NBD-Cer since the former photobleaches less rapidly than the latter.
Mitochondria	
Dim labeling	This probably means that the dye staining concentration or incubation period needs to be increased. If this does not help, perhaps this is due to a very low mitochondrial membrane potential. This in turn may be due to poor health of cells.
Swollen mitochondria; labeling of ER (fine reticular network throughout cell) in addition to mitochondria; cell death	These are indications that there is too much dye in the cells. The solution is to decrease dye staining concentration.

tion of the C₆-NBD-Cer or BODIPY-Cer labeled cells, it is crucial to check that there is minimal cross-over of the fluorescent Cer into the channel used for revealing the fluorescent antibody staining. BODIPY-Cer labeled cells usually cannot be used in double-labeling experiments, since BODIPY-Cer emits light at multiple wavelengths due to excimer formation.

Mitochondria

Since the first report of fluorescent dye staining of mitochondria (Johnson et al., 1980), many dyes have been found that have similar properties. Generally, these dyes are all small, lipophilic cationic molecules. The lipophilicity allows them to cross the plasma membrane, and the positive charge causes them to be accumulated in the mitochondria, which have a high negative membrane potential.

Rhodamine 123, the first fluorescent dye for mitochondria (Johnson et al., 1980), is used to stain cells at 10 µg/ml for 10 min followed by a brief wash and mounting in dye-free growth medium (Chen, 1989). The stock solution used for this dye is 1 mg/ml in water. Because the staining is observed in dye-free medium, mitochondrial staining eventually decreases due to leakage from the cells. The leakage rate is highly dependent on cell type. In some cases,

the dye is retained for over an hour, but in others, it leaks out within 10 min.

Several rhodamine and dicarbocyanine dyes have similar staining properties to those of rhodamine 123, although rhodamine 123 may be less toxic (Chen, 1989). Two examples are rhodamine 6G and the dicarbocyanine dye DiOC₆(3). The staining concentrations are lower for several of these dyes, and some of them can be left in the medium because they have a relatively low background. Other reasons for using one of these other dyes are brightness, slower bleaching rate, or convenience of fluorescence spectrum. Rhodamine 123 has an advantage in that it does not stain the ER at high doses, whereas most of the other dyes do. The dye described in Basic Protocol 3, TMRE, is well documented for quantitative measurements of the mitochondrial membrane potential (Loew et al., 1993).

Several other mitochondrial dyes are available for studying mitochondrial properties in interesting ways. The MitoTracker series of dyes can be fixed in place once they have been stained (Poot et al., 1996; Poot, 1998). JC-1 dyes change fluorescence after they form "J aggregates" which is potential dependent (Smiley et al., 1991). The CM-H2TMRos dye is nonfluorescent until it becomes oxidized in

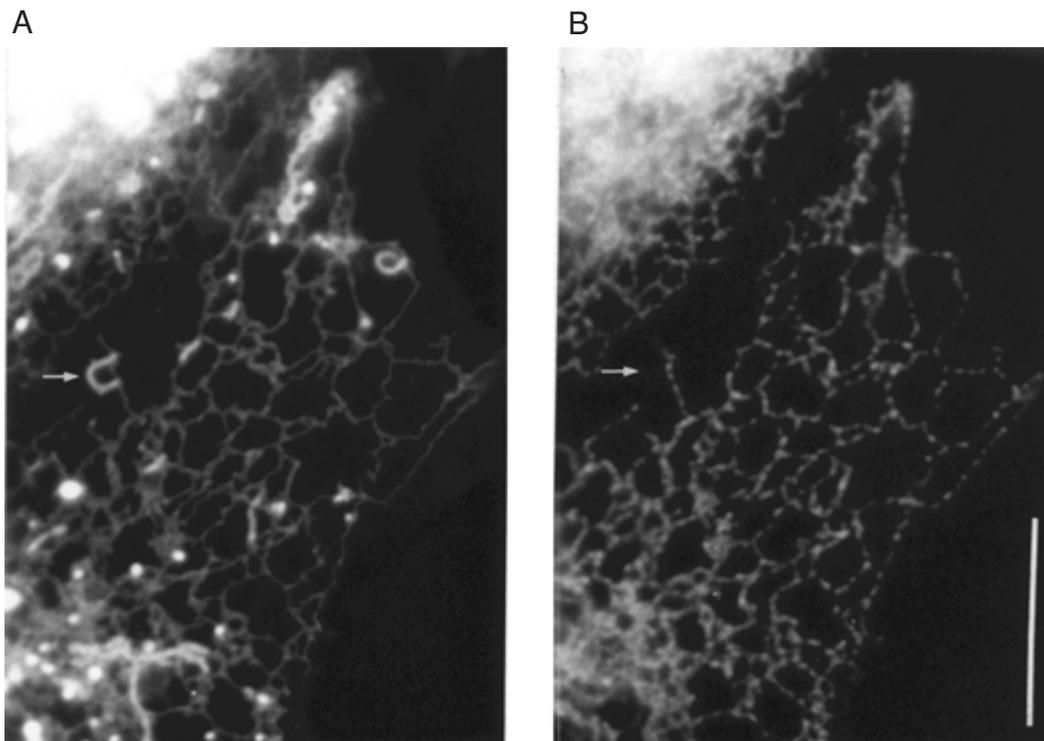


Figure 4.4.3 CV-1 cell double-labeled with (A) DiOC₆(3) and (B) immunofluorescent staining of BiP, a protein in the ER. Bar is equal to 10 μm.

the mitochondria. Nonyl acridine orange is thought to bind to cardiolipin rather than respond to membrane potential. A good place to get a more detailed overview of the different kinds of mitochondrial stains is the Molecular Probes catalog (Haugland, 1996).

Troubleshooting

Table 4.4.1 lists some problems that may be encountered in fluorescent staining the ER, Golgi complex, and mitochondria along with some possible causes and solutions.

Anticipated Results

Endoplasmic reticulum

The ER is best seen in the thin peripheral region of cultured cells, where it has a two-dimensional distribution. In some cell types, this peripheral region is very narrow or nonexistent, in which case DiOC₆(3) will probably not be of use. The ER pattern differs considerably from cell type to cell type, and also from cell to cell in a population. It is usually a network of tubes, but can also contain sheets or membrane cisternae. The overall pattern often seems to mirror underlying stresses or morphogenetic processes of the cytoplasm, and can be anything from a closely spaced isotropic meshwork to a

highly unrestrained pattern (seen more often in primary cultures).

Figure 4.4.3 shows a CV-1 cell (from African green monkey kidney), double-labeled with DiOC₆(3) (panel A) and immunofluorescence of BiP (panel B), a protein in the ER. Note that DiOC₆(3) stains a continuous network as well as mitochondria (arrow). Anti-BiP labels a vesiculated network, but does not label the mitochondria. The cells were first fixed lightly with glutaraldehyde (0.025% for 5 min), stained with DiOC₆(3), photographed, then permeabilized with -20°C methanol and processed for immunofluorescence (Terasaki and Reese, 1992). The vesiculated network seen in immunofluorescence was caused by the methanol permeabilization.

Golgi complex

Once conditions for specific labeling of the Golgi complex have been optimized, the localization and dynamics of the Golgi complex can be studied. Golgi membranes usually appear as a ribbon-like structure near the microtubule organizing center adjacent to the nucleus (Rogalski and Singer, 1984; Cole et al., 1996). Frequently, thin tubule processes interconnect the larger Golgi structures (Cooper et al., 1990). Because the normal distribution of Golgi mem-

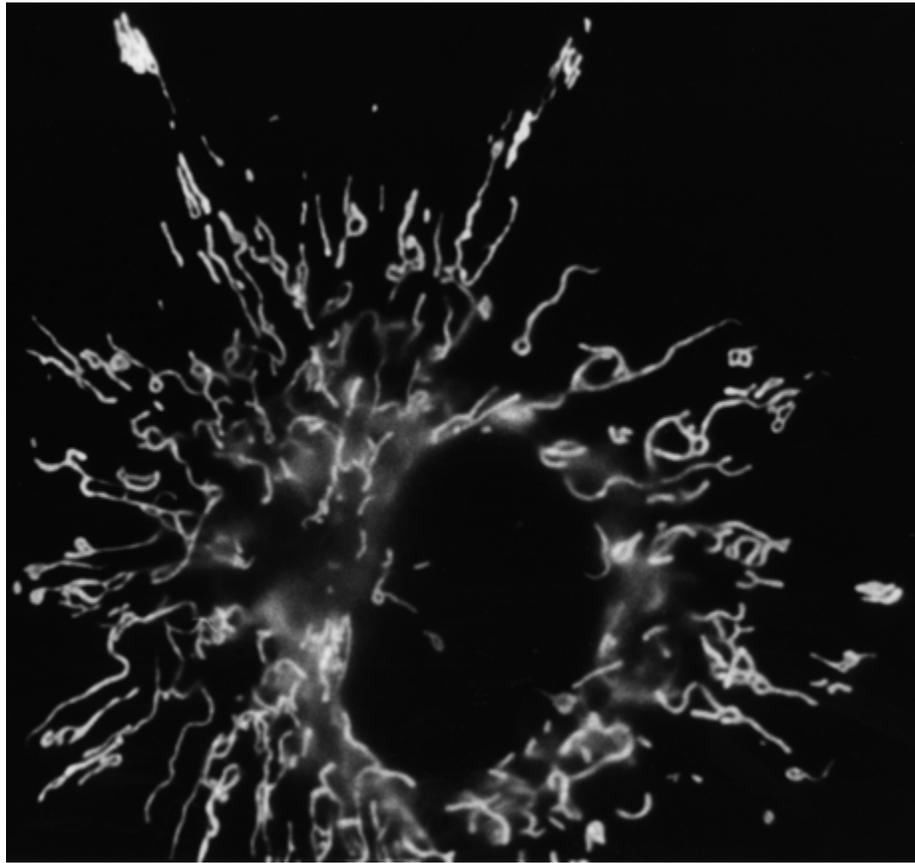


Figure 4.4.4 Labeling of the mitochondria in a CV-1 cell.

branes within cells represents a dynamic steady state of forward and recycling pathways connecting the Golgi and ER, any condition that perturbs forward traffic into the Golgi complex without a corresponding effect on recycling will result in redistribution of Golgi material to the site of perturbation (Cole et al., 1996). One example where the Golgi complex undergoes such reorganization is during microtubule disruption, which causes extensive fragmentation of the Golgi into hundreds of elements scattered throughout the cell (Turner and Tartakoff, 1989). Microtubules are known to serve as tracks for delivery of ER-derived transport intermediates into the centrosomal Golgi region (Presley et al., 1997; Scales et al., 1997) and are not as important for Golgi-to-ER traffic (Cole et al., 1996), therefore when net forward transport into the Golgi is blocked during microtubule depolymerization, Golgi proteins accumulate at ER exit sites and regenerate short Golgi stacks at these sites (Cole et al., 1996).

Mitochondria

Labeling of the mitochondria in a CV-1 cell (African green monkey kidney) is shown in

Figure 4.4.4. The appearance of the mitochondria can be very variable. In some cell lines, they are short, in others very long. Mitochondria are often aligned with cytoskeletal elements (Summerhayes et al., 1983).

Time Considerations

This is a rapid staining procedure once all the equipment and materials are in place. Equilibration time for staining is ~10 min.

The time required for making the chamber and getting the microscope set should be 5 to 10 min. Staining the ER with DiOC₆(3) is very rapid as compared to immunofluorescence. A certain amount of preparation is required, but once everything is in place, fixation, staining and microscopy can be done in 20 min or so.

The entire labeling procedure for the Golgi complex can be performed in ~1 hr. Although samples can be subsequently fixed and used for double-labeling studies with antibodies, they usually cannot be stored for very long (due to signal loss from photobleaching), so immediate use in imaging studies is recommended.

Once the equipment and reagents are prepared, mitochondrial staining is rapid.

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Confocal microscopy produces sharp images of structures within relatively thick specimens (up to several hundred microns). It is particularly useful for examining fluorescent specimens. Thick fluorescent specimens viewed with a conventional widefield fluorescent microscope appear blurry and lack contrast because fluorophores throughout the entire depth of the specimen are illuminated, and fluorescence signals are collected not only from the plane of focus but also from areas above and below. Confocal microscopes selectively collect light from thin (~1- μm) optical sections representing single focal planes within the specimen. Structures within the focal plane appear more sharply defined than they would with a conventional microscope because there is essentially no flare of light from out-of-focus areas. A three-dimensional view of the specimen can be reconstructed from a series of optical sections at different depths.

The confocal microscope is the instrument of choice for examining fluorescence-stained cells in tissue slices or small, intact organisms such as *Drosophila* (Fig. 4.5.1A,B) and zebrafish embryos. It is also useful for localizing fluorescent-tagged molecules in dissociated cells (Fig. 4.5.1C,D). Its sensitivity even allows fluorescence in living specimens to be monitored, making it feasible to follow the movements in living cells of fluorescent probes such as the green fluorescent protein (GFP; Fig. 4.5.1D). In addition, some types of confocal microscopes can be configured to perform photobleach experiments (Fig. 4.5.1D) and to photoactivate "caged" molecules (molecules that are inactive until released with UV illumination).

Biologists use confocal microscopy in a number of creative ways that are beyond the scope of this article. The information presented herein is intended to provide background and practical tips needed to get started with confocal microscopy. An excellent source of theoretical and technical information is the *Handbook of Biological Confocal Microscopy* (1995; edited by J. Pawley). Also recommended are *Cell Biological Applications of Confocal Microscopy* (1993; edited by B. Matsumoto), a good source of practical information; *Confocal Microscopy* (1990; edited by T. Wilson), for theoretical background; and *Video Microscopy* (1997; Inoué and Spring) for fundamentals of microscopy.

BASIS OF OPTICAL SECTIONING

Confocal microscopes accomplish optical sectioning by scanning the specimen with a focused beam of light and collecting the fluorescence signal from each spot via a spatial filter (generally a pinhole aperture) that blocks signals from out-of-focus areas of the specimen. The physical basis of optical sectioning in fluorescence confocal microscopy is illustrated in Figure 4.5.2. A point light source (typically a laser) evenly illuminates the back focal plane of the objective, which focuses the light to a diffraction-limited spot in the specimen. The irradiation is most intense at the focal spot, although areas of the specimen above and below the focal spot are also illuminated. Fluorescent molecules excited by the incident light emit fluorescence in all directions. The fluorescence collected by the objective comes to focus in the image plane, which is conjugate (confocal) with the focal plane in the specimen. A pinhole aperture in the image plane allows fluorescence from the illuminated spot in the specimen to pass to the detector but blocks light from out-of-focus areas.

The diameter of the pinhole determines how much of the fluorescence emitted by the illuminated spot in the specimen is detected, and the thickness of the optical section. From wave optics we know that a point light source in the plane of focus of an objective produces a three-dimensional diffraction pattern in the image plane. The cross section at the image plane is an Airy disk, a circular diffraction pattern with a bright central region. The radius of the bright central region of the Airy disk in the reference frame of the specimen is given by

$$R_{\text{Airy}} = 0.61\lambda/\text{NA}$$

where λ is the emission wavelength and NA is the numerical aperture of the objective (see UNIT 4.1 for a discussion of NA). At the image plane (the location of the pinhole aperture), the radius of the central region is R_{Airy} multiplied by the magnification at that plane (for a more complete explanation see Wilson, 1995).

Adjustment of the pinhole to a diameter slightly less than the diameter of the central region of the Airy disk allows most of the light from the focal point to reach the detector and reduces the background from out-of-focus areas by ~1000-fold relative to widefield microscopy (Sandison et al., 1995). The separation of the in-focus signal from the out-of-focus back-

Contributed by Carolyn L. Smith

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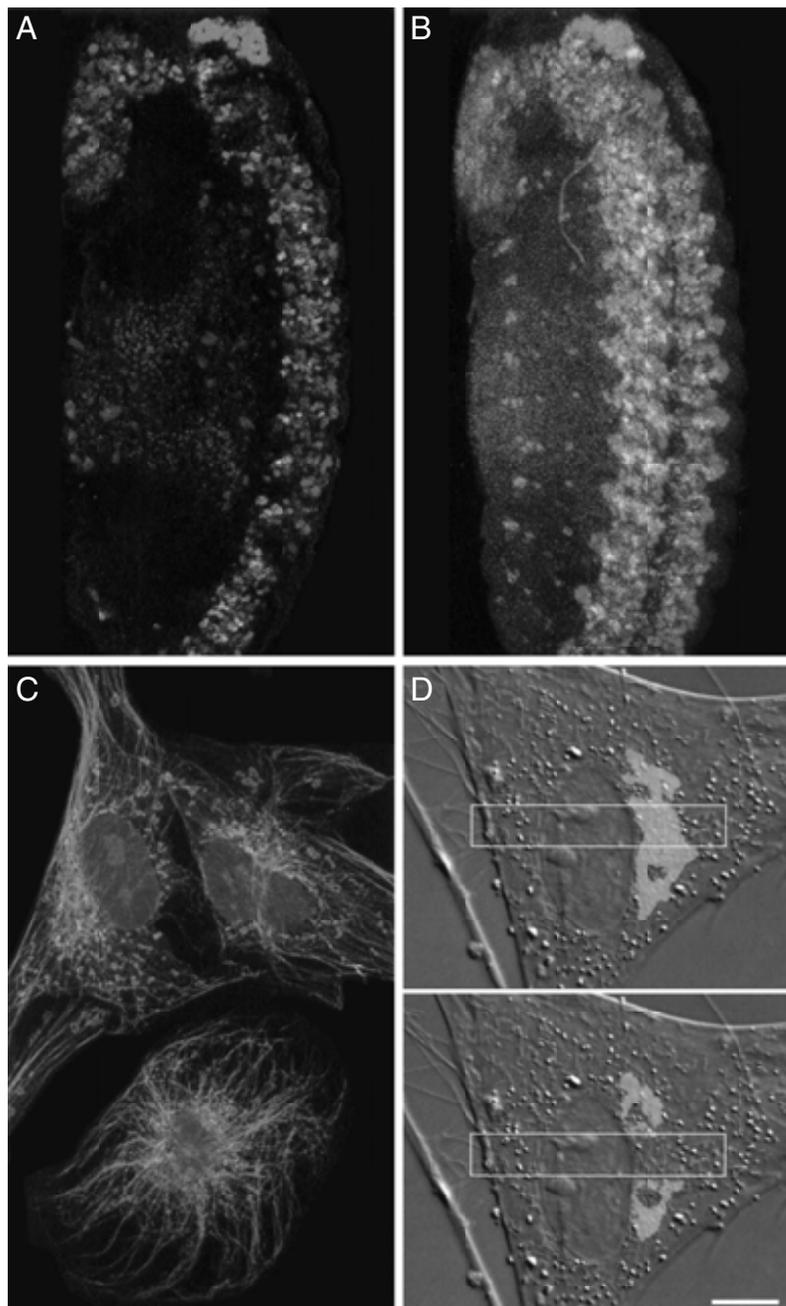


Figure 4.5.1 Applications of laser scanning microscopy. Top images (from W. Odenwald; see Kamabadur et al., 1998) show three-dimensional analysis of thick specimens. Different neuronal populations of an $\sim 250\text{-}\mu\text{m}$ -thick *Drosophila* embryo were immunolabeled with antibodies against three transcription factors. **(A)** Approximately $2.5\text{-}\mu\text{m}$ optical section collected with $25\times$, 0.8-NA objective using a detector pinhole diameter of ~ 1.3 Airy units. Labeled neurons in the plane of focus appear sharply defined, while those outside it are not visualized. **(B)** Projection (superimposition) of 65 optical sections collected at $2\text{-}\mu\text{m}$ intervals in the z axis. Neurons at different focal planes appear to overlap in this flattened image, but are distinct in a 3-D reconstruction. **(C)** Localization of intracellular structures. Dissociated rat fibroblasts were immunolabeled with anti-tubulin antibodies to visualize microtubules (green) and stained with fluorescent probes for mitochondria (MitoTracker, red) and DNA (DAPI, blue). The image is a projection of 20 optical sections collected at $0.3\text{-}\mu\text{m}$ intervals in the z axis with $100\times$, 1.4-NA objective. **(D)** Measuring molecular motility. In a living fibroblast expressing a Golgi membrane protein (galactosyltransferase) fused to GFP (S65T-GFP), GFP fluorescence (green) localized to the Golgi complex, shown superimposed on a DIC image of the cell. After the first image was collected, the boxed region (yellow) was scanned with full laser power; this photobleached the GFP in the boxed area as shown in the second image collected ~ 2 sec later. The rate of fluorescence recovery into the photobleached zone (not illustrated) indicated that the GFP-galactosyltransferase fusion is highly mobile in Golgi membranes. **See color figure.**

ground achieved by a properly adjusted pinhole is the principle advantage of confocal microscopy for examination of thick specimens (see Fig. 4.5.1A,B).

Point illumination and the presence of a pinhole in the detection light path also produce improved lateral and axial resolution relative to conventional microscopy (Table 4.5.1). The actual extent of improvement depends on the size of the pinhole. Near-maximal axial resolution is obtained with a pinhole radius $\sim 0.7 \times R_{\text{Airy}}$ whereas optimal lateral resolution is obtained with a pinhole smaller than $0.3 \times R_{\text{Airy}}$ (Wilson, 1995). However, a pinhole smaller than $\sim 0.7 \times R_{\text{Airy}}$ significantly reduces the total signal, a sacrifice that may not be worth the gain in resolution, especially when imaging dim samples. In fluorescence imaging, resolution also is influenced by the emission and excitation wavelengths (Table 4.5.1).

TYPES OF CONFOCAL MICROSCOPES

Several types of confocal microscopes are available, each having unique features and advantages. Those most commonly used for examining fluorescence specimens are laser-scanning confocal microscopes. These microscopes, as their name implies, use lasers as light sources and collect images by scanning the laser beam across the specimen.

Lasers provide intense illumination within a narrow range of wavelengths. The emission wavelengths of several types of lasers, together with the excitation spectra of familiar fluorophores, are illustrated in Figure 4.5.3. Mixed krypton-argon gas lasers are popular for multi-wavelength confocal microscopy because they emit at three well-separated wavelengths (488, 568, and 647 nm) that can be used to simultaneously image two or three fluorophores (e.g., FITC, lissamine rhodamine, and Cy5). The disadvantage of krypton-argon lasers is that

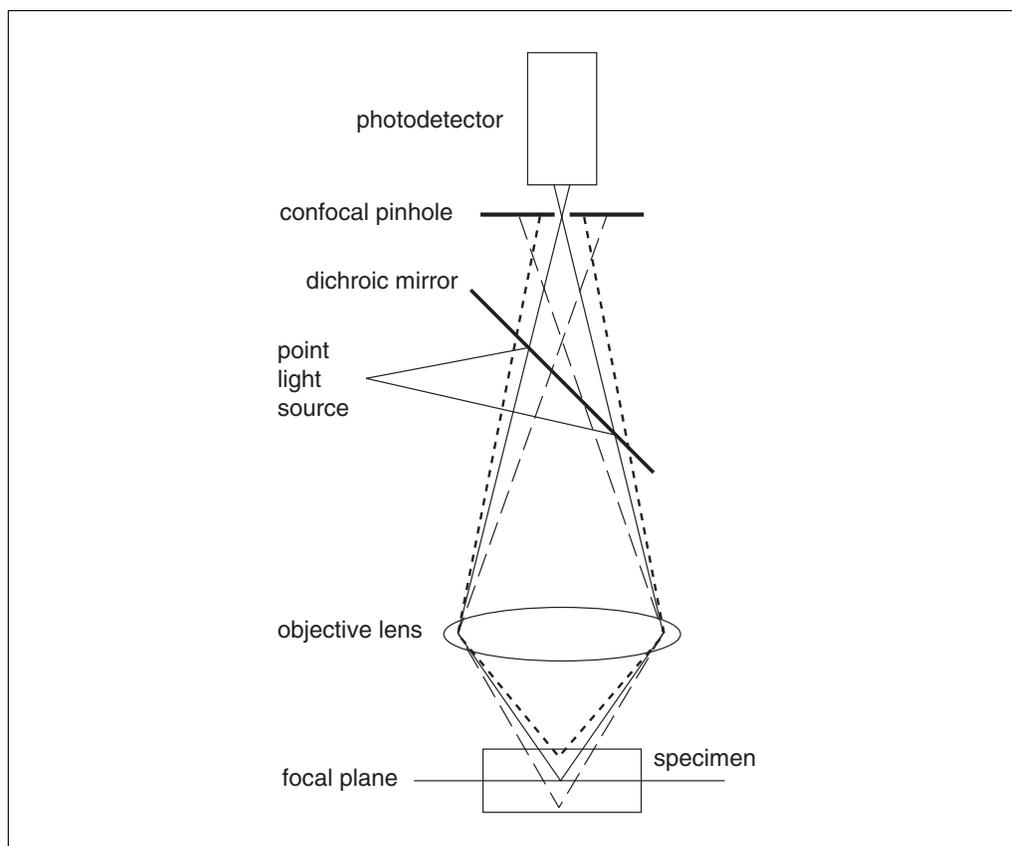


Figure 4.5.2 The basis of optical sectioning in confocal epifluorescence microscopy. Illumination from the point light source is reflected by the dichroic mirror and focused by the objective lens to a diffraction-limited spot within the specimen. Fluorophores within the focal spot as well as in the cone of light above and below it are excited, emitting fluorescence at a longer wavelength than the incident light. The fluorescence captured by the objective passes through the dichroic mirror because of its longer wavelength. The confocal pinhole allows fluorescence from the plane of focus in the specimen to reach the photodetector but blocks fluorescence from areas above and below the plane of focus. Redrawn from Shotton (1993).

Table 4.5.1 Theoretical Resolutions of Confocal and Conventional Microscopes^a

$\lambda_{\text{ex}}/\lambda_{\text{em}}$	Objective					
	10×, 0.4 NA, air		40×, 0.85 NA, air		60×, 1.4 NA, oil	
	Lat. res.	Ax. res.	Lat. res.	Ax. res.	Lat. res.	Ax. res.
<i>Confocal fluorescence microscope</i>						
488/518	0.55	4.50	0.26	0.99	0.16	0.56
568/590	0.64	5.17	0.30	1.09	0.18	0.64
647/677	0.72	5.88	0.34	1.28	0.21	0.72
<i>Conventional fluorescence microscope</i>						
518	0.79	6.48	0.37	1.43	0.24	0.93
590	0.90	7.38	0.42	1.63	0.28	1.06
680	1.04	8.50	0.49	1.88	0.32	1.22

^aData reprinted from Brelje et al. (1993) by permission of Academic Press. λ_{ex} and λ_{em} , excitation and emission wavelengths; lat. res. and ax. res., lateral and axial resolutions.

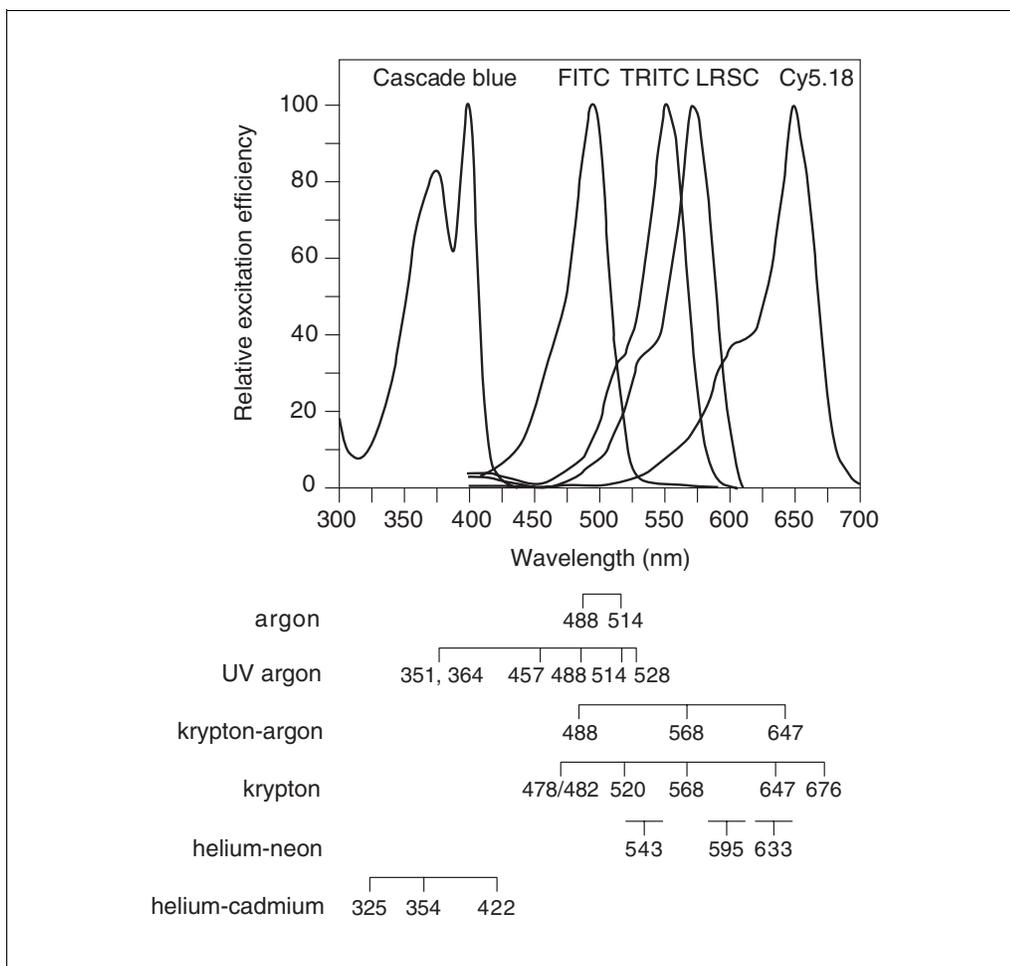


Figure 4.5.3 Comparison of the emission wavelengths of various lasers and the excitation spectra of representative fluorophores. The lasers most commonly used for laser-scanning confocal microscopy are air-cooled argon (488 and 514 nm), krypton-argon, and helium-neon lasers. UV argon lasers generally require water cooling and are more expensive. They may be configured to provide only UV wavelengths (351 nm and 364 nm) or both UV and longer wavelengths. Data for the excitation spectra of Cascade blue, fluorescein (FITC), tetramethylrhodamine (TRITC), lis-samine rhodamine (LRSC), and cyanine 5.18 (Cy5.18) are from Wessendorf and Brelje (1993) and were downloaded from the web page of Aryeh Weiss, <http://optics.jct.ac.il/~aryeh/Spectra>. Modified from Brelje et al. (1993).

their life spans are short (~2000 hr). Another way to achieve multiwavelength excitation is to combine the outputs of two or more lasers.

Several methods have been devised for scanning the sample with the laser beam to illuminate different positions in the specimen. The most common method employs a pair of galvanometer mirrors to both scan the laser beam across the specimen and collect the fluorescence emitted from the specimen (Fig. 4.5.4). One galvanometer mirror scans sequential spots along the *x* axis, and the second mirror moves from line to line in the *y* axis. The fluorescence emission is separated from the illuminating beam by a dichroic beam splitter and is directed to a photomultiplier tube which collects the fluorescence produced as each spot in the specimen is illuminated. The photodetector output is converted to a digital image that can be displayed on a monitor and stored as a digital image file for later analysis. Most laser-scanning confocal microscopes have 8-bit digi-

tizers that encode 256 gray levels, although some recent models have 12- or 16-bit digitizers. Collection of a full-size image (typically 1024 × 1024 pixels) takes ~2 sec. Laser-scanning microscopes that employ galvanometer mirror scanners sometimes are called “slow-scan” microscopes because of their relatively slow image acquisition rates. Slow-scan microscopes are available from several sources (Bio-Rad, Zeiss, Leica, Olympus, Nikon, Molecular Dynamics, and Meridian; see *SUPPLIERS APPENDIX*).

The movements of the galvanometer mirrors in laser-scanning microscopes are under the control of a computer, providing flexibility in the scanning pattern. For example, it is possible to “zoom” a region of interest (visualize it at higher magnification) by reducing the scan area and the distances between sample points. In addition, many laser-scanning microscopes have the ability to repetitively scan a single line or to “park” the scanner to monitor fluores-

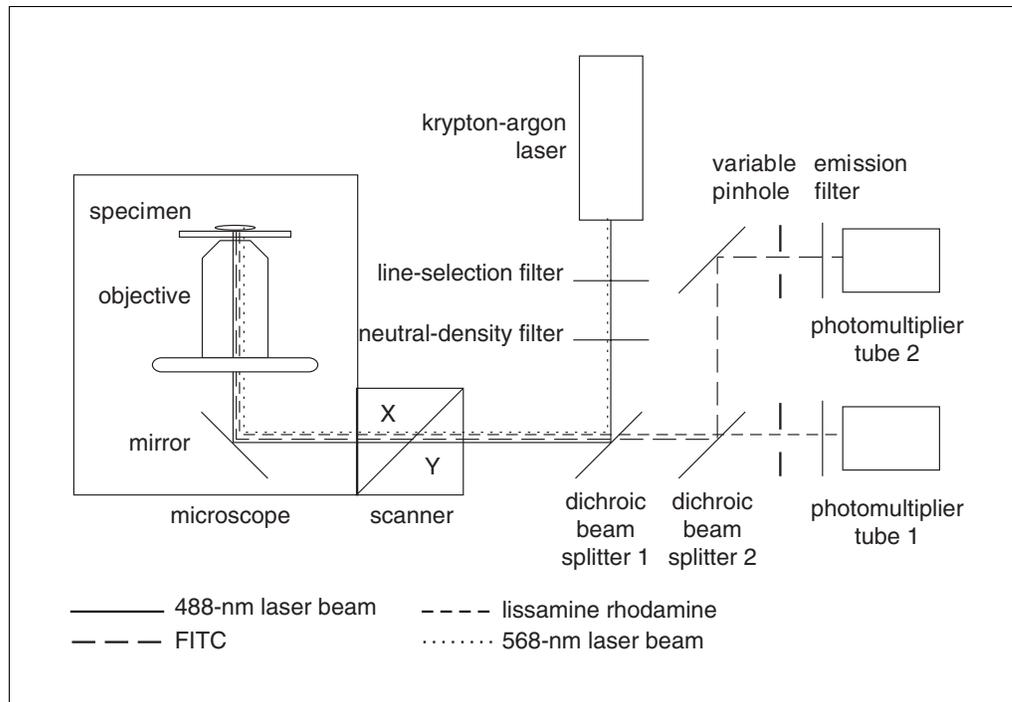


Figure 4.5.4 The light path of a laser-scanning confocal microscope set up for simultaneous imaging of FITC and lissamine rhodamine. The 488-nm and 568-nm lines of a krypton-argon laser are reflected by dichroic beam splitter 1 into the optical axis of the microscope. The scanner contains two galvanometer mirrors, which generate the *x* and *y* axis movements of the beam. The beam is reflected by a mirror into the objective which focuses the beam onto the specimen. The specimen is scanned line by line in a raster pattern. Fluorescence emitted by the specimen as each spot is illuminated travels the reverse path through the scanning system. The FITC fluorescence (peak at 520 nm) and lissamine rhodamine fluorescence (peak at 590 nm) pass through dichroic beam splitter 1 to dichroic beam splitter 2, which transmits the lissamine rhodamine fluorescence to photomultiplier tube 1 and reflects the FITC fluorescence to photomultiplier tube 2. A variable pinhole in front of each photodetector blocks light from out-of-focus areas of the specimen while allowing light from the illuminated spot to reach the detector.

cence at a single spot. The latter technique is particularly useful for studying rapidly changing fluorescence signals, such as those produced by a Ca^{2+} indicator in an active neuron.

Laser-scanning microscopes are available (from Noran, Life Sciences Resources, and Meridian; see *SUPPLIERS APPENDIX*) that can collect images at video rates (30 frames/sec) or faster. Several methods for achieving rapid scanning rates have been employed, such as acousto-optical deflection devices, rotating mirrors, or resonating mirrors (reviewed by Art and Goodman, 1993; Tsien and Bacsikai, 1995). The gain in imaging speed always comes at a cost, however. For example, rapid-scan confocal microscopes do not provide the degree of control over the scan pattern offered by top-of-the-line slow-scan microscopes, and some video-rate confocal microscopes are incapable of multiwavelength illumination. Video-rate microscopes that rely on slit apertures rather than pinhole apertures have slightly poorer lateral and axial resolution.

A type of rapid-scan confocal microscope that deserves mention because of its lower cost (among other reasons) uses a spinning disk with multiple pinholes (~200,000) to simultaneously illuminate and detect emission from many spots in the specimen. The light source can be a laser or a broad-spectrum lamp like that used for conventional epifluorescence microscopy. The principle advantage of this type of confocal microscope is that it is capable of collecting images very rapidly (up to 700 frames/sec at 5000 lines resolution; Kino, 1995). The images can be examined directly by eye or captured with a sensitive camera. The main disadvantage is that the disk transmits only ~1% of the available light because the holes in the spinning disk need to be widely spaced. A new type of spinning-disk confocal microscopy has recently become available that uses "microlenses" to improve optical throughput and achieve high-speed confocal imaging with better sensitivity (Ultra View; Life Sciences Resources; see *SUPPLIERS APPENDIX*).

Another form of laser-scanning microscopy that promises to be of great value uses two-photon (and three-photon) excitation to induce fluorescence emission (Denk et al., 1995). Two-photon excitation occurs when a fluorophore absorbs two photons, each having half the energy needed to raise the fluorophore to the excited state. The light intensities required for simultaneous absorption occur only at the focal point, so only fluorophores at the focal point are excited. Therefore, two-photon exci-

tation allows optical sectioning without a spatial filter in front of the detector. Moreover, since fluorophores outside the focal point are not excited, the specimen is less subject to photobleaching than in a conventional laser-scanning microscope. The wavelengths needed to excite standard visible light fluorophores by two-photon absorption are longer and penetrate tissue better than the wavelengths used for one-photon excitation, making it possible to look deeper into a specimen. In addition, UV fluorophores can be imaged without many of the problems that arise when UV wavelengths are used in conventional laser-scanning microscopes. A current drawback of two-photon confocal microscopy is the high cost of an appropriate laser (~\$100,000). Two-photon scanning microscopes are now available from commercial sources (Bio-Rad, Leica; see *SUPPLIERS APPENDIX*).

PRACTICAL GUIDELINES

Sample Preparation: Immunofluorescence in Fixed Specimens

Additional guidelines for sample preparation are discussed in *UNIT 4.3*.

Fixation

The best fixative is one that accurately preserves the three-dimensional geometry of the specimen. The standard fixative for fluorescence microscopy (2% to 4% formaldehyde in PBS) is not ideal because it can cause blebbing of the plasma membrane, vesiculation of intracellular membrane compartments, and other alterations in cellular morphology. Moreover, some commercial preparations of formaldehyde contain methanol, which shrinks cells. Techniques for optimizing formaldehyde fixation are described by Bacallao et al. (1995). The buffer should be chosen to match the osmolality and pH of the specimen. Fixatives containing 0.125% to 0.25% glutaraldehyde in addition to formaldehyde preserve cellular morphology better than those containing formaldehyde alone. Some investigators avoid using glutaraldehyde for fluorescence microscopy because it induces autofluorescence. However, autofluorescence can be reduced by treating the sample after fixation with NaBH_4 (1 mg/ml in PBS, pH 8.0, using two treatments of 5 min each for dissociated cells, longer for thicker samples). A more serious drawback of glutaraldehyde for immunofluorescence studies is that it destroys the antibody recognition sites of some antigens.

An alternative fixation technique that preserves tissue better than chemical fixation is rapid freezing followed by freeze substitution (Bridgman and Reese, 1984).

Choices of fluorophores

Criteria to consider in selecting fluorophores for fluorescence microscopy are described in *UNIT 4.2&APPENDIX 1E*. The only additional consideration for confocal microscopy is to choose fluorophores that can be excited by the wavelengths provided by the available lasers. However, it is not essential for the excitation spectrum peak to precisely match the laser wavelength because the lasers on most microscopes are sufficiently powerful to maximally excite fluorophores at off-peak wavelengths. For experiments that depend on imaging two fluorophores, it is best to select fluorophores whose excitation and emission spectra have minimal overlap. Good choices for multiwavelength imaging with a krypton-argon laser are: FITC/Oregon green/Alexa 488 (Molecular Probes) for excitation at 488 nm; lisamine rhodamine/Cy3/Texas red/Alexa 568 (Molecular Probes) for excitation at 568 nm; and Cy5 for excitation at 647 nm. UV fluorophores also are good for multicolor imaging (with absorption at 350 to 390 nm; some of the best dyes for DNA are UV fluorophores).

Control samples

Confocal microscopes rely on electronic image enhancement techniques that can make even a dim autofluorescence signal or nonspecific background staining look bright. In order to be able to distinguish a real signal from background it is essential to prepare appropriate control samples. For immunofluorescence experiments with one primary antibody, the appropriate control samples are unstained specimens and specimens treated with the secondary antibody but no primary antibody. Experiments with two primary and secondary antibodies require additional controls to test whether the secondary antibodies cross-react with the “wrong” primary antibody. Other control experiments may be required to verify the specificity of labeling.

Mounting the specimen

The mounting medium should preserve the three-dimensional structure of the specimen. PBS (*APPENDIX 2A*) or a mounting medium consisting of 50% glycerol/50% PBS preserves the shapes of cells quite well, but Mowiol and gelvatol cause a 10% decrease in height (Ba-

callao et al., 1995). Adding an antioxidant to the mounting medium helps to alleviate photobleaching. One of the best antioxidants is 100 mg/ml 1,4-diazabicyclo[2,2,2]octane (DABCO; Sigma; Bacallao et al., 1995). *n*-propyl gallate (Giloh and Sedat, 1982) and *p*-phenylenediamine (PPD; Johnson et al., 1982) also are effective antibleaching agents, but the former may cause dimming of the fluorescence while the latter may damage the specimen (Bacallao et al., 1995). ProLong, a new antifade reagent from Molecular Probes, hardens upon drying and is exceptionally effective in preserving the fluorescence of most fluorophores.

The choice of mounting medium should take into account the type of microscope objective that will be used to observe the specimen. In order for an objective to perform optimally, the mounting medium should have the same refractive index as the objective immersion medium. Table 4.5.2 gives the refractive indexes of standard objective immersion media and mounting media. Mismatches in the refractive indexes produce spherical aberration leading to loss of light at the detector, as well as decreased *z* axis resolution and incorrect depth discrimination. Image deterioration caused by spherical aberration increases with depth into the specimen. Significant losses of signal intensity and axial resolution are apparent at distances of just 5 to 10 μm when an oil immersion objective is used to examine a specimen in an aqueous medium (Keller, 1995).

Most microscope objectives are designed for viewing specimens through a glass coverslip of a specific thickness (typically 0.17 μm , a no. 1½ coverslip). Correct coverslip thickness is especially critical for high-NA (>0.5) dry objectives and water immersion objectives (Keller, 1995). Use of a coverslip that differs from the intended thickness by only 5% causes significant spherical aberration. High-NA dry and water immersion objectives typically have an adjustable collar to correct for small variations in coverslip thickness.

The specimen should be mounted as close to the coverslip as possible, especially for observation with immersion objectives, which have short working distances (~100 to 250 μm , depending on the type of objective). This also helps to avoid image deterioration due to spherical aberration. Fragile specimens should be protected by supporting the coverslip; for example, using a thin layer of nail polish, strips of coverslips, or a gasket made from a sheet of

Table 4.5.2 Refractive Indexes of Common Immersion and Mounting Media

Medium	Refractive index (RI)
<i>Immersion media</i>	
Air	1.00
Water	1.338
Glycerol	1.47
Immersion oil	1.518
<i>Mounting media</i>	
50% glycerol/PBS/DABCO	1.416 ^a
5% <i>n</i> -propyl gallate/0.0025% <i>p</i> -phenylene diamine (PPD) in glycerol	1.474 ^a
0.25% PPD/0.0025% DABCO/5% <i>n</i> -propyl gallate in glycerol	1.473 ^a
VectaShield (Vector Labs)	1.458 ^a
Slow Fade (Molecular Probes)	1.415 ^b
ProLong (Molecular Probes)	1.3865 ^{b,c}

^aData from Bacallao et al. (1995).

^bData from Molecular Probes.

^cRI for liquid medium (RI for solidified medium will be higher).

silicon rubber (Reiss; see *SUPPLIERS APPENDIX*). Sealing the edges of the coverslip—with nail polish or silicon vacuum grease (Dow Corning; see *SUPPLIERS APPENDIX*)—helps to prevent specimen desiccation and movement.

Living Specimens

Confocal microscopy of living preparations is challenging for several reasons. The specimen must be mounted in a chamber that keeps it healthy and immobile while at the same time providing access for the objective. For high-resolution transmitted-light imaging (e.g., by laser-scanning differential interference contrast microscopy), the chamber must be thin enough to accommodate a high-NA (oil immersion) condenser. Fluorescence signals in living specimens generally are weak and the illumination levels needed to detect them can be damaging to the specimen. Photobleaching inevitably is a problem for experiments that require collecting many images. Temperature fluctuations in specimens kept at nonambient temperatures make it difficult to maintain accurate focus.

A simple chamber for culture preparations grown on glass coverslips can be made by forming a well on a glass slide with a gasket cut from a sheet of silicon rubber or a plastic ruler. To prevent the well from leaking, it should be sealed

with silicon vacuum grease, a mixture of melted paraffin and petroleum jelly, or Sylgard (Dow Corning; see *SUPPLIERS APPENDIX*). The well is filled with medium and then the coverslip with attached cells is placed, cell side down, on top of the well. The preparation can be kept warm during observation on the microscope with a heated air blower—e.g., a hair dryer with variable power source or a commercial air-stream incubator (e.g., Neutek; see *SUPPLIERS APPENDIX*)—or with infrared lamps. More elaborate chambers, some of which have built-in heaters and ports for changing solutions, are available from commercial sources (see Terasaki and Dailey, 1995, for a partial listing of manufacturers). An important factor to consider in choosing a chamber is whether it maintains the desired temperature while in contact with an immersion objective that acts as a heat sink. One solution to this problem is to heat the objective as well as the chamber. A heated chamber and objective warmer designed for microscopy with a high-NA objective and condenser are available from Bioptechs (see *SUPPLIERS APPENDIX*).

Addition of an oxygen quencher to the medium can help to alleviate photobleaching of the fluorophores. Photobleaching not only leads to dimming of the signal but also to generation of oxygen radicals that can damage cells. Several oxygen quenchers have been re-

ported to be effective, including oxyrase (0.3 U/ml; Oxyrase [see *SUPPLIERS APPENDIX*]; Waterman-Storer et al., 1993); ascorbic acid (0.1 to 3.0 mg/ml; Sigma; Terasaki and Dailey, 1995); a mixture of Trolox (10 μ M; Aldrich) and *N*-acetylcysteine (50 μ M; Sigma; M. Burack and G. Banker, pers. comm.); and crocetin (Tsien and Waggoner, 1995).

Optimizing Imaging Parameters

Choice of objectives

High-NA objectives generally are preferable for fluorescence microscopy because they collect more light than low-NA objectives (brightness is proportional to NA^4). Most high-quality high-NA objectives have >80% transmission at visible wavelengths, but some have low transmission at UV wavelengths (Keller, 1995).

Water immersion objectives are the best choice for visualizing specimens in aqueous solutions (e.g., living specimens). Several microscope manufacturers recently have introduced high-NA water immersion objectives specifically designed for confocal microscopy of biological specimens. These objectives differ from previously available types of water immersion objectives in that they are intended for viewing specimens mounted under a coverslip. They have working distances of $\sim 250 \mu\text{m}$.

Oil immersion objectives can have higher NAs than water immersion objectives. Most have fairly short working distances ($\sim 100 \mu\text{m}$) although some recently introduced oil objectives have working distances of $\sim 200 \mu\text{m}$. A long-working-distance oil objective will be useful only if the specimen is mounted in a medium that matches the refractive index of immersion oil ($n = 1.518$). If an aqueous mounting medium is used, images from depths at more than $\sim 20 \mu\text{m}$ into the specimen will be noticeably degraded by spherical aberration. Also, distance measurements in the *z* axis will need to be corrected. The actual movement of the focal plane in the specimen (d_s) produced by a movement of the objective (d_{obj}) depends on the ratio of the refractive indexes of the specimen and immersion medium. A reasonable approximation (Majilof and Forsgren, 1993) of the relationship is given by:

$$d_s/d_{\text{obj}} = n_s/n_{\text{obj}}$$

Pinhole size

As was explained above (see Basis of Optical Sectioning), the size of the pinhole has a

critical influence on image quality. A pinhole with a radius equal to the radius of the first minimum of the Airy disk—which is approximately equivalent to the diameter at half maximal intensity (Amos, 1995)—will let most of the light from the plane of focus reach the detector, while blocking most of the out-of-focus flare. The lateral resolution will be $\sim 20\%$ better than that obtainable by conventional microscopy with the same optics (Centonze and Pawley, 1995), although not as good as can be achieved with a smaller pinhole. Lateral resolution continues to improve as pinhole radius is decreased down to a pinhole size of $\sim 0.2 \times$ Airy disk radius, but a pinhole this small excludes $\sim 95\%$ of the signal (Wilson, 1995). Axial resolution improves as pinhole size decreases, down to $\sim 0.7 \times$ Airy disk radius, then levels off. The best trade-off between signal intensity and resolution will depend on the characteristics of the sample and aims of the experiment.

Zoom factor

The zoom setting on a confocal microscope determines the size of the scan region and the apparent magnification of the image. A zoom factor of 2 will scan an area half as long and wide as a zoom factor of 1. Images are made up of the same number of samples (points along the horizontal axis, lines along the vertical axis) and are displayed on the image monitor by a fixed number of pixels regardless of the zoom factor. Therefore, the pixels in a zoom-2 image will represent areas within the specimen half as large in each dimension as the areas represented by the pixels at zoom 1. If the pixel size for an objective at zoom 1 represents $0.25 \mu\text{m} \times 0.25 \mu\text{m}$, then the pixel size at zoom 2 will be $0.125 \times 0.125 \mu\text{m}$. The pixel dimensions (referring to the specimen) are inversely related to the zoom setting.

For each objective, there is an optimal zoom setting which yields pixel dimensions small enough to take advantage of the full resolution of the objective but large enough to avoid oversampling. In order for the minimum resolvable entity to be visible on the display monitor, the pixel dimensions need to be smaller than (less than one-half) the optical resolution. However, if the pixel size is made too small by using a higher-than-optimal zoom factor, the specimen is subjected to more irradiation than necessary with an increased risk of photobleaching. The rate of photobleaching increases proportionally to the square of the zoom factor (Centonze and Pawley, 1995). A guideline for selecting an appropriate zoom factor derived from informa-

tion theory (the Nyquist Sampling Theorem) states that the pixel dimensions should be equal to the optical resolution divided by 2.3 (see Webb and Dorey, 1995). However, pixel dimensions smaller than this may produce more informative images.

Z axis sectioning interval

In order to study the three-dimensional structure of a specimen, images are collected at a series of focal levels at intervals determined by the commands sent to the focus motor. The most straightforward way to ensure that the reconstructed images have correct proportions in the x , y , and z axes is to collect optical sections at z axis intervals equal to the x, y pixel dimension. However, the interfocal plane interval needed to adequately sample the specimen in the z axis is not as small as the x, y pixel dimension because the axial resolution is poorer than the lateral resolution (see Table 4.5.1). The optimal interfocal plane interval (according to the Nyquist Sampling Theorem) is equal to the axial resolution divided by 2.3. Collecting images at shorter intervals results in oversampling with an increased risk of photobleaching.

Illumination intensity

Fluorescence emission increases linearly with illumination intensity up to a level at which emission saturates. Optimal signal-to-background and signal-to-noise ratios are obtained with illumination levels well below saturation (Tsien and Waggoner, 1995). The illumination intensity on a laser-scanning microscope can be adjusted by inserting neutral-density filters into the light path and/or by operating the laser at submaximal power. In general, the best images are obtained with illumination levels that are as high as possible without producing unacceptable rates of photobleaching.

PMT black level and gain

The contrast and information content of confocal images are influenced by the black level and gain of the photomultiplier tube (PMT) amplifiers. To obtain maximal information, the black level and gain should be adjusted to take advantage of the full dynamic range of the PMTs. The appropriate black level setting can be found by scanning while the light path to the PMT is blocked. The image that appears on the display monitor should be just barely brighter than the background, which is black (gray level = 0). To set the gain, scan the specimen and adjust the gain so that the brightest pixel in

the image is slightly below white (gray level = 255). Selecting black level and gain settings which ensure that all signals fall within the dynamic range of the PMT is important for quantitative imaging experiments. The software provided with many confocal microscopes includes a pseudocolor image display mode that facilitates selection of appropriate black level and gain settings by highlighting pixels with intensity values near absolute black and absolute white.

Averaging

Confocal images of dimly fluorescent specimens captured at typical scan rates (1 to 2 sec/frame for a slow-scan confocal microscope) appear noisy because of the small numbers of photons collected from each spot. In some instances, it may be possible to improve the signal-to-noise ratio by scanning the specimen at slower rates. Another way to obtain a better image is by summing and averaging the signals obtained in multiple scans (frame averaging). Some confocal microscopes provide a second averaging method (line averaging), in which individual lines are repeatedly scanned and averaged. Line averaging generally produces sharper images than frame averaging (which averages full frames) because there is less risk of blurring due to movements or changes in the specimen.

Image display

Commercial confocal microscope packages provide software for some types of image enhancement and display. The display options for three-dimensional datasets typically include “ z projections” (see Fig. 4.5.1B), which are two-dimensional displays formed by superimposition of stacks of optical sections, and stereoscopic views, which are made by combining two image stacks, one aligned in the z axis and the other with a displacement between successive images. Many systems also have the capability to compute cross-sections and projections of the specimen from varying angles. Computed projections for a sequence of view angles can be played as a movie in which the specimen appears to rotate around an axis. Such movies give the viewer a striking impression of the three-dimensional geometry of the specimen. Additional display options are available in various integrated software/hardware packages specifically designed for visualization and analysis of three-dimensional images.

Anticipated Results

Fluorescence in fixed specimens protected with an antifade agent is often sufficiently bright and resistant to photobleaching to make it possible to reconstruct three-dimensional images using imaging parameters that provide optimal resolution. Superb three-dimensional views may be obtained of structures as small and complex as a cell's cytoskeleton or the terminal arbor of an axon. The maximum depth in the specimen at which adequate images can be obtained depends on a number of factors (e.g., the match in refractive indexes of the immersion and mounting media, the wavelength of light, and the extent of scattering and absorption by the specimen). Under optimal conditions, it may be possible to image structures at depths near the limit allowed by the working distance of the objective; in practice, image quality usually deteriorates at depths in the range of a few hundred micrometers or less.

Although confocal microscopy on living cells is more difficult and damage to the tissue may preclude extensive three-dimensional reconstruction, the added time dimension and confidence in the reality of the images makes it well worth the effort. In addition, it is possible to study dynamic processes lasting for hours by collecting sequences of time-lapse images. Robust fluorophores such as certain variants of GFP (S65T, EGFP) can be imaged repeatedly with minimal loss of fluorescence (see, for example, Ellenberg et al., 1997). In addition, modern laser-scanning confocal microscopes provide a versatile optical bench and sophisticated specimen positioner which permit a wide range of experiments with the controlled application of laser light to living tissues. Current examples of these approaches are photobleaching (Cole et al., 1996; Wedekind et al., 1996) and release of caged compounds (Callaway and Katz, 1993; Svoboda et al., 1996). These are only the harbingers of many future applications of light probe physiology made possible by the versatility of the confocal microscope.

Resources Available via Internet

NIH Image, a powerful image analysis program for Macintosh computers developed by W. Rasband (Research Services Branch, National Institute of Mental Health, NIH), has many useful tools for analysis of confocal images. It can be downloaded from <http://rsb.info.nih.gov/nih-image/> or obtained via FTP from zippy.nimh.nih.gov. A version of NIH Image modified for operation under Windows also is available. Much information

about fluorescent probes can be obtained from the Molecular Probes Web Site at <http://www.probes.com/>.

Many topics of interest to confocal microscopists are discussed on the confocal e-mail listserver network. To subscribe to the list, send the message "subscribe confocal<your name>" to listserv@ubvm.cc.buffalo.edu.

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- Matsumoto, 1993. See above.
Good source of practical information.
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Excellent source of theoretical and technical information.
- Russ, J. 1995. The Image Processing Handbook. CRC Press, Boca Raton, Fla.
Excellent source of information about digital image processing.
- Shotton, D.M. (ed.) 1993. Electronic Light Microscopy. John Wiley and Sons, New York.
Covers many aspects of light microscopy, including confocal microscopy.
- Wilson, 1990. See above.
Good source of theoretical background information.

INTERNET RESOURCES

- zippy.nimh.nih.gov
Use to obtain NIH Image via FTP.
- <http://optics.jct.ac.il/~aryeh/Spectra>
Source of excitation and emission spectra for common fluorophores.
- <http://rsb.info.nih.gov/nih-image/>
Use to obtain NIH Image.
- <http://www.probes.com>
Molecular Probes web site, including product listings and much more.
- <http://www.mwrn>
Microworld Resources, a comprehensive list of microscopy products and vendors.
- listserv@ubvm.cc.buffalo.edu
Confocal e-mail listserv network.

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Immunoperoxidase Methods for Localization of Antigens in Cultured Cells and Tissues

This unit describes the localization of intracellular antigens by the immunoperoxidase staining technique at the electron microscopic (EM) level. These methods provide a potentially valuable alternative, or in some cases a complementary approach, to immunogold labeling. Labeling with immunogold provides a higher level of antigen resolution and can be made reasonably quantitative. However, immunogold labeling is usually best conducted on ultrathin, frozen sections, which requires a special microtome and an extremely high degree of specialized technical skill. In contrast, immunoperoxidase labeling methods are relatively simple in that no special equipment or skills are needed beyond those required for standard transmission EM of plastic-embedded samples. Furthermore, they provide the opportunity for enzymatic amplification of what might otherwise be weak immunoreactive signals. The immunoperoxidase method is limited by its inherently qualitative nature and by the potential for diffusion of the immunoreactive signal from the site of antigen location. These advantages and limitations make immunoperoxidase labeling particularly valuable for localizing intracellular antigens that are contained within membrane-bounded organelles. Under certain circumstances, however, the method can also be successfully applied to cytoplasmic and extracellular antigens.

The immunoperoxidase methods described here are typically referred to as preembedding labeling methods, meaning that the antibodies bind to antigens before the cells or tissues are embedded for sectioning. The methods are also of the indirect type, meaning that the antigen is localized by a two-step antibody binding protocol: antigens are first bound by primary antibodies, which are in turn bound by a secondary antibody that is covalently coupled with horseradish peroxidase (HRP). An electron-dense immunoreaction product at the sites of antigen-antibody binding is then generated in two steps: first, the bound HRP-conjugated secondary antibody catalyzes the formation of oxidized and polymerized diaminobenzidine (DAB) using H_2O_2 as an electron donor, and second, the oxidized and polymerized DAB is rendered electron dense by binding to reduced osmium tetroxide (OsO_4). Labeled cells are then embedded in plastic resin and sectioned for standard transmission EM.

This unit describes two protocols for immunoperoxidase staining: one specific for adherent, cultured cells (see Basic Protocol 1) and one specific for cells within tissue sections (see Basic Protocol 2). Three embedding methods are described for adherent cultured cells. (1) For antigens located anywhere in the cell except the endoplasmic reticulum (ER), cells can be easily detached using propylene oxide and embedded with a standard plastic embedding resin in a microcentrifuge tube (see Basic Protocol 1). (2) For antigens located in the ER, cells can be detached by scraping in ethanol before embedding (see Alternate Protocol 1). (3) Alternatively, to prepare sections through the full depth of the cultured monolayer, cells can be embedded while they are still attached to the culture dish (see Alternate Protocol 2).

STRATEGIC PLANNING

Preliminary Light Microscopy Experiments

Immuno-electron microscopy, by either immunogold or immunoperoxidase labeling, is generally a labor-intensive and time-consuming enterprise. The rate-limiting step is thin sectioning, which is required for EM observations. Therefore, it is strongly recommended that several preliminary studies establishing near-optimal staining parameters be carried

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at the light microscopic (LM) level, first by immunofluorescence staining (*UNIT 4.3*) and then by LM-level immunoperoxidase localization. Immunofluorescence is relatively rapid and simple, and it can be used to optimize a number of potentially important variables. For example, one can easily screen by immunofluorescence to optimize fixation time, detergent concentration for cell permeabilization, antibody concentrations, and incubation times. Experience has shown that experimental conditions (for these variables) that generate a bright immunofluorescence signal can generally be applied directly to the immunoperoxidase labeling protocol. Once these conditions are established, a few preliminary immunoperoxidase labeling experiments should be conducted at the LM level. This second series of experiments is valuable for establishing the optimal concentration of HRP-conjugated secondary antibody and the optimal DAB reaction time.

A large amount of time can be saved by optimizing conditions through these preliminary immunofluorescence and immunoperoxidase LM experiments rather than at the EM level. Also, the results obtained by the LM methods can serve as an invaluable comparison for those obtained by EM, either providing increased confidence that the antibodies are accurately localizing the antigen of interest or providing cause for concern. In other words, the results should be consistent irrespective of the method used. Finally, preliminary screening by immunofluorescence or LM immunoperoxidase labeling should be used to ensure that the primary antibody is of sufficiently high quality to generate a high signal-to-noise ratio. Meeting this goal may require production of an IgG fraction, affinity purification of the antibodies, or production of a high-titer ascites fluid for monoclonal antibodies. A successful immunoperoxidase localization at the EM level is very unlikely to be realized if the antibodies cannot first generate an easily detectable immunofluorescence and LM immunoperoxidase signal.

Monitoring the DAB Reaction

Monitoring development of the DAB reaction can easily be done with an upright light microscope and a low-power or long-working-distance objective lens (so it won't get wet), or with an inverted microscope. If using a standard upright microscope, place the petri dish on a microscope slide on the microscope stage to move the dish around. The development of the DAB reaction is best viewed by plain brightfield optics, rather than by phase contrast, because the formation of the contrasting brown/black reaction product is more easily detected.

For most antigens, by the time the reaction looks really good by LM, it will probably be overdone for EM, because the DAB reaction product may have diffused away from the immediate area of the antigen-antibody complexes. This is especially true for antigens contained within membrane-bounded organelles (e.g., the Golgi complex and endosomes), which can lyse following formation of extensive amounts of reaction product. However, antigens contained within the endoplasmic reticulum are sometimes more difficult to detect at the EM level and, therefore, require that the reaction product be quite dark and obvious at the LM level. Thus, it is sometimes difficult to know exactly how far to let the reaction go at the LM level in order to generate a well-contained signal by EM. Therefore, for each experimental condition, two to three dishes should be carried through the fixation and antibody steps, so that each can be subjected to the DAB reaction for a different period of time. The following sequence is suggested: first, react one dish until the reaction product is robust and obvious, then react another dish until the brown/black DAB reaction product is just barely visible by LM, and react a third dish to a point somewhere in between the first two. With experience, two dishes will usually suffice for each experimental condition. The optimal length of time for the DAB reaction can vary greatly from 2 to 30 min. Generally, if the reaction has not developed by ~30 min, something may have gone wrong.

Some special considerations apply to DAB reactions in tissue sections. The length of reaction time can be quite variable. It is best to not have an explosive reaction, as this leads to DAB diffusion in the tissue. It is better to have the reaction proceed slowly (typically, 5 to 30 min at room temperature). If reactions proceed very quickly (e.g., within 1 to 2 min), place the tubes on ice to chill the DAB substrate before adding H₂O₂, and perform the reaction on ice. During the reaction time, the tissue will probably turn brown or black, depending upon the antigen and antibody. Affinity-purified or monoclonal antibodies will often not turn sections as dark as those incubated with crude antiserum, but they give less background and the localization will be better. Monitor the reaction with a dissecting microscope. Some experience is required to know when the sections are dark enough. In the beginning, however, it is better to err on the side of excess so that there is some kind of signal. Therefore, react several sections until they are noticeably dark, and several others for a shorter period of time.

IMMUNOPEROXIDASE STAINING OF CULTURED CELLS

This protocol describes a method for the localization of antigens in cultured cells at the EM level by the immunoperoxidase staining procedure. The protocol has been specifically developed for adherent, monolayer cell cultures of established cell lines. However, cultured cells that grow in suspension can, in many cases, be attached to poly-L-lysine-coated dishes and then processed as described in this section. The protocol can also be easily applied to primary cells. In either case, specific conditions for culturing cells are left to the investigator (see *UNIT 1.1*).

Many of these steps can be modified to meet specific experimental needs (e.g., dish size and incubation times). All steps are conducted at room temperature unless otherwise indicated. This protocol describes one of three methods for removing the cells from the culture dishes. Selection of the appropriate method depends on whether (1) the antigen is located anywhere within the cell except the ER (this protocol), (2) the antigen is located in the ER (see Alternate Protocol 1), or (3) sections through the intact monolayer are desired (see Alternate Protocol 2).

Materials

- Cells of interest
- PLP fixative (see recipe), freshly prepared
- PBS, pH 7.4 (*APPENDIX 2A*)
- 0.005% to 0.05% (w/v) saponin (Sigma)/PBS
- Primary antibody in saponin/PBS with 0.1% (w/v) ovalbumin
- HRP-conjugated secondary antibody: horseradish peroxidase-conjugated Fab fragments of anti-rabbit IgG or anti-mouse IgG (Biosys) in saponin/PBS with 0.1% (w/v) ovalbumin
- Glutaraldehyde fixative: 1.5% (v/v) glutaraldehyde in 5% (w/v) sucrose/100 mM sodium cacodylate, pH 7.4
- 7.5% (w/v) sucrose/100 mM sodium cacodylate, pH 7.4
- 0.2% (w/v) DAB solution (see recipe), freshly prepared
- 3% (v/v) H₂O₂, freshly prepared from 30% H₂O₂ stock solution
- 1% (w/v) reduced OsO₄ solution (see recipe), freshly prepared and ice cold
- 100 mM sodium cacodylate, pH 7.4, ice cold
- 70%, 95%, and 100% (v/v) ethanol
- 100% propylene oxide, EM grade
- 1:1 (v/v) propylene oxide/plastic embedding resin (e.g., Spurr's)
- 100% plastic embedding resin (e.g., Spurr's)

BASIC PROTOCOL 1

Microscopy

4.6.3

35-mm disposable plastic petri dishes
Horizontal reciprocating shaker

NOTE: The propylene oxide detachment method of embedding can be somewhat tricky. It is advisable to practice on cells before attempting the immunoperoxidase staining (see step 18).

Prepare cells

1. Grow cells of interest to ~75% confluency in 35-mm disposable plastic petri dishes. Prepare two to three dishes for each experimental condition (see Strategic Planning and step 13).

It seems best to not do the immunoperoxidase protocol soon after trypsinization and replating. It is better to plate the cells at lower density and wait 2 to 4 days for them to repopulate the dish before immunoperoxidase staining.

Other dishes can be used for this procedure, but 35-mm dishes yield a reasonable number of cells for thin-sectioning, and larger dishes require more antibody.

2. Fix cells on the dish by replacing medium with 1 ml PLP fixative, taking care to prevent cells from drying out. Incubate 2 to 3 hr with gentle shaking on a horizontal reciprocating shaker.

Rotary shakers are acceptable; however, they may cause the center of the dish to dry out if small volumes of fluid are used (e.g., during antibody incubations).

3. Remove fixative and wash cells three times, 5 min each, with 1 to 2 ml PBS.
4. Permeabilize cells by removing PBS and adding 1 to 2 ml of 0.005% to 0.05% saponin/PBS. Incubate for 5 min with gentle shaking.

The minimal concentration of saponin required to allow antibody penetration should be determined by preliminary immunofluorescence experiments (see Strategic Planning). This concentration may vary depending upon the source of saponin and the type of cells. Nearly all cell types can be rendered optimally permeable using a concentration between 0.005% and 0.05%.

NOTE: *The saponin concentration used here should also be used in all subsequent antibody incubations and washes.*

Expose cells to primary antibody

5. Remove saponin/PBS and apply ≥ 0.5 ml primary antibody in saponin/PBS. Incubate ≥ 1 hr with gentle shaking.

For 35-mm dishes, ~0.5 ml is the minimal volume needed to cover and keep the cells from drying out. An appropriate antibody concentration should be determined by preliminary immunofluorescence experiments (see Strategic Planning).

Alternatively, cells can be incubated in primary antibody for ~30 min at room temperature, and then shifted to 4°C for overnight incubation. Dishes should be covered and placed in a sealed, humidified chamber (e.g., a Tupperware-type container). Overnight incubation is especially useful if the day has been long. The peroxidase signal also tends to be improved, but sometimes with increased background.

6. Wash cells three times, 5 min each, with 1 to 2 ml saponin/PBS.

Expose cells to secondary antibody

7. Remove wash solution and apply 0.5 to 0.7 ml HRP-conjugated secondary antibody. Incubate ≥ 1 hr with gentle shaking.

An appropriate dilution of the secondary antibody should be determined by preliminary immunoperoxidase staining experiments (see Strategic Planning). For the Biosys sec-

dary antibody, a good starting point is a 1:400 to 1:800 dilution. The Fab-HRP antibodies from Biosys are particularly good reagents. Their small size, relative to whole IgG-HRP or F(ab')₂-HRP conjugates, allow lower concentrations of saponin to be used for membrane permeabilization.

If importing the Biosys antibody into the U.S., it is necessary to obtain a Permit for Importation and Transportation of Controlled Materials and Organisms and Vectors (VS Form 16-6A) from the USDA. To obtain this permit, an Application for Permit to Import Controlled Material or Import or Transport Organisms and Vectors must be submitted. This application can be obtained from and submitted to USDA, APHIS, VS, Federal Building, Hyattsville, MD 20782 (Fax no. 301-436-8226).

8. Wash cells three times, 5 min each, with 1 to 2 ml saponin/PBS.
9. Wash cells three times over a period of 5 to 10 min with 1 to 2 ml PBS (each wash) to rinse out the saponin.

This is an important step because removal of saponin lessens the amount of DAB diffusion during the peroxidase reaction.

10. Fix cells by removing PBS, applying 1 ml glutaraldehyde fixative, and incubating for 30 to 60 min with gentle shaking.
11. Wash cells three times, 5 min each, with 1 to 2 ml of 7.5% sucrose/100 mM sodium cacodylate, pH 7.4.

Perform DAB reaction

12. Remove wash solution and apply 1 ml of 0.2% DAB solution.

CAUTION: DAB is carcinogenic. Wear gloves and work in fume hood when handling. Treat all DAB-containing solutions and contaminated supplies with bleach, and dispose according to institutional guidelines for hazardous chemicals.

13. Start the DAB reaction by adding 3.3 µl of 3% H₂O₂ to the dish. Quickly swirl to mix the H₂O₂ and DAB. Start timing and monitoring the reaction using an upright or inverted light microscope (see Strategic Planning). React one dish until the reaction product is robust and obvious, one dish until the brown/black DAB reaction product is just visible, and one to a point somewhere in between.

Start with a reaction taken to yield a robust product, and then scale back the time to give lesser amounts. The optimal length of time for the DAB reaction can vary greatly from 2 to 30 min. A nearly-optimal reaction time can be established by preliminary immunoperoxidase staining experiments (see Strategic Planning). With experience, this procedure can be reduced to two dishes/experimental condition.

14. Stop the reaction by removing DAB solution and quickly rinsing cells three times with 1 to 2 ml of 7.5% sucrose/100 mM sodium cacodylate, pH 7.4.

Osmicate the cells

15. In a fume hood, remove wash solution and osmicate cells with 1 ml ice-cold, 1% reduced OsO₄ solution. Cover petri dishes and place in a Tupperware-type container. Seal well and incubate for 1 hr at 4°C with gentle shaking.

CAUTION: Wear gloves when handling osmium. Perform this step in a fume hood to avoid osmium vapors. Be sure that the Tupperware-type container is well sealed—use Parafilm if unsure. Dispose of used osmium according to institutional guidelines for hazardous chemicals.

16. Return cells to the fume hood, remove osmium solution, and rinse cells quickly three times with 1 to 2 ml ice-cold 100 mM sodium cacodylate, pH 7.4. Allow cells to reach room temperature.

17. Dehydrate cells with a graded series of ethanol (70%, 95%, 100%) as typically done for conventional plastic embedding. Perform three or four quick rinses with ~2 ml of each ethanol solution.

Detach cells and embed in plastic

18. Remove ethanol and quickly add ~0.5 to 1.0 ml of 100% propylene oxide with a glass Pasteur pipet.

CAUTION: Perform embedding procedure in the fume hood.

19. Quickly score the bottom of the dish in a crude checkerboard pattern with the tip of the pipet. Immediately pipet the propylene oxide up and down to dislodge cells from the dish as the plastic dissolves.

The bottom of the dish starts to turn mottled in appearance and then turns white, and sheets of cells should be seen detaching from the dish.

20. Collect cells and place in a microcentrifuge tube.

If desired, the cells can be pooled from the dishes that were subjected to varying DAB incubation times to yield a single cell pellet. The advantage of combining cells is that only one pellet needs to be sectioned, giving a sampling of all reaction times in a single section. To identify which DAB reaction time gave the best EM-level staining, the cells from each dish should be placed in separate microcentrifuge tubes.

21. Microcentrifuge to obtain a tight pellet, using a horizontal-type rotor (e.g., Beckman model 11 microcentrifuge) for 2 to 3 min at the highest setting.

CAUTION: Be sure that the tubes are tightly capped because propylene oxide fumes are explosive.

22. Remove propylene oxide and wash the cell pellet twice with ~1 ml of 100% propylene oxide. Microcentrifuge again if pellet becomes disrupted.
23. Remove propylene oxide and replace with ~1 ml of 1:1 propylene oxide/plastic embedding resin. Incubate with gentle rotation for 30 min.
24. Remove 1:1 mixture and wash pellet twice with ~1 ml of 100% plastic embedding resin. Incubate overnight in 100% plastic embedding resin with gentle rotation.
25. Replace with fresh resin mix and polymerize according to manufacturer's specifications for the plastic.

Section and view the cells

26. Cut silver/gold sections by standard ultramicrotomy methods.
27. Stain thin sections with lead citrate only. Omit uranyl acetate to generate a good ratio of signal (DAB-OsO₄ deposits) to noise (rest of the cytoplasm). For details, see Brown et al. (1984).
28. Visualize the sectioned material by standard transmission EM.

Contrast of the electron-dense product can also be slightly increased by operating the EM at lower accelerating voltages (e.g., 60 versus 80 kV).

DETACHMENT AND EMBEDDING OF CULTURED CELLS FOR ANALYSIS OF ER ANTIGENS

ALTERNATE
PROTOCOL 1

This protocol is specifically designed to remove dehydrated cells from the dishes and embed them in plastic when antigens are localized to the ER. It avoids the use of propylene oxide, which tends to extract the DAB reaction product from the ER.

1. Expose the cells to primary and secondary antibody, fix the cells, stain them with OsO_4 , and dehydrate them (see Basic Protocol 1, steps 1 through 17).
2. With 100% ethanol still in the dishes, scrape cells from the dish with a small rubber policeman or cell scraper. Collect the detached cells and transfer them to a microcentrifuge tube.

If desired, the cells from each of the two to three DAB treatment times can be combined in one tube to prepare a single pellet containing cells from each of the treatment groups. To identify which DAB reaction time gave the best EM-level staining, keep the cells from each dish in separate microcentrifuge tubes.

3. Microcentrifuge the cells to obtain a tight pellet, using a horizontal-type rotor (e.g., Beckman model 11 microcentrifuge) for 2 to 3 min at the highest setting.
4. Remove the ethanol and embed directly in 100% plastic embedding resin with several changes of resin, including one overnight incubation. Polymerize the plastic according to the supplier's instructions.

CAUTION: *Perform embedding procedure in a fume hood.*

5. Cut silver/gold sections by ultramicrotomy, stain with lead citrate, and visualize the sections (see Basic Protocol 1, steps 26 through 28).

EMBEDDING OF CULTURED CELLS FOR ANALYSIS OF MONOLAYER SECTIONS

ALTERNATE
PROTOCOL 2

This protocol is used to prepare sections that span the full depth of the monolayer, allowing cells to be embedded, and thus antigens to be localized, while maintaining the attached cellular morphology. This method can be used whether or not the antigen is located in the ER.

Additional Materials (also see Basic Protocol 1)

Epon 812 resin
Embedding molds

1. Expose the cells to primary and secondary antibody, fix the cells, stain them with OsO_4 , and dehydrate them (see Basic Protocol 1, steps 1 through 17).
2. Rinse the cells twice more with 100% ethanol.
3. Remove the ethanol and rinse the cells three times with 100% Epon 812 resin.

CAUTION: *Perform embedding procedure in fume hood.*

For this method, Spurr's formulation should not be used because it is difficult to separate from the petri dish. The Epon 812 equivalent resin is recommended.

4. Incubate cells overnight in unpolymerized plastic resin.
5. Remove the resin, apply a thin layer (1 to 2 mm) of fresh 100% resin, and polymerize the resin according to the supplier's instructions.
6. Separate the hardened plastic resin from the dish using pliers or a hammer.

Microscopy

4.6.7

7. Use a razor blade to cut small squares (~5-mm²) of the plastic resin.
The squares should be small enough to fit in the embedding mold.
8. Place a square of the polymerized plastic into an embedding mold with the cell side facing upward.
9. Cover the square with a thin layer of unpolymerized plastic resin. Place another square with the cell side facing downward to make a sandwich.
If the polymerized plastic is thin enough, three to four layers can be sandwiched together.
10. Carefully overlay the sandwich with unpolymerized plastic resin to fill the mold and polymerize the resin according to the supplier's instructions.
11. Cut silver/gold sections by standard ultramicrotomy methods, orienting the resin blocks so the sections are cut perpendicular to the plane of the monolayer. Stain with lead citrate and visualize the sections (see Basic Protocol 1, steps 27 and 28).

**BASIC
PROTOCOL 2**

IMMUNOPEROXIDASE STAINING OF TISSUE SECTIONS

This protocol describes a method for immunoperoxidase staining of cells in tissue sections, and is useful for resolving both intracellular and extracellular antigens. Localizing antigens in cells within tissues can provide a valuable complementary data set to that obtained by immunoperoxidase localization in cultured cells (see Basic Protocol 1), because often a large number of different cell types can be examined. However, this method differs in several significant ways from that applied to cultured cells due to the inaccessibility of cells contained within tissues. A primary difference is that tissues must be sectioned prior to labeling, in order to allow antibody penetration into the cells. All steps are done at room temperature unless otherwise noted. This protocol requires expertise with a Cryostat-type tissue sectioner.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

- Animal tissue of interest
- PLP fixative (see recipe), freshly prepared
- PBS, pH 7.4 (*APPENDIX 2A*)
- 10% (v/v) dimethyl sulfoxide (DMSO) in PBS, 4°C
- Isopentane (2-methylbutane)
- Liquid nitrogen
- Tissue-Tek OCT compound (Baxter)
- 0.1% (w/v) ovalbumin/PBS
- Primary antibody in 0.1% ovalbumin/PBS with 0.02% (w/v) sodium azide (NaN₃)
- HRP-conjugated secondary antibody: horseradish peroxidase-conjugated Fab fragments of anti-rabbit IgG or anti-mouse IgG (Biosys) in 0.1% ovalbumin/PBS
- Glutaraldehyde fixative: 1.5% (v/v) glutaraldehyde in 5% (w/v) sucrose/100 mM sodium cacodylate, pH 7.4
- 7.5% (w/v) sucrose/100 mM sodium cacodylate, pH 7.4
- 0.2% (w/v) DAB solution (see recipe), freshly prepared
- 3% (v/v) H₂O₂, freshly prepared from 30% H₂O₂ stock solution
- 1% (w/v) reduced OsO₄ solution (see recipe), freshly prepared and ice cold
- 100 mM sodium cacodylate, pH 7.4, ice cold

70%, 95%, and 100% (v/v) ethanol
100% propylene oxide, EM grade
1:1 (v/v) propylene oxide/plastic embedding resin (e.g., Spurr's)
100% plastic embedding resin (e.g., Spurr's)
Large styrofoam box or container
Cryostat-type tissue sectioner
10 × 75-mm test tubes
Slow-moving shaker or mixer
Flat embedding molds

Fix tissue

1. Fix animal tissues by perfusion (if possible) with PLP fixative for 5 min.
2. Remove tissues from animal, immerse in ~5 ml PLP fixative, and carefully slice into small pieces of workable size (~2 mm³). Prepare extra pieces of tissue (at least three or four per tissue sample).

If desired, obtain and fix a variety of tissues from the animal to establish a tissue bank. Once they are frozen in liquid nitrogen (see step 15), tissues can be stored almost indefinitely and used when needed.

3. Continue to fix tissue pieces by immersion for 4 to 6 hr.
4. Wash tissue pieces four times, 15 min each, in ~5 ml PBS.

Freeze tissue

5. Cryoprotect the tissue pieces by placing in ~5 ml cold 10% DMSO in PBS for 1 hr at 4°C.
6. Fill a small (~50-ml) metal beaker or cup ~³/₄ full with isopentane.
7. Bore a “seat” slightly less than the diameter of the cup in the bottom of a large styrofoam box or container. Place the cup in the container so that it fits snugly in the seat.
8. Fill the container with liquid nitrogen just up to the level of the isopentane and wait for the isopentane to freeze (~10 to 20 min).
9. With a small metal probe (e.g., spatula blade), melt a small well in the frozen isopentane until it is large enough to hold a piece of tissue.
10. Wait a few seconds for the isopentane to cool (but not freeze). During this time, place a piece of tissue on the end of a wooden applicator stick.
11. Rapidly plunge the end of the stick with tissue into the well of liquid isopentane.

Within seconds, the tissue will turn white and be frozen throughout.

12. Leave in the isopentane for 5 to 10 sec.

Do not leave the tissue in longer or the isopentane will freeze with the tissue in it.

13. Remove the stick and place in the liquid nitrogen bath.

Tissues can be left there until all of the pieces have been frozen in a similar fashion. It may be necessary to occasionally remelt the frozen isopentane if doing several pieces.

14. With a scalpel equilibrated in liquid nitrogen, cut the tissue off the stick while immersed in liquid nitrogen being careful to keep fingers out of the liquid nitrogen.
15. Punch several small holes in a cryovial so that liquid nitrogen will bathe the tissue during storage.

16. With cold forceps, pick up the frozen tissue pieces and place in the cryovial. Store in liquid nitrogen.

Tissue frozen in this manner can be stored indefinitely in liquid nitrogen. This is a handy stopping point.

Cut sections on a Cryostat

17. Retrieve frozen tissue and keep in a liquid nitrogen bath until sectioned.
18. Remove one piece of tissue, mount in Tissue-Tek OCT compound, and assemble the chuck of a Cryostat-type tissue sectioner. Equilibrate the tissue to -18° to -20°C .
19. Cut ~ 10 - to 20 - μm -thick sections according to manufacturer's instructions.

Thinner sections provide better antibody penetration, but very thin ones are difficult to handle and tend to curl. For most tissues, 10 to 20 μm seems to be fine. Thicker sections are required for some tissues. For example, glomeruli tend to fall out of thin kidney sections, so kidneys should be cut at ~ 30 μm .

For the subsequent immunoreactions, three to five good sections per tissue sample are normally sufficient; however, the beginner might want to cut seven to nine because some may be lost along the way.

20. Place sections into 10×75 -mm test tubes (two to four per tube) containing 2 to 3 ml of 0.1% ovalbumin/PBS, and allow sections to come to room temperature.

Carry out antibody incubations

21. Allow the sections to sink to the bottom of the tube, carefully aspirate the ovalbumin/PBS, and gently add 200 to 300 μl primary antibody, avoiding any physical damage to the sections.

The approximate starting antibody concentration can be determined by preliminary immunofluorescence experiments using cultured cells (see Strategic Planning) or on 2- to 4- μm -thick cryostat sections. Good immunoperoxidase signals can be achieved in sectioned material by doubling the antibody concentration that gives a good immunofluorescence signal.

22. Seal tube with Parafilm and incubate overnight with gentle shaking or mixing.

Care must be taken to ensure that all sections remain immersed in the fluid and do not dry out.

23. Wash sections six times, 15 min each, with 1 ml of 0.1% ovalbumin/PBS with gentle shaking.
24. Remove wash solution and apply 200 to 300 μl HRP-conjugated secondary antibody. Incubate for 2 hr with gentle shaking.

An appropriate dilution of the secondary antibody should be determined by preliminary immunoperoxidase staining experiments (see Strategic Planning). For the Biosys secondary antibody, a starting point is a 1:400 to 1:800 dilution. The Fab-HRP antibodies from Biosys are particularly good reagents (for import information for this antibody, see Basic Protocol 1, step 7).

25. Wash sections four times, 15 min each, with 1 ml of 0.1% ovalbumin/PBS with gentle shaking.

Fix sections

26. Fix sections by removing ovalbumin/PBS, applying 1 to 2 ml glutaraldehyde fixative, and incubating for 60 min with gentle shaking.

27. Wash sections four times, 10 min each, with 1 ml of 7.5% sucrose/100 mM sodium cacodylate, pH 7.4, with gentle shaking.

Carry out DAB reaction

28. Remove wash solution and apply 1 ml of 0.2% DAB solution. Incubate for 1 min.

CAUTION: DAB is carcinogenic. Wear gloves and work in fume hood when handling. Treat all DAB-containing solutions and contaminated supplies with bleach, and dispose according to institutional guidelines for hazardous chemicals.

29. Start the DAB reaction by adding 3.3 μ l of 3% H₂O₂. Quickly swirl to mix the H₂O₂ and DAB. Start timing and monitor the reaction using a dissecting microscope. React several sections until they are noticeably dark, and several other sections for a shorter period of time.

The reactions can be carried out in the tubes and inspected with a dissecting microscope, or the sections can be transferred to a dish for viewing with an inverted microscope.

The optimal length of time for the DAB reaction should be between 5 and 30 min for tissue sections. A nearly-optimal reaction time can be established by preliminary immunoperoxidase staining experiments (see Strategic Planning).

30. Stop the reaction by removing DAB solution and rinsing sections four times quickly with 7.5% sucrose/100 mM sodium cacodylate, pH 7.4.

Osmicate the tissue

31. Place tubes on ice in a fume hood. Remove wash solution and osmicate tissue sections with 200 to 300 μ l of ice-cold, 1% reduced OsO₄ solution. Seal tubes with Parafilm and incubate for 1 hr at 4°C with gentle shaking.

CAUTION: Wear gloves when handling OsO₄. This step must be done in a fume hood to avoid osmium vapors. Use a small volume of osmium to reduce osmium waste. Dispose of used osmium according to institutional guidelines for hazardous chemicals.

32. Return tubes to the fume hood, remove osmium solution, and wash sections three times quickly with 1 to 2 ml ice-cold 100 mM sodium cacodylate, pH 7.4. Allow sections to reach room temperature.
33. Dehydrate sections with a graded series of ethanol (70%, 95%, 100%) as typically done for conventional plastic embedding. Perform three or four quick rinses with 2 to 3 ml of each ethanol solution.

Embed sections

34. Replace ethanol with two 1-vol changes of 100% propylene oxide.

CAUTION: Perform embedding procedure in a fume hood.

35. Replace propylene oxide with 1 vol of 1:1 propylene oxide/plastic embedding resin (e.g., Spurr's). Incubate with gentle mixing for 30 min.
36. Remove 1:1 mixture, add 1 vol of 100% unpolymerized plastic embedding resin, and gently mix overnight.
37. Replace resin with at least two 1-vol changes of fresh resin.
38. Transfer sections to flat embedding molds. Fill mold with resin and polymerize according to manufacturer's specifications for the resin.

Several sections can be stacked in one mold and then thin sections can be made through the entire depth of all sections for greater sample size.

39. Cut silver/gold sections, stain with lead citrate, and visualize the sections (see Basic Protocol 1, steps 26 to 28).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers see SUPPLIERS APPENDIX.

Diaminobenzidine (DAB) solution, 0.2% (w/v)

0.2% (w/v) 3,3'-diaminobenzidine tetrahydrochloride

7.5% (w/v) sucrose in 100 mM sodium cacodylate buffer, pH 7.4

Dissolve the appropriate amount of DAB in ~90% of the final desired volume of sodium cacodylate/sucrose buffer. Mix vigorously on a magnetic stirring plate in the dark (put a box over the stirring plate). Use 1 N NaOH to readjust the pH to ~7.2 to 7.4, checking by continuous monitoring with a pH meter. Bring to the final desired volume with sodium cacodylate/sucrose buffer. Filter by passing through a 0.2- μ m filter. Keep in the dark until use.

The DAB should dissolve in several minutes and the solution should appear clear if slightly brownish. The pH will drop to ~6.5.

CAUTION: DAB is carcinogenic. Wear gloves and work in fume hood when handling. Treat all DAB-containing solutions and contaminated supplies with bleach, and dispose according to institutional guidelines for hazardous chemicals.

Periodate/lysine/paraformaldehyde (PLP) fixative

Solution A: 0.2 M lysine-HCl/phosphate buffer

Dissolve 1.83 g lysine-HCl in 50 ml H₂O. With continuous monitoring with a pH meter, add 0.1 M Na₂HPO₄ dropwise until pH 7.4 is reached. Bring solution to 100 ml with 0.1 M sodium phosphate buffer, pH 7.4 (APPENDIX 2A). Store up to several weeks at 4°C.

Solution B: 8% (w/v) paraformaldehyde

Add 8 g paraformaldehyde to 80 ml H₂O while mixing on a magnetic stirring plate. Heat slowly with stirring until temperature reaches 60°C. Add 1 N NaOH dropwise until solution clears (~3 to 5 drops/100 ml). Bring to 100 ml with H₂O. After cooling, filter with Whatman no. 1 paper. Store up to several weeks at 4°C.

Fixative

Combine 3 parts solution A with 1 part solution B. Add sodium periodate (NaIO₄) to a final concentration of 0.01 M (21.3 mg/10 ml). Use the complete fixative at room temperature within 1 to 2 hr.

CAUTION: Prepare the paraformaldehyde in a fume hood and wear protective gloves.

Reduced osmium tetroxide (OsO₄) solution, 1% (w/v)

4% stock solution: Prepare 4% (w/v) OsO₄ in water and place in a glass container free of any organic contaminants. Store indefinitely at 4°C.

Working solution: Prepare a solution of 1% (w/v) OsO₄ in 100 mM sodium cacodylate, pH 7.4, by mixing one part 4% (w/v) OsO₄, two parts 200 mM sodium cacodylate, pH 7.4, and one part H₂O in a disposable plastic centrifuge tube. Chill on ice. Add solid K₄Fe(CN)₆ (potassium ferrocyanide) to a final concentration of 1% (w/v) and mix vigorously until it is dissolved and the solution has turned brown/black. Prepare fresh before use.

CAUTION: Osmium solutions must be contained in a properly vented fume hood, and protective gloves should be worn. Dispose of osmium and contaminated supplies according to institutional guidelines for hazardous chemicals.

COMMENTARY

Background Information

The ability to localize antigens, usually proteins and glycoproteins, at the EM level by immunocytochemical labeling methods has had a tremendous impact on our understanding of the subcellular and molecular organization of cells and on the dynamic processes carried out within them. Moreover, understanding the function of gene products is often aided by, and in many cases dependent on, knowing the subcellular location of the RNA or protein of interest. Not surprisingly, numerous immunocytochemical methodologies have been developed to couple the tremendous specificity of antibodies with staining methods that render antigen-antibody complexes detectable by either light or electron microscopy.

In order to be generally applicable for cell biological studies, all immunocytochemical protocols must overcome three common challenges: (1) allowing antibodies access to the intracellular environment, (2) preserving antigenicity, and (3) maintaining cellular morphology. Frustratingly, it is usually the case that solving one of these challenges only leads to problems with the others. For example, methods for maintaining excellent cellular morphology (e.g., glutaraldehyde fixation) are often counter-productive for preserving antigenicity. The protocols detailed in this unit strike a compromise that combines excellent morphological preservation with the power of antibody specificity and the signal amplification provided by enzyme cytochemistry via the peroxidase reaction (Graham and Karnovsky, 1966; Novikoff and Goldfischer, 1969; Novikoff, 1980).

Immunoperoxidase labeling at both the LM and EM levels has evolved over many years with important advances made by many laboratories (e.g., McLean and Nakane, 1974; Broadwell et al., 1979; Novikoff et al., 1979; Courtoy et al., 1980, 1982; Tougard et al., 1980; Louvard et al., 1982; Brown and Farquhar, 1984). The protocols detailed here derive from these efforts, and are useful for quite a variety of antigens. Not surprisingly, however, there are many variations of the immunoperoxidase labeling method, and it may be that some variations work better for particular antigen/antibody combinations. A few alternative procedures that readers might find useful or interesting are found in Brown and Farquhar (1989), Norgren and Lehman (1989), Saraste et al. (1995), Li et al. (1997), and Todd (1997). Con-

versely, DAB cytochemistry remains little changed since its early development and modification (Graham and Karnovsky, 1966; Novikoff and Goldfischer, 1969; Novikoff, 1980) and the application of ferrocyanide-reduced osmium to enhance electron density (Karnovsky, 1971).

Immunoperoxidase labeling at the EM level provides several important advantages over other methods. First, it is highly sensitive because the immunolabeled signal can be enzymatically amplified during the DAB reaction, and therefore it is quite useful for the detection of sparse antigens such as membrane receptors. Second, the morphological preservation of intracellular organelles and structures by PLP fixation (McLean and Nakane, 1974) can reach that of conventional glutaraldehyde-fixed samples. In this regard, the PLP fixative is particularly versatile for immunoperoxidase labeling at the EM level. Third, the method is fairly simple and does not require any special equipment or skills beyond those needed for standard transmission EM (TEM) of plastic-embedded samples. These three characteristics are in contrast to immunogold labeling of cryosections, which requires a special and expensive cryoultramicrotome and a high degree of specialized skill and training to generate frozen thin sections, does not provide for amplification of the immunolabeling signal, and typically generates samples in which membranes are negative images, making interpretation of results sometimes difficult.

Nevertheless, immunoperoxidase labeling has three important disadvantages when compared to immunogold. First, immunoperoxidase labeling is, by its nature, not quantitative. It gives only a qualitative picture (like immunofluorescence) of where an antigen is located in the cell. Immunogold labeling can, by rigorous statistical methods, provide important quantitative information about antigen distribution. Second, immunoperoxidase suffers from potential diffusion of the DAB reaction product away from the site of antigen-antibody binding. This diffusion leads to lower resolution than with immunogold labeling, possibly leading to erroneous conclusions about the actual site of antigen location. Third, double immunolabeling by immunoperoxidase is not generally possible, although one method for doing this has been described (Norgren and Lehman, 1989). For discussions of the limita-

tions of DAB cytochemistry see Novikoff (1973, 1980) and Courtoy et al. (1983).

Given its attractions and limitations, immunoperoxidase labeling at the EM level can be particularly useful for localizing molecules contained within intracellular membrane-bounded organelles, where antigens might be sparse (e.g., membrane receptors), but where they are sequestered within a compartment that prevents diffusion of the DAB reaction product into the cytoplasm. However, immunoperoxidase labeling can also be productively applied to antigens not contained within membranes, including both extracellular antigens (such as those in the extracellular matrix; Courtoy et al., 1980, 1982), and cell-surface antigens (Kerjaschki and Farquhar, 1983; Brown et al., 1984). In addition, immunoperoxidase can be useful for localizing certain cytoplasmic antigens, especially if they are localized to a specific region of the cell or if a low level of resolution is required (for example, when attempting to identify a particular cell type in a complex tissue or organ).

Critical Parameters and Troubleshooting

The most common problems with immunoperoxidase staining are the lack of a DAB reaction product signal or a reaction that leads to inappropriate diffusion of reaction product away from the sites of antigen-antibody binding. Without question, the DAB reaction is the most tricky step in this procedure. For this reason, two to three dishes of cultured cells (or multiple tissue sections) should be processed per experimental condition, using a range of DAB reaction times within each sample. However, with a little experience the reaction can become quite reliable and reproducible. A lack of signal at the EM level can virtually always be avoided by conducting the preliminary experiments for LM and fluorescence microscopy (see Strategic Planning). It cannot be over-emphasized how much time can be saved by spending a week or two on these preliminary experiments to establish important initial parameters.

Nevertheless, a weak or undetectable signal at the LM or EM level may result from one or more problems listed in Table 4.6.1. These problems are typically easy to diagnose and rectify by LM-level screening. When testing a new antibody for immunoperoxidase staining, be sure to include a few dishes for a known positive control antibody to distinguish between potential problems with the new primary

antibody and other subsequent steps. This positive control is especially important because occasionally an antibody may work particularly well by immunofluorescence on methanol- or formalin-fixed cells but will not give an immunoperoxidase signal on cells fixed with PLP.

Inappropriate diffusion of the DAB reaction product may be harder to avoid in the beginning because it is usually only detected by EM (Fig. 4.6.1). If the preliminary experiments indicate that the antigen under investigation is contained within a membrane-bounded organelle, then all the reaction product should be sequestered within that compartment. Diffusion of reaction product out of the organelle and into the cytoplasm may indicate one of several possible problems detailed in Table 4.6.1.

Once good reaction product is obtained at the EM level, photographic documentation should be obtained. Although EM film is relatively expensive, do not cut corners here. Expose a number of negatives to have many good choices when it comes time to picking the best, most-representative image. Also, if there is a particularly good image that really demonstrates the point you want to make, take several pictures, perhaps slightly altering the focus on a few, in case something happens to one of the negatives. It is usually much easier to find an EM negative than it is to find that same cell again.

Anticipated Results

At the EM level, the DAB reaction product should be noticeably electron dense—more so than any cellular structure including membranes, ribosomes, or heterochromatin (Fig. 4.6.1). The reaction product may have a slightly mottled appearance if filling a large membrane-bounded compartment. Oxidized DAB has an affinity for membranes, so even if an antigen is present throughout the lumen of a vacuolar organelle, the reaction product may not be uniformly distributed within that lumen. Rather, the DAB reaction product may diffuse and bind to the inner surface of the limiting membrane yielding a dark rim of staining. At the LM level, the DAB reaction product appears a brown/black color.

Time Considerations

Immunoperoxidase staining of cultured cells

The labeling protocol from the beginning of fixation to the start of embedding can be done in a single day; however, it will take at least 8

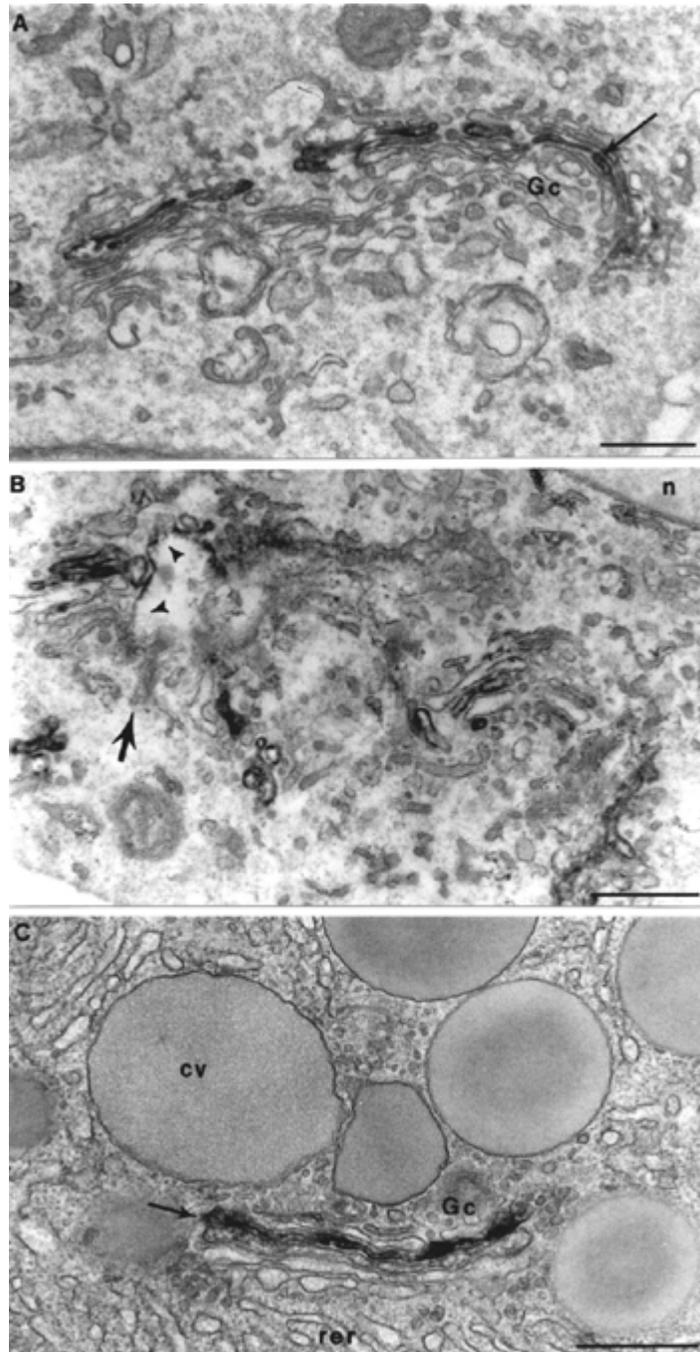


Figure 4.6.1 Immunoperoxidase localization of resident Golgi-complex enzymes in cultured cells and cells from tissue sections. **(A and B)** Rat clone 9 hepatocytes stained by the methods described in Basic Protocol 1 for cultured cells with antibodies to the medial Golgi enzyme α -mannosidase II. In **(A)**, the very electron-dense DAB reaction product (arrow) stands out in sharp contrast to the rest of the cytoplasm and fills mainly one or two cisternal elements of the Golgi stack. This example illustrates a well-preserved, immunolabeled cell whose reacted organelle membranes are morphologically intact. Note that the DAB reaction product remains sequestered within the lumen of the cisternae. In **(B)**, another clone 9 cell, reacted with the same antibody, shows significant damage resulting from cisternal elements that have become extremely dilated (“exploded”) and broken (arrowheads). As a consequence, the DAB reaction product leaks into the cytoplasm (arrow). In **(C)**, a portion of a rat pancreatic acinar cell is shown. The cell was labeled with antibodies against sialyltransferase, a *trans* Golgi enzyme, by the methods described in Basic Protocol 2. In this cell, the DAB reaction product also stands in sharp contrast to the cytoplasm and is sequestered within an intact cisternae (arrow). Abbreviations: cv, condensing vacuole; Gc, Golgi cisternae; n, nucleus; rer, rough endoplasmic reticulum. Scale bars, 0.5 μ m.

Table 4.6.1 Troubleshooting Guide for Immunoperoxidase Localization Protocol

Problem	Possible cause	Solution
Poor general morphology	Inadequate fixation time Fixative made incorrectly	Fix longer Follow recipe carefully; ensure that paraformaldehyde goes into solution
Organelles appear to be exploded, leaving holes in the cytoplasm (Fig. 4.6.1B)	DAB reaction too long or rapid	React for shorter period of time and/or slow the reaction down on ice
Weak or no DAB reaction product	Antibody titer and/or affinity low Cells not well permeabilized HRP-conjugated antibody no longer active H ₂ O ₂ not good	Prepare IgG fraction or affinity purify antibodies Increase saponin concentration or incubation time Check HRP activity by testing with small amount of DAB and H ₂ O ₂ Use fresh H ₂ O ₂ ; check H ₂ O ₂ in a test tube with DAB solution and 1-2 µl HRP-conjugated antibody to see if substrate turns dark brown/black
DAB reaction product diffused away from primary site of reaction and/or from membrane-bounded organelles that appear to have exploded (see above)	Samples over-reacted for DAB deposition Saponin not sufficiently washed out	React for shorter period of time; lower concentration of primary or secondary antibody Increase number of washes in PBS
Immunofluorescence indicates that antigen is a membrane protein, but immunoperoxidase results in a diffuse cytoplasmic stain near an organelle but not within the lumen	DAB has possibly leaked out through a compromised membrane (see above), or worse, the epitope recognized is on the cytoplasmic face of membranes	Not much can be done if the epitope is cytoplasmically oriented; try reacting for a shorter period of time and/or on ice
DAB reaction product seen by LM but not EM	OsO ₄ not properly reduced	Follow recipe for reduced osmium
General background staining	Used antiserum or antibody that is not specific	Use IgG fraction or affinity-purified antibodies; try increasing ovalbumin concentration to 1% when diluting antibody to reduce nonspecific binding
DAB not soluble	Used 3,3'-DAB free base	Use 3, 3'-DAB tetrahydrochloride
Cells will not detach from dish	Inferior quality propylene oxide	Use EM-grade propylene oxide

to 9 hr even in experienced hands and can be quite fatiguing. Also, keep in mind that this timeframe does not account for any experimental manipulations that might have been done to the cells before fixation. Therefore, the author recommends that on day 1 (following any experimental procedures) cells be fixed and processed up to and including incubation in the primary antibody, at which point the cells can be kept overnight as described in the protocol. The time from fixation to addition of the primary antibody will take minimally ~3 to 4 hr. The author does not recommend incubating

cells overnight in the secondary antibody, because this gives a slightly higher nonspecific background labeling. Thus, on day 2, cells are processed through incubation with the secondary antibody, the DAB reaction, osmication, and the start of embedding. This process will take minimally ~4 to 5 hr. Infiltration of embedding plastic will proceed overnight and all the next day (day 3), and another overnight incubation is required for heat-activated polymerization. Thus, cells are ready for thin sectioning, staining, and visualization on day 4.

Immunoperoxidase staining of tissue sections

Immunoperoxidase labeling of tissue sections can conveniently be done in five days.

Day 1: Fix and freeze tissues. This will take anywhere from 6 to 8 hr depending on how many tissue samples are being processed. Tissues can be stored frozen indefinitely, until used to complete the protocol.

Day 2: In the afternoon, cut the cryostat sections. Apply the first antibody and incubate overnight. These steps can take anywhere from 1 to 4 hr depending on the level of experience with microtomes and the number of samples being processed. With experience, days 1 and 2 can be combined.

Day 3: Complete the procedure from application of secondary antibody to start of embedding sections (4 to 6 hr). Allow embedding to proceed overnight.

Day 4: Continue embedding during the day and perform heat-activated polymerization overnight.

Day 5: Thin section and stain tissue for visualization.

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Cryo-Immunogold Electron Microscopy

UNIT 4.7

This unit describes subcellular localization of proteins/antigens using high-resolution cryo-immunogold electron microscopy (see Basic Protocol), which allows study of topological biochemistry at the ultrastructural level. This is the most sensitive procedure for immunodetection of antigens on ultrathin sections prepared from chemically fixed cells (see Support Protocol 1) or tissues (see Support Protocol 2), since aldehyde fixation is the only denaturation step. The omission of harsh organic solvents (such as those used for plastic embedding) ensures better preservation of protein antigenicity. Fixed material is embedded in gelatin (see Support Protocol 3), cryosectioned (see Support Protocol 4), and mounted on Formvar-coated grids (see Support Protocol 5). Although the basic principles and variations are described in these protocols, the reader is encouraged to consult a microscopy textbook and to work with an experienced cryomicroscopist.

IMMUNOGOLD LABELING

The precise reaction conditions used for immunogold labeling may vary depending on the antibody, and this is determined by trial and error (a useful reference is Raposo et al., 1997). Described below is a typical labeling protocol during which the sectioned material is exposed first to antibody, then to protein A–gold particles. If the primary antibody does not bind to protein A, incubation with a secondary antibody that does bind protein A can be added. All procedures are carried out at room temperature.

**BASIC
PROTOCOL**

Materials

Gelatin storage plate with cryosections mounted on Formvar-coated grids (see Support Protocol 4)

PBS (APPENDIX 2A) containing 0.15 M glycine

PBS (APPENDIX 2A) containing 1% (w/v) BSA

Primary antibody in PBS/1% BSA

PBS (APPENDIX 2A)/0.1% (w/v) BSA (see recipe for 10% BSA stock)

Secondary antibody in PBS/1% BSA (optional)

10-nm protein A–gold particles at OD 0.1 (commercially available from Cell Biology, Medical School, Utrecht University, The Netherlands; fax: 31-30-254-1797) in PBS/1% BSA (see recipe for 10% BSA stock)

PBS (APPENDIX 2A) containing 1% (v/v) glutaraldehyde

Uranyl oxalate solution (see recipe; optional)

Methyl cellulose/uranyl acetate solution (see recipe)

37°C hot plate or incubator

Forceps

Stainless steel loop slightly larger than grids attached to P1000 (“blue”; 1000- μ l) pipet tips

1. Place the gelatin storage plates containing the cryosections (see Support Protocol 4, step 9) for 20 min on a 37°C hot plate or in an incubator maintained at 37°C.

The gelatin will melt and the grids will start floating to the top. Also, residual 10% gelatin that was introduced into the sample during the embedding step is melted away at this point.

2. Lift the floating grids with a pair of forceps and transfer them onto 100- μ l droplets of PBS/0.15 M glycine laid out on a sheet of Parafilm. Wash four times, each time by floating the grids for 3 min on fresh 100- μ l droplets of PBS/0.15 M glycine.

All subsequent treatments (washings and incubations) involve the transfer of the grids onto fresh droplets of medium that are laid out on Parafilm. 100- μ l droplets should be used for all solutions except the antibody and protein A–gold, for which 10- μ l droplets are used.

Contributed by Peter J. Peters

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Microscopy

4.7.1

Supplement 2

3. Wash grids twice, each time by floating for 5 min on droplets of PBS/1% BSA.
4. Incubate grids for the appropriate period of time at room temperature on 10- μ l droplets of primary antibody diluted to an appropriate concentration in PBS/1% BSA.

The appropriate dilution and incubation times vary with the antibody used. Incubation time ranges from 30 to 100 min. Antibody titer is usually in the range of 0.2 μ g/ml but must be titered for each antibody, at the EM level, in pilot experiments.

5. Wash the grids four times, each time by floating for 2 min on droplets of PBS/0.15 M glycine, then perform a final wash in PBS/0.1% BSA.
6. *Optional:* When a primary antibody that does not react with protein A (Table 7.2.1) is used, incubate grids with a secondary (bridging) antibody that binds protein A, diluted in PBS/0.1% BSA, for 20 min.
7. Wash grids five times, each time by floating for 2 min in droplets of PBS/0.1% BSA.
8. Incubate grids for 20 min on 10- μ l droplets of 10-nm protein A–gold particles (at an OD of 0.1) in PBS/1% BSA.
9. Wash grids seven times, each time by floating for 2 min on droplets of PBS/0.1% BSA.
10. Incubate grids for 5 min on droplets of PBS/1% glutaraldehyde.

For double immunogold labeling, after this step wash the grids with PBS/0.15 M glycine (step 2) and repeat the entire procedure from that point on with the second primary antibody. For double-labeling experiments, use protein A with different sizes of gold particles (10 and 15 nm).

11. Wash grids seven times, each time by floating for 2 min on droplets of distilled water.
12. *Optional:* Incubate grids for 5 min on droplets of uranyl oxalate solution.
13. Quickly wash grids by floating for 1 min on droplets of methyl cellulose/uranyl acetate solution, then floating 5 min in fresh droplets of methyl cellulose/uranyl acetate solution.
14. Retrieve the grids with a stainless steel loop of diameter slightly larger than the grid, attached to a P1000 pipet tip.
15. Drain away the excess of methyl cellulose/uranyl acetate solution by touching the loop at an angle of 45° to a filter paper.
16. Air dry grids at room temperature or under warm air by placing the pipet tips upside-down in a pipet tip rack.

A film of methyl cellulose/uranyl acetate is left on the grid after drying. This is important to give optimal contrast and to preserve the integrity of membrane structures.

17. Examine grid by electron microscopy at 80 kV with a small aperture.

SUPPORT PROTOCOL 1

FIXATION OF CELLS FOR IMMUNOGOLD LABELING

The fixation step is critical for cryo-immunogold electron microscopy. The purpose of fixation is to immobilize all subcellular structures and antigens in their most native form. It is important, however, to maintain maximum antigenicity. Three different fixatives are commonly used—paraformaldehyde (PFA), glutaraldehyde (GA), or a combination of both (PFA/GA). It is advantageous to the investigator to first determine which of these fixatives is best suited for maintaining optimal antigenicity within the proper subcellular compartment.

Materials

Cultures of adherent or suspension cells

Culture medium

2× fixative: 2× PFA (see recipe), 2× GA (see recipe), or 2× PFA/GA (see recipe),
room temperature

Storage solution (see recipe)

Rubber policeman

10-ml centrifuge tubes

Screw top microcentrifuge tubes

1. Add fresh medium to cells the day before the experiment.

Adherent cells should be 70% to 80% confluent; the density of cells in suspension should be $\sim 1 \times 10^6$ cells/ml. The total volume of growth medium should be 5 ml.

2. Add 5 ml of 2× fixative to 5 ml of a suspension of cells that have just come out of the incubator. Incubate the cells 24 hr (for PFA) or 1 hr (for GA or PFA/GA) at room temperature. Do not disturb cells during fixation period.

CAUTION: Fixatives are corrosive to the skin, nose, and eyes, therefore it is advisable to work in the hood with a double set of gloves.

It is advisable not to wash cells in PBS or any other medium before fixation, in order to avoid artifacts.

3. For adherent cells: Scrape cells into the 10 ml of medium/fixative using a rubber policeman.
4. Transfer cells to 10-ml tubes and centrifuge 5 min at 1000 to 2000 × g, room temperature. Discard supernatant.
5. Carefully resuspend pellets in 1 ml storage solution and transfer cells into screw-top microcentrifuge tube for storage. Store at 4°C in an airtight container.

Fixed cells can be shipped at room temperature.

FIXATION OF TISSUE FOR IMMUNOGOLD LABELING

Pieces of tissue—biopsy, dissected organs, or embryos—can also be fixed for immunogold labeling.

Materials

Tissue of interest

1× fixative: 1× PFA (see recipe), 1× GA (see recipe), or 1×PFA/GA (see recipe)

Storage solution (see recipe)

Razor blades, alcohol cleaned

Glass vials with screw caps

High-precision tweezers

1. Add biopsy tissue, dissected organs, or embryos immediately to 2 ml of 1× fixative (GA, PFA/GA or PFA) in a glass vial with a screw cap.

CAUTION: Fixatives are corrosive to the skin, nose, and eyes, therefore it is advisable to work in the hood with a double set of gloves.

Perfusion fixation of tissue is preferable for achieving good morphology. It is normally used for small animals such as mice and rats (see APPENDIX 3).

SUPPORT PROTOCOL 2

Microscopy

4.7.3

2. Cut tissue into 3 × 3–mm pieces using clean (alcohol-rinsed, air-dried) razor blades and high-precision tweezers.

Use of a dissecting microscope helps prevent mechanical damage to the tissue. Perform this operation in a fume hood.

3. Fix tissue at room temperature for 24 hr (for PFA) or 2 hr (for GA or GA/PFA).
4. After fixation, transfer tissue to storage solution in glass vials. Store at 4°C in an airtight container.

EMBEDDING CELLS FOR IMMUNOGOLD LABELING

Cells and loose tissue are embedded in gelatin to facilitate handling during the rest of the procedure. It is advisable to have a dissecting microscope with “cold” light optics permanently present in a cold room. Care should be taken never to allow the samples to dry out. Gelatin allows solidification of a cell suspension, so that it becomes, temporarily, a piece of material that can be handled relatively easily. The gelatin will melt once the ultrathin cryosections are prepared for immunolabeling (see Basic Protocol). Sucrose infiltration prevents the material from ice-crystal damage during freezing in liquid nitrogen.

Materials

Fixed cells (see Support Protocol 1) or tissue (see Support Protocol 2) in storage solution

PBS (*APPENDIX 2A*) containing 0.15 M glycine

10% gelatin (see recipe), 37°C

2.3 M sucrose in 0.1 M sodium phosphate buffer, pH 7.4 (see *APPENDIX 2A* for buffer)

Petri dishes

Razor blades, alcohol-rinsed and air-dried

Dissecting microscope with cold light optics (Leica)

1-ml vials

End-over-end rotator

High-precision tweezers

Aluminum specimen holders (Leica), roughened with sandpaper, soap-cleaned, and dust-free

1. Centrifuge cells 1 to 5 min at 5000 × g, room temperature, and pipet off storage solution. Wash twice, each time by adding 1 ml PBS/0.15 M glycine, centrifuging again, and removing the supernatant.
2. Resuspend cells in 1 ml of 10% gelatin (prewarmed to 37°C), then incubate 10 min at 37°C.
3. Microcentrifuge cells 2 min at 10,000 × g, room temperature, then remove excess gelatin before it solidifies.

For tissues, wash twice, each time for 5 min, in PBS/0.15 M glycine. Incubate 30 min each in 2.5% and 10% gelatin, then transfer the tissue/gelatin mix to the lid of a petri dish as in step 4.

Alternatively, it is possible to allow the gelatin to solidify at this stage and cut the microcentrifuge tubes, with the sample, just above the pellet. If this is done, add cold PBS/0.15 M glycine to the solidified material, keep at 4°C, and transfer the cells/gelatin pellet to a droplet of cold PBS/0.15 M glycine. After this, proceed to step 8.

4. Transfer the cells/gelatin mixture on top of a lid from a petri dish.

5. Place the dish on ice.

The droplet will begin to solidify.

6. Just before the gelatin solidifies, place a small petri dish on top of the droplet.

This creates a flattened piece of solidified gelatin with cells, which can be handled relatively easily.

7. Remove the top dish and add cold PBS/0.15 M glycine to the solidified material and keep at 4°C.

This will prevent the gelatin from melting.

8. Cut the cells/gelatin into slices of 0.5 mm and then cut the slices into 0.5-mm cubic blocks, using clean (alcohol rinsed, air dried) razor blades, under a dissecting microscope.

This step is best carried out in a cold room, to ensure that the gelatin does not melt and that the sample does not dry out, and for easy handling with sucrose.

9. Transfer the cubic block to a 1-ml vial containing 2.3 M sucrose.

10. Rotate sample in sucrose for at least 2 hr in an end-over-end rotator in the cold room.

11. Working under the dissecting microscope and using high-precision-grade tweezers with a minimum of force, transfer the samples, along with a minimum of sucrose, from the vial to very clean aluminum specimen holders that have been roughened with sandpaper.

12. Remove excess sucrose by capillary action on the forceps or using clean filter paper, and transfer holders to small containers filled with liquid nitrogen.

Samples can be stored in liquid nitrogen and are ready for ultrathin cryosectioning (see Support Protocol 4).

CRYOSECTIONING FOR IMMUNOGOLD LABELING

The procedure for ultrathin cryosectioning is time-consuming and can still be considered an art. It requires a lot of training and weekly practice; learning this skill is comparable to learning a musical instrument for professional performance. However, within a week one can train personnel to prepare usable sections with state of the art equipment. The sample blocks that are stored in liquid nitrogen (see Support Protocol 3) can be used repeatedly.

The preparation of glass knives for ultrathin cryosectioning has been described in detail elsewhere (Griffiths, 1993); one may also consult the instructions provided by the manufacturer. High-quality diamond knives have also been developed; however, it is advisable that the latter should be used by the more experienced microscopist.

Materials

Embedded cells or tissue (see Support Protocol 3)

2.3 M sucrose in 0.1 M sodium phosphate buffer, pH 7.4 (see APPENDIX 2A for buffer) or 3:2 mixture of 2.3 M sucrose and 2% (v/v) methyl cellulose
2% gelatin (see recipe) in a small petri dish

Ultramicrotome (Leica) with cryochamber and antistatic devices (Diatome)

Glass or diamond knife (Diatome or Drukker)

Trimming knife

Eyelash mounted on a wooden stick

1.5-mm diameter stainless-steel loop mounted on 15-cm wooden stick

Carbon- and Formvar-coated copper grids (see Support Protocol 5)

SUPPORT PROTOCOL 4

Microscopy

4.7.5

1. Set the ultramicrotome to -100°C . Insert and secure specimen and trimming knife in the ultramicrotome as described by the manufacturer.

Trimming of the sample is a prerequisite for making ultrathin sections. The end result is a perfectly trimmed rectangular block from which ultrathin cryosections are made.

2. Trim the sample with a glass knife or trimming device by making sections from the short side of the sample block. Maintain the temperature setting of the microtome at -100°C .
3. Trim sections of $0.1\text{-}\mu\text{m}$ thickness at a speed of 50 mm/sec . Rotate the sample at 90° angles and trim along each side to end up with a shiny rectangular block of $\sim 0.2 \times 0.3\text{ mm}$.

At this step, it is advisable to check whether the trimmed block is suitable for further sectioning, by cutting a few $0.25\text{-}\mu\text{m}$ thin cryosections. These can then be picked up (step 6) and evaluated by light microscopy. Toluidine blue staining or immunocytochemistry (UNITS 4.3 & 4.6) may be performed on these sections. If necessary, one can trim the sample even further after the light microscope examination.

4. For ultrathin sectioning, set and maintain the temperature setting of the cryochamber, knife holder, and specimen holder of the microtome at -120°C .

Fluctuations in temperature should be $<1^{\circ}\text{C}$.

5. Cut ultrathin sections of 50-nm thickness (for PFA fixed material) or 65-nm (for PFA/GA-fixed material) at a speed of 1.6 mm/sec using clean glass or diamond knives.

Sections should be lined up adjacent to each other so as to form "ribbons" of 5 to 6 sections. During sectioning, sections can be manipulated with an eyelash mounted on a wooden stick. This can be used to carefully flatten out any curled or wrinkled sections or position them onto the mounting material of the diamond knife.

6. Retrieve sections from the cryochamber using a droplet of 2.3 M sucrose created on a 1.5-mm stainless steel loop attached to a 15-cm wooden stick. Draw the droplet toward the face of the ribbon sections until the sections attach.

It is preferable, but more difficult, to use a 1:1 mixture of 2.3 M sucrose/ 2% methyl cellulose to retrieve the sections.

Just before freezing, the droplet should be very gently pushed onto the section so that the sections will gently attach to the droplet.

7. Quickly remove the loop carrying the ribbon of sections from the cryochamber and let the sections thaw.
8. As soon as the sections are thawed, place them onto carbon- and Formvar-coated copper grids.
9. Place grids coated side down onto 2% gelatin in a small petri dish which is itself in a wet petri dish on ice ("gelatin storage plate").

Alternatively store sections in a closed box at 4°C .

PREPARATION OF CARBON- AND FORMVAR-COATED COPPER GRIDS

Carbon- and Formvar-coated copper grids are used for mounting sections of samples for immunogold labeling.

Materials

Formvar (Merck)
Chloroform, analytical grade
Glass microscope slides
Glass-stoppered Erlenmeyer flask accommodating ≥ 100 ml
Coplin jar
100-mesh copper grids
Address labels
Carbon-coating device (BOC Edwards)

1. Rub the fingertip (“body-grease”) along the surface of a glass microscope slide, then gently rub the surface with lens paper. Make sure that the slide is dust-free.

The slides are now ready for coating.

2. Dissolve 1.1 g Formvar in 100 ml chloroform in a glass-stoppered Erlenmeyer flask while stirring. Transfer the Formvar solution gently into a Coplin jar.
3. Place the slide for 70% of its length into Formvar solution for 2 min.
4. Remove the slide and allow it to air dry for 1 min.

The rate at which the slide is removed will determine the thickness of the Formvar coat (optimum thickness ~50 nm).

5. Insert the slide slowly into a glass container of distilled water and allow the film to float on the surface.

Formvar films will float on the surface and should have a grayish (interference color) appearance (due to the 50-nm thickness).

6. Overlay clean 100-mesh copper grids onto the Formvar film, with the rough surface of the grid facing the Formvar film.
7. Remove Formvar-coated grid from the water surface by laying the grids over the surface of a glass slide, one side of which is covered with a white “address label,” which allows the grids to adhere onto the slide.

8. Let the grids air dry.

9. Evaluate the quality of the Formvar-coated grids by viewing them under an electron microscope.

The film should be dust free, without crevices, holes, or tears, and should not collapse under a high-energy beam (80kV without objective aperture).

10. Coat the grids the next day with a layer of evaporated carbon under high vacuum.

This may be done according the equipment manufacturer’s instructions.

The desired thickness is critical and can be judged by evaluating the color of the address label relative to a noncoated address label. There should be only a minor change in color.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

BSA stock solution, 10% (w/v)

Prepare 10% (w/v) bovine serum albumin (BSA, fraction V; Sigma) in distilled water containing 0.02% sodium azide. Adjust pH to 7.4 and centrifuge 1 hr at $100,000 \times g$, 4°C. Retain supernatant and store up to 1 month in 1-ml aliquots at 4°C in closed tubes.

The BSA fraction V is prepared by heat treatment and organic solvent precipitation.

Gelatin, 2% and 10% (w/v)

Prepare 2% and 10% (w/v) gelatin (Merck) solutions in 0.1 M sodium phosphate buffer, pH 7.4 (APPENDIX 2A) containing 0.02% azide. Store 2% gelatin in small petri dishes in a closed box; store 10% gelatin in small vials up to 1 month at 4°C.

Glutaraldehyde (GA) fixative, 2×

2.5 ml 0.4 M PHEM buffer (see recipe)

2.5 ml 8% glutaraldehyde (EM grade; commercially available)

Prepare immediately before use

The final concentrations are 0.2 M PHEM and 4% glutaraldehyde.

Methyl cellulose/uranyl acetate

Methyl cellulose stock: Add 2 g methyl cellulose (Sigma, 25 centipoise), with stirring, to 98 ml distilled water that has been prewarmed to 90°C. Cool on ice while stirring, until temperature has dropped to 10°C. Stir overnight at low speed in the cold room. Let the solution mature for 3 days in the cold room. Centrifuge 95 min at $97,000 \times g$ (29,000 rpm in a Beckman 45 Ti rotor), 4°C, then divide the supernatant into 10-ml aliquots and store up to 3 months at 4°C.

To prepare methyl cellulose/uranyl acetate: Carefully mix 1 ml of 4% uranyl acetate (see recipe) and 9 ml of methyl cellulose stock. Store up to 1 month at 4°C in a dark container.

CAUTION: *When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by your local radiation safety officer (also see APPENDIX 1D).*

Paraformaldehyde (PFA) fixative, 2×

Thaw an aliquot of 16% paraformaldehyde stock solution (see recipe) in 60°C water.

When solution is clear, mix the following:

10 ml 0.4 M PHEM buffer (see recipe)

5 ml 16% paraformaldehyde stock (see recipe)

5 ml H₂O

Prepare immediately before use

The final concentrations are 0.2 M PHEM buffer and 4% PFA.

Paraformaldehyde/glutaraldehyde (PFA/GA) fixative, 2×

Thaw an aliquot of 16% paraformaldehyde stock solution (see recipe) in 60°C water.

When solution is clear, mix the following:

10 ml 0.4 M PHEM buffer (see recipe)

5 ml 16% paraformaldehyde stock solution (see recipe)

5 ml 8% glutaraldehyde (EM grade)

Prepare immediately before use

The final concentrations are 0.2 M PHEM, 4% PFA, and 0.4% GA.

Paraformaldehyde (PFA) stock solution, 16%

Dissolve 32 g paraformaldehyde in 200 ml water. To help dissolution, add 0.34 g Na₂CO₃ to the PFA solution and stir until the solution is clear. Heat the solution on a hot plate until the paraformaldehyde is completely dissolved, but do not heat above 60°C. Divide into aliquots in 10-ml tubes and store up to several years at –20°C in a sealed box.

CAUTION: *PFA is toxic, so it is necessary to prepare the solution in a fume hood.*

PHEM buffer, 0.4 M

100 ml 240 mM PIPES
100 mM HEPES
8 mM MgCl₂
40 mM EGTA
Adjust pH to 6.9 with NaOH
Store up to several years at –20°C

Storage solution

Thaw an aliquot of 16% paraformaldehyde stock solution (see recipe) in 60°C water. When solution is clear, mix the following:
5 ml 0.4 M PHEM buffer (see recipe)
0.6 ml 16% PFA stock solution (see recipe), thawed and clear
14 ml H₂O

The final concentrations are 0.1 M PHEM and 0.5% PFA.

Uranyl acetate, 4%

Prepare a 4% (w/v) stock of uranyl acetate in distilled water and adjust pH to 4 with HCl. Store up to 1 month at room temperature; filter through Millipore filter before use.

CAUTION: *When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by your local radiation safety officer (also see APPENDIX 1D).*

Uranyl oxalate solution

Mix 1 vol of 4% uranyl acetate (see recipe) and 1 vol of 0.15 M oxalic acid. Adjust pH to 7 with 25% ammonium hydroxide.

COMMENTARY

Background Information

The pioneer in cryosectioning was A. Kent Christensen, who devised a cryosectioning bowl with a knife holder in its depth, on an ultramicrotome (Christensen, 1971). He was the first to demonstrate some ultrastructural details of frozen sections between ice crystals. However, rapid strides in the field of ultrathin cryosectioning came from the work of S. Tokuyasu, who used material embedded in sucrose (Tokuyasu, 1973). Hence, cryosectioning is also referred to as the Tokuyasu technique. He introduced high-molar sucrose solution to infiltrate aldehyde-fixed material as a way to prevent ice-crystal formation and thus maintain water in an amorphous state at low temperature.

In addition, he introduced a new method to retrieve the ultrathin cryosections from a dry knife at –100°C. This was done by approaching the section with a droplet of high-molar sucrose that was about to freeze, and then quickly retrieving the section, on the droplet, from the cryochamber. Another creative aspect of his work was that he used ferritin as an electron-dense marker to visualize the antibody that was bound to the antigen on the ultrathin cryosection. By using uranyl as a negative stain, he could visualize the ultrastructural details of membranes. However the use of ferritin as an immunomarker was not satisfactory, and air-drying artifacts were a serious drawback. These artifacts were later overcome by Tokuyasu by

the introduction of methyl cellulose at the step when sections are dried in air.

The pioneers in immunogold labeling of ultrathin plastic sections were Romano and Romano (1977) and Roth et al. (1978), who developed procedures to couple staphylococcal protein A-coated colloidal gold particles for use in ultrastructural localization of intracellular antigens. The latter study also introduced quantitative immunocytochemistry at the subcellular level. This technique is still in use for detection of bulk proteins, but lacks the high sensitivity of immunodetection, since the antigenicity is often destroyed by the procedure.

It was Slot and Geuze (1981) who introduced the immunogold labeling procedure on ultrathin cryosections and laid the basis for the procedure outlined in this unit. The use of different sizes of gold particles made the technique very powerful for localizing antigens at the ultrastructural level. Later Griffiths et al. (1982) modified the uranyl staining procedure to enhance contrast of the ultrathin cryosections. Another major improvement in modern ultrathin cryosectioning was the development of a high-quality ultramicrotome and diamond knives. Embedding of cells with gelatin in the cold before sucrose infiltration simplifies sectioning (Peters, 1991). Today, it is possible to localize three different antigens on ultrathin cryosections using glutaraldehyde as a blocking step between labeling procedures (Slot et al., 1991). More recently, Liou et al. (1996) showed that the structural integrity of cryosections was greatly improved by substituting a mixture of methylcellulose and sucrose for the sucrose that Tokuyasu introduced. Furthermore, the preservation of ultrastructural details is maximal compared to that achieved by other techniques, such as plastic embedding. Therefore, this procedure has become the first choice for high-resolution detection of antigens at the subcellular level.

Critical Parameters and Troubleshooting

In immunogold staining of ultrathin cryosections, there are many steps where things can go wrong. Unfortunately, whether this has happened can be determined only at the end of the procedure, by electron microscopy. The most commonly encountered problems are: low-quality sections, absence of specific labeling, high background labeling, colabel artifacts, dirty grids, and weak contrast.

Bad sectioning is often due to unsuitable preparation of the specimen or instability in the cryomicrotome. Dilutions of fixatives must be prepared just before they are used for specimen fixation. Specimens that have dried out during preparation, or the presence of residual sucrose around the gelatin block, may cause sectioning problems. Also, loose attachment of the specimen holder or knife holder to the cryomicrotome will cause problems. Contamination of specimen and knife, which can result from condensation of ice crystals, must be avoided.

Nonspecific labeling is encountered mainly because the antibody does not recognize the antigen after aldehyde fixation. This can be tested by evaluating the antibody on aldehyde-fixed material by FACS (fluorescence cell sorter analysis) or immunofluorescence microscopy (UNIT 4.3), prior to testing the antibody at the EM level.

Overexpression of the antigen (protein) in a control cell that does not express the antigen endogenously is the best quality control for specificity.

Background labeling often results because an excessive concentration of protein-A gold was used or because the antibody titer was too high. For the protein-A gold concentration it is best to follow the instructions of the manufacturer. For most applications, the antibody titer is used in the range of 0.2 to 2 $\mu\text{g/ml}$. It is best to try different dilutions and evaluate the specific signal-to-background ratio. Polyclonal, monoclonal, and recombinant Fab antibodies can be successfully employed. It is important to use a bridging antibody that binds specifically and efficiently to protein-A gold as an intermediate step if the primary antibody does not bind protein A efficiently.

Colabel artifacts are often encountered when double-labeling procedures are carried out. In this case, the two sizes of gold particles are always adjacent to each other, at <20 nm apart, and this is often mistaken for colocalization. The cause of colabeling is not always understood, but often the antibody that is used for the detection of the second antigen binds to the antibody that was used to detect the first antigen. Also, protein-A gold that is used in the second step may still bind to the antibody used in the first step. Glutaraldehyde incubation after the first immunolabeling should overcome the later problem. It is important to always check using a single label first.

Dirt on the grid is usually caused by phosphate contamination of solutions. Also, partial drying out of droplets during the immunoincu-

bation can result in dirty grids. Always keep the grids under a petri dish with a wet tissue during incubation and never touch the Parafilm where droplets are being applied.

A screening of antibodies by FACS or light microscopy before and after aldehyde fixation, and an evaluation of specificity by immunoblots (*UNIT 6.2*) and immunoprecipitation

(*UNIT 7.2*) may speed successful labeling on cryosections. Control cells that are transfected with or without the specific protein are very instrumental as a positive control.

Protein-A gold particles of 10 and 15 nm are routinely used. So far this author has not seen a major significance of 1-nm (silver-en-

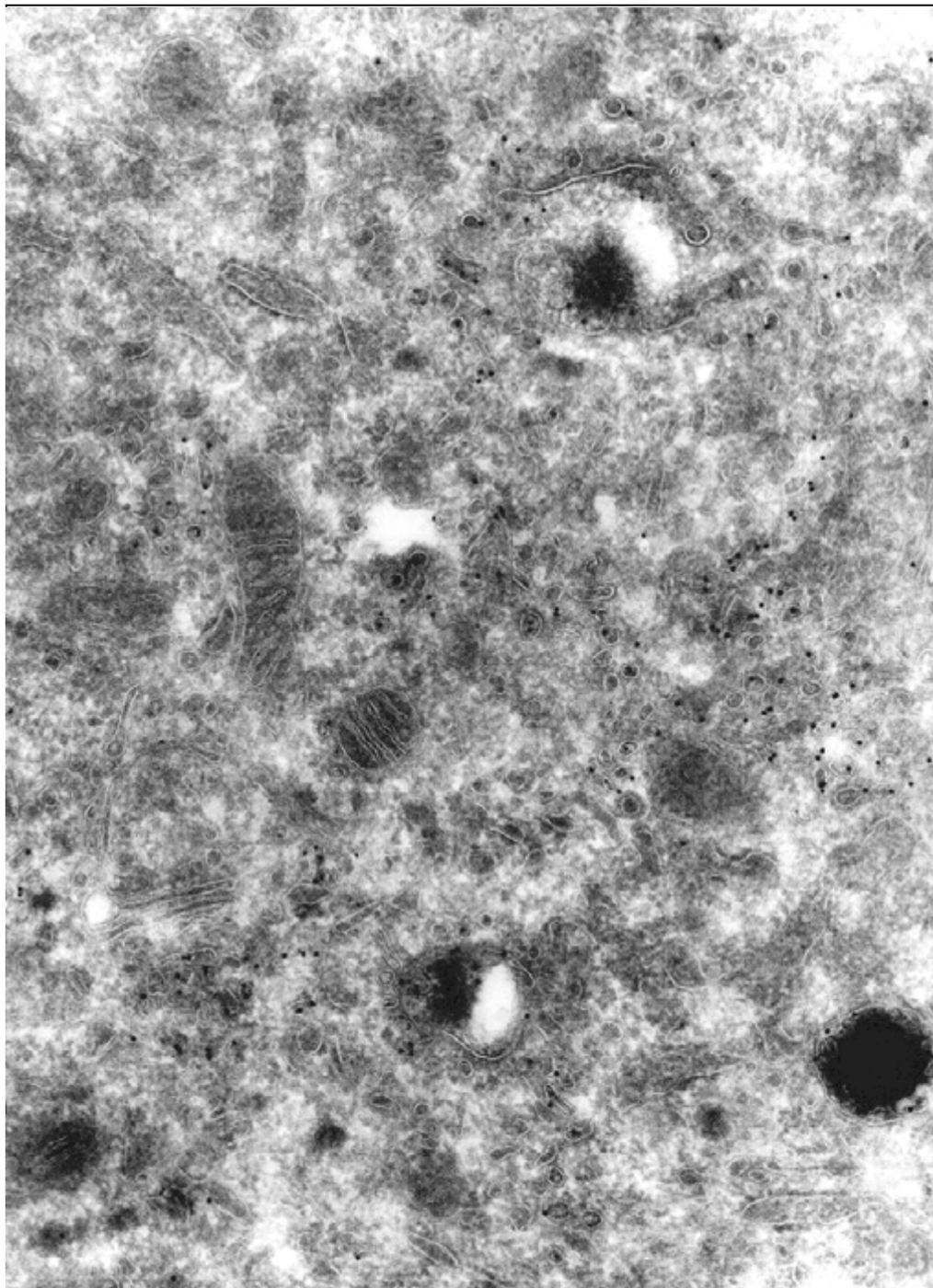


Figure 4.7.1 EM micrograph of an ultrathin cryosection of NRK cells transfected with furin construct and labeled with anti-furin antibodies and 15-nm protein-A gold (For more information see Voorhees et al., 1995).

hanced) gold, nor does the author favor Ig-coated gold particles.

Transmission electron microscopy is carried out at 80 kV with the smallest objective aperture. Exposure time of film is between 0.5 and 1 sec. Damage to sections can be reduced by never evaluating sections without objective aperture.

Anticipated Results

Successful immunogold labeling should allow the detection of specific antigens at the subcellular level. Figure 4.7.1 shows an EM micrograph of a NRK cell labeled with anti-furin antibodies and 15-nm protein-A gold.

Time Considerations

On average, it takes about one week to test and evaluate newly fixed cells with a new antibody. Often, however, multiple antibodies need to be tested at varying dilutions and under varying experimental conditions.

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Key References

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Raposo et al., 1997. See above.

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Contributed by Peter J. Peters
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Amsterdam, The Netherlands

The methods outlined in this protocol could not have been developed without the input of all members of the Geuze/Slot Laboratory in Utrecht. The author is also grateful for the help of Elly van Doneslaar and Crislyn D'Souza Schorey.

Correlative Video Light/Electron Microscopy

UNIT 4.8

This unit describes newly developed methods that allow the examination of living cells by time-lapse analysis with the subsequent identification of the just-observed organelle under an electron microscope.

**BASIC
PROTOCOL**

Many cellular functions, such as intracellular traffic, cytokinesis, and cell migration crucially depend on rapid translocations and/or shape changes of specific intracellular organelles. To understand how such functions are organized and executed *in vivo*, it is important to observe in real time in living cells such dynamic structures as a budding transport carrier, an elongating microtubule, or a developing mitotic spindle, but to have the degree of spatial resolution afforded by electron microscopy (EM). Most suitable for this is a conceptually simple, yet powerful, method called correlative video light/electron microscopy (CVLEM), by which observations of the *in vivo* dynamics and the ultrastructure of intracellular objects can indeed be combined to achieve the above-mentioned result. This unit describes this methodology, illustrates the type of questions that the CVLEM approach was designed to address, and discusses the expertise required for successful application of the technique.

The CVLEM procedure includes several stages: (1) transfection of living cells with an appropriate green fluorescent protein (GFP) fusion protein, (2) observation of structures labeled with GFP in living cells, (3) fixation, (4) immunolabeling for EM, (5) embedding, (6) identification of the cell in the resin block, (7) sectioning, and (8) EM analysis. During the first step, cells have to be transfected with the cDNA encoding the GFP fusion protein whereby the structure of interest can be discovered in living cells (Lippincott-Schwartz and Smith, 1997). In this way, it is possible to gain information about the structure, including its dynamic properties (*i.e.*, motility, speed and direction, changes in size and shape) and life cycle. At the end of this stage, it is necessary to stabilize the cell structure by addition of a fixative, allowing one to capture the fluorescent object at the moment of interest. As GFP is not visible under an electron microscope, immunostaining is required to identify the GFP-labeled structure at an EM level. This protocol uses immunogold and immunoperoxidase protocols to perform staining for EM. Usually the immunogold protocol (Burry *et al.*, 1992) is suitable for labeling the vast majority of antigens; the immunoperoxidase protocol should be used only to label antigens residing within small membrane-enclosed compartments, because the electron-dense products of the peroxidase reaction tend to diffuse from the place of antibody binding (Brown and Farquhar, 1989; Deerinck *et al.*, 1994). Once stained, the cells must be prepared for EM by traditional epoxy (or other) embedding techniques, and the cell and the structure of interest must be identified in EM sections. Finding an individual subcellular structure in a single thin section can be complex, and sometimes impossible, simply because most of the cellular organelles are bigger than the thickness of a routine EM section and may lie in a plane different from the plane of a random section. Analysis of serial EM sections of the whole cell is thus required to identify the structure previously observed *in vivo*. An example of such an identification is shown in Figure 4.8.1. Finally, EM analysis of serial sections can be supported by digital three-dimensional serial reconstruction or high-voltage EM tomography.

Microscopy

4.8.1

Contributed by Roman S. Polishchuk and Alexander A. Mironov

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Supplement 11

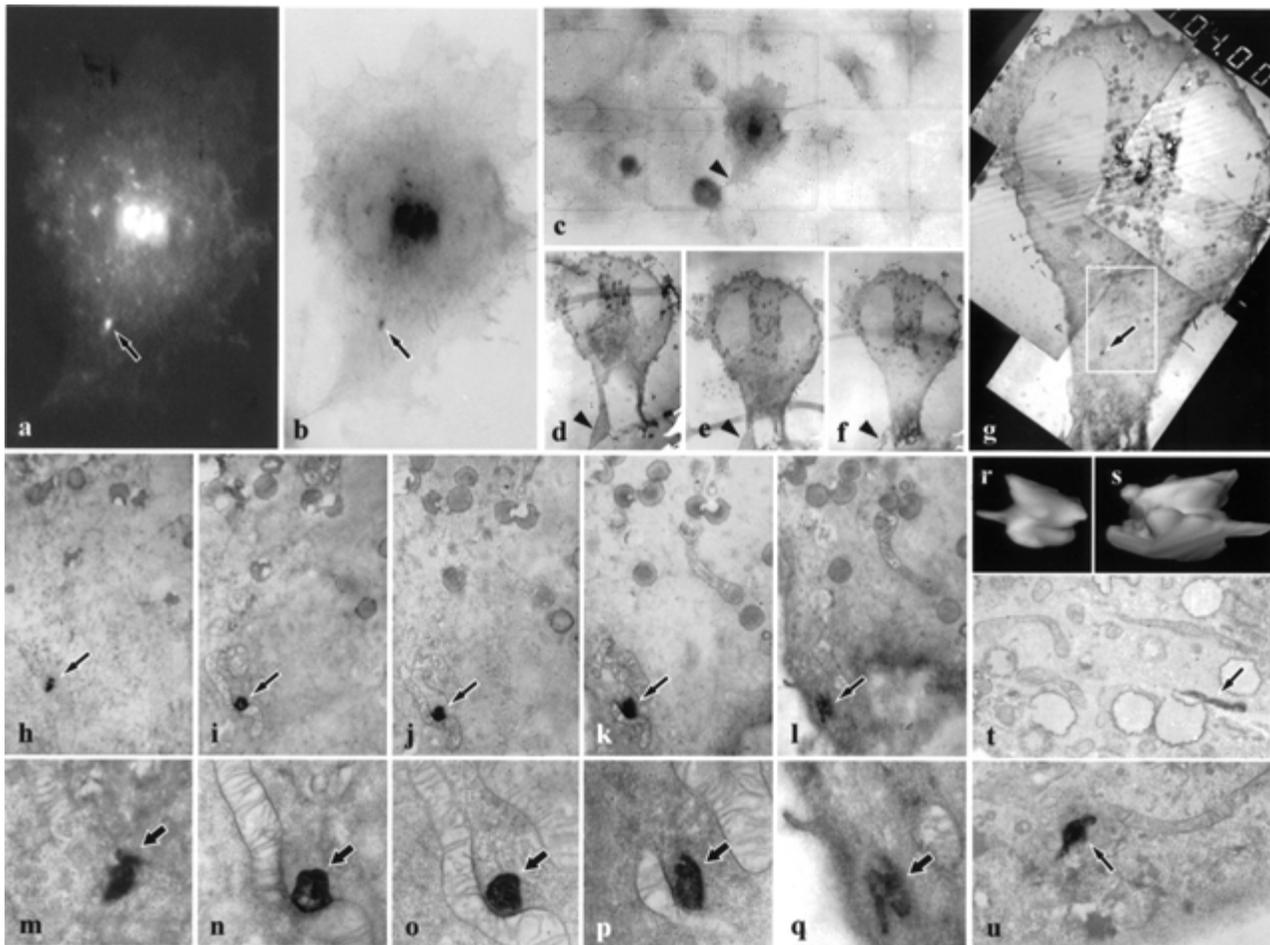


Figure 4.8.1 In vivo dynamics and ultrastructure of an individual Golgi-to-plasmalemma carrier (GPC) studied using CVLEM. The GPC in (a) (arrow) was observed in vivo using time-lapse confocal microscopy. After fixation, the cells were immunoperoxidase labeled and embedded in epoxy resin. The structure in (b) (arrow) was identified as the fluorescent GPC shown in panel a by the computer-aided superimposition of the two images using the CELLocate coordinates [an example of which is shown in (c)]. The general pattern of immunoperoxidase labeling in (b) coincided with the fluorescent pattern of vesicular stomatitis virus glycoprotein–GFP (VSVG–GFP) in (a). Serial sections of the cell were then produced (d through f), and the first section displaying the transport intermediate [(g); arrow] was captured at low magnification (f, g). Note that the sections contain structures that are helpful for identification [e.g., protrusion in (c through f); arrowheads]. (h through q) represent a series of consecutive 200-nm sections containing the GPC (arrow). The field shown in (h through l) is the area of the cell identified by a white box in (g). Three-dimensional reconstruction characterizes the GPC as an elongated sacculi with a short tubular protrusion (r, s). Other GPCs identified using the same approach appeared either as tubules [(t); arrow] or sacculi with short tubules [(u); arrow]. The bar in panel u represents the following lengths in the other panels: 9 μm (a, b), 31 μm (c), 12.2 μm (d–f), 6 μm (g), 2.1 μm (h–l), 700 nm (m–q), 320 nm (r, s), 1.2 μm (t, u).

Materials

- Cells of interests
- cDNA encoding an appropriate GFP fusion protein
- Fixative A (see recipe)
- Fixative B (see recipe)
- PBS (APPENDIX 2A)
- Blocking solution (see recipe)
- Primary antibody to label structure of interest
- Monovalent Fab fragments of secondary antibody conjugated with horseradish peroxidase (HRP; Rockland) or Nanogold (Nanoprobes)
- 1% (w/v) glutaraldehyde in 0.2 M HEPES buffer, pH 7.3

Correlative Video
Light/Electron
Microscopy

4.8.2

Gold enhancement mixture (see recipe)
DAB solution (see recipe)
2% (w/v) OsO₄ (Electron Microscopy Sciences) in water
3% (w/v) potassium ferrocyanide in 0.2 M cacodylate buffer
0.2 M cacodylate buffer, pH 7.4 (see recipe)
50%, 70%, 90%, and 100% (v/v) ethanol
Epoxy resin (see recipe)
8-mm resin cylinder prepared in advance from a cylindrical mold
35-mm MatTek petri dishes with CELLocate coverslip and map of CELLocate grid (MatTek)
Inverted fluorescence microscope
Multiphoton microscope, laser-scanning confocal microscope, or digitalized fluorescence inverted microscope capable of acquiring a time-lapse series of images by computer
60°C oven
Ultramicrotome with sample holder, glass knife, and diamond knife
Eyelashes
Pick-up loop (Agar)
Adjustable-angle laboratory clamps
Slot grids covered with carbon-formvar supporting film (Electron Microscopy Sciences, Agar)
Electron microscope
Software for three-dimensional reconstruction from serial sections
Additional reagents and equipment for transfection (*APPENDIX 3A*)

Transfect cells

1. Plate cells of interest at 50% to 60% confluence on a 35-mm MatTek petri dish with the CELLocate coverslip attached to its bottom.

The CELLocate coverslip contains an etched grid with coordinates that allow the localization of the cell of interest at any step in the preparation.

To facilitate the process of locating the visualized cell of interest in the resin block, the cells should be plated at 50% to 60% confluence.

2. Transfect cells with cDNA encoding an appropriate GFP fusion protein using any method described in *APPENDIX 3A*.

If electroporation is used, steps 1 and 2 should be reversed.

The authors have successfully used procollagen I–mannosidase II–, and vesicular stomatitis virus glycoprotein (VSVG)–GFP constructs for transfection.

Observe labeled structures in living cells

3. Place the dish under an inverted fluorescence microscope, select a transfected cell of interest, and identify its position relative to the coordinates of the CELLocate grid.
4. Draw (or photograph) the position of the cell on the map of the CELLocate grid.
5. Observe the dynamics of the GFP-labeled structures in the selected living cell using a multiphoton microscope, laser-scanning confocal microscope, or digitalized fluorescence inverted microscope that allows acquisition of a time-lapse series of images by a computer.

Fix cells

6. At the moment of interest, while still acquiring images, add fixative A to the cell culture medium at a ratio of 1:1 fixative/medium.

Fixation usually induces the fast fading of GFP fluorescence and blocks the motion of labeled structures in the cell.

7. Stop acquiring images and keep cells in fixative for 5 to 10 min.

During this time it is useful to acquire a Z series of images of the cell.

8. Wash with 2 ml fixative B and leave the cells in this fixative for 30 min.

Immunolabel cells for EM

For EM with Nanogold:

- 9a. Wash cells three times for 5 min with 2 ml PBS.

- 10a. Incubate cells 30 min in 2 ml blocking solution.

- 11a. Incubate cells overnight with primary antibody diluted in blocking solution.

The dilution depends on the antibody; in general, the concentration of antibodies for EM should be 5- to 10-fold higher than for immunofluorescence.

- 12a. Wash cells six times for 2 min with 2 ml PBS.

- 13a. Dilute Nanogold-conjugated Fab fragments of the secondary antibody 1:50 (v/v) in blocking solution and add it to the cells; incubate 2 hr.

- 14a. Wash cells six times for 2 min with 2 ml PBS.

- 15a. Fix cells 5 min with 1 ml of 1% glutaraldehyde in 0.2 M HEPES buffer, pH 7.3.

- 16a. Wash cells three times for 5 min with 2 ml PBS and then again three times with distilled water.

- 17a. Incubate cells 6 to 10 min with 0.5 ml gold enhancement mixture.

- 18a. Wash cells three times for 5 min with 2 ml distilled water.

For EM with HRP:

- 9b. Wash cells three times for 2 min with 2 ml PBS.

- 10b. Incubate cells 30 min in 1 ml blocking solution.

- 11b. Incubate cells overnight with primary antibody diluted in blocking solution.

The dilution depends on the antibody; in general, the concentration of antibodies for EM should be 5- to 10-fold higher than for immunofluorescence.

- 12b. Wash cells six times for 2 min with 2 ml PBS.

- 13b. Dilute HRP-conjugated Fab fragments of the secondary antibody 1:50 (v/v) in blocking solution and add it to the cells; incubate 2 hr.

- 14b. Wash cells six times for 2 min with 2 ml PBS.

- 15b. Fix cells 5 min with 1 ml of 1% glutaraldehyde in 0.2 M HEPES buffer, pH 7.3.

- 16b. Wash cells three times for 5 min with 2 ml PBS.

- 17b. Incubate the cells with 1 ml DAB solution.

- 18b. Wash cells three times for 2 min with 2 ml PBS.

Embed cells

19. Incubate cells in 0.5 ml of a 1:1 (v/v) mixture of 2% OsO₄ and 3% potassium ferrocyanide in 0.1 M cacodylate buffer on ice for 1 hr.
20. Wash cells with 2 ml distilled water.
21. Dehydrate cells in a series of ethanol solutions: 50% (10 min), 70% (10 min), 90% (10 min), and three times in 100% (10 min each).
22. Incubate cells 1 to 2 hr in 1:1 (v/v) epoxy resin/ethanol, room temperature.
23. Incubate cells in pure epoxy resin for 1 hr at room temperature and then overnight (≥12 hr) in a 60°C oven.

Isolate cell of interest in the resin block

24. Put a droplet of fresh epoxy resin on the block where the examined cell is located. Use the fresh resin as glue to attach the block of embedded cells to the flat base of an 8-mm resin cylinder. Return the assembly to the 60°C oven for an additional 16 hr.
25. Carefully lift the resin from the petri dish and cover glass.

It is easiest to remove the resin by gently bending the cylinder to and fro.

If the CELLocate cover glass cannot be detached from the embedded cells, incubate the sample in a plastic tube with hydrofluoric acid (HF; Sigma) for 30 to 60 min to dissolve the glass. Do not use a glass tube, as it will be dissolved by the HF. Wash with water and monitor the completeness of glass dissolution under a stereomicroscope. If glass is not dissolved, repeat treatment with HF and wash again with water. Incubate sample 60 min in 0.1 M PBS or 0.2 M HEPES buffer, pH 7.3, to neutralize the HF, wash with water, and dry.

If desired, embedded specimens can be stored for an unlimited time at room temperature before sectioning and analysis.

Prepare EM sections

26. Trim the resin block to give a pyramid of ~3 × 3 mm size with the cell of interest in its center.
27. Put a sample holder into an ultramicrotome so that the segment arc of the ultramicrotome is in the vertical position. Secure a glass knife in place.
28. Bring the sample as close to the knife as possible.
29. Rotate the knife stage to align the bottom edge of the pyramid parallel to the knife edge.
30. Tilt the segment arc and the knife to adjust the gap between the knife edge and the surface of the sample.

The gap is visible as a bright band if all three lamps of the ultramicrotome are switched on. It must be identical in width during the up and down movements of the resin block to ensure that every point of the sample surface containing the cell of interest is at the same distance from the knife edge.

31. Turn the specimen holder 90° to the left or to the right and trim the edges of the resin block to leave a narrow pyramid (≤100 μm wide) with its long axes parallel to the knife edge.

The pyramid should be as narrow as possible and the cell of interest should be in its center. An experienced person can trim a pyramid directly with a razor blade.

32. Turn the specimen holder 90° back and lock it in exactly the same position as before.

This is very important.

33. Replace the glass knife with a diamond knife and position the latter towards the pyramid.

34. Make serial sections according to the instructions for the ultramicrotome.

35. Stop the motor. Use two eyelashes to divide the band of the sections into pieces suitable in size for collection with a pick-up loop.

Each piece should be small enough to fit completely inside the inner circle of the loop without touching it.

36. Pick up a band of sections by touching the pick-up loop to the surface of water containing the band, making sure the band is completely inside the inner circle of the loop and does not touch it.

37. Raise the loop with the sections on it, and fix the loop inside the lab clamps near the stereomicroscope of the microtome.

The loop should be visible under the stereomicroscope of the ultramicrotome.

38. Take a formvar-coated slot grid and gently touch sections on the water (do not touch the loop) with the carbon-coated surface of the grid.

39. Very slowly move the slot grid away from the loop.

If the movement is slow enough, the water is eliminated from the surface of the supporting film, and only a very small droplet of water remains on the grid, which does not hinder the placement of the grid directly into the grid container. Grids can be stored an unlimited time at room temperature.

Perform EM analysis

40. Place the slot grid under the electron microscope and identify the cell, using the traces of the coordinated grid filled with the resin on the first few sections.

41. Take consecutive photographs (or acquire the images with a computer using a video camera) from the serial sections until the organelle of interest (just observed by light microscopy) is no longer seen.

42. Using software designed for three-dimensional reconstruction, align the images and make a three-dimensional model according to the instructions for the software.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Blocking solution

100 ml PBS (APPENDIX 2A) containing:

0.5 g BSA

0.1 g saponin

0.27 g NH₄Cl

Store up to 3 months at 4°C

Cacodylate buffer, 0.2 M

2.12 g sodium cacodylate

100 ml water

Adjust to pH 7.4 with 1 N HCl

Store up to 6 months at 4°C

Diaminobenzidine (DAB) solution

10 mg DAB
20 ml 0.1 M Tris·Cl, pH ~7.6 (*APPENDIX 2A*)
Prepare fresh
Just before use add 13.3 µl of 30% H₂O₂

Epoxy resin

Combine 20 g epoxy embedding resin (Fluka), 13.0 g dodecenylsuccinic acid (DDSA), and 11.5 g methyl nadic anhydride (MNA) in a test tube. Heat in a 60°C oven for 2 to 3 min and then vortex well to mix the components. Add 0.9 g 2,4,6 Tris (dimethylaminomethyl) phenol (DMP-30) and immediately vortex again. Store 1-ml aliquots up to 6 months at -20°C.

Fixative A (0.1% glutaraldehyde, 4% paraformaldehyde)

Add 1.25 ml of 8% (w/v) glutaraldehyde to 100 ml of fixative B (see recipe). Store up to 2 to 3 days at 4°C.

Fixative B (4% paraformaldehyde)

Dissolve 4 g paraformaldehyde powder in 100 ml 0.2 M HEPES buffer, pH 7.4, while stirring and heat to 60°C. Add a few droplets of 1 N NaOH to form a clean solution. Store up to 3 months at 4°C.

Gold enhancement mixture

Mix equal volumes of solutions A (enhancer; green cap) and B (activator; yellow cap) of a Gold-enhance kit (Nanoprobes) and wait 5 min. Add equal volumes of solution C (initiator; purple cap) and then solution D (buffer; white cap) and mix. Prepare ~400 µl final reagent per dish.

A convenient method is to use equal numbers of droplets from each bottle.

COMMENTARY

Background Information

Correlative light/electron microscopy (CLEM) was developed several years ago, and has been used when the analysis of immunofluorescently labeled structures required higher resolution than can be achieved using light microscopy (Tokuyasu and Maher, 1987; Powell et al., 1998). In spite of its potential, CLEM has not been used very often, probably because the possibility of correlating two static images—one fluorescent and one electron microscopical—is of interest only in a limited number of situations. The real gains from CLEM come from its combination with the kind of dynamic observations obtainable from GFP video microscopy in living cells (i.e., from its use in CVLEM). The CVLEM approach is potentially valuable in any area of cell biology where elucidating the three-dimensional ultrastructure of individual dynamic cellular objects at times of choice can be informative (Mironov et al., 2000). For example, the growth of a subset of microtubules can be visualized *in vivo* (Perez et al., 1999). By CVLEM, it is now possible to study at EM resolution the environ-

ment in which they are growing, as well as the interactions of the microtubule tips with other cytoskeletal elements or intracellular organelles, at various stages of tip growth. In addition to cytoskeletal dynamics, CVLEM could also be useful for studying cell division and cell-cell interactions (although the specific questions to be addressed are best left to the specialists). CVLEM has been applied successfully to characterize the ultrastructure of membrane carriers transporting secretory proteins from the Golgi complex to the cell surface (Polishchuk et al., 2000).

One limitation, and, at the same time, attraction, of CVLEM is its complexity. The use of this technique is demanding, and to master its various steps requires a whole array of skills. However, microscopy is developing fast both in the field of live-cell imaging and in the field of EM, and new powerful technologies are rapidly becoming available in user-friendly versions. This should make the use of CVLEM appealing to a number of cell biologists. Through the use of fluorescent proteins of different colors, two or more marker proteins can

be observed simultaneously (Ellenberg et al., 1999). This will allow analysis of the interactions between different organelles and organelle subdomains (Ellenberg et al., 1999; Pollok and Heim, 1999). Combining these and other methods with the quickly developing electron microscope tomography (Ladinsky et al., 1994) will increase the subtlety and range of the questions that can be answered by CVLEM. While we wait for the microscopy of the future, endowed with the magical power to show cellular structures with EM resolution in vivo in real time, CVLEM offers a useful chance to look deeper inside living cells.

Critical Parameters

Taken together, all the steps of CVLEM represent quite a long procedure (see Time Considerations) and require significant effort by a researcher or technician. Hence, it would be especially disappointing to lose such tour-de-force experiments due to small problems in specimen handling. To apply CVLEM successfully, several important parameters should always be taken into account by the experimenter.

Fixation/labeling protocol. Preliminary experiments should be carried out to understand whether the antibodies selected for labeling of a GFP–fusion protein work with the immunoperoxidase protocol. Many antibodies that give perfect results for immunofluorescence do not work for immunoperoxidase staining, because the glutaraldehyde used in most EM fixatives tends to cross-link amino groups of antigen epitopes, therefore decreasing the antigenicity of the target protein and restricting the penetration of antibodies across the cytosol. However, a decrease or an absence of glutaraldehyde in the fixative can result in poor preservation of ultrastructure of the intracellular organelles. If immunoperoxidase labeling is not successful, it may be possible to optimize the concentration of glutaraldehyde in the fixative, or to use the periodate/lysine/paraformaldehyde fixative described by Brown and Farquhar (1989). In addition, it is important to select an immunoperoxidase or immunogold protocol to label the structure of interest. To label epitopes of a GFP–fusion protein located in the cytosol, only the immunogold protocol should be used; for other epitopes, HRP labeling is also suitable.

Locating the cell of interest. It is extremely important to be able to find the cell of interest at any step in the CVLEM procedure. Only cells located on the grid of the MatTek petri dish can be selected for time-lapse observations. The position of the cell of interest on the grid should

be noted, otherwise the cell will be difficult to locate again. Low-magnification images showing the field surrounding the cell of interest can greatly help when trimming the resin block around the cell and when locating the cell under the electron microscope. In this case, neighboring cells can be used as landmarks to identify the cell of interest. It is more convenient to work with cells that are plated at a lower confluence (e.g., 50% to 60%). It is also useful to have fluorescent and phase-contrast images of the target cell, because particular structures (e.g., microvilli, pseudopodia, inclusions) can also be used to help find both the cell and the structure of interest.

Section thickness. The thickness of serial sections should be ~50 nm (or less) for very precise three-dimensional reconstruction, ~80 nm for routine work, or ~250 nm for electron microscope tomography.

Anticipated Results

Once considerations for specific localization of the cell and the organelle have been optimized, the precise three-dimensional structure of the organelle and its connections with other organelles at different moments of its life cycle can be identified.

Time Considerations

The entire CVLEM procedure requires 4 to 5 days. During the first day, cells must be transfected with cDNA. The next day, it is possible to make observations in living cells, fix them, and start the immunolabeling. The third day is required to complete the immunolabeling and resin embedding of the cells. During the fourth experimental day, the cell of interest is identified in the resin block and cut into serial sections. On the fifth day, EM analysis is performed. If desired, it is possible to store embedded specimens indefinitely before sectioning and analysis.

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INTRODUCTION

Polarization microscopy, also called polarized light microscopy, allows one to nondestructively follow dynamic, anisotropic organization of living cells and tissues at the microscopic as well as submicroscopic levels.

Since, in polarization microscopy, light waves are used to probe the specimen, spatial resolution of the image is limited to $\sim 0.2 \mu\text{m}$, even using objective and condenser lenses with numerical apertures approaching 1.4. Nevertheless, the brightness of each resolved area in the image measures the polarization optical property, most commonly birefringence, of the corresponding minute area in the specimen. The birefringence directly reflects the anisotropic organization of the microscopically unresolvable, fine structure—namely, the submicroscopic, molecular, and atomic lattice arrangements of the specimen within each diffraction-limited image area.

Thus, e.g., the assembly and disassembly dynamics of spindle microtubules and the developmental changes in sperm chromatin could be revealed, and the arrangement of the atomic lattice in developing biocrystalline spicules, as well as the packing arrangement of DNA molecules in sperm chromosomes, could be unveiled with polarization microscopy at levels even finer than those conventionally seen with electron microscopy.

Examples in this unit were chosen mostly from those particularly familiar to the author and which have been used successfully by many generations of students and co-workers. Beyond the biological material mentioned below, or those that are commonly used for studying living cell behavior and fine structure of their organelles, a treasure trove of suggestions for other living cells appropriate for study can be found throughout an article by Belar (1928).

The first part of this unit touches on some general procedures, and outlines the background optical theory and relevant literature sources for polarization microscopy. The unit then examines several basic examples for using the polarizing microscope, emphasizing the study of living cells or products, starting with those that can be carried out with relatively simple equipment and proceeding to examples that require more advanced instruments. For instructions on setting up and operating a polarization microscope see Murphy (2001), Old-

enbourg (1999), and manufacturer's instructions.

For more comprehensive discussions and an annotated list of references on polarized light and birefringence phenomena, see Inoué (1986, especially Appendix 3). For additional information, see Bennett (1950), Jenkins and White (1957), Hartshorne and Stuart (1960), and Hecht (1998).

Types and Transmission Axis of Polars

Polarizers, or polars, range from plastic-sheet "Polaroids" to calcite polarizers of many designs. Although very expensive and fragile, the calcite polarizers provide the theoretically maximum transmission (i.e., nearly 50% of the incoming nonpolarized light) over a wide wavelength range. Their extinction factor (EF; see in Example 5 below) also tends to be an order of magnitude better than the sheet Polaroids. On the other hand, light transmitted through the calcite polars has to be collimated into parallel beams in order to avoid astigmatism. They are used in expensive polarization optical systems requiring the highest EF and minimum loss of light. Some new sheet polarizers such as "Polacolor" and those available from Spindler and Hoyer are reported to have both high transmission for white light and high EFs.

Sheet Polaroids generally contain a stretched sheet of polyvinyl alcohol, a long-chain polymer whose backbone can be aligned by heating and stretching the polymer sheet that has been cast from an aqueous solution. Commonly they are stained with polyiodide micelles, whose conjugated backbones lie parallel to the aligned polyvinyl alcohol chains. It is the conjugated backbone of the polyiodide that gives rise to the *dichroism* of these Polaroid sheets (Inoué, 1986). They can be made into large sheets and can easily be cut into various shapes with a pair of scissors. This is the material used for Polaroid sunglasses. As polars in microscopes, their soft and somewhat wavy surfaces are protected by mounting them with index-matched cement between antireflection-coated glass cover plates.

The transmission axis (E-vector direction of the transmitted polarized light) for Polaroid sunglasses is oriented vertically in order to reduce the glare from horizontal surfaces, such as the road, water, or hood of a car. Light

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reflected from such (nonmetallic) horizontal surfaces contains a high proportion of polarized light whose E- (electric-) wave oscillates horizontally and hence is cut out by the Polaroid sunglasses. Thus, the Polaroid sunglasses become convenient, quick references for establishing the E-vector transmission direction of other polars.

Birefringence

Light traveling inside a birefringent material is split into two waves that travel at different speeds. The slower wave suffers greater refraction than the faster wave since the refractive index experienced by each wave is inversely proportional to its velocity of propagation.

The two waves vibrate in planes that lie perpendicular to each other, as dictated by the material's optical axes, and to the direction of propagation of the light ray. For a ray traveling in a particular direction through a birefringent material, the electric (E-) vector direction encountered by the slow wave is called the slow axis, and that of the fast wave the fast axis.

Optical Axes and Optic Axis

Broadly speaking, crystals can be classified into six groups depending on their axes of symmetry. All crystals, except those in the cubic group (e.g., NaCl, KCl), are birefringent, that is, they transmit light whose E-vectors, oscillating in different planes, travel at different velocities (Hartshorne and Stuart, 1960; Inoué, 1986). However, each crystal type has a unique single axis, or two axes, along which light is transmitted at equal speeds regardless of the direction of the E-vector. In other words, light propagating along such an axis does not suffer birefringence. Such an axis is called the optic axis of the crystal or the birefringent material.

In addition to the optic axis, a crystal generally has three principal optical axes (one of which may correspond to the optic axis). Between crossed polars, when the optical axes of a crystal come to lie parallel to the polarizer or analyzer, the entering polarized light is not split into two components, thus encountering no birefringence, and so the crystal appears dark. This extinction is seen intermittently in a swimming pluteus larva of a sea urchin, when the long axis of one of the biocrystalline, skeletal spicules comes to lie along the polarizer or analyzer transmission direction (see Example 1; Fig. 4.9.1).

In the younger, gastrula-stage sea urchin embryo, the triradiate precursor of the spicule may appear not to be birefringent unless viewed

from its side. That is because the optic axis of these biocrystals, made of calcite, lies perpendicular to the plane of the triradiate spicule.

With the background between crossed polars made gray by turning a compensator from its extinction orientation, regions of the birefringent specimen whose axes coincide with the transmission axes of the polars appear with similar brightness to the background, i.e., neither brighter nor darker than the background. Four such regions are commonly seen, e.g., on the fertilization envelope, asters at the spindle poles, and starch grains (see Examples 3 and 4 and Fig. 4.9.4).

Sign of Birefringence and Coefficient of Birefringence

Depending on the crystal type, light may travel faster or slower along the optic axis compared to other axes. The former type of crystal is known to be positively birefringent (e.g., quartz, collagen fiber), and the latter negatively birefringent (e.g., calcite, DNA thread; Inoué, 1986). In addition to its sign, each crystal type exhibits a unique amount of birefringence, the coefficient of birefringence, which is the *retardance*-per-unit thickness of crystal (see discussion of Retardance, below). In fact, the signs of birefringence and refractive indices for light traveling along the axes of pure crystals are so unique that they are used in crystallography for identifying their composition and lattice type.

Traditionally, in biological polarization microscopy, the expressions positive and negative birefringence are used relative to some obvious frame of reference (see Examples 3 and 4) rather than to the crystal axis of the object. For example, the fertilization envelope is said to be positively birefringent relative to its tangent, meaning the E-wave oscillating parallel to the tangent of the fertilization envelope (the obvious outline seen outside the equator of the egg; Fig. 4.9.4) is retarded (refracted) more than the E-wave oscillating perpendicular to the envelope. Since the fertilization envelope is a hollow sphere, the axis of symmetry (and, hence, optic axis for any unit area of the envelope) should be its radius rather than its tangent. So, according to the crystallographic definition, the envelope has a negative birefringence. On the other hand, if one were to make a thread of the material that makes up the envelope, the axis of symmetry would be along the length of the thread, so that the traditional nomenclature commonly used in biology does have its utility.

Retardance

Light waves traveling through a birefringent specimen are split into two orthogonally polarized components, one being refracted more, or traveling slower, than the other (Bennett, 1950; Inoué, 1986). The phase difference between these two waves as they emerge from the specimen is known as their retardance, or birefringence retardation. The retardance (Δ) is, in fact, the difference in refractive indices ($n_e - n_o$) experienced by the “extraordinary” and “ordinary” waves multiplied by the thickness (d) of the specimen. Thus $\Delta = (n_e - n_o) \times d$.

The retardance can be expressed in fractions of wavelengths or in nanometer units; e.g., $\lambda/4$, or 136.5 nm (= 546 nm/4), for a quarter-wave plate for the mercury green line. A mitotic spindle may show a retardance of 2.7 nm, or only 1/200 wavelength for the mercury green line.

Intrinsic, Form, and “Accidental” Birefringence

Birefringence of a specimen may arise from anisotropy (difference in physical property exhibited for different directions in the same material) of structural organization at various submicroscopic levels that are all too fine to be resolved with the light microscope. If the ultrastructure of the specimen is anisotropic at a level usually detectable with the electron microscope, but not with the light microscope (e.g., stacks of cell membranes, such as in the Schwann sheaths of nerve or the outer segments of the retina, parallel-oriented filament systems such as the F-actin thin filaments in muscle, microtubules in flagellar axonemes, and spindle fibers), they exhibit an optical anisotropy known as form birefringence. Form birefringence arises from the parallel array of filaments or membranes whose refractive index differs from that of the imbibing medium and therefore, disappears when the refractive indexes of the two match. For filaments, the sign of form birefringence is positive, whereas for membranes it is negative.

When the birefringence arises from anisotropy within the molecules themselves, it is not altered by the refractive index of the imbibing medium, and the material is said to possess intrinsic birefringence. The regularly aligned polypeptide chain in collagen fibers and in the myosin filaments of muscle A-bands, the regularly aligned DNA protein in mature sperm head, and the biocrystalline calcite in the skeletal spicules of sea urchin embryos are some examples of material that exhibit intrinsic birefringence.

While intrinsic birefringence tends to be considerably stronger than form birefringence, the two may contribute more or less equally in some biological structures, either with the same or opposite signs of birefringence. For example, the positive intrinsic birefringence of lipid molecules, or of membrane proteins, may overcome the negative form birefringence introduced by the lipid bilayers when the refractive index of the imbibing medium approaches that of the bilayers (Ambronn and Frey, 1926; Schmidt, 1935, 1937; Frey-Wyssling, 1953).

In addition to the naturally observed intrinsic and form birefringence, one may encounter birefringence induced by flow or by external forces such as stretch or compression or by the application of electrical, magnetic, or other fields. The induced birefringence, in turn, may reflect the intrinsic or form birefringence of the aligned molecules or submicroscopic particles, or may reflect distortion of the electron orbits within the substance itself. In this last regard, strain birefringence can be induced even in naturally isotropic substances such as glass, isotropic crystals, or well annealed plastics. In fact, the strain birefringence in the objective and condenser lenses can be high enough to interfere with observation of small specimen retardances, and special strain-free objectives (designated with P or Pol) are sometimes supplied to minimize such effects.

Edge Birefringence

The edges of objects, viewed with polarization microscopy using high-NA objective lenses, may display what appears to be a double birefringent layer. While in some cases these may represent the birefringence due to membrane fine structure or its molecular organization, in other cases they can be caused merely by the presence of a sharp refractive index gradient at the edges of the object. The object can be purely isotropic; the only condition required is that there exist a sharp gradient of refractive index at the boundary between the object and its surrounding. Such birefringence is called “edge birefringence.”

As detailed by Oldenbourg (1991), with edge birefringence the inner, thin “birefringent layer” shows a slow axis (axis with greater refractive index) parallel to the boundary and an outer thin “birefringent layer” with its slow axis perpendicular to the boundary when the refractive index of the object is greater than its surroundings. When the refractive index of the object becomes equal to its surroundings, the edge birefringence disappears. When the re-

fractive index of the object becomes less than its surroundings, the directions of the slow axes are reversed.

As with edge birefringence, form birefringence also disappears when the refractive index of the rodlets or platelets matches that of the imbibing medium. But the sign of form birefringence does not change below and above the matched index. The slow axis direction is unchanged (relative to the specimen axis) above and below index match point. In contrast, with edge birefringence the signs of birefringence (in the two layers on both sides of the optical boundary) are reversed in media whose refractive index is greater than the index match point compared to the situation in media below the match point. In contrast to both edge and form birefringence, the retardance due to intrinsic birefringence is unaffected by the refractive index of the imbibing medium.

It is important to distinguish edge birefringence from form or intrinsic birefringence since edge birefringence reflects an optical effect that has nothing to do with the molecular or fine structural anisotropy that we are probing.

COLOR IN POLARIZED LIGHT

Interference Colors

Many crystals that are normally colorless, including the biocrystalline spicules of sea urchin plutei, show in vivid color when observed in white light between crossed polars (Fig. 4.9.2). The color reflects the birefringence retardation (or retardance, i.e., the phase difference) that the two components of the orthogonally polarized light components suffered in passing through the crystal. For example, if the retardance were 550 nm, or a full wavelength for green light, the two orthogonal components would add back together to form the initial state (i.e., of plane polarized light as before entering the crystal) upon exiting the crystal. Thus, green light is extinguished by the analyzer. Wavelengths other than 550 nm are elliptically polarized and are partially transmitted by the analyzer. Thus, the complementary color of green, i.e., red, is seen through the analyzer (Inoué, 1986).

Similarly, for an object giving a retardance of 490 nm, full wavelength for blue light, blue is extinguished and its complementary color yellow is seen through the analyzer. For a retardance of 568 nm, full wavelength for yellow light, yellow is extinguished and its complementary color blue is seen through the analyzer.

For a retardance of 600 nm, full wavelength for red light, red is extinguished and its complementary color green is seen through the analyzer. These interference colors thus measure the retardance of the specimen.

For a cylindrical object like the pluteus skeletal spicule, light would travel the furthest distance across its middle and suffer the most retardation, so that the axis of the spicule would appear with the highest-order interference color. Thus, the axis may appear green, surrounded by blue, then red, then yellow, then gray, etc., as light travels through decreasing thickness towards the outer margin of the cylindrical biocrystal and is thus retarded, proportionately, less and less.

Dichroism

In addition to optical anisotropy that is manifested as birefringence (difference of refractive index for light waves whose E-waves are oscillating in different planes), one may also encounter dichroism (difference of absorbance, or of absorption spectrum, for light waves whose E-waves are oscillating in different planes). Whereas a birefringent material simply changes the phase of polarized light, a dichroic material transmits light to different degrees, depending on the wavelengths and the angle between its axes and the plane of the entering polarized light.

Polaroid films serve as effective polars by taking advantage of the aligned polyiodide chains that show a strong dichroism for most wavelengths in the visible spectrum. The conjugated electrons that make up the backbone of the polyiodide chains strongly absorb the (E-) component of the visible light waves that are polarized parallel to that direction, whereas they absorb little of the component that is polarized at right angles to the backbone.

The dichroism of Polaroid sheets that extends over much of the visible wavelength range is, however, an exception. Most dichroic materials (or pleochroic materials, i.e., many-colored instead of showing two different colors, depending on the vibration direction of the polarization of light) exhibit two different absorption curves depending on the orientation of their chromophores relative to the plane (of the E-wave) of polarized light.

DNA in mature sperm is a biological material that shows prominent dichroism in ultraviolet (UV) light. The conjugated bonds in the purine and pyrimidine bases of B-form DNA, regularly oriented at right angles to the DNA backbone as in steps of a spiral staircase, give

rise to a UV dichroism with a dichroic ratio of 4, spanning the wavelength range of ~250 to 380 nm. This dichroism is present because the conjugated bonds in the bases (that resonate in the UV range) lie at right angles to the B-form DNA backbone. So the UV polarized at right angles to the backbone is absorbed four times more effectively than UV that is polarized parallel to the DNA backbone (Wilkins, 1951; Inoué and Sato, 1966). This gives rise to the UV dichroism that has a negative sign (i.e., greater absorbance perpendicular to the backbone compared to parallel to the backbone).

The same conjugated bonds give rise to the much greater polarizability of the electrons, and, hence, to higher refractive indexes for visible light waves whose E-fields lie in the plane of the bases compared to fields along the backbone of DNA. That gives rise to the strong negative birefringence of B-form DNA in the visible wavelength range (Inoué and Sato, 1966; Inoué, 1986). The author took advantage of this dichroism and birefringence, which are both abolished upon polarized UV microbeam irradiation (possibly by dimerization of the thymidine bases), to analyze the detailed packing arrangement of DNA and to uniquely visualize chromosomes, taking advantage of a needle-shaped head of an insect sperm (Fig. 4.9.7; Inoué and Sato, 1966).

Chromophores with such high dichroic ratios can be recognized by a change in light transmittance or absorbance, or of color, as the orientation of a single polar (a polarizer in the absence of an analyzer, or an analyzer in the absence of a polarizer) is changed.

Cell inclusions with regularly oriented natural chromophores—e.g., retinal rods and cones (Schmidt, 1935), sickled blood cells (Perutz and Mitchison, 1950; Hárosi, 1981), and chloroplasts (Breton et al., 1973; Hsu and Lee, 1987)—may also show some degree of dichroism. Cell inclusions, or products with regularly oriented fine structure that have been stained with dyes, may likewise be dichroic.

When the dichroic ratio is low, as in these latter examples, it may require a sensitive spectrophotometer to detect a change in color, or wavelength dependence of light transmittance, as the specimen orientation is changed relative to the orientation of the polarized light (Hárosi and MacNichol, 1974). In such cases, the dichroism may still be detected visually if one uses both a polarizer and an analyzer and orients one of the two a few degrees from the crossed position. The complementary dichroic colors can then be seen visually by turning the

polar or compensator in opposite directions from the extinction orientation.

Using the dichroic fluorescence of tetramethylrhodamine that was used to sparsely decorate actin filaments, Kinoshita and coworkers were able to demonstrate, and measure, the speed of rotation of individual actin filaments around their own axis as the filaments are propelled by myosin molecules coated on the surface of a microscope coverslip. Through video contrast enhancement of the fluorescence microscope images taken in polarized light, they were able to show the cyclic brightness change in the dichroic fluorescent dye as the single actin filament, <10 nm in diameter, glided along the lawn of myosin heads (Kinoshita, 1999).

The sensitivity for measuring the orientation of a dichroic fluorescent molecule, such as green fluorescent protein, can be increased several fold by using parallel polars instead of a single polarizer or analyzer (Inoué and Goda, 2001).

Colloidal Gold, Pinholes

Spindle microtubules in *Haemaphysalis* endosperm cells decorated with 20 nm-diameter colloidal gold appear as dark black lines when observed in a compensated field between crossed polars in monochromatic light. These same microtubules show in striking colors ranging from red to purple, green, orange, and gold when they are observed in white light with the polars off-crossed by a few degrees and the compensator orientated to different directions (Inoué and Spring, 1997, Color Plate III). These colors are attributed to anisotropy of Tyndall light scattering (or di-Tyndallism) by the linear array of colloidal gold particles that are arranged quite densely (not contacting each other, yet acting as an effective conductor, or antenna, for the high-frequency electromagnetic light waves) along the lengths of each microtubule.

Examined at high magnification, pinholes in thin metal film, evaporated on coverslips that are used to test the performance of microscope objective lenses, can also each appear with striking saturated colors when examined in polarized white light. Light from the pinholes becomes alternately extinguished as the microscope stage is rotated. In other words, each pinhole is acting as a polarizer that appears to have a narrow transmission wavelength. No doubt, each pinhole is not completely circular but must be elongated, since they are generally produced by some random dust particle or impediment to sputtering or high-vacuum depo-

sition of the metal film. Thus, each “pinhole” acts as an anisotropic nonconductor in the thin metal film.

In fact, a very narrow crack in a thin metal film preferentially transmits light that is polarized with its E-vector oriented perpendicular to the narrow crack and whose wavelength reflects the local width of the submicroscopic crack (S. Inoué, unpub. observ.). The situation is somewhat the converse of the aligned polyiodide chains or of the colloidal gold-decorated microtubules, in that instead of a submicroscopic thread-shaped conductor, one has a thread-shaped non-conductor surrounded by a conducting film.

Anomalous Birefringence

Stained or colored biological tissue and cell components often exhibit “anomalous birefringence,” i.e., the color observed in white light between crossed polars actually changes with adjustment of the compensator. In other words, the birefringence, or the retardance observed, is a function of the wavelength of light. Such a color change, or “anomalous birefringence,” is observed even when the retardance of the object is a small fraction of the wavelength of light (Perutz and Mitchison, 1950).

Colorless objects, on the other hand, generally show birefringence that changes only slightly with variation of wavelengths. What color is observed for a colorless object is primarily due to interference colors when the retardance approaches a wavelength or more, as described earlier (Fig. 4.9.2).

On the other hand, with strongly absorbing materials such as a dye or stain, the refractive index drops sharply on the shorter wavelength side and rises precipitously on the longer wavelength side of the absorption peak (“anomalous dispersion”; Jenkins and White, 1957; Hecht, 1998). Since the wavelength-specific absorption of light in a dye or stain is commonly brought about by conjugated ring structures, the birefringence associated with such an anisotropic structure would also be expected to show a strong dispersive character (wavelength dependence). The anisotropic dispersive character would then give rise to anomalous birefringence. Objects exhibiting anomalous birefringence would also be expected to show some dichroism as well.

These several wavelength-dependent polarization optical effects (as with polarization of fluorescence, a topic not covered here) are of considerable potential interest as potent tools for analyzing dynamic changes within, or in-

teractions among, biological molecules and fine structures. Further studies on the physical optical bases of these phenomena should lead to their wider application in cell biology.

OPTICAL ROTATION

Optical rotation (also known as circular birefringence) is a polarization optical effect that may (rarely under the microscope) induce an object to become bright between crossed polars. Unlike (linear) birefringence, the brightness due to optical rotation does not depend on the regular alignment of the molecules but on their internal asymmetry and thus is observed even in solutions of the particular molecules. Optical rotation may also be seen in certain crystalline substances, such as quartz and other material viewed along the helical axes of the molecules that make up the crystal.

An object exhibiting optical rotation does not undergo extinction between crossed polars as the microscope stage revolves. Instead, the object can be made darker by turning the polarizer or analyzer away from their crossed orientation. Except in pure monochromatic light, as a polar is turned, these “optically active” objects or solutions acquire a brownish or bluish hue instead of undergoing complete extinction. The color reflects a high degree of wavelength dependence that is characteristic of optical rotation. Such wavelength dependence of optical rotation is known as optical rotatory dispersion. The infrared optical rotatory dispersion, e.g., of myosin and related proteins in solution, has been used to determine the degree of helix content of protein molecules (Szent-Györgyi et al., 1960).

VIDEO MICROSCOPY

As in many other modes of microscopy, the capabilities of polarization microscopy have been substantially enhanced by the application of video and electronic image processing, analysis, and device control. As described in Allen et al. (1981) and Inoué (1981b) and summarized, e.g., in Shotten (1993), and Inoué and Spring (1997), video microscopy can effectively enhance image contrast, subtract background, reduce noise, and allow image capture with better corrected higher-NA objective lenses and even with relatively short exposures.

The new LC PolScope introduced by Oldenbourg, provides striking, high-contrast images of weakly birefringent cellular regions and does so independently of specimen axis orientation. The recent system introduced by N. Allen and co-workers selectively enhances (or

suppresses) contrast in differential interference contrast (DIC) microscopy and should also be applicable for bringing out birefringent regions of the specimen.

Oldenbourg's LC PolScope

Oldenbourg's LC PolScope (which can be adapted to most research-grade microscopes outfitted for polarization microscopy) provides striking, high-contrast images of weakly birefringent objects. Furthermore, the system has the unique attribute of providing maps of birefringence distribution that is independent of orientation of the specimen axis within the focal plane.

The new system uses circularly, instead of linearly polarized light and two electronically controlled liquid crystal compensators instead of a mechanically driven conventional compensator (Oldenbourg and Mei, 1995; Oldenbourg, 1996). Four video images are acquired into computer memory in rapid succession. In acquiring each of these four images, the computer provides appropriate voltages that adjust the retardances of the two compensators. The LC PolScope uses no mechanically moving compensators and records the images in perfect register. From the set of four images, the retardance map is immediately calculated by the computer and displayed on the monitor.

The calculated image generated with the LC PolScope is, in fact, a map of birefringence distribution whose intensity is directly proportional to the retardance of each specimen point. In contrast, with images obtained by standard polarization microscopy, the intensity of each image point is proportional to the square of the retardance of the specimen point. The LC PolScope can also display a map of slow-axis orientations for pixels for each image point.

The LC PolScope has been used to display the dynamic distribution of microtubules in newt lung epithelial cells undergoing mitosis (see on-line video accompanying Inoué and Oldenbourg, 1998) and the highly dynamic behavior of actin filaments and network in cultured growth cones of *Aplysia* neurons (Kato et al., 1999) with unprecedented clarity.

The LC PolScope system is available from CRI (Cambridge Research and Instrumentation).

Nina Allen's Compensator

In another recent development, Holzwarth et al. (1997, 2000) also use a voltage-driven liquid-crystal compensator for DIC observations with video microscopy. They use crossed

linear polars rather than circular polarizers as in Oldenbourg's system. Appropriate voltages, applied by a computer to the compensator, generate an additive and a subtractive shift to the phase difference introduced by the Wollaston or Nomarsky prism. Each of these images is stored in computer memory and subtracted from each other. The difference image displays the DIC effect alone, while subtracting away stationary background noise and image features that did not originate from interference between the two sheared rays.

Although designed for use in DIC and still retaining the orientation-dependent contrast characteristic of images observed between crossed linear polarizers, Allen's new compensator applied to polarization microscopy should also effectively bring forth contrast in weakly birefringent regions of the specimen. Conversely, the modified system could selectively suppress contrast due to birefringence by adding (rather than subtracting) the two images stored in computer memory and selectively bring forth image features not based on optical anisotropy.

Centrifuge Polarizing Microscope

Living cells centrifuged on an isopycnic gradient at several thousand $\times g$ (earth's gravitational field) tend to stratify with their lighter components packed at the centripetal pole and heavier inclusions packed at the centrifugal pole of the cell (Harvey, 1940; Zalokar, 1960). In the process, cytoskeletal elements, membranes, and organelles tend to become aligned or deformed. To study the fine structure and function of stratified cell components and to learn about the mechanical linkages of the organelles to the membranes and cytoskeletal elements, the author of this unit has developed a centrifuge polarizing microscope (CPM; Inoué et al., 1998, 2001a).

The CPM allows video-rate polarization microscope observations of the spinning specimen, with image resolution of 1 μm , in living cells suspended in an isopycnic solution or crawling on a glass substrate. Birefringence retardations can be detected and measured down to 1 nm in cells that are exposed to up to 10,500 $\times g$.

With the CPM, the author has found striking changes in the conformation of endoplasmic reticulum immediately following fertilization of sea urchin eggs, has followed the process of Ca^{2+} -induced nuclear-envelope breakdown and meiotic spindle formation in a marine annelid egg, measured the climbing forces generated

by myosin mutants of slime mold amoebae, and observed the enigmatic formation and rapid precipitation of dense “comets” in a concentrated solution of a thread-shaped virus (Inoué et al., 2001b).

APPLICATIONS OF POLARIZATION MICROSCOPY

Example 1: Examining Biocrystals

Hard tissue generated in living organisms often contains, or is made up of, biocrystals. Biocrystals are indistinguishable (in terms of their atomic lattice and, hence, intrinsic optical properties) from inorganic crystals, yet their morphology is primarily determined by their biological function and seldom reflects the habits (e.g., cleavage faces) of the natural crystal.

A common form of biocrystal, such as the skeletal spicules found in embryonic sea urchins (or the plates of their adult forms), is based on calcite, a highly birefringent, negative uniaxial material that is the main ingredient of chalk.

The birefringence, growth, and morphogenesis of calcareous biocrystals, such as the skeletal spicules generated by the mesenchyme cells in the gastrula- and pluteus-stage sea urchin larvae, can be studied with a simple polarizing microscope that can be easily assembled.

All one needs is a polarizer, inserted in the illumination path before the condenser of the microscope, and an analyzer (crossed relative to the polarizer) placed after a low (10× or 20×) power objective lens. In fact, both of these “polars” may already be present on the microscope. If the microscope is equipped for DIC,

simply slide out the Nomarsky prism and set the condenser turret to the bright-field position. Close down the condenser iris so that the condenser NA becomes just below half that of the objective NA. Make sure the polars are carefully crossed so that, with the lamp brightness turned up, the blank field becomes completely (or very) dark in Köhler illumination (see *UNIT 4.1*).

When live gastrula or pluteus larvae are observed between crossed polars, the pair of triradiate or skeletal spicules should alternately twinkle like stars as the ciliated embryo swims about (see Fig. 4.9.1). The thicker parts of the skeletal spicule may show brilliant interference colors when observed in white light (see Fig. 4.9.2).

One can even make these observations using a dissecting microscope of the type that allows transillumination of the specimen. Simply attach a polarizer below the specimen-support glass plate and an analyzer below the objective lenses. The polars can be sheet Polaroids, perhaps those cut to appropriate shape from plastic Polaroid sunglasses. With the analyzer transmission axis oriented horizontally (i.e., turned 90° with respect to the way Polaroid sunglasses would normally be oriented), the polarizer mounted below the glass specimen support plate can be oriented vertically (north-south) by rotating the support plate to achieve extinction. Note that it is important to observe the specimen in a glass container, not in a plastic Petri dish, since most plastic lab ware exhibits very strong birefringence that can overwhelm the specimen birefringence.

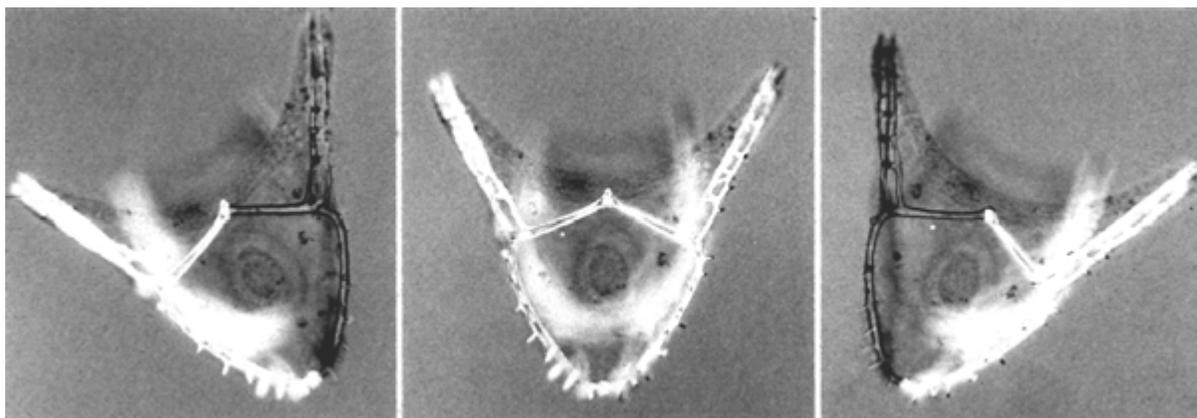


Figure 4.9.1 Pair of skeletal spicules in sea urchin pluteus larva. As the ciliated pluteus swims about in the field of the polarizing microscope, the skeletal spicules alternately shine and become extinguished. Despite its complex shape, the whole of each spicule is extinguished at once when its (calcite) crystal axes come to lie parallel to the axes of the crossed polars. Such extinction pattern is characteristic of biocrystals (from Okazaki et al., 1980).

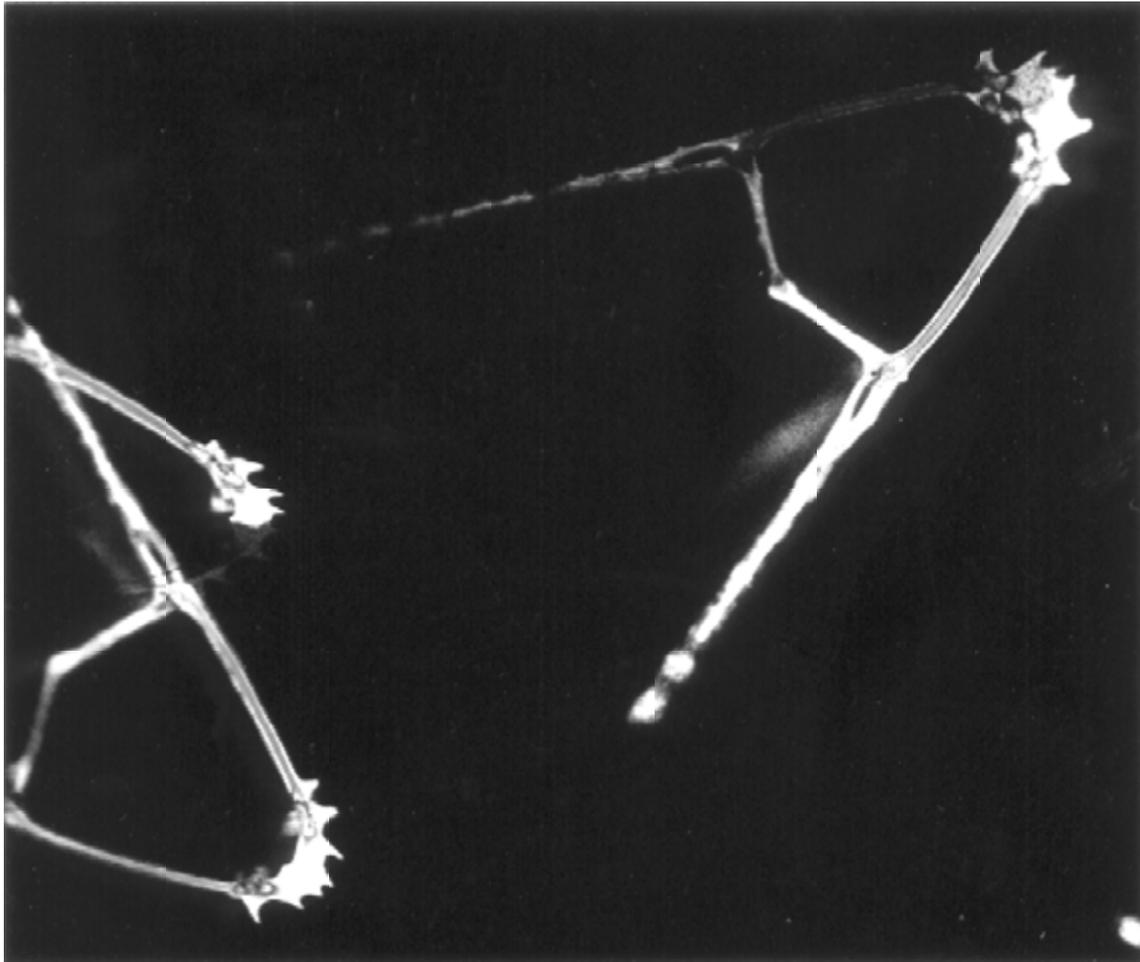


Figure 4.9.2 Skeletal spicules isolated from sea urchin pluteus larvae observed between crossed polars in white light. The calcite spicule is so strongly birefringent (coefficient of birefringence = -0.172) that a region just over $3\text{-}\mu\text{m}$ thick already introduces a 580-nm retardation. In that region, yellow light exiting the spicule is extinguished by the analyzer so the spicule takes on a bluish hue. Figure courtesy of Jan Hinsch, Leica. **See color plate.**

While appearing to be a rudimentary exercise, consideration of the polarization optics and close observations of the image of the specimen can, in fact, reveal the directions and homogeneity of optical axes of the biocrystals, yield their sign and coefficient of birefringence, relate the growth of the biocrystal to the activity of the mesenchyme cells, and produce other useful information (Okazaki and Inoué, 1976).

In addition to the calcareous skeletal spicules that consist of a single biocrystal in echinoderm embryos, most bone and teeth contain an array of biocrystals (Schmidt, 1924).

Example 2: Examining Collagen, Muscle, and Histological Preparations

Many transparent organisms and tissue sections exhibit birefringent regions that can be seen with simple polarizing microscopes. The

wavy collagen fibers and cross-striated muscle in animal tissues (see Fig. 4.9.3), and cellulose fibrils in the walls of plant cells (e.g., see Green, 1963), stand out prominently sometimes amidst bright crystalline inclusions. Likewise, the keratin layers in hair (which can vary with animal species) and synthetic fibers show strong birefringence.

Some of the crystalline inclusions seen in the background were formed in life, while other randomly scattered crystals could have developed in the medium in which a fixed specimen is embedded. Others, which at first glance may appear to be simple crystals (with simple optical axes as in biocrystals), upon closer inspection may turn out to have complex (more or less radially symmetric) extinction patterns. These may be starch grains with concentric lamellar structures that show characteristic extinction

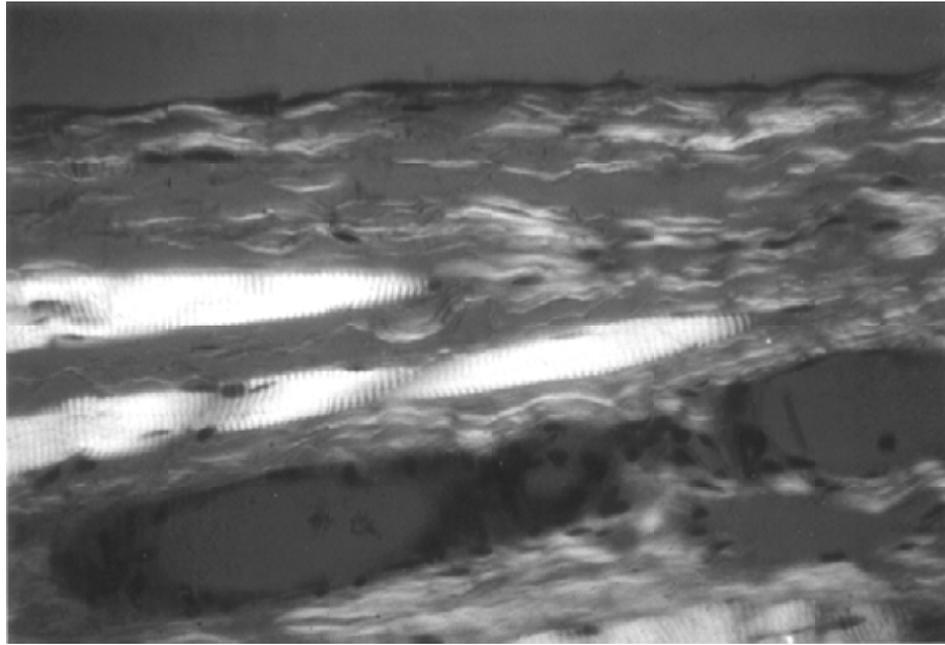


Figure 4.9.3 Tissue section showing birefringent cross-striated muscle and collagen fibers. A typical H & E-stained tissue section observed between crossed polars in the presence of a Brace-Köhler compensator. The birefringent A-bands of the cross-striated muscle fibrils and the wavy axes of the birefringent collagen fibers stand out. **See color plate.**

patterns (see Frey-Wyssling, 1953). On a larger scale, a more or less similar pattern can be seen in the shells of embryonic gastropods (Schmidt, 1924).

Example 3: Preparing to Study Spindles and Asters

In contrast to the structures described above, which may show interference colors between crossed polars and tend to show retardances of the order of many hundreds of nanometers, other structures in living cells display a very weak birefringence retardation that amounts to only a few to a fraction of a nanometer. These include the arrays of microtubules that make up the spindle fibers, astral rays, and flagellar and ciliary axonemes, the arrays of actin and intermediate filaments, various membrane systems, and chromatin in sperm heads.

Unlike the examples mentioned above, whose birefringence is mostly based on the anisotropy of their intramolecular bonds (intrinsic birefringence), many of the intracellular filament and membrane arrays exhibit form birefringence that is based on their ultrastructural geometry.

In order to detect and study the weak birefringence common to intracellular structures, one needs a polarizing microscope equipped with polars yielding a high extinction factor

(see Example 4), strain-free optics, a Brace-Köhler compensator, and a bright light source (Swann and Mitchison, 1950; Inoué and Dan, 1951).

While the test specimen and protocol described below are useful for checking the performance of a polarizing microscope equipped with the highest-NA lenses, in this protocol for observing fertilization and spindle and aster formation in marine eggs, it is recommended that one use a 10× to 20× objective lens with the condenser iris partly closed so that $NA_{\text{cond}} = 0.3 \times NA_{\text{obj}}$.

To see whether a microscope has been adequately equipped and adjusted for studying weak birefringence of intracellular structures, one can try out a convenient test specimen prepared as follows. On a clean microscope slide place a few drops of saliva. Place a clean coverslip on top and observe in Köhler illumination (see *UNIT 4.1*) using a bright light source. Make sure the polars are fully crossed and that the microscope is equipped with a Brace-Köhler compensator.

If the microscope is adequately equipped and adjusted, the buccal epithelial cells (the so-called “spit cells” that are present in the saliva) should appear brighter or darker than the gray background depending on the orientations of the cell and the compensator. The gray back-

ground is introduced by the compensator and varies with the orientation of its axes between the crossed polars, as described below.

If the spit cells show up in this fashion, both brighter and darker than the gray background depending on their orientation, the polarizing microscope should be ready for other applications. However, before removing the test slide, go through the following exercises, which will give further insight into the submicroscopic structure of the cell structure that is being examined.

When a spit cell in focus is brighter than the background, the retardances of the cell and the compensator are added together and introduce a greater phase difference, thus making the specimen appear brighter. Since the slow axis of the birefringent spit cells (i.e., the long axis of their keratin fibrils) is generally oriented along the long axis of the cell, they appear bright when that axis lies in the same quadrant as the slow axis of the compensator.

When the slow axes of the specimen and compensator lie in opposite quadrants, their retardances cancel (subtract from) each other, so that the specimen appears darker than the background field.

Using these criteria, record which quadrant of the compensator contains its slow axis. Some cells appear bright in some regions and dark in others. There the keratin fibrils are not uniformly aligned parallel to the long axis of the cell, but in different directions depending on the part of the cell.

Note also that the cells (or cell regions) in certain orientations appear neither brighter nor darker than the field. With the compensator removed (or brought to its extinction orientation), those cell regions should appear as dark as the extinguished background. Those are cells, or cell regions, in which the keratin fibrils are oriented exactly parallel or perpendicular to the polarizer and analyzer axes. Verify this explanation by revolving the microscope stage or by observing a cell that is flowing and changing its orientation slowly.

Just as the specimen becomes dark when its optical axes, in the absence of a compensator, coincide with those of the polars, the background for the whole field becomes dark when the compensator optical axes are oriented parallel to the axes of the polars. These extinction orientations show the optical axes, respectively of the compensator or specimen, which relate directly to the orientations of their atomic bonds or fine structure.

In testing for the adequacy of the polarizing microscope through these exercises, the apparatus is also set up to establish the optical axes and signs of birefringence of the weekly birefringent specimen. Through such an exercise, we can interpret the nature and alignment of the submicroscopic molecular or fine-structural arrays that are themselves much too small to be resolved with the light microscope.

Example 4: Examining Fertilization, and Spindle and Aster Formation, in Egg Cells

Elevation and hardening of fertilization envelope

Collect eggs and sperm from appropriate species of gravid sea urchins or sand dollars (Costello et al., 1957; Schroeder, 1986; Lutz and Inoué, 1986). Use species with optically clear eggs such as *Lytechinus variegatus*, *Lytechinus pictus*, or *Echinarchnius parma*.

Wash the eggs in filtered seawater three times, each time by allowing the eggs to settle, changing the seawater above the layered eggs, and again letting the eggs settle. Add a dilute suspension of sperm to a portion of the washed, unfertilized eggs (save some unfertilized eggs in a larger dish). Then, quickly make a preparation using a clean slide and coverslip (with a coverslip fragment or small piece of Scotch tape placed under one edge to prevent squashing the egg). Observe the elevation of the fertilization envelope with the polarizing microscope. In the first 3 to 5 min after fertilization, note how the fertilization envelope elevates and becomes increasingly birefringent.

Note also that the parts of the envelope that lie in the same quadrants as the slow axis of the compensator become quite bright while parts of the envelope in the other quadrants become darker than the gray background (Fig. 4.9.4). In other words, the slow axis for the envelope lies parallel to its surface, or the envelope shows a “tangentially positive birefringence.” As the birefringence of the envelope rises, the middle (crescent-shaped) region of the darker part of the envelope may become brighter. For that region, the birefringence retardation of the envelope has become greater than that of the compensator. This effect is more pronounced the smaller the retardance introduced by the compensator. In fact, with the compensator oriented in its extinction orientation, all four quadrants of the envelope will shine equally brightly.

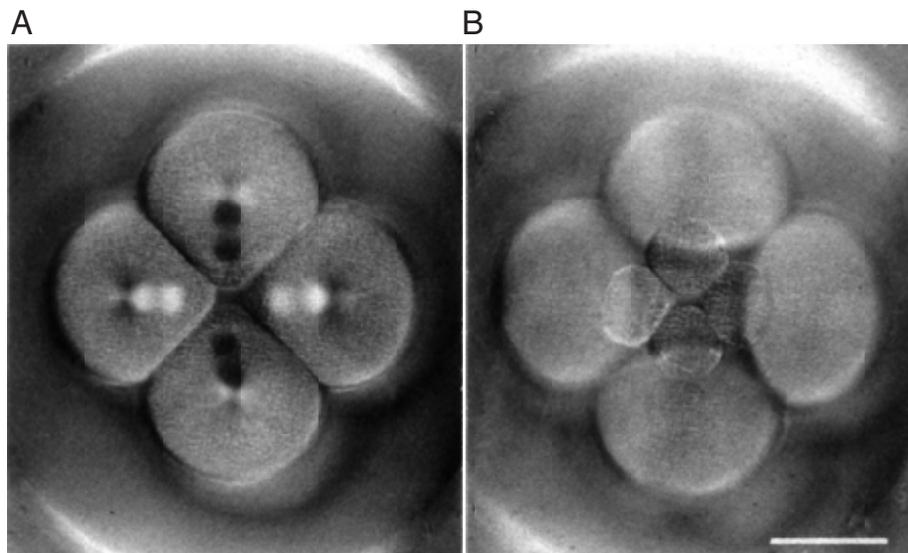


Figure 4.9.4 Birefringence of fertilization envelope and fourth-division mitotic spindles in a sand dollar egg. **(A)** The tangentially positive birefringence of the fertilization envelope and the radially positive birefringence of the mitotic spindle and aster microtubules show clearly in this transparent cleavage stage egg of *Echinarchnius parma*. The egg is viewed near its vegetal pole, to which the vegetal fourth-division spindles have converged. **(B)** The asymmetric location of the vegetal pole spindles has given rise to four small micromeres, whose descendants give rise to the gametes and the mesenchyme cells that pattern the pair of skeletal spicules. Compensator slow axis is oriented horizontally.

This rise in positive birefringence of the fertilization envelope reflects the deposition of a fibrous inner layer that strengthens the envelope. In fact, it is possible to strip off the envelope by passing the fertilized eggs through a fine-meshed bolting cloth, within 3 to 4 min after fertilization, after the fertilization envelope has risen sufficiently, but before it has hardened (as judged by its strong birefringence).

Mitotic spindle and asters: Assembly and disassembly of mitotic microtubules

Make a fresh preparation ~40 min to 2 hr after fertilization (~15 to 20 min before the onset of first cleavage, depending on the species and temperature). In this preparation made at the appropriate time, one should see the birefringent asters and the mitotic spindle gradually appearing.

Note how both the asters and spindle initially appear as small, weakly birefringent structures that may be barely detectable.

Depending on the orientation of their fibrillar components (microtubules) and the compensator setting, portions of the asters and spindle may appear bright or dark. In fact, each aster will generally appear as a radial structure with two dark and two bright quadrants since the microtubules radiate out from the centrosome.

Over the next several minutes, the asters and spindle gradually grow in size and birefringence, until the spindle reaches full metaphase. At anaphase, the clear gap in birefringence that develops between the two half spindles by full metaphase gradually increases in length as the chromosomes are moved polewards.

Starting with the first appearance of the tiny spindle and asters, make time-lapse video records of the events every 5 to 15 sec apart, making sure that a time-of-day signal and audio records of any comments are included. Keep a record of room temperature near the microscope. If possible, record also the orientation of the compensator that just extinguishes the darkest part of the spindle. Choose spindles whose long axes lie in the plane of focus and that are oriented at 45° to the transmission axes of the polars. This exercise may be easier if observations are made on eggs in their second division where two spindles should be oriented parallel to the first cleavage plane.

Depending on the species being used and on the microscope, the chromosomes themselves may or may not be visible. Switching to DIC or phase contrast may help see the chromosomes in favorable cases (see Figure 1 in Salmon and Segal, 1980).

As the spindle approaches telophase and the half-spindle birefringence diminishes, the asters

generally grow to their maximum size and birefringence. Shortly thereafter, the cell cleaves in a plane bisecting the anaphase spindle.

The astral rays and fibers in the spindle display a positive birefringence along the length of their fibrils or of their microtubules. Since virtually all of their birefringence can be accounted for by the form birefringence of their constituent microtubules (Sato et al., 1975), the rise and fall and distribution of their birefringence directly reveals the change in concentration and distribution of oriented microtubules, which themselves are too small to be resolved with the light microscope.

Observation with a polarizing microscope of changes in the spindle and astral birefringence, associated with the natural progress of cell division or in cells exposed to cold or mitosis-inhibiting chemicals such as colchicine, have laid an important foundation for our present understanding of the submicroscopic organization and events in mitosis, as well as the assembly properties of the mitotic, and other labile, microtubules (Inoué, 1952a, 1952b, 1964, 1981a; Inoué and Sato, 1967; Inoué and Salmon, 1995).

Example 5: Using Polarization Microscopy at Maximum Resolution

So far, these examples have dealt with observations and measurements of weak birefringence that could be made with lower-power microscope objective lenses. In order to gain more image resolution, one must not only use higher-power objective lenses, but objective and condenser lenses with higher numerical apertures (NAs). That is because the minimum distance (d) between two small points that can just be resolved is given by the following relationship (see *UNIT 4.1* for further detail):

$$d = 1.22 \times (\text{wavelength of light}) / (\text{NA of objective} + \text{working NA of condenser}).$$

While the exercises described in Examples 1 to 4 can be carried out with a simple polarizing microscope equipped with objective lenses that have relatively low NAs, they become difficult or impossible when one attempts the same procedures with lenses that have NAs of >0.5 . That is because a considerable amount of background light leaks through the crossed polars at high NAs, no matter how carefully the system is adjusted. The extinction factor $\{EF = (\text{Intensity with parallel polars}) / (\text{Intensity with crossed polars})\}$ that may be as high as 10^4 for low-power objectives which are clean and free

of strain birefringence, may drop to as low as 10^2 for some highest-NA objectives. It takes place even with carefully selected strain-free lenses and is generally caused by two factors: the differential transmission of the polarized components of light in and perpendicular to the plane of incidence and their phase differences, both introduced at oblique optical interfaces that lie between the polarizer and analyzer.

In simple terms, light must be refracted at high angles of incidence by the objective and condenser lenses, and the slide and coverslip surfaces, in order to gain the high NA needed for obtaining the high image resolution. In polarization microscopy, that very fact makes the EF drop precipitously at higher NAs. In other words, without some special remedies as described below, the detection of very weak birefringence and generation of very high image resolution are mutually incompatible.

Fortunately, there are a few remedies, although these are somewhat difficult to implement, that can help alleviate these problems. To understand these remedies, first observe the appearance of the back aperture of the objective lens. This can be done by replacing the ocular of the microscope with a telescope used for adjusting a phase-contrast system. Or, if your microscope is equipped with a Bertrand lens, insert and focus the Bertrand lens so that it, together with the ocular, makes up a telescope that images the back aperture of the objective lens. Alternatively, remove the ocular and peek down directly at the back aperture of the objective lens.

Confirm that you have indeed focused on the back aperture plane. There you should see the image of the condenser iris, whose size changes as the condenser iris is opened and closed. With proper Köhler illumination, if you open and close the field diaphragm rather than the condenser iris, only the brightness of the back aperture and not the size of the iris image should change.

In the absence of a compensator, observe the objective lens back aperture as the polarizer and analyzer are crossed and slightly off-crossed. If the lenses are free of strain birefringence and birefringent dirt or dust particles, the objective back aperture should show a dark cross flanked by brighter quadrants between the arms of the cross when the polars are exactly crossed. The arms of the dark cross lie along the transmission axes of the polars (Fig. 4.9.5A).

When the polarizer or analyzer is slightly off-crossed, the dark arms of the cross turn into two dark V shapes located in opposing quad-

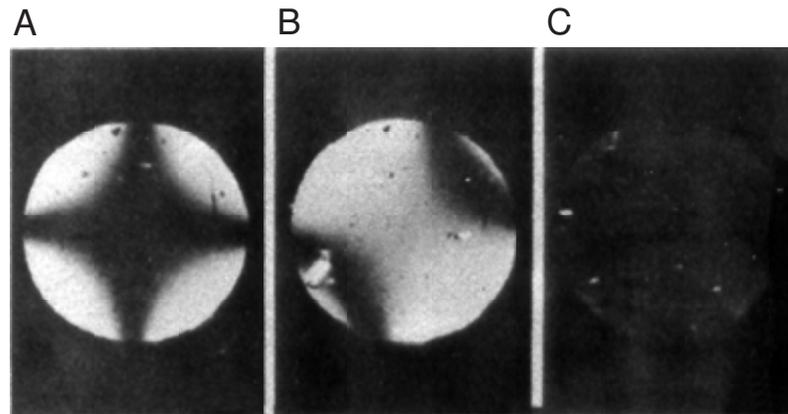


Figure 4.9.5 Patterns observed at the back aperture of polarizing microscope equipped with high-NA ($\times 97/1.25$) strain-free, matched condenser and objective lenses. **(A)** The back aperture shows a dark cross between crossed polars in the absence of a birefringent specimen or a compensator, if the objective and condenser lenses are free from *lateral* strain birefringence. The lenses may, however, still be introducing *radial* birefringence! **(B)** As the analyzer (or polarizer) is turned, the cross opens up into two dark Vs that should remain dark up to the edge of the aperture if there is no contributing birefringence. Turning the polar in the opposite direction reverses the quadrant in which the Vs appear. (If the Vs do not move out to the periphery of the aperture but fade rapidly as a polar is turned, the contrast of the Vs can generally be restored by adjusting a compensator. That region of the aperture is then introducing elliptically polarized light, either because a lens suffers radial strain birefringence or because of the nature of the anti-reflection coating used on the lens.) **(C)** Extinction pattern of the same lens pair shown in A and B, but equipped with a polarization rectifier. The whole back aperture (up to the maximum NA), as well as the microscope field, is now well extinguished. (From Inoué and Hyde, 1957.)

rants of the back aperture (see Fig. 4.9.5B). Those V-shaped regions are where the plane of the incident polarized light has been rotated (by lower transmission of the polarized light vector vibrating normal to the plane of incidence compared to that vibrating parallel to the plane of incidence).

As the polars are further off-crossed, the V-shaped regions move further out, but may become faint and no longer be very dark. At this point a Brace-Köhler compensator inserted at the appropriate orientation can restore the darkness of the Vs. In other words, light rays traversing the objective and condenser lenses (and slide and coverslip), at angles and directions corresponding to those regions that have now become darkened at the back aperture of the objective lens, act as though those particular rays experienced birefringence or had suffered a phase difference and become elliptically polarized. This elliptical polarization is commonly due to the anti-reflection coating applied to the lens surfaces. As with the rotation of polarized light, it also increases with the angle of incidence and the departure of the plane of incidence from the vibration plane of the polarized light incident at each air-glass interface.

The net result of all of these polarization-altering effects at the lens (and slide and

coverslip) surfaces is the dark cross at extinction and the two sets of Vs that move further out into opposing quadrants.

The polarized light modified in this fashion (in the four quadrants between the arms of the dark cross) is transmitted by the analyzer crossed with the polarizer and, hence, gives rise to the large amount of background “stray light” found with high-NA lenses.

To minimize this stray light that drowns the low-contrast image of weakly birefringent specimens, a simple, quick fix is to close down the condenser iris and use an objective lens with a lower NA. However, one loses image resolution as well as the amount of image brightness needed to see or record the images. Also, the depth of field can become unacceptably deep and make for a confusing image.

Oil-immersion objective and condenser lenses tend to depolarize the light less and hence, introduce less stray light compared to “high dry” objective lenses that have the same NA. That is because up to four air-glass interfaces, where the rays suffer the steepest angle of incidence, are essentially eliminated by oil immersion between the objective lens and coverslip, and condenser lens and slide.

Video enhancement can also help to a certain degree. By analog or digital processing, the

unwanted background stray light can be offset (suppressed) and the image contrast can be raised so that the optical problems can be overcome to some degree (Allen et al., 1981; Inoué, 1981b; Inoué and Spring, 1997).

A proper remedy for the depolarization of light is accomplished by using objective and condenser lenses equipped with polarization rectifiers. By using “rectified” lenses, one can, in fact, restore much of the polarization states of the light passing each region of the objective lens back aperture (Fig. 4.9.5C) and thus achieve high extinction for the full aperture even when using high-NA lenses (Inoué and Hyde, 1957).

In addition to allowing the detection and measurement of very weakly birefringent minute objects, rectification gets rid of the diffraction anomaly that is introduced in images of weakly birefringent objects. The diffraction anomaly, which arises from the different states of polarization in different quadrants of the aperture plane, can even reverse the contrast of weakly birefringent minute objects and prevent proper interpretation of the specimen’s fine structure (Inoué and Kubota, 1958; Inoué and Spring, 1997).

Unfortunately, rectified lenses, which used to be available from American Optical, and from Nikon, are currently not commercially available. However, the following two alternative approaches can provide some relief of the problems discussed.

The first uses two opposite-handed circular polarizers instead of crossed linear polarizers to extinguish the field (Huxley, 1960). An advanced version of this type of polarizing microscope is Oldenbourg’s “LC PolScope.”

As a final alternative, one can gain some improvement of image resolution combined with elevated extinction by using an appropriate mask at the aperture plane. The mask, placed at the back aperture of the objective lens and/or at the plane of the condenser iris diaphragm, would be shaped so as to transmit light along the dark arms of the cross, while cutting out the modified polarized light that would have passed through the rest of the aperture.

Such masks would definitely raise the EF, making the background much darker and allowing detection of very low birefringence retardances. Such masks would also effectively provide high NAs in two orthogonal directions, thus yielding high resolution for objects that diffract light in those directions. The accompanying modifications of the point-spread function may be a slight penalty to pay in order to

gain high extinction and freedom from anomalous diffraction at very high NAs.

Example 6: High-Resolution Polarization Microscopy of Meiotic Mitosis and Spermiogenesis

Using a polarizing microscope as described in Example 5, it is possible to use high-NA objective lenses (combined with high effective NA of the condenser) to examine the detailed distribution of birefringence as in the mitotic spindle of a dividing cell or the coil-of-a-coil packing arrangement of DNA in certain chromosomes.

Some cell types are more suited for such observations: such as single-layered tissue cells that remain flat instead of rounding up during mitosis (McIntosh et al., 1975), cells without excessive light scattering or birefringent inclusions, cells with large and clearly visible chromosomes, and cells in which different mitotic and developmental stages are well synchronized. Some examples include: newt lung epithelial cells (Rieder and Hard, 1990; Oldenbourg, 1999), endosperm cells of the African blood lily (Inoué and Bajer, 1961), some symbiotic protozoa in the wood-eating cockroach (Inoué and Ritter, 1978), spermatocytes of crane flies (Forer, 1965), grasshopper spermatocytes (Fig. 4.9.6 and Zhang and Nicklas, 1996), and maturing spermatids (Fig. 4.9.7 and Inoué and Sato, 1966). The extensive article (in German) by Belar (1928) is a treasure trove of cell sources available for observing various features of cells in the living state as well as after fixation and staining.

The following describes a method for preparing grasshopper spermatocytes and spermatids for high-resolution polarization microscopy. The following materials are required:

- Healthy male grasshoppers, preferably of *Dissosteira*, *Chortophaga*, or related species that fly well for several meters at a time, rather than those that only hop or fly for short distances (for some reason, the spermatocyte mitochondria, which are birefringent, are better aligned in the former group of grasshoppers and interfere less with detailed observation of spindle birefringence);
- Grasshopper Ringer’s solution (see Table 4.9.2 and preparation instructions below);
- Clean Syracuse watch glasses;
- Bio-cleaned slide and coverslips (see Table 4.9.1 and instructions for bio-cleaning below);
- A pair of sharp iridectomy scissors;
- A sharp pair of stainless-steel watchmaker’s forceps;

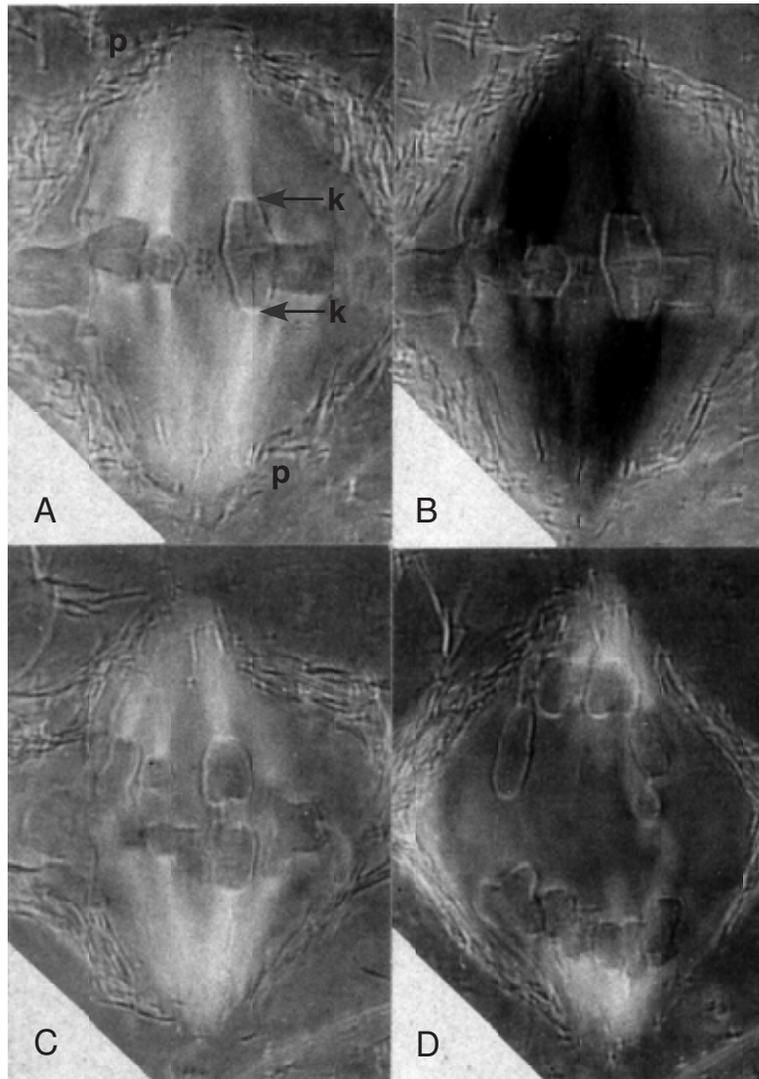


Figure 4.9.6 Meiosis-1 spindle of grasshopper spermatocyte observed with rectified optics. The positive birefringence of the bundle of microtubules that make up the chromosomal spindle fibers shows prominently. The kinetochores (k) also appear to show the same sign of birefringence as the microtubules, but it has not yet been established whether this reflects fine-structural anisotropy of the kinetochore, or primarily the edge birefringence of the chromosome at the kinetochore. The mitochondria also show a longitudinally positive birefringence with overlapping edge birefringence. The compensator slow axis lies parallel to the spindle fibers in panels (A), (C), and (D), so that the positively birefringent microtubule bundles appear brighter than the background. (C) The compensator orientation is reversed so that the microtubule bundles appear in reverse contrast. (From Nicklas, 1971.) For online version of time-lapse series of this and other cells in mitosis, see <http://www.molbiolcell.org> and Inoué and Oldenbourg (1998).

Sharp, stainless-steel dissecting needles;
 A sharp hypodermic needle mounted on a wooden handle;
 Pasteur pipets with rubber bulbs;
 Small balls of absorbent cotton;
 6- to 8-mm-wide strips of filter paper;
 Mineral oil or other innocuous oils such as Halocarbon oil 27;
 Valap (see *UNIT 13.1*);
 70% ethanol;

Dissecting microscope with trans-illumination.

Preparation instructions for several of these items, along with some parameters critical to performing the experiments described here, are included in the discussion of Ensuring that Cells are “Happy” While They Are Being Observed Under the Microscope, below.

Let the male grasshopper hold onto a small wad of absorbent cotton or a small piece of tissue, so as to absorb the brown fluid expelled

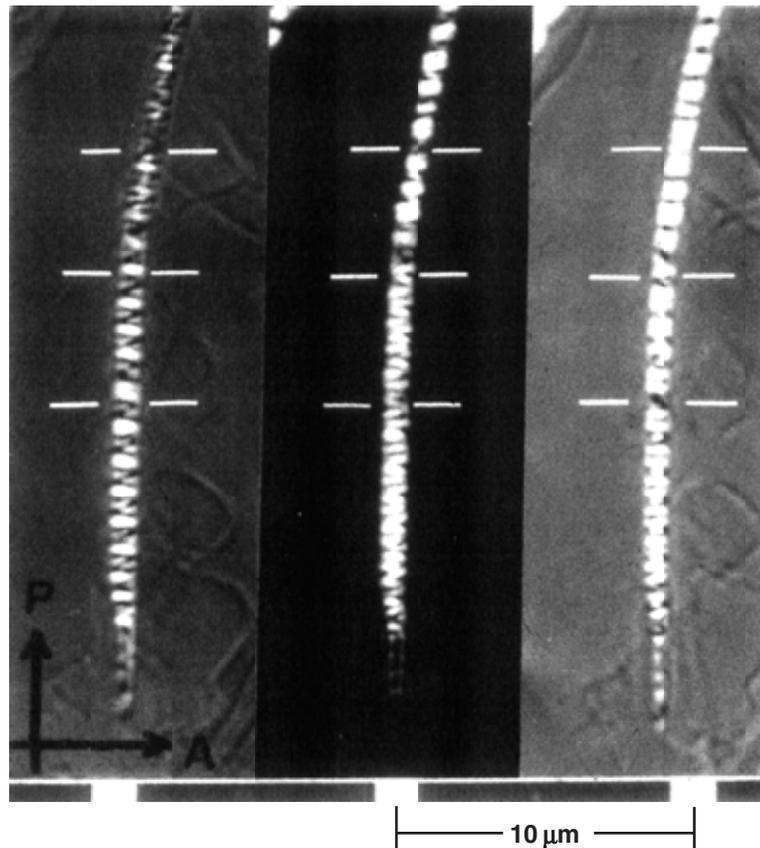


Figure 4.9.7 Live sperm head of cave cricket viewed with a high-resolution polarizing microscope. The sperm head immersed in DMSO (dimethyl sulfoxide) is viewed at three compensator settings using rectified optics ($\times 97/1.25$ NA). P, A: transmission axes of the polarizer and analyzer. The compensator slow axis is oriented perpendicular to the sperm axis in the middle panel, turned $\sim 5^\circ$ clockwise in the left panel and $\sim 4^\circ$ counterclockwise in the right panel. The helical regions of the DNA, wound in a coil of coil within the chromosomes, appear bright or dark depending on their slow axis orientations relative to that of the compensator. Bars indicate junctions of chromosomes that are packed in tandem in the needle-shaped sperm head. This is the first (and virtually only) mode of microscopy by which the packing arrangement of DNA and the chromosomes have been clearly imaged in live sperm of any species. From Inoué and Sato (1966).

from its mouth. Lift and clip off the wings at their bases. Then, holding onto the grasshopper with his hind legs folded under the abdomen, insert one blade of the iridectomy scissors pointing up and forward at around the third or fourth from the last abdominal segment as shown in Figure 4.9.8. Make an incision that covers four or five segments along the back of the abdomen. Then gently squeeze the lower part of the abdomen until the pair of testes (Fig. 4.9.9; usually colored bright yellow, orange, or red) pop out from the incision. Cut the ducts and place the testes in the saline (grasshopper Ringer's) solution in a watch glass.

Under the dissecting microscope, use a watchmaker's forceps and sharp needle to peel off the colored layer of fat and the Malpighian tubules that surround the testes. They can be

toxic to the isolated spermatocytes if not removed from the final preparation. Freed from the covering material, the whitish lobes of the testes should now look like a bundle of bananas. With a piece of absorbent cotton moistened with 70% ethanol, wipe clean the iridectomy scissors, forceps, and needle used, and then transfer the lobe bundles of the testes to a clean Syracuse watch glass containing fresh saline solution.

Again, under the dissecting microscope, use sharp dissecting needles to separate a testis into small bundles containing three to four intact lobes. Cut apart the bundles where they bunch together, i.e., at the proximal end of the lobes where they share a common sperm duct.

Using the Pasteur pipet, transfer one small bundle of lobes together with a small drop of

saline onto a clean slide. Place a clean coverslip on top and remove excess solution with a small piece of filter paper or tissue paper until the lobes are just starting to be flattened.

Observe the preparation under the polarizing microscope using a low-power (10 \times) objective lens. In each intact lobe, there should be located, from the duct end towards the distal end, more or less in the following order, bundles of mature sperm heads, immature sperm heads,

and sperm tails, then follicles containing spermatids, secondary spermatocytes, primary spermatocytes, and, finally, spermatogonial cells (Fig. 4.9.10). The birefringence of the heads of the mature and immature sperm, as well as of some of the spermatid, and perhaps of the bundle of sperm tail, should be quite prominent.

By using a Brace-Köhler compensator, establish the sign of birefringence (direction of

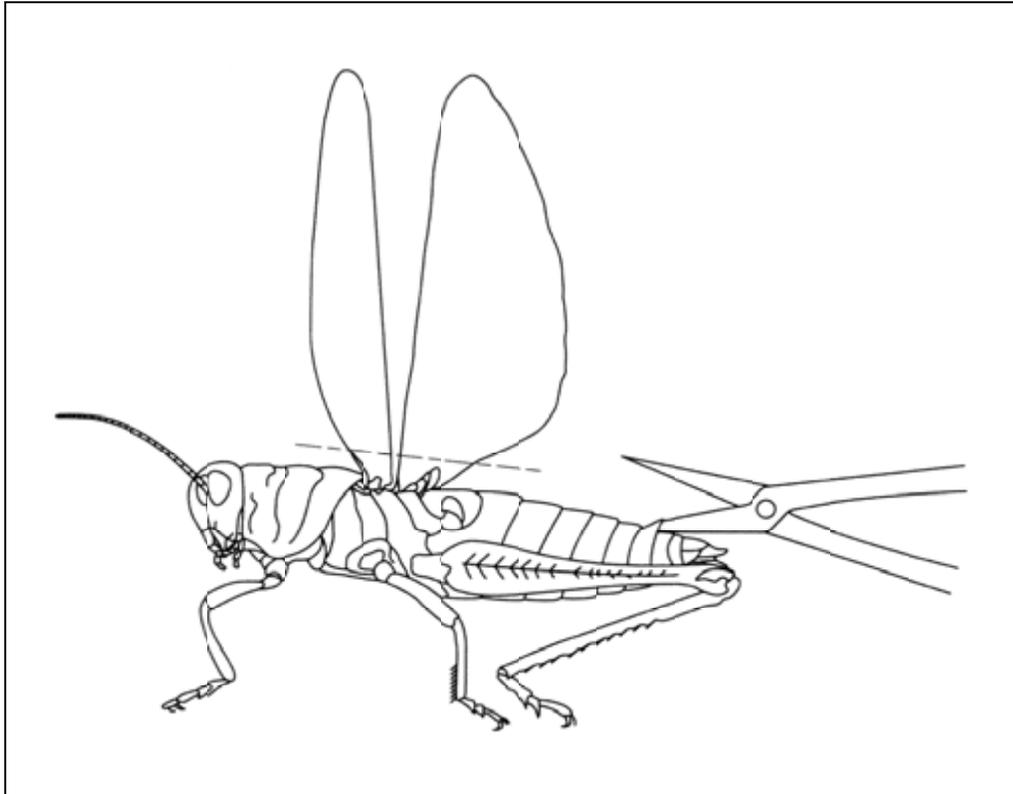


Figure 4.9.8 Dissecting a male grasshopper to expose testes (also see Fig. 4.9.9 and instructions in text).

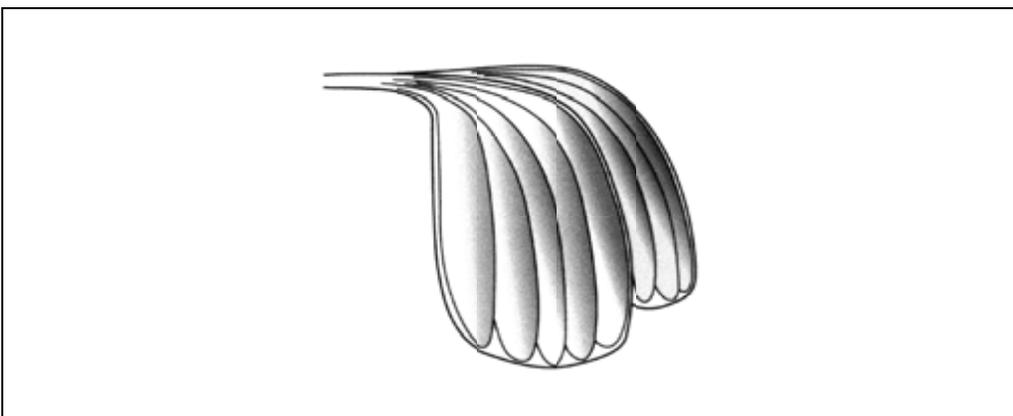


Figure 4.9.9 The pair of testes extracted from a grasshopper. The bundle of lobes in each testis, made like a bunch of bananas and held together where they share a common sperm duct, are covered by a brightly colored sheath. The distal ends of the lobes splay apart when the testicular sheath is peeled off.

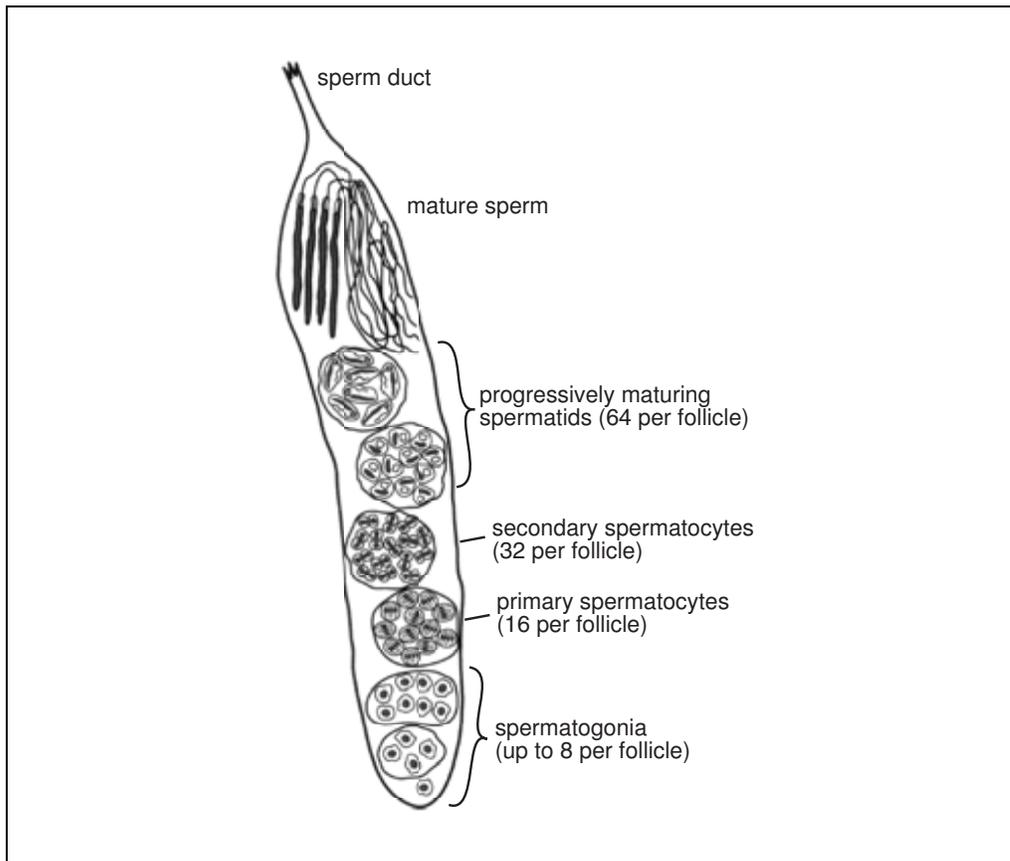


Figure 4.9.10 Arrangement of follicles and cells (not to scale) in the lobe of a grasshopper testis. This highly schematic diagram illustrates one of the many lobes, which are arranged like a bunch of bananas, in a grasshopper testis. The cells, developing synchronously within each follicle in the lobe, are progressively more mature from the distal end of the lobe to the proximal end where the lobes are joined by the sperm ducts. For a photograph of section of fixed lobes, see Figure 1 in Belar (1929).

the slow axes of these structures relative to their long axes). Note that the contrast due to sperm tail birefringence, which is mostly due to the form birefringence of their axonemal microtubules, is reversed from that of the sperm head, which is almost exclusively due to the intrinsic birefringence of the DNA molecules aligned parallel to the length of the sperm head.

Next, make a fresh preparation for observing the spindle fibers and chromosomes and individual sperm with greater resolution. This exercise may require some practice, since a combination of care and speed of preparation is important. Also, do not be frustrated if, at first try, primary spermatocytes at just the right stage of meiotic mitosis are not found. There are a number of follicles at different stages of spermiogenesis in each testicular lobe, and the divisions of the 16 primary spermatocytes in each follicle are synchronized, but you may not come across a lobe that contains a follicle at the right stage. Usually, it is not necessary to try more than two or three lobes.

Using a pair of watchmaker's forceps, transfer a bundle of three to four testicular lobes onto a bio-cleaned microscope coverslip. The coverslip should be resting on a clean microscope slide or some other appropriate holder that lifts it off from the lab bench or other surfaces, which may be a source of lint or other birefringent dust. In making the transfer, be sure to hold onto the duct ends of the lobes and not the other, free end. During the transfer, touch the lobes gently to the clean, dry rim of the watch glass to remove much of the saline solution.

Using a hypodermic needle with a very sharp tip as a knife, cut the lobes (now resting on the coverslip) near their free ends. Then, holding onto the duct end of the lobes with a watchmaker's forceps, draw the lobes in a spiral a few millimeters in radius, making sure that their free, cut ends trail on the surface of the coverslip. As you draw the spiral, cells should flow out together with the testicular fluid, from the cut ends of the lobes onto the surface of the

coverslip. Those near the cut end flow out first, then those closer to the ducts that you are holding onto. Pick up and discard the residue of the lobes. The result is a spiral-shaped thin film of cells, more or less arranged in order from least differentiated cells to mature sperm (see Fig. 4.9.10).

Quickly place a drop of oil (Halocarbon 27 or light mineral oil) on the spiral, invert the coverslip, and cover the preparation by contacting onto a clean slide. Do not use any spacers. Remove the excess oil by contacting a strip of filter paper to the rim of the coverslip, then seal the preparation with Valap.

The slide is now ready for observation of the spindle and chromosomes in meiotic divisions and different stages of sperm maturation. The observations can be carried out on the same live cells for many hours. Not only are the cells arranged and clustered, more or less in the order of stages of spermiogenesis, but they should also lie in reasonably flat monolayers making them suitable for observations with high-NA lenses using polarization microscopy, phase contrast, or DIC.

Note, however, that the testicular lobes, from the time they are removed from the saline medium, and especially the very thin spread of cells in the spiral, could almost instantly lose water by evaporation before being covered by the Halocarbon oil. Thus, some practice and speed of operation becomes important for the last phases of the preparation described above.

In successful preparations observed in high-extinction polarization microscopy, one finds meiosis-I spindles (which are considerably larger than those in meiosis-II) with a moderate degree of positive birefringence, somewhat rounded spindle poles (in contrast to being very pointed), and chromosomal spindle fibers that appear somewhat fluffy (rather than too sharp as though drawn as a line). Such cells can be expected to complete meiosis-I as well as meiosis-II in several hours (see Inoué, 1964).

The process of sperm maturation, on the other hand, is very slow, so that one is not likely to see the actual transition of single spermatids from one stage to the next. The successive stages of sperm maturation, including the rise of negative birefringence in the spermatid nucleus associated with their dehydration and shape changes, should, however, be neatly displayed in chronological order on the slide, so that it should not be difficult to visualize the sequence of events.

Example 7: Examining Meiotic Spindles and Chromosomes

Note that in grasshopper spermatocytes, it can take several hours between the time when an apparently mature spindle is formed and the beginning of anaphase. During that interval, known as prometaphase, chromosomes oscillate back and forth to both sides of the metaphase plate (Deitz, 1969). Concurrently, the associated spindle fibers change the strength of their birefringence. With time-lapse cinematography or video, one can also observe considerable fluctuation of the spindle birefringence (the so-called “Northern Lights Phenomenon”; Inoué, 1964), reflecting the dynamic growth and shortening of their constituent microtubules (Mitchison and Kirschner, 1984; Walker et al., 1988).

The sex chromosomes, in particular, may be found to travel all the way from one spindle pole to another. The oscillatory behavior of these prometaphase chromosomes, and the associated stretching and contraction of their kinetochores to which the chromosomal spindle fibers are attached, may be observed more clearly by switching from polarization to DIC or phase-contrast microscopy (Salmon and Segal, 1980; Nicklas et al., 1995).

Once full metaphase is established, and all of the chromosomes have lined up on the metaphase plate, a signal is released and the chromosomes synchronously enter anaphase (Nicklas et al., 1995). Note the change in distribution and strength of birefringence of the spindle fibers and astral rays as anaphase progresses. What can one infer about the behavior of the microtubules from these observations?

Note also, as anaphase progresses, that another bundle of thread-shaped, birefringent elements lines up along the long axis and surrounding the spindle, to be pinched apart into two as the cell cleaves. Note the sign of birefringence of these mitochondrial threads.

In each daughter cell that results from the division of the spermatocytes, the mitochondria come together to form a dense, round body, the Nebenkern, which has a diameter similar to the reconstituted daughter nucleus and is often mistaken for the latter. The Nebenkern shows a higher refractive index than their surrounding cytoplasm, whereas the daughter nuclei themselves tend to have a lower refractive index than the surrounding cytoplasm. Using DIC optics, verify these refractive index differences by observing the shadow-cast appearances at the edges of these structures.

Example 8: Examining The Sperm and Maturing Spermatids

The needle-shaped, mature sperm heads in grasshoppers show a very strong birefringence whose character is reversed from that of the sperm tail and the acrosome at the tip of the head. Establish the sign of birefringence of these three regions.

Also note that, unlike the nuclei in spermatocytes (and many other cells), the refractive index of the sperm head is very high, reflecting the loss of water and condensation of its chromatin during spermatid maturation.

Choose a series of spermatids starting with those that are nearly mature to very young ones. As maturation progresses, the sperm nucleus becomes more and more elongated, while its birefringence increases. The coefficient of birefringence of the mature sperm is, in fact, extremely high, amounting to -2×10^{-2} , approximately that of a thread of pure DNA (Schmidt, 1941; Wilkins, 1951; Inoué and Sato, 1966).

Note that before the spermatid starts to lose water and become elongated, the nucleus is almost nonbirefringent. Most cell nuclei show very little birefringence (with the exception of those in sperm and in certain Dinoflagellates; Schmidt, 1932, 1937; Cachon et al., 1989), despite the fact that they contain a large quantity of DNA.

In maturing spermatids, note also that the axis of birefringence of the sperm head is tipped this way and that and is not totally parallel to the elongated axis of the head, as in the fully mature sperm. In some insects, such as the cave cricket, the axes of negative birefringence, even of the mature sperm head, can still be resolved and seen not to be uniform. Instead, they are disposed more or less in a zigzag fashion (Fig. 4.9.7), reflecting the packing arrangement of their DNA backbone and chromosomes. The detailed packing arrangement of the DNA bases, including at levels far smaller than the resolution limit of the polarizing microscope, was determined by irradiation of the live sperm head with a microbeam of polarized UV and by quantitatively measuring changes in the birefringence and their axes for each resolvable area (Inoué and Sato, 1966).

Ensuring that Cells Are “Happy” While They Are Being Observed Under the Microscope

To observe the behavior or fine structure in living cells under a microscope, there are several factors that demand special attention. This is especially true when observations are to be made for extended periods of time and under

conditions that require brilliant illumination, as in polarization microscopy, to visualize weakly birefringent objects. On the other hand, birefringence can be an especially effective monitor for the physiological state, and “happiness,” of the cell.

Biological material

Start with healthy organisms and cells. Clearly, one cannot expect to gain healthy cells from diseased or necrotic plants or animals or gain normal fertilization and development when the gametes are not fully ripe.

Culture medium

Be alert to toxic contaminants, especially those that can affect the cells at low concentrations. Heavy metals such as copper, zinc, and silver, which can be present in trace amounts even in conventional reagent-grade sodium chloride, can be sources of trouble since NaCl is used in high concentrations in artificial seawater and physiological saline. Likewise, for making culture media, distilled water made in glass or quartz stills (with adequate vapor, i.e., water droplet, suppression) is preferred over that from metal stills. Deionizers remove trace metals, but one should be careful that the water is not contaminated by mold or bacteria (which can grow in the columns or tubing) and their products including surfactants (which show up as long-lasting foam after the water is shaken or bubbled).

Slide and coverslips

Cells prepared between a slide and coverslip are exposed to a large area of the glass surface relative to the volume of the medium bathing the cell. In addition, the cells are likely to be contacting the glass surface directly. Thus, slight contaminants on the glass surface can disproportionately affect the cells being observed. Eliminate surfactants and heavy metals that may affect the well-being of the cells as well as birefringent contaminants that may interfere with observations by polarization microscopy (see below).

Oxygen supply

In addition to changes in pH of the medium, anoxia (and hyperoxia in the case of some obligatory anaerobes; Ritter et al., 1978) can interfere with proper health and division of cells grown in the confined space between the slide and coverslip. Tissue culture cells are commonly observed through special flow-through microscope chambers where the culture me-

dium is appropriately oxygenated and its pH and temperature are monitored or controlled. Alternatively, developing marine embryos and dividing protists can be observed continuously for many hours using a micro-chamber containing an equilibrated gas phase formed between a slide and coverslip (see Inoué and Spring, 1997). Microscope preparation of cells covered with a layer of nontoxic oil (even under the coverslip) can survive for many hours since many oils, especially silicone and fluorocarbon oils, dissolve gases, including oxygen, very effectively. Halocarbon oils, such as Kel F-10, FC-47, and Voltalef that previously were used to cover cultured cell smears, e.g., of insect spermatocytes, are no longer available. Instead, the Halocarbon oils 27 or 200 (Halocarbon Products; see *SUPPLIERS APPENDIX*), used for immersing developing *Drosophila* embryos, would appear to be reasonable, currently available alternatives. The manufacturer recommends that fresh oils be bubbled with air before use for a day or two to replenish the oxygen that was evacuated during manufacturing.

Temperature

While slides containing cultured cells of mammalian origin generally require heating, most invertebrate cells may need to be kept cool, i.e., no warmer than moderate room temperature. For observing such cells, be especially careful with the illumination used for polarization microscopy. A source with high brightness is needed in order to see or record the weak birefringence in the cell. But most high-brightness sources (not just quartz halogen or other tungsten filament lamps, but also mercury and xenon arc lamps) produce a huge quantity of infrared. Be sure to use a good quality heat-cut filter, and, if practical, a high-transmission (>65%) narrow-band-pass (± 15 nm) green interference filter. That is because the preparation can be heated up with the infrared and far red portions of the spectrum, and cells can react adversely to the blue end of the spectrum (Langford and Inoué, 1979).

In addition, use Köhler illumination and keep the illuminated area to the smallest practical size by adjusting the opening of the field diaphragm. Even using a laser beam, it is not easy to heat up a portion of the specimen with a diffraction-limited small spot of light because water carries away the heat very effectively. On the other hand, the light from an unfiltered incandescent or arc source can rapidly heat up the specimen under the microscope if the illumination covers a large area of the specimen.

The birefringence of the mitotic spindle (as well as its shape) can be a sensitive thermometer that reflects the temperature of the specimen, which is otherwise quite difficult to monitor closely. At lower temperatures, the spindle birefringence drops, while at higher (physiologically compatible) temperatures the birefringence rises (showing enhanced polymerization of tubulin) following a remarkably reproducible curve (Inoué et al., 1975; Nicklas, 1979).

Drying

With cells mounted between slide and coverslip in an aqueous medium be especially alert to drying and increased tonicity of the medium. Valap (see *UNIT 13.1*) is an effective material that can be applied to seal cells mounted in aqueous solutions between the slide and coverslip. The distribution of birefringence in the spindle can indicate the tonicity of the medium. In hypertonic media the chromosomal spindle fibers tend to appear as sharp pencil-drawn lines that converge sharply to the spindle poles (a sign of poor health, although partially reversible), while in less concentrated media, they appear fluffier.

Bio-clean slides and coverslips

In preparing cells for long-term observations under a microscope, one needs to be especially careful to prepare media with the appropriate pH and degree of oxygenation, free of heavy metals. It is especially important to use glassware that is "biologically clean." Since the cells being observed under a microscope are bathed in a very small volume of medium and their surfaces contact a disproportionately large area of the glass surface (and the concentration of any contaminant can become exceptionally high), the slide and coverslips in particular must be bio-clean.

Bio-clean glassware should be free from soap and detergent in addition to even minute amounts of heavy metal, fixatives, and other toxic contaminants or residues that may have been left or adsorbed onto the glass surface. Glassware (including slides and coverslips) and plasticware that have been acquired new from suppliers (except specially packaged pipet tips and capped centrifuge tubes, etc.) should not be assumed to be bio-clean. Table 4.9.1 shows the sequence of procedures used in the author's laboratory for preparing bio-clean glassware and slides and coverslips (Lutz and Inoué, 1986).

Glassware once used with fixatives should be separated by marking with clear, indelible F

Table 4.9.1 Procedures for Bio-Cleaning Glassware (after Lutz and Inoué, 1986)

Step	Procedure
1	Fill 500-ml beaker with hot tap water and enough detergent to make the solution sudsy.
2	Drop 30 to 50 slides or coverslips <i>individually</i> into the beaker. Try to position the slides in a criss-cross arrangement in the beaker.
3	Soak for 15 to 30 min.
4	Place beaker in ultrasonic washer for 3 to 5 min.
5	Rinse many times in tap water to remove the majority of the detergent.
6	Using stainless-steel forceps, transfer slides or coverslips individually into another beaker containing tap-distilled water. Repeat this individual transfer/rinse ten times. Sonicate beaker 3 to 5 min before the last transfer.
7	Individually transfer slides or coverslips into a beaker containing glass-distilled water. Repeat this ten times. Sonicate beaker for 3 to 5 min before the last transfer.
8	Individually transfer slides or coverslips into bioclean storage jars filled with 80% ethanol for storage.
9 ^a	Before use, remove slide or coverslip from alcohol using clean forceps and wipe dry with a Kimwipe folded twice (four thicknesses). Do not rub.

^aIf possible, use a centrifuge (“Spin Dry” available from Technical Video) to avoid the possibility of lint associated with Step 9.

marks, and not mixed with bio-clean glassware, since it may be very difficult to get rid of some ingredients of fixatives.

In addition to preparing bio-clean slides and coverslips, keep their surfaces, as well as optical surfaces of the microscope that lie between the polarizer and analyzer, free from birefringent dirt and dust particles. In particular, be careful to prevent pieces of lint (including cellulose fibers, which often have birefringence retardations close to a wavelength or more) from falling on to, or becoming attached to, the surfaces of slides and coverslips. Such fibers can drastically lower the EF of the whole polarization optics and completely interfere with observation of weak birefringence of intracellular structures. Since clean glass surfaces tend to attract lint particles electrostatically, avoid laying your slide or coverslip directly on a bench top, or on the surface of the glass plate that supports the specimen on a dissecting microscope. Instead, place the slide and coverslip on a spacer, such as a fresh applicator stick bent into a V shape, or prop it up by supporting one end by a couple of millimeters off the surface. Note that Kleenex and lens tissue used to wipe the slide or coverslip can also be a source of cellulose fibers. To avoid wiping, use a centrifuge to spin off the alcohol used for the final rinse of the slide and coverslip (see Figures 3 to 11 in Inoué and Spring, 1997).

Grasshopper Ringer’s solution

Table 4.9.2 provides the composition of saline solutions that can be used for preparing the

grasshopper testes and for observing meiosis in their spermatocytes for many hours (Nicklas et al., 1979). Note that the final tonicity of the medium (plus testicular fluid) may vary somewhat, depending in part on the atmospheric humidity at the time the contents of the testicular lobe are streaked onto the coverslip. Thus, Belar advises either breathing onto the preparation with one’s mouth wide open or blowing with lips nearly closed, depending on whether the preparation should be made more or less dilute before sealing the preparation. As noted before, the morphology and birefringence distribution in the spindles are, in fact, sensitive indicators of the cell’s health and whether the preparation is or is not appropriate for long-term observation of the division events.

Valap

Valap is a low-melting-point, nontoxic, waxy material used for sealing coverslips. Valap is made by simmering a 1:1:1 mixture of vaseline, lanolin, and paraffin (using indirect heat to prevent overheating or catching on fire) for several hours (also see *UNIT 13.1*). Use of beeswax instead of paraffin, or Valab, yields a somewhat more adhesive sealant. A major advantage of these sealants is that they can be applied directly to seal the edges of coverslips covering wet specimens.

Valap and Valab can be applied either from a melt using a small brush or with a warm spatula or heating wire made by passing current (e.g., from a low-voltage-microscope lamp transformer) through an ~5-inch length of U-

shaped nichrome wire fastened to an insulated holder such as an electric plug fixture (Fig. 4.9.11). Be careful not to pass too much current through the heating wire (if it smokes, it is too hot) since the sealant can spit, and small droplets of this highly birefringent aggregate that land on the coverslip can ruin the extinction of the polarization optical system.

After applying the sealant, examine all four sides of the coverslip with a dissecting micro-

scope or magnifying glass to make sure that there are no imperfect seals or pinholes, since water evaporates rapidly from even a very small hole in the seal.

Effect of fixation

Polarization microscopy, carried out on living cells that continue to divide, develop, or otherwise undergo normal physiological or developmental activities, provides a good

Table 4.9.2 Composition of Insect Ringer Solution^a

Stock solution^b

500 mM PIPES
 27 mM KCl
 67.5 mM CaCl₂
 2.5 mM MgCl₂
 Adjust pH to 6.8 to 6.9 with 10 N NaOH^c

^aThis is the solution currently recommended by Nicklas et al. (1979) and supercedes the earlier formula of Niklas (Nicklas and Staehly, 1967) as well as those of Belar (1929, footnotes on pp. 364 and 387). In his 1929 article, Belar describes and illustrates with photographs of live cells the appearance of pseudopodia, chromosomes, and mitochondria of spermatocytes exposed to media with different degrees of tonicity. He also warns that the preparation be kept from being heated by (the infrared rays from) the light source. See Inoué, 1952b, for effect of temperature on the birefringence of spindle fibers (polymerization of spindle microtubules), which serves as a sensitive intracellular thermometer.

^bPrepare fresh in glass-distilled water each month and store in refrigerator.

^cOn day of use, dilute 5 ml of stock solution to between 27 and 38 ml (start with 32 ml) with glass-distilled water. The final optimum concentration depends on the extent of evaporation during culture preparation and the physiological state of the animals; the required dilution usually does not change over a period of a week or two.

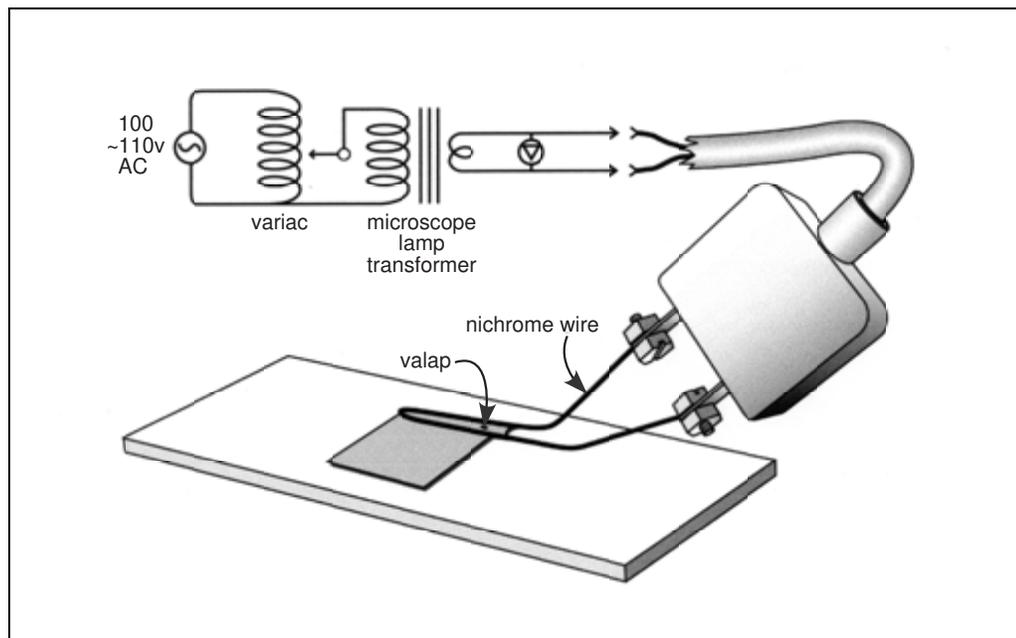


Figure 4.9.11 Electric heater for sealing coverslips with Valap. A 5-in.-long piece of ~20-G nichrome wire is bent as shown and attached, via a convenient handle (e.g., a two-pronged AC plug), to a variable voltage transformer (e.g., one used to power a low voltage microscope lamp). The melted Valap, that has formed a bridge by capillarity between the arms of the nichrome wire loop, is transferred to and painted along the edges of the cover slip.

baseline for understanding the submicroscopic organization and its changes in these cells. That baseline is an important yardstick to heed in considering whether the fine structure of the cell has remained intact when one fixes a cell, or tissue, in preparation for electron microscopy, cytochemistry, or other technique.

When the same cell loses, gains, or changes its birefringence appreciably upon fixation, one needs to wonder whether the fixation itself may not have abolished, created, or altered the fine structure of the cell component. Note that the refractive index of the medium in which the fine structure is immersed also affects the form birefringence of the structure.

For example, prior to introduction of glutaraldehyde as a fixative, spindle and other labile microtubules tended to disappear with standard osmium fixation (Inoué, 1993). Even with glutaraldehyde fixation, there is the possibility that not all of the labile microtubules are preserved (Sato et al., 1975). Given the preparative techniques used for electron microscopy today, we may still be unaware of the loss in fine structural organization encountered by some of the cell's labile filament or membrane systems (e.g., see Burgos et al., 2000).

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Fluorescent Speckle Microscopy (FSM) of Microtubules and Actin in Living Cells

This unit describes the execution of an epifluorescent microscopic method, fluorescent speckle microscopy (FSM), that allows visualization of assembly/disassembly dynamics, movement, and turnover of macromolecular assemblies *in vivo* and *in vitro*. Microinjection or expression in living cells of fluorophore-conjugated proteins followed by their incorporation into cellular structures and visualization by fluorescence microscopy has yielded much information about protein localization and dynamics (Wang, 1989; Prasher, 1995). However, this method has been limited by high-background fluorescence from unincorporated and out-of-focus incorporated fluorescent proteins and difficulty in detecting movement of fluorescent structures because of their uniform labeling. These problems have been partially alleviated by use of more cumbersome methods of laser photobleaching and photoactivation of fluorescence in which a portion of molecules in a structure is marked and the behavior of the molecules in the marked region is monitored (Wolf, 1989; Mitchison et al., 1998). In contrast, FSM uses a very low concentration of fluorescent subunits that co-assemble with endogenous unlabeled subunits giving a structure with a speckled appearance in high-resolution fluorescence images (Waterman-Storer et al., 1998; Fig. 4.10.1). The appearance, disappearance, and movement of speckles stand out to the eye in time-lapse FSM. These changes in fluorescent speckle pattern correspond to the assembly, disassembly, and movement of the structure and can be quantitated (Waterman-Storer et al., 1999). FSM images are captured using conventional wide-field fluorescence light microscopy and digital imaging with a low-noise, cooled charge-coupled device (CCD) camera. FSM provides information about protein dynamics throughout the field of view, as opposed to within a small marked region of the cell as is the case for laser photobleaching and photoactivation of fluorescence techniques. FSM also significantly reduces out-of-focus fluorescence and greatly improves visibility of fluorescently-labeled structures and their dynamics in thick regions of living cells (Waterman-Storer et al., 1998). Finally, the low level of fluorescent protein used in FSM is much less likely to perturb cellular protein balance thereby reducing the chances of artifactual changes in cell behavior due to the effects of protein “overexpression.”

This unit will focus on the utilization of FSM for observing microtubule and actin cytoskeletal dynamics *in vivo*. For microtubules, which can easily be distinguished in fluorescence light microscopy as single filaments both *in vivo* and *in vitro*, the fiduciary marked filaments seen in FSM will allow one to unequivocally distinguish between microtubule translocation as powered by motor proteins and microtubule treadmilling, in which one end of the polymer assembles concomitant with polymer disassembly at the other end (Waterman-Storer and Salmon, 1997). Microtubule FSM also allows observation of microtubule behavior within mitotic spindles containing many hundreds of microtubules and in the central regions of interphase cells. For actin, FSM can be used for studying the assembly dynamics and retrograde movement of actin meshworks in the leading lamella of migrating cells (Waterman-Storer et al., 1998).

STRATEGIC PLANNING

General

Successful FSM requires knowledge and experience in high-resolution epifluorescence microscopy, basic tissue culture and biochemistry skills, and considerable skill in single-cell microinjection and handling of cells for live-cell imaging. This unit provides detailed information in basic protocols for designing a digital microscope system for obtaining

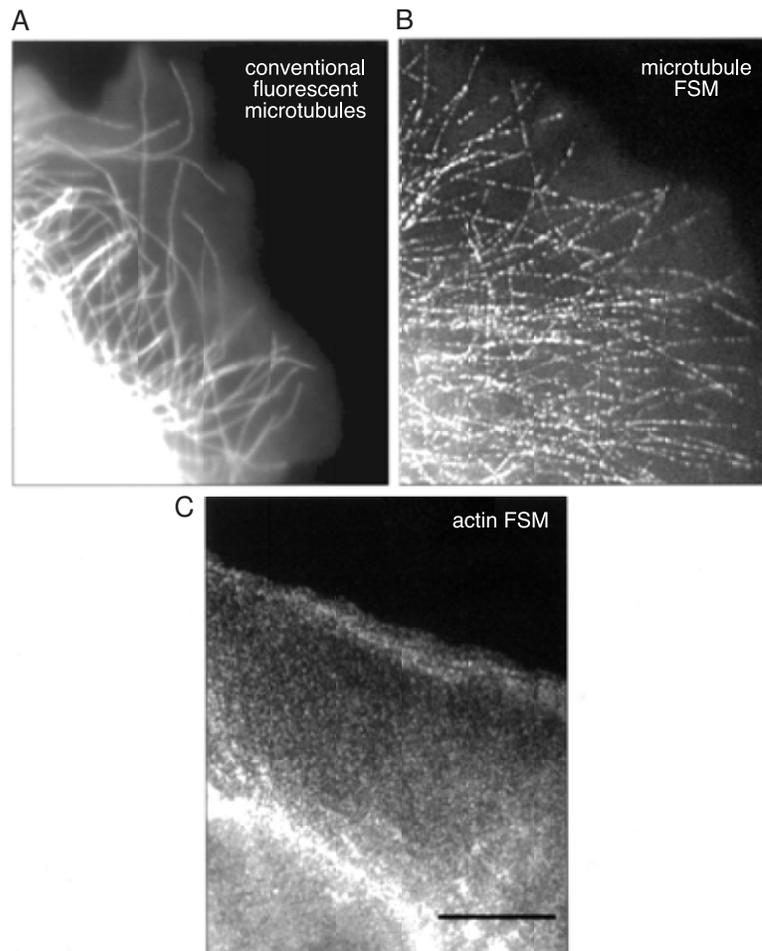


Figure 4.10.1 FSM images of actin (**C**) and microtubules (**B**) in the lamellae of living epithelial cells. A comparison of conventional image of fluorescent microtubules (**A**), labeled with X-rhodamine tubulin, in which labeled tubulin makes up ~10% of the cellular tubulin pool with microtubule FSM (**B**), X-rhodamine tubulin, in which labeled tubulin makes up 0.5% of the tubulin pool. In the conventional image (**A**), there is a high background fluorescence that masks details in the central regions of the cell, and the microtubules are continuously labeled along their lengths, devoid of fiduciary marks. In the FSM image (**B**), the background fluorescence is low, there is no loss in the ability to see the full length of the individual microtubules, and they are covered with fiduciary marks. The images were taken on the same imaging system with the same objective lens. The exposure time for (**A**) was ~200 msec while that for (**B**) was 1500 msec. (**C**) FSM image of a cell injected with low level of X-rhodamine labeled actin. Note the speckled appearance of the lamella, which is made up of a very dense meshwork of cross-linked actin filaments. Bar = 10 μm , all images are at the same magnification.

time-lapse FSM images (see Basic Protocol 1), tips for success in live-cell fluorescence microscopy (see Basic Protocol 2), and basic quantitative analysis of FSM images (see Basic Protocol 3); and support protocols for fluorescent labeling of tubulin (see Support Protocol 1) and actin (see Support Protocol 2). Please refer to *UNIT 4.2* for information on basic fluorescence microscopy, *UNIT 1.1* for cell and tissue culturing, and details of how to carry out single-cell microinjection.

Prior to performing FSM imaging, good quality fluorescently labeled protein must be prepared. Labeled proteins that have given good results in FSM applications can be obtained commercially from Cytoskeleton, Inc.. However, if imaging cytoskeletal dynamics is going to be ongoing in the laboratory, it is much more economical to prepare the proteins oneself. For making fluorescent tubulin, unlabeled tubulin must first be made.

Tubulin purification requires a source of fresh pig or cow brains, and can be prepared by the method described in *UNIT 13.1*, Support Protocol 3. Fluorescent labeling of actin requires a source for muscle acetone powder. Although the powder is available from Sigma, better quality acetone powder can be obtained from a laboratory that specializes in actin biochemistry (generally, these laboratories store large quantities in freezers), or alternatively, it can be prepared by the method of Pardee and Spudich (1982).

Choice of Fluorophore

Support Protocols 1 and 2 describe preparation of tubulin and actin, respectively, that is covalently bound to a succinimidyl ester derivative of the fluorophore of choice. The Molecular Probes catalog is an excellent source of information about the chemistry of fluorophores. Succinimidyl ester derivatives react with lysine residues that are accessible on the surface of the protein. For both tubulin and actin, there is more than one lysine available for reaction, thus, there is the opportunity for higher dye-to-protein ratios, and therefore, brighter fluorescence. For tubulin labeling, succinimidyl esters have, historically, given the greatest success in yield and functional product capable of polymerization and depolymerization (Hyman et al., 1991). Actin labeled with 5-iodoacetamide, which reacts with a single cysteine residue (cys374) on actin, has been well characterized (Wang and Taylor, 1980), however, the dye-to-protein ratio is lower than labeling on lysines.

In choosing the “color” (excitation and emission spectrum) of the fluorophore for FSM imaging, there are two things to keep in mind—the phototoxicity effects on cells of particular wavelengths and endogenous cellular auto-fluorescence. Cells are sensitive to and damaged by short wavelengths; these effects are manifested as retraction of the cell edge, rounding up, and eventually death. Thus, fluorophores that excite at <450 nm (e.g., Coumarin, pyrene) should not be used. Even wavelengths between 450 and 500 nm that excite fluorescein-like dyes (e.g., FITC, Oregon green, Bodipy FL, Alexa 488, or Cy-2) can produce phototoxicity. However, excitation wavelengths beyond the visible (>700 nm, infrared) should be avoided, as well, due to heating effects. The best choice in terms of minimizing phototoxic effects are yellow/orange (570 to 600 nm, tetramethylrhodamine, TAMRA, Cy-3, Bodipy TMR, Alexa 546), orange/red (600 to 630 nm, Lissamine rhodamine, X-rhodamine, Alexa 568, Texas red), or far-red emitting dyes (>630 nm, Cy-5). Of these, the longer wavelength exciters are better, although emission of dyes such as Cy-5 are very difficult, if not impossible, to see by eye, and thus, are problematic to work with. The author’s best success with FSM has come from using the X-rhodamine, Alexa 568, or Texas red fluorophores.

Cellular auto-fluorescence is caused by flavo-proteins that excite in the UV and blue, and emit in blue and green (450 to 550 nm) wavelengths. This auto-fluorescence contributes to background when using fluorescein (FITC) or fluorescein-like dyes, and it “dilutes” the speckle contrast. The author has found this effect to be particularly problematic for actin FSM, in regions of the cell where actin filaments make up cross-linked meshworks that appear as uniform, dense fields of speckles in FSM images (Fig. 4.10.1). If the use of green emitters is unavoidable, as is the case in the author’s laboratory where microtubules and actin are labeled with spectrally distinct fluorophores and monitored by dual wavelength FSM, green emitters can be used with some success for microtubule FSM.

From this discussion, it is apparent that low-level expression of green fluorescent protein (GFP) fusion proteins are not optimal for FSM. First, there is the problem of cellular autofluorescence. Second, the large size (23 kDa) of the GFP moiety, often results in a sizable proportion of the expressed fusion protein losing its ability to functionally incorporate into the cytoskeleton, contributing to background fluorescence. A third problem is the lack of a suitable method for preventing photobleaching of GFP. Finally,

the brightness (quantum efficiency) of the GFP fluorophore is much lower than chemical fluorophores. For studying a microtubule-binding protein, the author has alleviated this last problem to a large degree by fusing multiple (3 to 5) GFP molecules in tandem, joined by short flexible linkers, to the microtubule binding domain of the protein and selecting cells for imaging that express very low levels of the fusion protein (Faire et al., 1999). However, this approach would be unadvisable for actin or tubulin, which would surely be rendered dysfunctional by the incorporation of a 75- to 125-kDa attachment.

**BASIC
PROTOCOL 1**

DESIGNING A MICROSCOPE SYSTEM FOR TIME-LAPSE DIGITAL FSM

As opposed to a step-by-step protocol, in this section, the basic components needed to set up an FSM system are discussed, giving the recommendations for critical elements required in each type of component (Fig. 4.10.2). It is *imperative* that one reads the Background Information on the theory of FSM image formation before one attempts to understand how and why particular components are chosen for the imaging system.

After reading the discussion of FSM image formation (see Background Information), it is clear that there are three key elements of the microscope imaging system to ensure successful FSM imaging. First, the epifluorescent microscope should be as efficient as possible at collecting photons and should be capable of high-resolution, high-magnification imaging. This is achieved with high-quality, 1.4-NA oil-immersion objective lenses with minimal lens elements. Additionally, this requires a simple optical path between the specimen and camera with as few intervening components as possible. Second, the camera should be highly sensitive, low noise, and should have high enough spatial resolution to match the resolution of the microscope. These properties are met by several cooled CCDs available on the market today. Finally, since fluorophores are subject to photobleaching, light exposure to the specimen should be kept to a minimum. This is accomplished through the use of an electronically controlled shutter on the epi-illuminator that only opens during the time of camera exposure. In addition, a computer with a digital image acquisition board and software for controlling the shutter and image acquisition timing are needed to integrate the system.

Materials

- Upright or inverted epi-fluorescent microscope and optics including:
 - Epi-illuminator
 - High-magnification objective lens (e.g., 60 \times , 63 \times , or 100 \times)
 - Excitation filter, emission filter, and dichromatic mirror
 - Electronically controlled shutter
 - Cooled CCD camera
 - Computer, digital image acquisition board, and software for control of shutter and image acquisition
- Microscope stand

Upright or inverted epi-fluorescent microscope and optics

1. The microscope stand should be of biological research quality, with a substantial mass that resists vibration and is not subject to temperature-induced expansion and contraction.

If possible, the microscope should be mounted on a vibration isolation table. The focusing system should be of high quality, with a means by which to lock focus during long-term time-lapse imaging, since slight shifts in focus result in changes in speckle intensity that can be artifactually interpreted as cytoskeletal dynamics.

2. The microscope should be equipped with a high quality epi-illuminator including a 100-W HBO mercury arc lamp, and a lamp housing with a parabolic mirror that

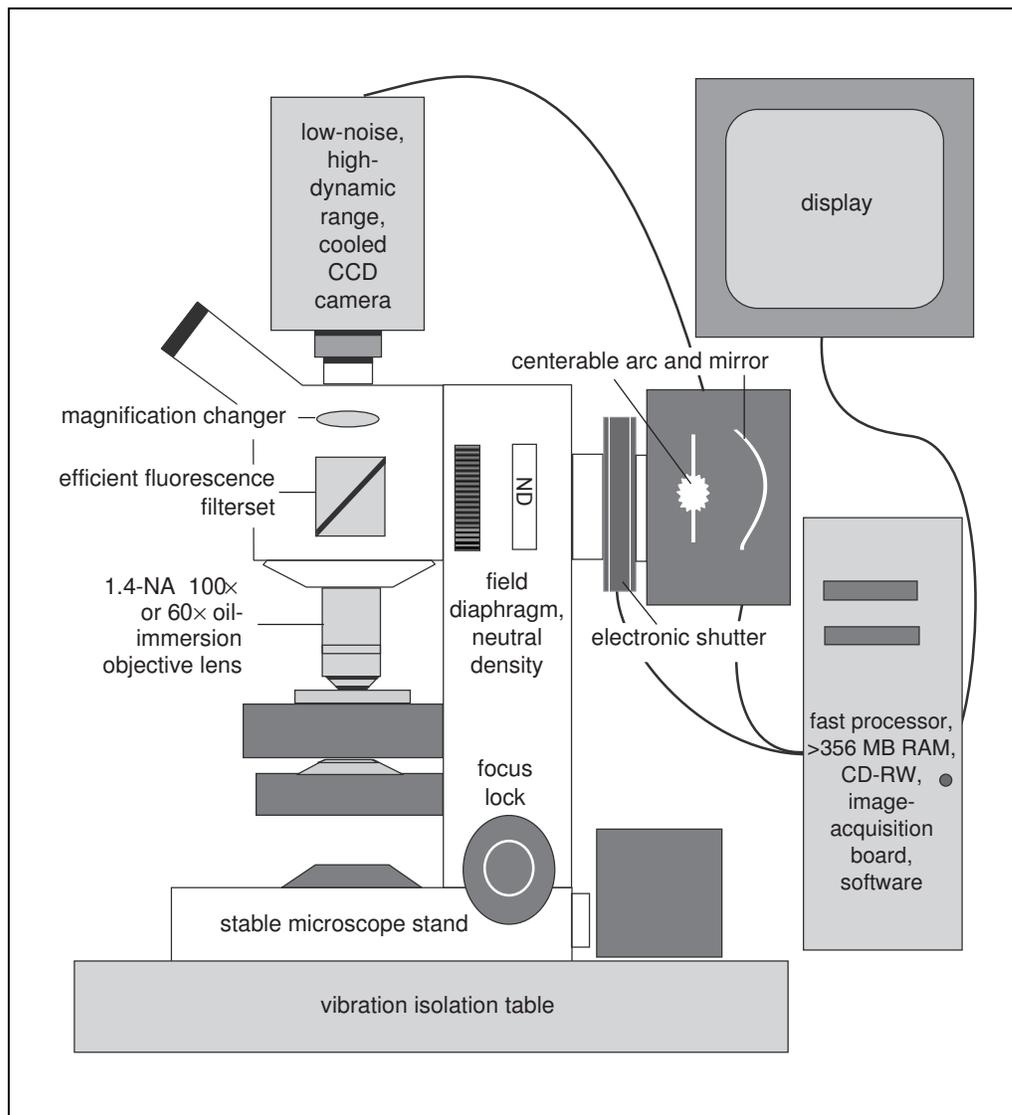


Figure 4.10.2 Diagrammatic view of the components of an upright FSM imaging system. See Basic Protocol 1 for details.

allows manual control of bulb and mirror centration for proper alignment of Köhler epi-illumination.

It is very helpful, though not necessary, to have manual control of centration and size of the field diaphragm of the epi-illuminator. Closing down the field diaphragm to just the area of the specimen being imaged reduces photo-damage to the whole specimen and reduces out-of-focus fluorescent flare in the image. Similarly, it is helpful if the epi-illuminator is equipped with slots in which to insert neutral density filters to control specimen illumination. An infrared blocking filter can also be included in the illumination path to minimize exposure of the specimen to damaging heat.

3. The objective lens (which also acts as the condenser in epi-illumination) should allow the highest resolution possible, thus, one with the highest numerical aperture available, i.e., 1.4-NA oil-immersion; and magnification of 60 \times , 63 \times , or 100 \times , the choice being dependent on the spatial resolution of the camera detector (see Matching Microscope and Detector Resolution below).

Keep in mind that 60 \times or 63 \times lenses are often more efficient at transmitting light than 100 \times lenses. However, never sacrifice system resolution for this small gain in light detection. The

lens should not contain contrast-forming elements such as phase rings that block transmission of photons. The lens should be corrected for chromatic aberration (apochromatic) and should be highly efficient at passing the wavelengths of light being imaged, which depends on the choice of fluorophore. Ask the manufacturer for information on the spectral efficiency of the lens.

When imaging a small specimen, it is not necessary that the lens be flat-field corrected (plan-corrected). Indeed, often objectives specifically designed for efficient epi-fluorescence (Super Fluor-, Nikon; Fluor-, or Fluor- lenses) are not plan-corrected because this correction adds glass elements to the lens, and the more glass elements, the more places to lose photons. However, for a flat, wide-field view, the author has had good luck using DIC-grade plan apochromat objectives.

4. The excitation and emission filters and a dichromatic mirror should be as efficient as possible for exciting the fluorophore, separating the excitation from emission, and collecting the emission of the fluorophore of choice. The use of long-pass filters rather than band-pass filters may maximize this efficiency.
5. The path from the objective to the detector should be simple and contain as few intervening components as possible to allow maximum photon collection. Remove analyzers, wave plates, and Wollaston prisms that are used for various modes of polarization microscopy. Optovars or magnification changers should be optimally removed; however, if needed to match the microscope resolution to the detector resolution (see below), this is a source of light loss that will have to be tolerated.
6. The camera port should utilize the most direct path from the specimen. For an upright microscope, this would be the one port directly over the objective, and for an inverted configuration, a bottom or “Keller” port underneath the microscope is best. Remove prisms from the light path that split the image between the ocular and camera port.

The bottom port requires a hole in the table upon which the microscope is seated, in order to accommodate the camera. If this is not possible, a side camera port, which requires one mirror to direct the image to the camera is better than a front port that requires at least two mirrors.

Electronically-controlled shutter

7. The electronically-controlled shutter should be mounted with proper adapters in the light path between the lamp-house and the epi-illuminator.
8. The shutter should be mirrored on the surface facing the lamp to reflect heat away from the specimen.
9. The shutter should operate quietly, quickly, reliably, and without excessive vibration. Obtain a shutter that can be actuated from a software triggered pulse via the serial or parallel port of the computer.

Cooled CCD camera

10. The camera should be a scientific-grade slow-scan cooled CCD camera; a video-rate CCD camera equipped with a cooling device cannot be substituted. The choice of camera will be discussed in terms of the choice of the CCD chip itself and then the electronics of the camera in which the chip is housed. Various combinations of the two are available from different camera manufacturers. For a general introduction and in-depth discussion of CCD cameras, see Inoué and Spring (1997).

The CCD chip

11. *Spatial resolution.* The spatial resolution is determined by the physical size of the silicon photodiodes or “pixels” on the CCD chip. These currently range in size from ~6 × 6– to 30 × 30–µm. The larger the pixel size, the more magnification from the

microscope will be needed to ensure resolution-limited images. Thus, smaller pixel size is better for FSM as it will not require photon-robbing magnification changers or Optovars in the light path. The total number of pixels making up the CCD and the pixel size will determine the imaging area.

12. *Spectral sensitivity.* Different types of CCD's have specific probabilities at any given wavelength of converting a photon to a photoelectron that is counted as signal by the camera, i.e., quantum efficiency (QE). For example, a Sony HyperHad CCD has ~45% chance of converting a green photon to a photoelectron (45% QE for green light) and a ~25% QE for red light. Thus, this CCD is much more efficient at detecting green fluorescence than red. Manufacturers supply graphs of the wavelength versus QE for their available CCDs. A CCD should be chosen that has spectral sensitivity for the fluorophore of choice.
13. *Illumination geometry.* CCDs can be illuminated from their front or back sides. Illumination from the front requires that the light pass through substrate materials to reach the photosensitive area, reducing QE. Back-illuminated CCDs are physically thinned to allow illumination directly on the back side of the photosensitive surface, making them much more sensitive (also much more fragile and expensive). However, because of the thinning process, there are limits to the size of the pixels, usually around $\sim 25 \times 25$ - μm , with the smallest currently available at 13×13 - μm . Thus, one has to weigh whether the increased sensitivity is worth the loss in having to put an Optovar in the image path, as well as whether one can afford the expense. For FSM applications, sensitive front-illuminated CCDs have worked quite well.
14. *Readout geometry.* Once photons are converted to charge in the array of pixels, the charges must be read out to an image acquisition board so that the image can be reconstructed in the computer by assigning a gray value to the relative charge at each pixel position. Charges are transferred out of the CCD in three basic ways. Full-frame readout occurs as each row of pixel charges is transferred serially out of the CCD one row after another. This type is the slowest and introduces the most noise into the image, although, this type of readout is still acceptable for FSM if other camera electronics do not introduce sources of noise. In contrast, in frame-transfer and interline-transfer CCDs, either the entire pixel charge array or whole rows of pixels are rapidly transferred to an array of pixels that are masked from light. The charges are then read out from the masked area while the imaging area is being exposed to light again. These types are much faster and less noisy than the full-frame readout. Frame-transfer CCDs tend to be more expensive and interline-transfer CCDs suffer from lower resolution, although this problem has been overcome in recent years. Thus, all three geometries are acceptable for FSM, although the increased speed of the interline-transfer CCD may offer an advantage for increasing image-acquisition rate to observe actively ruffling cells or microtubule motor activity.

Camera electronics

15. A CCD with the lowest temperature cooling system within the means of one's budget should be chosen. Heat on the CCD can be interpreted by the photodiodes as light, thus, contributing to image noise. Different camera manufacturers will house the same CCD in cameras with different degrees of cooling ranging from 20°C below room temperature to -50° or -60°C. Do not choose the least expensive CCD because heat is an avoidable source for noise that can easily mask the very faint FSM signal.
16. *Readout speed.* In general, the faster the readout speed, the more error is introduced during charge transfer, which translates to noise in the image. Speeds in modern cameras range from 100 kHz in some low-noise back-illuminated cameras, to 14 to

15 MHz in interline- and frame-transfer cameras. For quantitative FSM imaging of cytoskeletal dynamics, image acquisition rates of 1 to 2 images/sec may be required, which cannot be accomplished by the slower cameras. Here, a reasonable compromise of speed and low noise must be sought, but it is recommended not to buy a camera much slower than 1 mHz.

17. *Dynamic range.* Although the total number of photons that can be absorbed by each pixel before it is saturated with charge is set for a given CCD, the number of gray levels this amount of charge is divided up into for display is not fixed. It can be represented by 8, 10, 12, 14, or 16 bits of information per pixel, corresponding to 256, 1024, 4096, 16,384, or 65,536 (2^8 , 2^{10} , 2^{12} , 2^{14} , or 2^{16}) gray levels, respectively. For FSM imaging of very dim specimens, it is important to have the biggest dynamic range possible (again, within a reasonable budget). This is required so that there will be finer gradations of gray within the very small portion of the dynamic range that is utilized. Successful FSM has been achieved using 10-bit cameras, although ≥ 12 bits/pixel is recommended.
18. *Subarraying and binning.* Being able to read out only a specified portion of the CCD (subarraying) can increase image acquisition speed for imaging small areas of a cell, but it is not necessary. Binning, in which the charges in a group of pixels are combined and read out as a single pixel to increase sensitivity should not be done in FSM, as this effectively increases pixel size and decreases CCD resolution.

Computer, digital image acquisition board, and software for control of shutter and image acquisition

19. A computer with the fastest processor and most random access memory (RAM) affordable should be used. Time-lapse FSM image series are large files often on the order of ≥ 100 MB, and computer “horsepower” is necessary to view and manipulate these.

The author recommends ≥ 356 MB of RAM.

20. *File storage.* A compact disc read/write (CD R/W) device is the most economical choice recommended to archive the large files generated by time-lapse FSM. The fastest write speed available within budget should be chosen.
21. Use the image acquisition board recommended by the camera and software manufacturer, making sure that the board can handle the bit depth of the camera. Many cameras come with their own boards.
22. Software should be capable of time-lapse digital image acquisition and triggering the shutter during camera exposure. The software should allow easy viewing of time-lapse series as movies, with control of play-back rate and adjustment of brightness and contrast in the entire image series. Basic image processing including the ability to perform low-pass filtering and image arithmetic (subtraction, multiplication, etc.) is required. The software should provide the ability to perform quantitative analysis of intensity, position, and distance.

The author has used MetaMorph (Universal Imaging) with outstanding success. However, NIH-Image freeware (<http://rsb.info.nih.gov/nih-image/>) is also very versatile and many free macros are available.

Matching microscope and detector resolution

23. The key to actually achieving resolution-limited images of fluorescent speckles is matching microscope and detector resolution. Magnifying the diffraction-limited spot to the size of 3 pixels on the CCD is required so that the CCD does not limit

imaging system resolution or produce aliasing between pixels. This is called the Nyquist sampling criterion. Any magnification over this value does not contain any more information and simply reduces the area of the specimen that is imaged. The magnification (M) required to achieve this is given by

$$M = 3(P_{\text{width}})/r$$

where P_{width} is the width of a pixel on the CCD and r is the size of the diffraction-limited spot. Thus, for red fluorescence with a resolution limit of 0.27 μm (for red fluorescence, see Background Information) and a camera with 6.7- μm pixels, the magnification required to satisfy the Nyquist criterion is 74.4 \times . Thus, either a 100 \times objective or a 60 \times with a 1.25 \times Optovar should be used, whichever transmits more light.

TIME-LAPSE FSM IMAGING OF THE CYTOSKELETON IN LIVING CELLS

This protocol describes how to handle living cells for FSM imaging. Included are basic recommendations for microinjection, detailed instructions for prevention of photobleaching, and image acquisition. Photobleaching of chemical fluorophores (not GFP) is dependent on dissolved oxygen in the medium. Thus, photobleaching can be substantially prevented by removal of dissolved oxygen, in this case, with a commercial product, Oxyrase, and by then sealing the imaging chamber from contact with air (Waterman-Storer et al., 1993).

Materials

- ~0.5 mg/ml labeled cytoskeletal protein (tubulin or actin, see Support Protocols 1 or 2, respectively)
- Cultured tissue cells grown on 22 \times 22-mm glass coverslips in small plastic petri dishes
- Valap (UNIT 13.1)
- Buffered filming medium (see recipe)
- Oxyrase EC (Oxyrase Inc.)
- Microultracentrifuge (Optima TL or TLX, Beckman Instruments; or Discovery M120, Sorvall)
- 0.6-ml microcentrifuge tubes
- Swinging bucket rotor for microultracentrifuge with adapters for holding 0.6-ml microcentrifuge tubes (for the Beckman TLS-55 rotor, the standard thick-walled 1.4-ml polycarbonate tubes are used; and for the Sorvall, custom adapters are needed)
- Microinjection needles (see recipe)
- Microloader pipet tips (Eppendorf or equivalent) or narrow-gauge syringe (narrow enough to fit in the bore of the microinjection needle, Hamilton Company)
- Single-cell microinjection system capable of controlled backpressure of 0.1 to 20 psi and with a precision micromanipulator for injection of single cells, mounted on an inverted microscope equipped with a long-working-distance phase-contrast condenser and a 40 \times dry phase-contrast objective lens with a working distance long enough to focus through the plastic petri dish and coverslip.
- Microscope stage incubator (optional)
- 1 \times 3-in. glass microscope slides
- Scotch double-stick tape (3M)
- Cotton swabs
- FSM imaging system (see Basic Protocol 1)

BASIC PROTOCOL 2

Microscopy

4.10.9

Prepare labeled cytoskeletal protein

1. Cool the microultracentrifuge and rotor to 4°C.
2. Rapidly thaw and immediately place on ice a 3- μ l aliquot of ~0.5 mg/ml labeled cytoskeletal protein.
3. Pellet insoluble protein in the sample. Cut off the lid of the 0.6-ml microcentrifuge tube containing the labeled protein and place the tube in the polycarbonate adapter for the swinging bucket rotor. Make sure to place a balance tube in the opposite bucket. Microultracentrifuge 20 min at 77,000 \times g (Beckman TLS-55 rotor at 30,000 rpm), 4°C.

Do not microultracentrifuge any faster, as this will crush the microcentrifuge tube.

4. During the microultracentrifugation, locate the cultured tissue cells to be injected on the microscope stage using a 40 \times phase-contrast objective lens.

All manufacturers correct their high-resolution optics for the thickness of a no. 1.5 coverslip.

Cell choice is obviously dependent on the biological question; however, it is advisable to choose flat, large, well-spread cells as these are better for microinjecting and imaging. PtK1 are a favorite for mitosis studies, while Swiss 3T3 are a favorite for cell locomotion studies.

5. Following microultracentrifugation, promptly remove the sample from the microultracentrifuge and transfer to ice.

Microinject cells with labeled protein

6. Backload a microinjection needle with ~0.5 μ l of protein solution using either a microloader pipet tip or a narrow-gauge syringe.

Be careful not to touch the insoluble pellet when retrieving the solution from the microcentrifuge tube.

7. Microinject several cells in the center of the coverslip using ~0.5 to 1.5 psi constant pressure.

The constant flow from the needle helps to keep it from clogging.

It is not recommended to transiently increase the pressure during microinjection, as this will result in too much fluorescent protein in the cells, ruining the speckle effect.

8. Return the cells to a 37°C, 5% CO₂ incubator for 1 hr to recover from microinjection and to allow incorporation of labeled protein into the cytoskeleton.

9. During this time, place filming medium in a 37°C water bath, melt Valap on a hot plate set on low; and turn on the microscope, camera, and stage incubator to allow temperatures to equilibrate.

A microscope stage incubator is only required for cells from warm-blooded species. An incubator can be as simple as a home hair blow dryer custom-fitted with a rheostat for control of the heating coils and a thermometer taped to the stage.

Mount cells for imaging

10. Just prior to mounting the specimen, prepare 1 ml of buffered filming medium containing 20 to 30 μ l Oxyrase EC.

The amount of Oxyrase the specific cell type can tolerate must be determined empirically. Some cells are more sensitive to oxygen deprivation than others. For sensitive cells (such as PtK1), make sure the filming medium is prepared with high glucose medium, so that extra glucose will be available for anaerobic metabolism.

11. Prepare a filming chamber by placing two 3 × 30–mm strips of double-stick tape side by side 1 cm apart and parallel with the long axis onto a 1 × 3–in. glass microscope slide to form a channel where the cells will be.
12. Retrieve the coverslip containing the injected cells from the dish of medium, dry the back side and the edges that will be placed on the tape strips, and place the coverslip, cell-side down onto the tape so that the area containing the injected cells lies between the strips of tape.

Press the coverslip into place to form a good seal with the tape.

13. Add Oxyrase-containing filming medium to the cell “chamber” by pipetting it against the edge of the coverslip between the strips of tape. Exchange the medium several times by wicking away medium with filter paper from the opposite open end of the chamber while pipetting.

Be sure not to introduce any bubbles into the chamber during pipetting as the presence of air in the chamber will cause problems with photobleaching.

14. Carefully dry the edges of the specimen chamber (do not introduce bubbles) and use a cotton swab to apply melted Valap along all edges of the coverslip to seal it completely to the slide.

Observe mounted cells

15. Allow the slide to equilibrate to the proper temperature on the microscope stage for 10 to 15 min. Turn the room lights off to allow one’s eyes to adapt to the dark.
16. Use epifluorescent illumination to locate the fluorescent-injected cells.

The cells should be quite dimly fluorescent and may not be visible with a low-power, low-NA objective lens, therefore, one may have to search with an oil-immersion lens. After locating the cells, switch to high-magnification, high-NA objective lens. At this point, be careful about illuminating the specimen and causing photobleaching—look only as long as needed to get a cell in focus and centered in the field of view then quickly shutter the light. Use of neutral density filters to attenuate illumination during focus and centration may also be helpful in preserving the fluorescence in the specimen.

Cytoskeletal polymers are excluded from the nucleus; cells with fluorescent nuclei are likely dead. Dimly fluorescent individual microtubules should be visible by eye, while fluorescent actin should be rather diffuse throughout the cell and perhaps brighter in stress fibers.

17. Pick a dim cell to start with because microinjection will result in cells of varying fluorescent brightness.

With practice, one will be able to judge by eye the brightness required for good FSM.

18. Illuminate only the area of interest by adjusting the field diaphragm of the epi-illuminator.

Optimize camera conditions and acquire time-lapse images

19. Optimize exposure time. First, make sure the camera is set to the maximal dynamic range possible and set the image display to “autoscale.” Without autoscaling, the image will likely appear black since the FSM image is expected to use a very small portion of the low end of the dynamic range of the camera. Then, take a 500-msec “dark image” image with the illuminating light shuttered to get a measurement of the background gray level of the camera. Next, be sure the illumination shutter is set to synchronize with the camera exposure. Then, take images of the fully illuminated specimen (no neutral density filters), trying to get the specimen signal to ~10% higher in gray level value than the background.

For example, if the background gray level in a 14-bit (16,384 gray level) camera is 500, try for an exposure that will give the specimen a gray level of ~550. The sample should be dim enough that this very small amount of signal above the noise should require a rather long exposure time. A good starting point is 500- to 1000-msec exposures, but upwards of 2000 msec may be required.

The time of exposure will vary depending on the camera used, the brightness of the sample, the age of the arc lamp, and the tolerance of the specimen to light. Signs of photo-damage include cessation of cell motility, retraction of cell lamellipodia, or blockage of mitotic progression.

20. Optimize focus. Take exposures while slightly adjusting the fine focus until the best focused–fluorescent speckles can be seen. Then, lock the focus.

If the actin network does not appear speckly, or if individual microtubules do not appear discontinuously labeled along their lengths, there is too much labeled protein in the cell. Find another, more dimly labeled cell.

21. Acquire time-lapse image series. For following actin movement, acquire images every 10 to 30 sec. For following microtubule dynamics and movement, acquire images at 3- to 10-sec intervals. Image as long as is needed or until the sample is photobleached.

BASIC PROTOCOL 3

QUALITATIVE AND QUANTITATIVE ANALYSIS OF TIME-LAPSE FSM IMAGE SERIES

Methods are presented to extract measurements of speckle movement and lifetime to determine polymer velocities and turnover times.

Materials

Stage micrometer (Fisher)
Image analysis software

1. Calibrate the pixel-to-distance conversion factor. Take an image of a 10- μ m stage micrometer using the objective lens used for image acquisition.

The number of pixels in 10- μ m is used to calculate the pixel-to-distance conversion factor. Most software packages will allow this factor to be input so that measurements made are automatically reported in micrometers as opposed to pixels.

2. Adjust the brightness and contrast of the image series to maximize the speckle appearance.
3. Play the time-lapse series as a movie, paying attention to the movement, appearance, and disappearance of fluorescent speckles.
4. To enhance FSM images, perform an “unsharp mask” filter on each image in the time-lapse series (Waterman-Storer et al., 1999).

This consists of performing a 9×9 - or 10×10 -low-pass filter on the image and then multiplying by a constant of 0.5. This low-passed, scaled image is then subtracted from the original image, and the result is multiplied by a constant to scale it to the same average gray level as the original image. This series of operations greatly enhances speckle appearance.

5. To measure speckle movement rates in complex fields of speckles such as the mitotic spindle or the actin-rich lamella of a migrating cell, perform kymograph analysis (Waterman-Storer et al., 1999; Fig. 4.10.3).

There are two ways to make kymographs of speckle motion in an image series. In the first method, a narrow rectangular cursor is drawn on the image stack, with the long axis of the

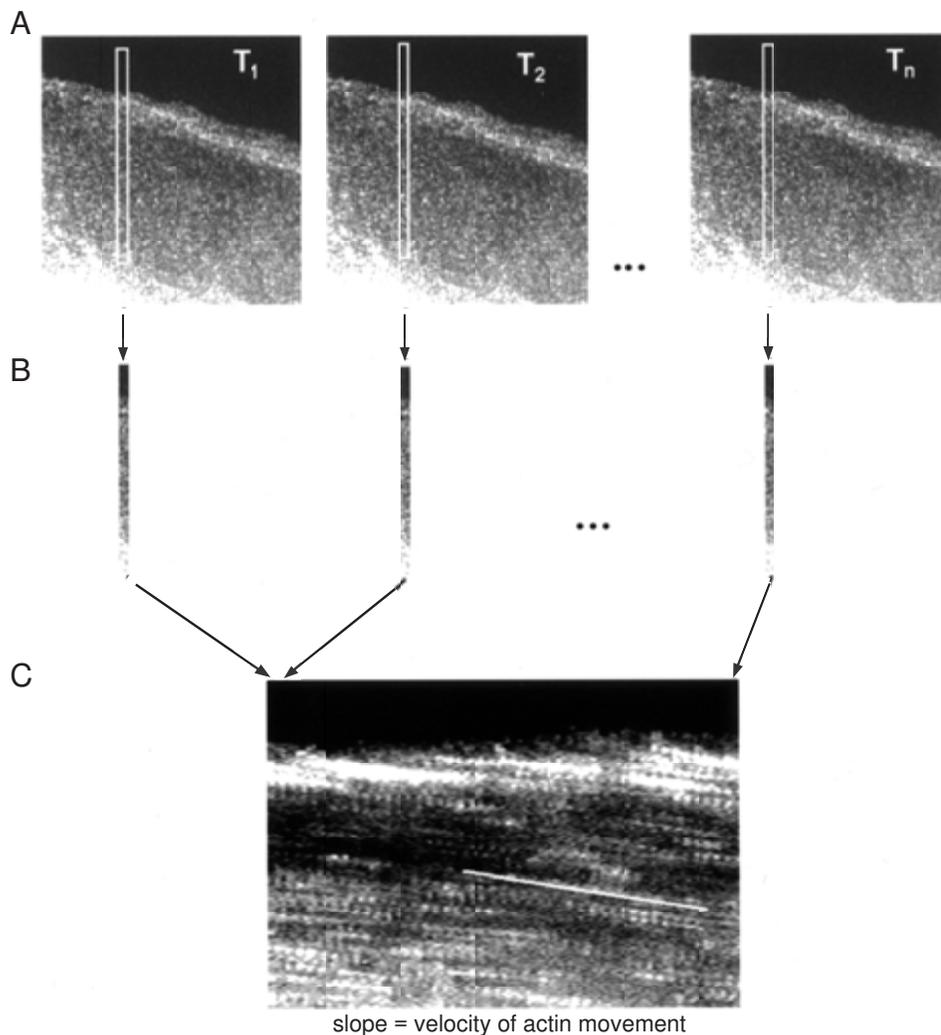


Figure 4.10.3 Construction of a kymograph. **(A)** A long thin rectangular region whose long axis is parallel to the direction of speckle movement is selected in each image in a time-lapse series (T_1 - T_n). **(B)** This region is extracted from each image and pasted side-by-side in a montage to form the kymograph **(C)**. As speckles move along the axis of the rectangular region in the original image series over time, they appear as oblique streaks on the kymograph, the slope of which correspond to the velocity of speckle movement (see Basic Protocol 3).

rectangle aligned with the direction of speckle movement determined by viewing the series as a movie. The rectangle should be 2 to 4 pixels in width and the length of the area desired for motion analysis. This region of interest is then copied from each image in the time-lapse series, and pasted side-by-side in a montage to form the kymograph picture. By eye, the speckles within the rectangular region that are moving along its long axis produce oblique white lines in the kymograph, the slope of which yields the velocity of motion. The disadvantage of this method is that it only measures speckle movements in either horizontal or vertical directions.

The second method requires the image processing software to possess a “kymograph function.” The MetaMorph system is used as an example. The point-to-point line drawing function in MetaMorph is used to draw a line in any direction along the direction of movement for motion analysis on the first image in the time-lapse series. This has the advantage that the line can curve with the curvature of motion. The “kymograph” routine in MetaMorph samples pixel intensity values along this line for each image in a series and

montages these values into an image where the horizontal direction is the pixel values measured along the line, and the vertical direction is lines from subsequent images in the time series. Measurements of distance in the horizontal direction are calibrated in micrometer units. Measurements of distance in the vertical direction must be calibrated to the total time of the number of images in the series. For example, if the images are recorded at 10-sec intervals and there are 50 images in the series, then the vertical distance of the kymograph corresponds to 500 sec. Velocities are determined by the slopes of the oblique trajectories of speckle movements in the kymographs.

6. Use single-speckle motion tracking to track the movement of single cytoskeletal polymers.

Currently, this can only be done by hand, but software for automated tracking is being developed. Commercially available automated tracking software is incapable of tracking single speckles in a dense field of similar speckles, which in addition are often fuzzy, ill-defined, tiny, and subject to intensity changes due to slight shifts in focus. Hand tracking is done by measuring the distance of the speckle from its origin at time zero in each frame of the time-lapse series. This can often be done semi-automatically using software functions such as “track points” in MetaMorph. The distance over time can be used to calculate instantaneous velocities (change in distance from frame n to frame $n + 1$) or average velocities by performing regression analysis of the distance versus time data.

7. Measure speckle lifetime to give a value for the lifetime of the cytoskeletal filament, i.e., its rate of turnover.

In theory, the intensity value of a single pixel at the center of a fluorescent speckle could be recorded at every point in time and the lifetime of the speckle determined from this “fluorescent life history.” However, this is currently very difficult in practice because of problems with photobleaching, shifts in focus that affect speckle intensity, and minute movement of speckles in living specimens. Again, image analysis software that takes these problems into consideration is currently being developed.

SUPPORT PROTOCOL 1

PREPARATION OF FLUORESCENTLY LABELED TUBULIN FOR FSM

This is a modification of a protocol developed by Hyman et al. (1991; also see the Mitchison laboratory Web page, <http://iccbweb.med.harvard.edu/mitchisonlab/Pages/label.html>). In this method, tubulin is first polymerized to bury the sites of tubulin dimer-dimer interactions to mask them from being labeled, then polymerized tubulin is reacted with fluorophore. The succinimidyl ester derivative of the fluorescent dye reacts with lysine residues at a high pH. Tubulin is readily denatured at high pH, so the longer the labeling reaction is allowed to proceed, the lower the yield, therefore, do not exceed the recommended reaction time. The reaction is then quenched by lowering the pH, and the tubulin is cycled for a temperature-dependent round of depolymerization and re-polymerization (see *UNIT 13.1* for more information on the biochemistry of tubulin) to select for the assembly competent labeled tubulin dimers before finally being depolymerized in microinjection buffer and frozen for storage. This method takes ~7 hr and should yield ~10 mg of labeled tubulin, which should be useful for 6 to 8 months frozen at -80°C .

Materials

- 10-ml aliquots of phosphocellulose-purified tubulin in column buffer (CB; totaling 40 to 60 mg of tubulin; *UNIT 13.1*)
- CB/BRB-80 conversion buffer (see recipe)
- 100 mM GTP (see recipe)
- Glycerol
- Labeling buffer (see recipe)
- High-pH cushion (see recipe)
- Quench (see recipe)

Low-pH cushion (see recipe)
Succinimidyl-ester derivative of fluorescent probe of choice
Anhydrous DMSO
Injection buffer (IB; see recipe)
1× BRB-80 (see recipe for 10×)
1 M MgCl₂ (APPENDIX 2A)

37°C water bath
100- and 10-ml graduated cylinder
Parafilm
Ultracentrifuge (with a Beckman 70.1 Ti rotor; or equivalent)
13.5-ml ultracentrifuge screw-cap polycarbonate tubes
Manual pipet-pump type pipettor (VWR or equivalent)
1.6-ml microcentrifuge tubes
Microultracentrifuge (Beckman Optima TLX with TLA-100.4 rotor or Sorvall RC M120 GX with S100AT4 rotor)
5.1-ml microultracentrifuge polycarbonate tubes
7-ml dounce homogenizer with “B” pestle
Spectrophotometer with a small-volume quartz cuvette
0.6-ml UV-impermeant microcentrifuge tubes

Prepare tubulin

1. Thaw three to four 10-ml aliquots of phosphocellulose-purified tubulin (frozen off the column in CB; UNIT 13.1) by immersing tubes in a 37°C water bath and agitating gently and continuously until they are fully thawed. As soon as the solutions are fully thawed, transfer tubes to ice and pool the aliquots into a 100-ml graduated cylinder on ice.
2. Add 1/20 vol CB/BRB-80 conversion buffer and add 100 mM GTP for a final concentration of 1 mM. Allow tubulin to bind GTP for 5 min on ice.
3. Add 1/3 vol glycerol, cover the cylinder with Parafilm, and mix very well by gentle inversion.
4. Incubate 40 min in a 37°C water bath to allow tubulin to polymerize, gently mixing every 10 min.

Make sure the water level in the bath is as deep as the liquid level in the cylinder so that the temperature is constant throughout the whole solution.

5. During this incubation prewarm the ultracentrifuge with a Beckman 70.1 Ti rotor, labeling buffer, high-pH cushion, Quench, low-pH cushion, and several 13.5-ml ultracentrifuge screw-cap polycarbonate tubes to 37°C.

The rotor can be prewarmed by putting it in a watertight plastic bag and submersing it in warm water (in a separate 37°C water bath from the polymerization reaction). The centrifuge may only warm up to ambient temperature. If this is the case, so long as the rotor is at the proper temperature, the microtubules should be intact.

Isolate microtubules

6. At the end of the incubation, add 3 ml warm high-pH cushion to each warm 13.5-ml ultracentrifuge tube. Slowly layer the polymerized tubulin onto the cushion by using a manual pipet-pump to slowly drip the tubulin solution down the side of the ultracentrifuge tube, being careful not to mix the tubulin with the dense cushion. Divide the microtubule solution evenly among the ultracentrifuge tubes. Balance pairs of filled ultracentrifuge tubes to within 0.01 g, and remember to include the centrifuge cap.

7. Ultracentrifuge the microtubules 1 hr at $185,000 \times g$ (Beckman 70.1 Ti rotor at 52,000 rpm), 37°C .
8. During the ultracentrifugation, make up 100 mM succinimidyl-ester derivative of fluorescent dye of choice in anhydrous DMSO in a 1.6-ml microcentrifuge tube. Warm to 37°C and vortex it well to get as much as possible into solution.
9. Just before microtubule ultracentrifugation is over (step 7), microcentrifuge the insoluble fluorescent dye solution for a few minutes at maximum speed, room temperature. Promptly collect the supernatant and transfer it to a new 1.6-ml microcentrifuge tube.
10. After the ultracentrifugation, note the position of the clear/pearlescent pellet in each tube. Aspirate the supernatant to half the height of the cushion, and wash the cushion with 1 to 2 ml of warm labeling buffer. Then, completely aspirate the supernatant off, rinse the pellet gently with 1 ml warm labeling buffer, and aspirate again. Immediately add 500 μl warm labeling buffer to each tube, and return the tubes to the 37°C water bath.
11. Resuspend each pellet by pipetting up and down in warm labeling buffer. While resuspending one pellet, keep the remaining tubes in the 37°C water bath, and use the same pipet tip for all tubes.

Careful and thorough resuspension is key to high labeling efficiency.

The pellets are dense and sticky. Use a cut-off tip (cut ~5 mm off the end) on a 1-ml pipetman, starting with the 500 μl in each tube. Dial the pipetman in to 300 μl , and pipet liquid up and down over the pellet until it is fully resuspended with no chunks present. Be patient, avoid frothing the solution, and do not poke the tip into the pellet as chunks of the pellet will get stuck inside the tip and will be lost.

12. After the first 500 μl , pool the resuspended pellets in one tube in the 37°C water bath, and go back and rinse each tube thoroughly with another 300 μl of warm labeling buffer. Pool all together, a total of 2 to 3 ml, and keep in the 37°C water bath.

Label microtubules

13. Add fluorophore dye solution (step 9) to the resuspended microtubules to 10 mM final concentration, mix well by gentle vortexing, and incubate 30 min at 37°C . Gently vortex every few minutes throughout the labeling reaction, being careful not to froth the solution.
14. During the labeling incubation, warm up the microultracentrifuge with rotor and several 5.1-ml microultracentrifuge polycarbonate tubes to 37°C .
15. Stop the labeling reaction by adding an equal volume of Quench.
16. Put 1 ml of low-pH cushion into each 5.1-ml microultracentrifuge tube, and layer 2 ml of the labeled polymer on top. Microultracentrifuge microtubules 30 min at $200,000 \times g$ (Beckman TLA-100.4 rotor at 70,000 rpm), 37°C .
17. During the centrifugation, cool a 7-ml dounce homogenizer, pestle, and 10 ml of injection buffer (IB) on ice and warm up a few milliliters of distilled water to 37°C .
18. After centrifugation, aspirate the supernatant to 1/2 cushion volume, wash the cushion with a few drops of warm distilled water, and then aspirate the remaining cushion.

Depolymerize labeled microtubules

19. Immediately add 500 μl cold IB to each tube. Incubate 10 min on ice to let depolymerization initiate before beginning to resuspend the pellets. Pool resuspended

pellets into the dounce homogenizer on ice, rinse each tube with an additional 200 μ l of cold IB, pool all.

This pellet will be horribly sticky. Do not attempt to fully resuspend the pellet in the microultracentrifuge tube, but instead try to partially resuspend as much solids as possible and transfer them into the homogenizer. As always, be patient and do not introduce bubbles into the solution.

20. Allow the microtubules to depolymerize for 30 min on ice, slowly raising and lowering the pestle in the homogenizer the entire time, being careful not to introduce bubbles and keeping the homogenizer immersed in ice.
21. During depolymerization, chill the microultracentrifuge, a few 5.1-ml microultracentrifuge tubes, and the rotor to 2°C.
22. Transfer the resuspended pellets to cold 5.1-ml microultracentrifuge tubes and clarify the depolymerized tubulin by microultracentrifuging 10 min at 415,000 \times g (Beckman TLA-100.4 rotor at 100,000 rpm), 2°C.

Polymerize labeled microtubules

23. Collect the supernatants and pool in a 10-ml graduated cylinder at room temperature. Add 1/10 vol of 10 \times BRB-80, 1 M MgCl₂ to a final concentration of 4 mM, 100 mM GTP to a final concentration of 1 mM, and finally 1/3 vol glycerol. Mix well by inversion.
24. Polymerize microtubules by incubating the graduated cylinder, covered with Parafilm, for 45 min in a 37°C water bath, with occasional mixing.
25. During the incubation, warm up the rotor, microultracentrifuge, and two 5.1-ml microultracentrifuge tubes to 37°C.
26. Just prior to termination of incubation, put 0.5 ml of low-pH cushion in each warm 5.1-ml microultracentrifuge tube. After the incubation, layer the polymerized microtubules on each low-pH cushion.
27. Microultracentrifuge microtubules 30 min at 200,000 \times g (in the TLA-100.4 rotor at 70,000 rpm), 37°C.
28. During this microultracentrifugation, chill 5 ml IB on ice and warm 5 ml of 1 \times BRB-80 to 37°C.
29. After the microultracentrifugation, aspirate the supernatant to 1/2 cushion volume, wash the cushion two times with a few drops of warm 1 \times BRB-80, then aspirate the supernatant completely, wash the pellet very gently two times with warm 1 \times BRB-80 to remove all residual glycerol (which is toxic if microinjected into cells).
30. Cover pellets with 200 μ l of cold IB and incubate 5 min on ice before resuspending the pellets. During this time, chill the microultracentrifuge and rotor to 4°C.
31. Resuspend the pellets in a total of 300 μ l per tube by pipetting, using the second 100 μ l as a rinse. Pool the resuspended pellets in a clean, chilled microultracentrifuge tube and cover it with Parafilm.

These pellets should not be as sticky as the last ones and should be fairly easy to resuspend by pipetting.

Depolymerize microtubules

32. Depolymerize the resuspended microtubules by incubating 30 min on ice.

33. Clarify the depolymerized microtubules by microultracentrifuging for 5 min at $415,000 \times g$ (in the TLA-100.4 rotor at 100,000 rpm), 4°C .
34. After microultracentrifugation, transfer the supernatant to a new 1.6-ml microcentrifuge tube on ice.

Calculate concentration of tubulin and fluorophore

35. Calculate concentration of tubulin, the fluorescent dye-to-protein ratio, and the percent yield of the preparation. Measure the absorbance of a 1:100 dilution (in water) of the tubulin solution at 278 nm (A_{278}) and at the excitation maximum of the fluorophore in a quartz cuvette, using water to blank the spectrophotometer. Calculate the concentration of tubulin using the extinction coefficient for tubulin $\epsilon = 115,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 278 nm and Beer's law:

$$\text{Concentration} = \text{dilution factor} \times A_{278}/\epsilon$$

36. Calculate the concentration of fluorescent dye similarly, using the extinction coefficient supplied by the fluorescent dye manufacturer (contact technical service for information).

The fluorescent dye-to-protein ratio is simply the ratio of the molar concentrations. For calculation of the percent yield, determine the total amount of labeled tubulin obtained from the concentration and volume and use the molecular weight of the dimer, 110 kDa to convert to milligrams to compare to the original amount of tubulin labeled.

37. Adjust the concentration of labeled tubulin to 20 to 30 mg/ml with IB, and drop freeze 3- μl aliquots in 0.6-ml UV-impermeant microcentrifuge tubes in liquid nitrogen for storage at -80°C .
38. For use for microinjection, rapidly thaw an aliquot of concentrated labeled tubulin and transfer immediately to ice. Dilute to 0.5 mg/ml with cold IB, and drop freeze 3- μl aliquots for use within 2 weeks.

**SUPPORT
PROTOCOL 2**

PREPARATION OF FLUORESCENTLY LABELED ACTIN FOR FSM

This is a modification of a protocol developed by J.S. Sanger and J.W. Sanger (Turnacioglu et al., 1998). In this protocol, actin is labeled on lysines with a succinimidyl ester derivative of the fluorescent dye of choice. The labeling is carried out on actin filaments, so that the dye does not bind to regions of the actin monomer that are involved in filament formation, and then the labeled actin is taken through rounds of depolymerization, polymerization, and depolymerization to select for functional labeled molecules, similar to the protocol for labeling tubulin. However, actin polymerization is not temperature dependent like tubulin, but salt dependent with polymerization driven by high-salt concentrations and depolymerization occurring at low-salt concentrations (Pardee and Spudich, 1982). Thus, the depolymerization portion of the cycle occurs very slowly (i.e., over 3 days) by dialysis to remove salt, and should not be rushed. This protocol takes a total of 7 days and should yield 20 to 30 mg of labeled actin, which is good for >1 year when stored at -80°C .

Materials

- Muscle (rabbit psoas or chicken breast) acetone powder
- 1 M KCl (APPENDIX 2A)
- 1 M MgCl_2 (APPENDIX 2A)
- 100 mM ATP (see recipe)
- Succinimidyl ester derivative of the fluorescent probe of choice, pre-equilibrated to room temperature
- Anhydrous DMSO

1 M sodium bicarbonate (see recipe), freshly prepared
1 M NH₄Cl (see recipe)
G-buffer, 4°C (see recipe; ATP should be added just prior to use)
50-ml beaker
Refrigerated ultracentrifuge with rotor (e.g., Beckman 70.1 Ti rotor)
13.5-ml screw-cap ultracentrifuge tubes
50-ml and 1-liter graduated cylinder
Spectrophotometer and quartz cuvette
1.6-ml microcentrifuge tubes
Aluminum foil
Refrigerated centrifuge with rotor (e.g., Sorvall SS-34 rotor)
50-ml open-top polycarbonate centrifuge tubes (or equivalent)
7-ml dounce homogenizer with type “B” pestle
Pretreated glycerol-free cellulose dialysis tubing (see recipe), ~2 cm diameter,
20,000 MWCO (i.e., Spectra/Por 6, Fisher)
Dialysis tubing clips
0.6-ml UV-impermeant microcentrifuge tubes

Extract and polymerize actin monomers from muscle

1. Add 1 g of muscle acetone powder to 25 ml chilled deionized, distilled water in a 50-ml beaker. Extract the actin monomers by mixing 30 min at 4°C. During extraction, cool the ultracentrifuge and rotor to 4°C.

Use a magnetic stirrer and stir plate in the cold room.

Muscle acetone powder is commercially available from Sigma, but actin preparations from commercial sources will not give the best yields. A better source is from a laboratory that purifies actin regularly (generally, these laboratories store large frozen quantities) or to prepare the powder oneself using the method of Pardee and Spudich (1982).

2. Transfer to 13.5-ml ultracentrifuge tubes and clarify the extract by ultracentrifuging 1 hr at 185,000 × g (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.

Be sure to balance paired tubes by weight to within 0.01 g.

3. After the centrifugation, carefully collect supernatant in a 50-ml graduated cylinder. Determine the initial amount of actin monomers by measuring the absorbance at 290 nm (A_{290}) of a 1:10 dilution and using the extinction coefficient of actin $\epsilon = 0.62 \text{ M}^{-1} \text{ cm}^{-1}$ in the equation for Beer's law (see Support Protocol 1, step 35).
4. Polymerize the actin by adding 1 M KCl (final 0.1 M), 1 M MgCl₂ (final 2 mM), and 100 mM ATP (final 1 mM). Transfer to a 50-ml beaker and stir 30 min at room temperature.
5. During the polymerization, dissolve the fluorophore in anhydrous DMSO to a concentration of 100 mM in a 1.6-ml microcentrifuge tube. Warm fluorescent dye solution for 10 min to 37°C and vortex vigorously.
6. Clarify the dye solution by microcentrifuging 5 min at maximum speed, room temperature. Promptly transfer the supernatant to a fresh 1.6-ml microcentrifuge tube and keep at room temperature.
7. After polymerization, raise the pH of the actin solution to ~pH 9 for the labeling reaction by adding 1/5 vol of 1 M sodium bicarbonate.

Label actin

8. Add fluorescent dye solution to the protein solution at a ratio of 5 mole fluorophore to 1 mole actin. Add a stir bar to the beaker, cover the beaker completely with aluminum foil, and stir 45 min at room temperature.
9. After 45 min, add a second aliquot of fluorophore, equal to the amount of the first addition, re-cover the beaker, and continue stirring for an additional 45 min, room temperature.

During this time, chill the super-speed centrifuge and rotor to 4°C.

10. Following labeling, quench the reaction by adding 1 M NH₄Cl to a final concentration of 50 mM. Stir 10 min, 4°C.
11. Transfer the solution to 50-ml super-speed centrifuge tubes and pellet large aggregated material by centrifuging 10 min at 12,000 × *g* (Sorvall SS-34 rotor at 10,000 rpm), 4°C.
12. Collect the supernatant and transfer to new 50-ml screw-cap ultracentrifuge tubes. Balance the tubes to within 0.01 g and ultracentrifuge the F-actin 60 min at 185,000 × *g* (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.
13. Note the position of the pellet in the ultracentrifuge tube and aspirate the supernatant. Resuspend pellet in 5 ml of G-buffer.
14. Pool pellets in a 7-ml dounce homogenizer and homogenize slowly on ice to resuspend, being careful not to introduce bubbles; then thoroughly rinse the ultracentrifuge tubes with an additional 1 ml of G-buffer.

Depolymerize the F-actin

15. Transfer the resuspended pellets into pretreated dialysis tubing (clipped at one end to form a bag) that has been rinsed with G-buffer. Squeeze out the bubbles and seal the opposite end with another clip. Place the dialysis bag in a 1-liter graduated cylinder containing 1 liter G-buffer and a magnetic stir bar. Cover the entire cylinder with aluminum foil, and stir for 3 days in a cold room, changing the buffer once every day.
16. Collect the dialyzed actin, transfer it to 13.5-ml ultracentrifuge tubes, and clarify the actin solution by ultracentrifuging 1 hr at 185,000 × *g* (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.
17. Collect the supernatant containing G-actin into a small beaker and discard the pellet.

Polymerize G-actin to F-actin

18. Polymerize G-actin into F-actin by addition of 1 M KCl (final 0.1 M), 1 M MgCl₂ (final 2 mM), and 100 mM ATP (final 1 mM); and mix 60 min at 4°C.
19. Transfer the solution to an ultracentrifuge tube and pellet the F-actin by ultracentrifuging 60 min at 185,000 × *g* (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C.
20. Aspirate the supernatant and resuspend the pellet in 1 to 2 ml of cold G-buffer in the dounce homogenizer. Transfer to a dialysis bag.
21. Dialyze the resuspended pellet in 1 liter G-buffer in a graduated cylinder covered with aluminum foil for 3 days, 4°C, changing the buffer once every day.

Add ATP fresh to each change of dialysis buffer

22. Following dialysis, transfer the dialysate to an 13.5-ml ultracentrifuge tubes and clarify the G-actin by ultracentrifuging 1 hr at $185,000 \times g$ (Beckman 70.1 Ti rotor at 52,000 rpm), 4°C .

Analyze actin

23. Collect the supernatant and determine the concentration, total protein, percent yield, and fluorophore-to-actin ratio of the final product (see Support Protocol 1, step 35 for tubulin labeling).

The extinction coefficient for actin at 290 nm is $0.62 \text{ M}^{-1} \text{ cm}^{-1}$, the molecular mass of actin monomer is 45 kDa, and the extinction coefficient for the fluorophore is supplied by the manufacturer.

24. Drop-freeze the labeled actin in 10- μl aliquots in 0.6-ml UV-impermeant microcentrifuge tubes and store at -80°C .
25. For use for microinjection, rapidly thaw an aliquot of concentrated labeled actin and transfer immediately to ice. Dilute to 0.5 mg/ml with cold IB, and drop freeze 3- μl aliquots for use within 2 weeks.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Ammonium chloride (NH_4Cl), 1 M

5.35 g NH_4Cl
100 ml distilled H_2O
Store up to 6 months at room temperature

ATP, 100 mM

8.5 ml H_2O
0.55 g Na_2ATP
pH to ~ 7.0 with NaOH
Bring to final volume to 10 ml with H_2O
Dispense into 200- μl aliquots
Store up to 1 year at -20°C

BRB-80, 10 \times

400 mM K PIPES, pH 6.8 (see recipe)
5 mM MgCl_2 (APPENDIX 2A for 1 M)
5 mM EGTA (UNIT 13.1)
Store up to 1 year at 4°C

Buffered filming medium

14.8 g/liter Leibovitz L-15 medium powder lacking phenol red (Sigma)
7 mM HEPES, sodium salt
1 \times antibiotic/antimycotic (100 \times , Life Technologies; penicillin/streptomycin/amphotericin B)
10% (w/v) fetal bovine serum (FBS, APPENDIX 2A)
Adjust pH to 7.2 and sterile filter
Store up to 3 months at 4°C

CB/BRB-80 conversion buffer

1.18 M K PIPES, pH 6.8 (see recipe)
11 mM MgCl₂ (APPENDIX 2A for 1 M)
Store up to 6 months at room temperature

G-buffer

2 mM Tris·Cl, pH 8.0 (APPENDIX 2A)
0.2 mM CaCl₂ (APPENDIX 2A)
Add just before use:
0.2 mM ATP (see recipe for 100 mM)
0.5 mM 2-mercaptoethanol
Store up to 1 day at 4°C

Glycerol-free cellulose dialysis tubing, pretreated

Boil 10 min in 10 mM EDTA. Store in sealed jar for up to 1 year at 4°C. Rinse well with water before use.

GTP, 100 mM

Check molecular weight of GTP lot to determine the amount required for 10 ml of a 100 mM solution
8.5 ml distilled H₂O
1 ml of 1 M MgSO₄ (APPENDIX 2A)
Adjust pH to 7.0
Distilled H₂O to 10 ml
Dispense into 200- μ l aliquots, store up to 1 year at -20°C

HEPES, 1 M (pH 8.6)

119.15 g HEPES (free acid)
Distilled H₂O to 400 ml
Add solid KOH a few pellets at a time while mixing until the pH is ~8.4
Add concentrated KOH dropwise to achieve pH 8.6
Distilled H₂O to 500 ml
Sterile filter and store up to 1 year at 4°C

High-pH cushion

0.1 M HEPES, sodium salt, pH 8.6 (add from 1 M stock; see recipe)
1 mM MgCl₂ (APPENDIX 2A)
1 mM EGTA (UNIT 13.1)
60% (w/v) glycerol
Store up to 6 months at room temperature

Injection buffer (IB)

50 mM potassium glutamate
0.5 M MgCl₂ (APPENDIX 2A)
Store up to 2 years at -20°C

Labeling buffer

0.1 M HEPES, sodium salt, pH 8.6
1 mM MgCl₂ (APPENDIX 2A)
1 mM EGTA (UNIT 13.1)
40% (w/v) glycerol
Store up to 6 months at room temperature

Low-pH cushion

1 \times BRB-80 (see recipe for 10 \times)
60% (w/v) glycerol
Store up to 6 months at room temperature

Microinjection needles

For Eppendorf systems, standard femtotip microinjection needles work well. For home-pulled needles, a blunt-tipped needle with a ~0.5- to 1.0- μm i.d. tip opening should be used. Either needle type should be pre-coated inside and out with hexamethyldisilazane (HMDS, Pierce Chemical). This is done by propping the needles on a piece of modeling clay in a petri dish, putting a couple of drops of HMDS on the bottom of the dish, quickly covering the top, and putting the dish in the fume hood overnight to allow the HMDS to vaporize and coat the needles. This treatment greatly reduces needle-clogging problems. Needles may be tested by watching flow of a 1 mg/ml fluorescent dextran solution from a needle immersed in culture medium using a 40 \times lens and epifluorescent illumination. At 1- to 2-psi needle pressure, the solution should flow *very* slowly from the needle, i.e., a small “cloud” of fluorescence as big as ~1/4 the field of view should take 2 to 4 sec to form.

PIPES, 1.5 M (pH 6.8)

226.8 g PIPES (free acid)

H₂O to 400 ml

Add solid KOH a few pellets at a time while mixing until the pH is ~6.6

Add concentrated KOH dropwise to achieve pH 6.8

Distilled H₂O to 500 ml

Sterile filter and store up to 1 year at 4°C

Quench

2 \times BRB-80 (see recipe for 10 \times)

100 mM potassium glutamate

40% (w/v) glycerol

Store up to 6 months at room temperature

Sodium bicarbonate, 1 M

8.40 g NaHCO₃

100 ml distilled H₂O

Make up fresh and keep at room temperature

COMMENTARY

Background Information

Principles of FSM image formation

FSM was originally characterized for imaging fluorescent microtubules, thus it is important to understand the basics of microtubule assembly and structure in order to understand FSM images of microtubules. Similar principles apply to FSM images of actin.

Microtubules dynamically assemble in cells from a cytoplasmic pool of α/β tubulin dimers (reviewed by Desai and Mitchison, 1997). Dimers of 8 \times 5-nm are oriented head-to-tail along the 13 protofilaments that comprise the 25-nm diameter cylindrical wall of a microtubule (Fig. 4.10.4). There are 1625 dimers in 1 μm of microtubule length. Microtubules grow by dimer association with their ends. Thus, microtubules can be fluorescently labeled by attachment of chemical fluorophores to puri-

fied tubulin dimers, then incorporation of the fluorescent dimers into the ends of a microtubule polymer. As seen in Figure 4.10.1, microtubules assembled from a low fraction (~0.5%) of fluorescent tubulin appear speckled in high-resolution images, while microtubules assembled from higher fractions (~10%) of labeled tubulin appear continuously labeled along their lengths.

The resolution of fluorescent microtubules in the light microscope depends on the emission wavelength of the fluorophore (Inoué and Spring, 1997). Resolution limit in wide-field fluorescence microscopy is given by

$$r = 0.61\lambda/NA_{\text{obj}}$$

where λ is the wavelength of emission light and NA_{obj} is the numerical aperture of the objective lens. In practice, this equation means that the image of a single fluorophore will be the same

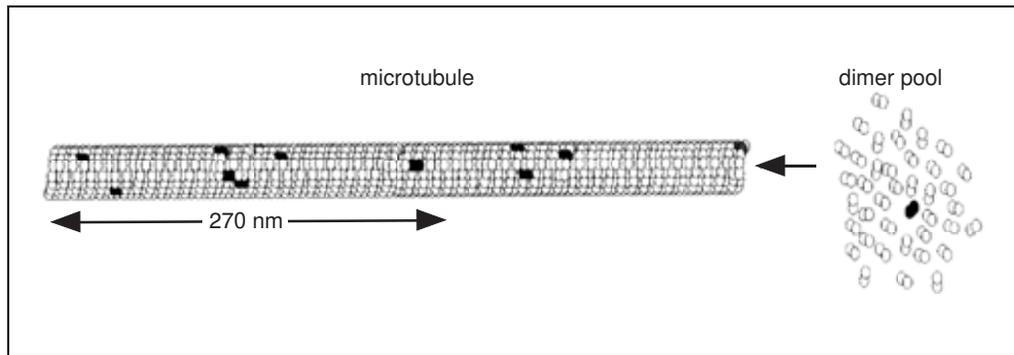


Figure 4.10.4 Stochastic growth model for how microtubules get fluorescent speckles. See Background Information. R (red fluorescence) = $0.61\lambda/NA_{\text{obj}} = 270 \text{ nm} = 440$ tubulin dimers; $M = FN = 4.4$ for 1% labeled tubulin; $SD = [Nf(1-f)]^{0.5} = 2.15$; $C = SD/M = 0.48$; as mean, M , decreases, standard deviation, SD , increases, and speckle contrast, C , increases.

size as the image of however many fluorophores that can fit in a resolution-limited spot, only the single fluorophore will be much dimmer than the group. For example, resolution is ~ 270 nm for the 620-nm fluorescence from tubulins conjugated to the X-rhodamine fluorophore. Thus, the image of a linear, 25-nm wide, red fluorescent microtubule is a linear series of resolution-limited 270-nm-wide fluorescent spots along the microtubule, with the intensity of the resolution-limited spots related to the number of fluorophores within each spot.

Analysis of the assembly of purified tubulins *in vitro* (Waterman-Storer and Salmon, 1998) has shown that fluorescent speckles in microtubule FSM images are generated by the normal stochastic association of fluorescently labeled and unlabeled tubulin dimers with growing microtubule ends (Fig. 4.10.4). Each time a dimer is added to the microtubule end, the probability that it will be a dimer with a fluorophore depends on the fraction (f) of labeled dimers in the tubulin pool. If, for example, $f = 1\%$, then each time a dimer is added to a microtubule end, there is a 1 in 100 chance it will have bound fluorophore. Over many microns of microtubule length ($1625 \text{ dimers}/\mu\text{m}$), the mean number of fluorescent dimers (M), in 270 nm, the size of the resolution-limited spot, is:

$$M = fN$$

where f is the fraction of labeled fluorophores and N is the number of dimers in 270 nm of microtubule, or 440 dimers. For a situation in which $f = 1\%$, the average number of fluorescent dimer per 270 nm is $M = 4.4$. In FSM images, the speckle pattern along the microtubule is produced by variations from the mean in the number of fluorophores per resolution-

limited spot. The standard deviation (SD) for a stochastic process is

$$SD = (Nf(1-f))^{0.5}$$

which is approximately the square root of the mean for small values of the fraction of labeled tubulin. For a mean value of 4.4, $SD = 2.1$. This high standard deviation relative to the mean explains how microtubules get fluorescent speckles as a result of a high variability in the number of fluorescent tubulin subunits per resolution-limited spot. The author defines speckle contrast (C) as:

$$C = SD/M$$

At low fractions of fluorescent dimer, the standard deviation is high relative to the mean, producing more contrasting speckles. For the microtubule FSM image seen in Figure 4.10.1, the fraction of labeled tubulin of $\sim 0.5\%$ or less is estimated. At this concentration, the number of fluorophores per diffraction-limited spot has been calculated to be between 0 and 7 (Waterman-Storer and Salmon, 1999).

The above analysis also does not consider the effects of background fluorescence on speckle contrast. As the fraction, f , of fluorescently labeled tubulin decreases, background fluorescence also decreases, giving less out-of-focus fluorescence and higher speckle contrast in the FSM images.

Critical Parameters and Troubleshooting

The critical parameters for setting up a time-lapse FSM imaging system will not be discussed here as Basic Protocol 1 dealt with this in detail. Similarly, troubleshooting hardware and software problems is virtually impossible without knowledge of the specific components

of the imaging system. These problems are best dealt with by consultation with the manufacturer as problems arise.

For successful live-cell FSM imaging, the most critical components are proper fluorescent protein concentration, successful suppression of photobleaching, and high quality, well labeled, fully functional protein. Fluorescent speckles cannot be detected if the concentration of fluorescent protein in the cell is too high. Although, the needle concentrations recommended have been used successfully, the amount of protein that gets incorporated into the cell depends on the microinjection technique. Tubulin can be diluted with IB and actin can be diluted with G-buffer to alleviate problems of overly bright cells. Conversely, if there is not enough labeled protein, the camera exposures required to capture an image will be excessively long and may result in motion artifacts in the image. This can easily be solved by increasing the needle concentration.

Photobleaching problems can occur if the imaging chamber is not fully sealed, if there are air bubbles present in the chamber, or if the potency of the Oxyrase is wearing out. Make sure the edges of the coverslip are dry when attaching it to the tape and use Oxyrase that is stored for <1 year at -80°C . Labeled tubulin or actin that is incompetent for polymerization due to denaturation or blockage of protein-protein interaction sites by dye binding will not contribute to forming fluorescent speckles by incorporating into microtubules or actin filaments, but it will surely contribute to soluble background fluorescence, making the dimly fluorescent speckled structure difficult to detect above the background. If the background is too high and the protein is not incorporating into polymers, the labeling will have to be carried out again.

The points to pay attention to during labeling of fluorescent tubulin to increase yield and produce well-labeled, functional protein is to not over-label the protein by extending the labeling reaction time, to save time by having solutions and centrifuges pre-equilibrated to the proper temperatures, and to be meticulous in resuspending pellets and collecting supernatants. After the first microtubule polymerization and centrifugation, when resuspending the microtubules for labeling, keeping the pellets warm and using warm buffer for resuspension is essential. This is a step where much tubulin can be lost if it is allowed to depolymerize prior to labeling. After centrifuging the labeled microtubules, resuspension of the labeled micro-

tubule pellet and depolymerization is another step where yield can be reduced. If the pellet is not completely resuspended, the microtubules will not depolymerize and will be lost in the subsequent clarification step—thus, be patient and homogenize slowly during the entire depolymerization incubation. A final key point to note is that small amounts (<15 mg) of starting material when carrying out these protocols should not be used. Yield for the protocols is knowingly very low, and if small amounts are used to start with, one may end with no labeled product.

Actin labeling is quite easy because one begins with such a large amount of material and actin is such a stable protein that it is difficult to be unsuccessful. One key parameter is the quality of the acetone powder. Actin will not be readily extracted from poor powder. Use the most finely powdered or “fluffiest” powder within the supply. Do not include hard chunks or dark-colored particles in the starting material for the prep as these will not have been fully acetone extracted. It is not recommended to speed the depolymerization steps by decreasing dialysis time. This will cause large losses of protein, which will fail to depolymerize and subsequently be lost in the pellet of the clarification centrifugation. One may change dialysis buffers more often, but do not decrease dialysis time. In fact, one may increase the dialysis for up to 5 days if necessary. When changing dialysis buffers, always add the ATP fresh to the buffer.

The labeling efficiency of tubulin or actin should not have dramatic effects on imaging. However if a very poor labeling is achieved (<0.5 dye per protein), the large amount of protein microinjected to get good images may have some effects on the balance of polymer/monomer in the cell.

Anticipated Results

When live cells are injected with low levels of well-prepared fluorescently labeled cytoskeletal proteins and imaged on an efficient epifluorescent microscope at high resolution with a sensitive, low-noise cooled CCD that is matched in resolution to the microscope optics, FSM images like those in Figure 4.10.1 should be obtained. In time lapse, microtubule FSM should reveal the assembly dynamics of speckled microtubule ends at the cell periphery as microtubules undergo dynamic instability, growing at 1 to 10 $\mu\text{m}/\text{min}$ and shortening at 5 to 20 $\mu\text{m}/\text{min}$, with frequent switching between these states. The speckles on microtubules

should remain constant in pattern and only change after depolymerization and repolymerization. In this regard, keep in mind that very slight shifts in focus can change the intensity of speckles dramatically. For time-lapse actin FSM, actin speckles should appear along the leading edges of migrating cells and move towards the cell center at 0.1 to 2 $\mu\text{m}/\text{min}$. In addition, within the lamella actin network, fluorescent speckles should appear and disappear as actin within the meshwork turns over. Actin in stress fibers and bundles will appear as linear arrays of speckles, and contractility can be seen as speckles move within these bundles.

Time Considerations

Relative to preparing fluorescent proteins, performing time-lapse FSM of living cells requires little time and is dependent on the biological process of interest. The cells will need to be microinjected, to recover for ~1 hr after microinjection, to be mounted for live-cell microscopy, and then to be imaged on the microscope for as long as is required by the biological process of interest. Fluorescent labeling of tubulin requires 1 full day, provided tubulin has already been purified according to UNIT 13.1, Support Protocol 3. Fluorescent labeling of actin takes 7 days with the first day, in which the labeling is carried out, being the most labor intensive, with ~6 hr required. Day 2 and 3 need 10 min each, 4 hr on day 4, 10 min on both days 5 and 6, and 2 to 3 hr on day 7. The method should yield 20 to 30 mg of labeled actin. Both of these fluorescent proteins are good stored at -80°C for a reasonably long time, although labeled actin appears to be more stable (>1 year) than tubulin (6 to 8 months).

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Two-Photon Excitation Microscopy for the Study of Living Cells and Tissues

The effective sensitivity of fluorescence microscopy, especially with thick samples, is limited by out-of-focus flare. This limitation is greatly reduced in a confocal microscope (UNIT 4.5; White et al., 1987), which uses a confocal pinhole to reject out-of-focus background and produce unblurred imaging of thin (<1 μm) optical sections. Alternatively, deconvolution microscopy, using a conventional microscope, digitally reconstructs an image using the measured point spread function of the optics (Agard et al., 1989; Carrington et al., 1990; Holmes et al., 1995; McNally et al., 1999). Two-photon excitation microscopy (also known as nonlinear optical microscopy or two-photon laser scanning microscopy; Denk et al., 1990) is another alternative to confocal and deconvolution microscopy that provides clear advantages for three-dimensional imaging. In particular, two-photon excitation excels at imaging of living cells, especially within intact tissues such as brain slices, embryos, whole organs, and even animals. This unit will describe the basic physical principles of two-photon excitation and discuss the advantages and limitations of its use in laser-scanning microscopy. Practical considerations for this technique will be highlighted in order to illustrate the utility of this technique and demonstrate some of its physical limitations. Finally, selected applications of two-photon excitation microscopy will be discussed, in order to illustrate how this technique has made possible experiments that could not have been performed otherwise.

Before performing any optical-sectioning experiment, careful consideration should be given to selecting the technique that is best suited to answer the biological question. For fluorescence microscopy on relatively thick samples, two-photon excitation often provides the most attractive solution, although complementary three-dimensional fluorescence microscopy methods each have particular benefits that make them better suited for certain experiments.

Confocal microscopy uses a pinhole to reject out-of-focus background fluorescence. Thus, this technique allows three-dimensional sectioning into thicker tissues. However, the excitation light generates fluorescence, and

thus photobleaching and phototoxicity, throughout the specimen, but it only collects signal from within the plane of focus. This can cause significant photobleaching and phototoxicity problems, especially in live samples. Also, the penetration depth in confocal microscopy is limited by absorption of excitation energy throughout the beam path and by sample scattering of both the excitation and emission photons.

Deconvolution techniques often provide the best solution for samples with relatively low out-of-focus background or for samples with low signal levels (McNally et al., 1999). Since deconvolution methods use conventional wide-field microscopes for image acquisition, the excitation intensity is generally kept low. Thus, deconvolution is usually effective for imaging monolayers of living cells. It is important to realize, though, that many so-called deconvolution methods are simply nonlinear data filters that do not generate quantitative data. Only true, constrained, iterative deconvolution methods provide quantitative data that can be used for further analysis. However, deconvolution on wide-field fluorescence microscopes provides limited penetration into thick samples, as a result of increased out-of-focus background and light scattering. Also, because of the heavy computation required, the deconvolved images cannot give immediate feedback during the experiment.

Two-photon excitation provides three-dimensional sectioning without absorption (and thus photobleaching and phototoxicity) above and below the plane of focus. Consequently, it offers increased depth penetration over confocal microscopy and can be less phototoxic to live samples. Thus, two-photon excitation microscopy is used, in preference to these other techniques, for experiments that require deep penetration into live tissue or intact animal samples. However, because the photophysics involved with two-photon excitation is different from conventional fluorescence excitation, deleterious effects are occasionally observed with two-photon excitation of certain fluorophores, which in turn limits the applicability of this method for optical sectioning in thin samples.

TWO-PHOTON EXCITATION

Principles of Two-Photon Excitation

Two-photon excitation is a relatively old concept in quantum optics. It was first proposed theoretically by Maria Göppert-Mayer in her doctoral dissertation (Göppert-Mayer, 1931) and observed experimentally shortly after the invention of the laser (Kaiser and Garrett, 1961). Thus, much of the theoretical and experimental background is well understood. Two-photon excitation arises from the simultaneous absorption of two photons in a single quantized event. Since the energy of a photon is inversely proportional to its wavelength, the two photons should be about twice the wavelength required for one-photon excitation. For example, a fluorophore that normally absorbs ultraviolet light (~350 nm) can also be excited by two red photons (~700 nm) if they reach the fluorophore at the same time (Fig. 4.11.1). In this case, “the same time” means within $\sim 10^{-18}$ sec. Because two-photon excitation depends on simultaneous absorption, the resulting fluorescence emission depends on the square of the excitation intensity. This quadratic dependence gives rise to many of the significant advantages associated with two-photon excitation microscopy (see Two-Photon Excitation in Laser-Scanning Microscopy). To obtain a significant number of two-photon absorption events (where both photons interact with the fluorophore at the same time), the photon density must be approximately a million times what is required to generate the same number of one-photon absorptions. This means that extremely high laser powers are required to generate significant two-photon-excited fluorescence. These powers are easily achieved by focusing mode-locked (pulsed) lasers, where the power during the peak of the pulse is high enough to generate significant two-photon excitation, but the average laser power is fairly low (Denk et al., 1995). In this case, the resulting two-photon-

excited state from which emission occurs is the same singlet state that is populated during a conventional fluorescence experiment. Thus, fluorescent emission after two-photon excitation is exactly the same as generated in normal one-photon excitation (Denk et al., 1995).

Another nonlinear optical process, three-photon excitation, may also prove useful for biological experiments (Maiti et al., 1997). Three-photon excitation works in much the same way as two-photon excitation, except that three photons must interact with the fluorophore at the same time. Because of the quantum-mechanical properties of fluorescence absorption, the photon density required for three-photon excitation is only about ten-fold greater than what is needed for two-photon absorption (rather than another million-fold greater). This makes three-photon excitation attractive for some experiments. For instance, an infrared laser (~1050 nm) can bring about three-photon excitation of an ultraviolet-absorbing fluorophore (~350 nm) and simultaneously produce two-photon excitation of a green-absorbing fluorophore (~525 nm). Three-photon excitation can also be used to extend the region of useful imaging into the deep ultraviolet (i.e., by use of 720-nm light to excite a fluorophore that normally absorbs at 240 nm). This can be useful, since ultraviolet wavelengths below ~300 nm are very problematic for regular microscope optics. Higher-order nonlinear effects are also possible and have been experimentally demonstrated (e.g., four-photon absorption), although it is unlikely that they will find any immediate application in biological research.

Two-Photon Excitation in Laser-Scanning Microscopy

The powerful advantages of using two-photon excitation in laser-scanning microscopy arise from the basic physical principle that the absorption depends on the square of the excitation intensity. In practice, focusing a single

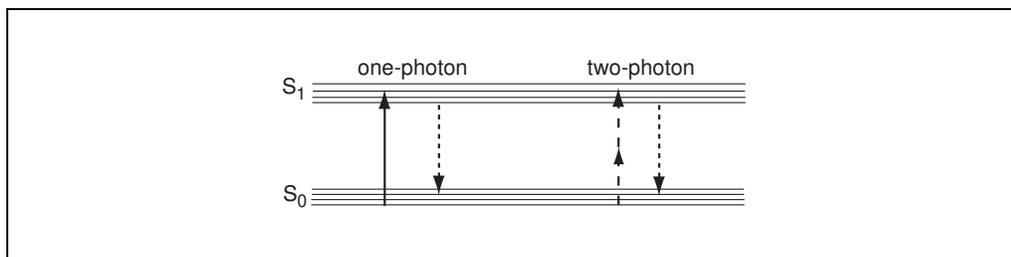


Figure 4.11.1 Jablonski diagram of a single light blue photon (solid black arrow) being absorbed to generate an excited state (left). Simultaneous absorption of two photons of red light (dashed arrows) can produce the identical excited state.

pulsed laser through the microscope optics generates two-photon excitation. As the laser beam is focused, the photons become more crowded, and the chance of two of them interacting simultaneously with a single fluorophore increases. In fact, the focal point is the only place where the photons are crowded enough to generate an appreciable amount of two-photon excitation. This is illustrated in Figure 4.11.2. Above the focal point, the photon density is not high enough for two photons to be within the absorption cross-section of a single fluorophore at the same time. However, at the focal point, the photons are so crowded that it is possible to find two of them within the absorption cross-section of a single fluorophore simultaneously. In practice, two-photon excitation microscopy is made possible not only by crowding the photons spatially (by focusing in the microscope), but also by concentrating the photons in time (by using the pulses from a mode-locked laser). This gives the needed excitation intensities for two-photon excitation,

but the pulse duty cycle (the duration of the pulse divided by the time between pulses) of 10^{-5} limits the average input power to <10 mW, which is just slightly greater than what is used in confocal microscopy. The pulse durations are considered ultrashort, typically from ~ 100 femtoseconds to ~ 1 picosecond (10^{-13} to 10^{-12} sec), but from the perspective of the fluorophore (where the absorption event is on the 10^{-18} sec time scale), they appear to be quite long in duration.

The localization of two-photon excitation to the focal point provides most of the advantages over confocal microscopy. In a confocal microscope, fluorescence is excited throughout the sample, but only signal from the focal plane passes through the confocal pinhole, so background-free data can be collected. By contrast, two-photon excitation only generates fluorescence at the focal plane, so there is no background, and no pinhole is required. This dramatic difference between confocal and two-photon excitation microscopy can be demon-

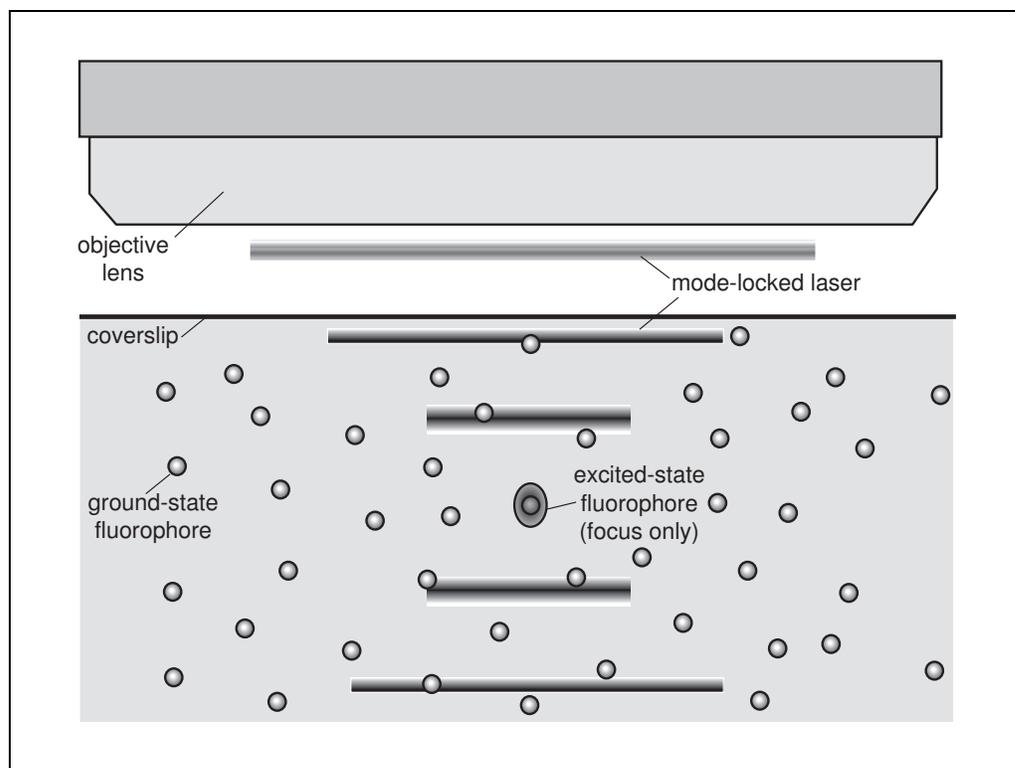


Figure 4.11.2 Demonstration of photon crowding that occurs at the focus of a microscope. As the pulses of red laser exit the objective lens and pass through a sample containing fluorophores (small circles), there is nearly zero probability of two photons passing simultaneously within the cross-section of a single fluorophore located outside the focal point. This is true even during the peak of each laser pulse. However, because of the extremely high photon density at the focal point, it is possible for two photons to interact simultaneously with a fluorophore, which becomes excited. To reach a sufficiently high concentration, the light is crowded both spatially (to the focus of the objective lens) and temporally (through the pulses of the mode-locked laser).

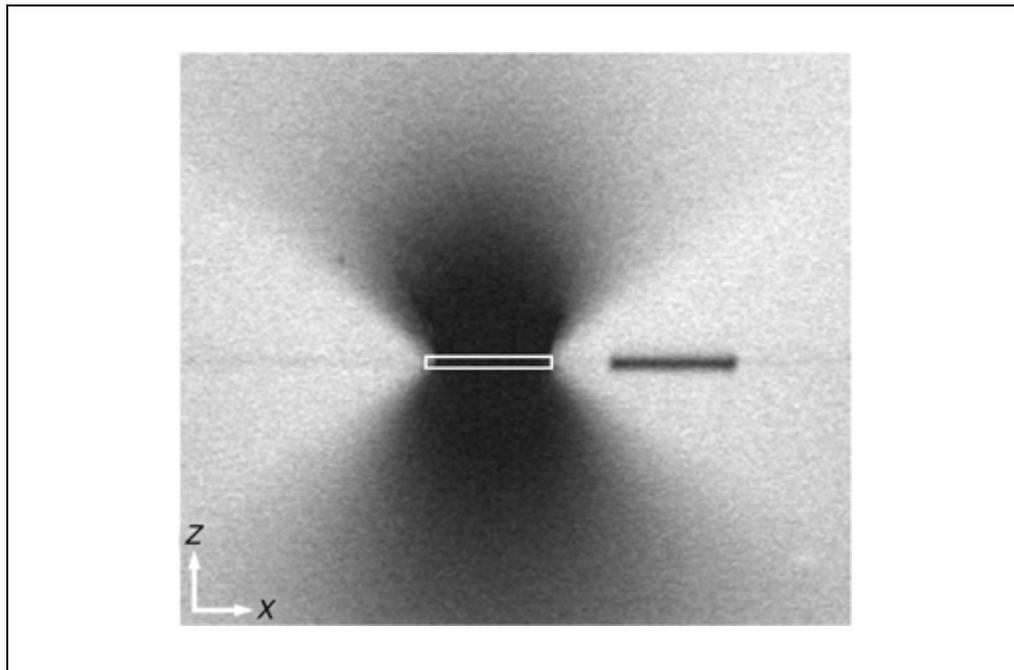


Figure 4.11.3 Photobleaching (x - z) profiles caused by one-photon and two-photon excitation. The x - z profile of the excitation pattern in a confocal microscope (left side pattern) formed by repeatedly scanning a single x - y optical section in a thick film of fluorescein-formvar until fluorescence was completely bleached. The white box represents the focal plane or the region from which data was collected by the confocal microscope. Nearly uniform bleaching occurred both above and below the focal plane. The same excitation pattern is shown for two-photon excitation (right side pattern). Two-photon excitation causes no photobleaching (excitation) outside the focal plane.

strated by imaging the photobleaching patterns of each method. Figure 4.11.3 shows the x - z photobleaching pattern that arises from repeated scanning of a single x - y plane (or the image plane) in a fluorescein-stained formvar film. The confocal microscope laser excites fluorophores above and below the focal plane (shown by the white box); hence bleaching is observed in these areas (Fig. 4.11.3; left). In contrast, two-photon excitation only occurs at the focal plane and confines bleaching to this area (Fig. 4.11.3; right).

The localization of excitation yields many advantageous effects. First, the three-dimensional resolution of a two-photon excitation microscope is identical to that of an ideal confocal microscope. Second, because there is no out-of-focus absorption, more of the excitation light penetrates through the sample to the plane of focus. This can lead to greatly increased sample penetration—generally at least two to three times deeper than is possible with confocal microscopy (Centonze and White, 1998). Third, as shown in Figure 4.11.3, use of two-photon excitation minimizes photobleaching and photodamage, two of the most important limitations in fluorescence microscopy of living cells and tissues. Although damage to cells

owing to interactions with light is poorly understood, decreasing photodamage will lead to extended viability of the biological samples under investigation. Since practical experience indicates that the red excitation light alone does not affect cell viability, it is likely that most of the photodamage is associated with two-photon absorption and thus occurs only at the focal plane.

Two-photon excitation microscopy does not require a pinhole to obtain three-dimensional resolution, which allows flexible detection geometries. The geometries for both descanned- and nondescanned-detection two-photon excitation are shown in Figure 4.11.4. In the descanned geometry, the emitted light (represented by the dotted pattern) returns through the same path as the excitation light hitting the scanning mirrors before passing through the confocal pinhole to the detector. In confocal microscopy, this geometry is necessary to block the detection of out-of-focus emission. Nondescanned beam paths provide more alternatives: (1) a dichroic mirror, located directly after the objective lens, reflects the emitted light through a transfer lens to a detector placed in a plane conjugate to the objective rear aperture (“conjugate plane detector” in Fig. 4.11.4); (2) the

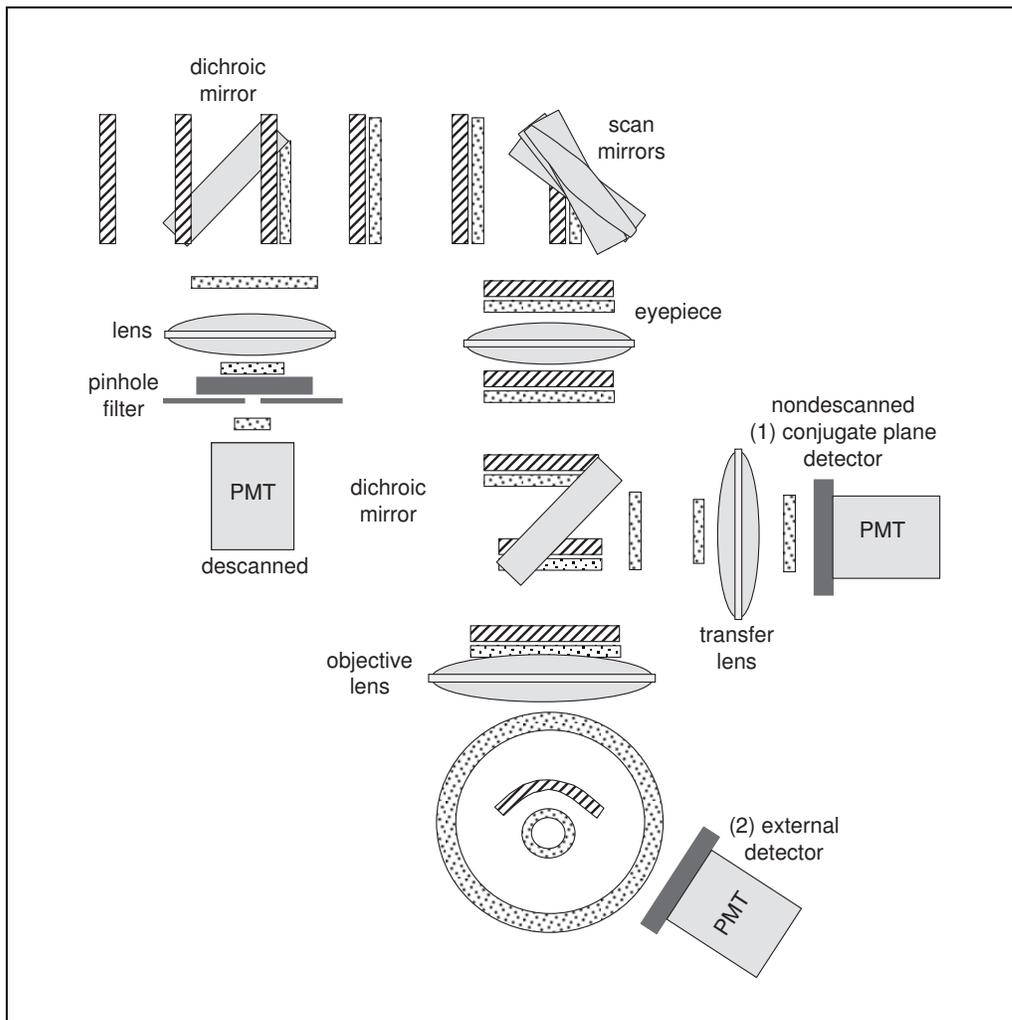


Figure 4.11.4 Descanned and nondescanned detection geometries used with two-photon excitation microscope. The incoming laser light is raster scanned (x and y scan mirrors), and is focused onto the sample by the objective lens. The descanned detection path returns through the excitation beam path, passing back through the scanning mirrors, and it is focused through the confocal pinhole to the photomultiplier tube (PMT). With nondescanned detection, the fluorescence: (1) returns through the objective lens and then is reflected by a dichroic mirror through a transfer lens that focuses the light so that the back aperture of the objective is conjugate to the front face of the PMT, or (2) is detected directly from the sample using an external PMT.

emitted light is collected, without passing through the objective lens, directly from the sample using an external detector (“external detector” in Fig. 4.11.4); or (3) the emitted light is reflected by a dichroic mirror to a charged-coupled device (CCD) camera at the intermediate image plane, in order to collect the wide-field image (not shown in Fig. 4.11.4). This last type of geometry is used for fast data acquisition systems using two-photon excitation. Although it is possible to use descanned detection for two-photon excitation, using a nondescanned alternative (listed as 2 above) is recommended to fully utilize the depth penetration of this technique. The nondescanned path allows for collection of more scattered photons, re-

quires fewer optical elements (e.g., mirrors, lens), and reduces the distance over which dust particles in the air will interfere with the fluorescence signal. Therefore, using nondescanned detection with two-photon excitation dramatically increases collection efficiency and is a must for maximal depth penetration into living tissue.

PRACTICAL CONSIDERATIONS FOR TWO-PHOTON EXCITATION MICROSCOPY

Mechanism of Deep Sectioning

As mentioned above, the most powerful advantage of two-photon excitation microscopy

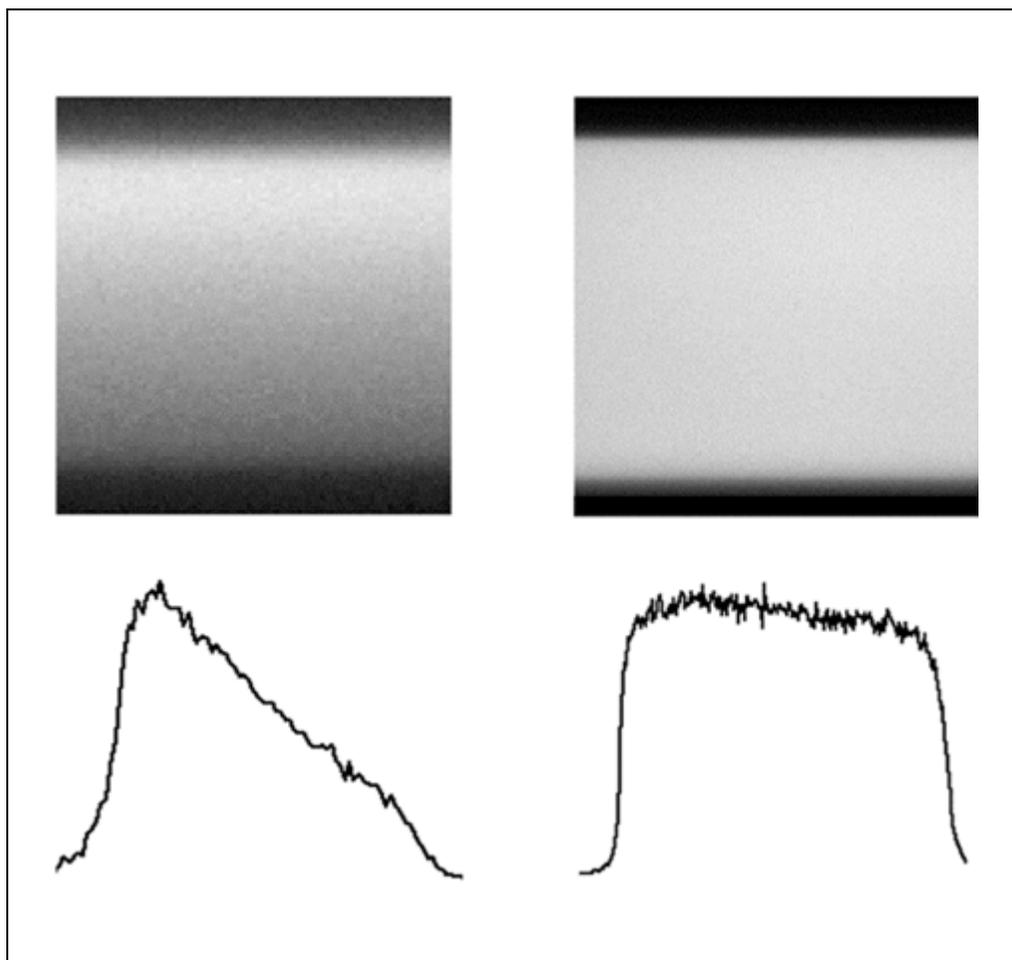


Figure 4.11.5 Top: x - z images of fluorescence and z -profiles from a rhodamine-stained polymer film (no scattering). Bottom: Intensity profiles from z -scan of x - z scan. Because there is no out-of-focus absorption in two-photon excitation, much more excitation light reaches deep into the sample so the signal is brighter and the imaging depth larger.

is its ability to provide superior optical sectioning deep into thick samples. Thus, it is important to understand how this increased depth of penetration is achieved. There are three physical mechanisms that combine to allow this increased effectiveness in thick samples: (1) lack of out-of-focus absorption allows more of the excitation light to reach the sample; (2) the red and infrared light used in two-photon excitation is scattered less than bluer light; and (3) the effects of light scattering are less detrimental to two-photon microscopy than confocal microscopy. These three points can be considered separately.

The first point is that the lack of out-of-focus absorption in two-photon excitation microscopy allows more of the excitation light to reach the focal plane. In a confocal microscope, excitation photons are absorbed by any fluorophores along the excitation light path, so fewer photons reach the focus, thus decreasing the signal. This effect becomes worse if the sample contains fluorophores throughout, as shown in

Figure 4.11.5. The polymer sample shown in this figure is nonscattering but contains a uniform distribution of a high fluorophore concentration. In this figure, the top of the x - z scan is closest to the objective lens, and the fluorescence intensity is plotted as a function of depth into the sample (z distance) for each x - z scan. In the case of one-photon excitation (confocal microscopy), the intensity shows a steady decrease with penetration depth as the excitation light is absorbed before it reaches the deeper focal planes. In contrast, two-photon absorption only occurs in the focal plane, so there is no absorption of excitation light by fluorophores between the objective lens and the focal plane. Thus, all of the excitation reaches the focal plane, which keeps the fluorescence signal constant throughout the depth of the polymer. This dramatic difference between confocal and two-photon excitation is shown on the right in Figure 4.11.5, where the intensity is relatively stable with penetration depth.

The second point is that the redder excitation light used in two-photon excitation microscopy is less scattered by the sample than is bluer excitation light used in conventional excitation. Biological tissue can be considered as a medium with a nonuniform index of refraction. Light traveling through such a medium is scattered off in various directions. In fluorescence microscopy, the incoming excitation light can be scattered away before it reaches the focal plane, and the resulting fluorescence can also be scattered as it passes back through the sample before it reaches the detector. Both of these effects will reduce the collected fluorescence signal. Because of the irregular distribution of material within biological samples, it is not possible to calculate the scattering behavior precisely. However, the simplest approximation, Rayleigh scattering, gives a minimal estimate of the fraction of light scattered in such a system. For this case, the amount of scattered light is inversely proportional to the fourth power of the light's wavelength ($1/\lambda^4$). Using this estimate, 488-nm (one-photon) light would be expected to scatter ~7-fold more than 800-

nm (two-photon) light. Thus, even more of the two-photon excitation laser light can reach the focal plane, which further increases the depth of penetration into the sample. In practice, scattering from tissue structures is always greater than is predicted by the Rayleigh approximation, but it is always the case that longer (redder) wavelengths are scattered less than shorter (bluer) wavelengths. On the detection arm of the microscope, the emitted fluorescence is identical regardless of whether it was generated using one- or two-photon excitation, so scattering of fluorescence emission affects both methods equally.

The third point is that any scattering of the excitation or fluorescence light does not affect signal collection as significantly as it does in confocal microscopy. This is due simply to the physics of image formation in two-photon excitation microscopy. While the lack of out-of-focus absorption and the differences in scattering both allow for increased excitation light to reach the focal plane deep within intact tissue, this third point actually allows for increased image contrast with two-photon excitation. The

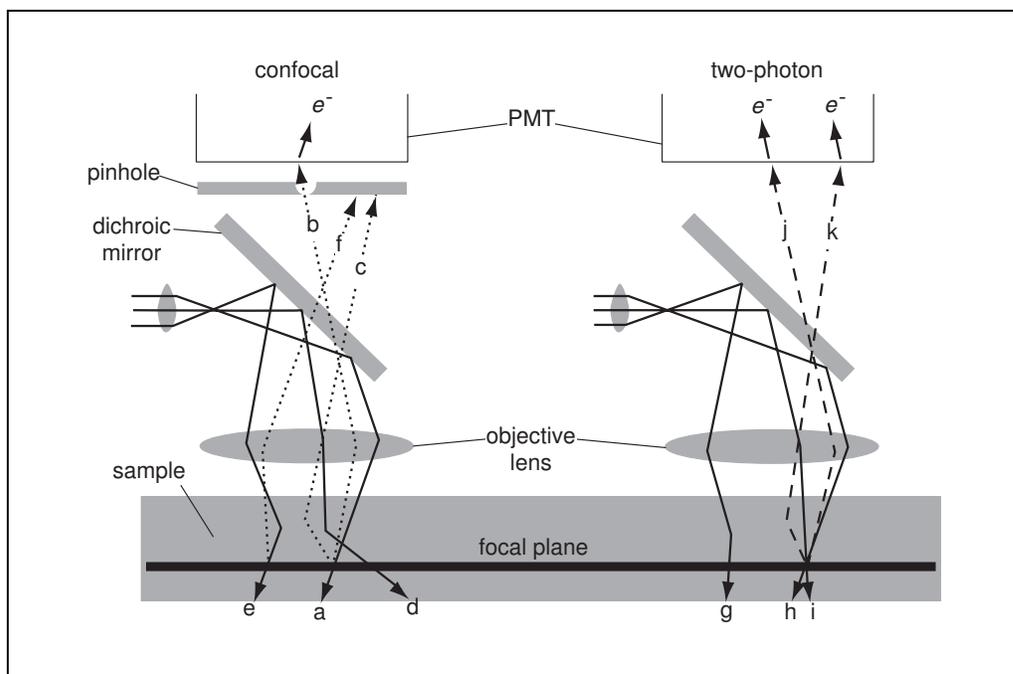


Figure 4.11.6 In confocal microscopy (shown on the left), excitation light reaches the focus (a), and fluorescence from the focus is collected and passes through a pinhole (b). Fluorescence that is scattered (c) does not pass through the pinhole, thus reducing signal. Any scattering of the excitation beam (d and e) can cause fluorescence (f), which may add background to the image. This fluorescence is inefficiently collected but can cause a background fog. For two-photon excitation microscopy (shown on the right), any scattering of a single excitation photon (g) will not cause background, since the chance of two photons scattering to the same place at the same time is essentially zero. Scattering and out-of-focus absorption are reduced with two-photon excitation, resulting in an increase in the amount of excitation light reaching the focus (h and i). Unscattered fluorescence is collected by the PMT (j). Further, because no pinhole is needed, any scattered fluorescence photons can still be collected, thus greatly increasing the collected signal (k).

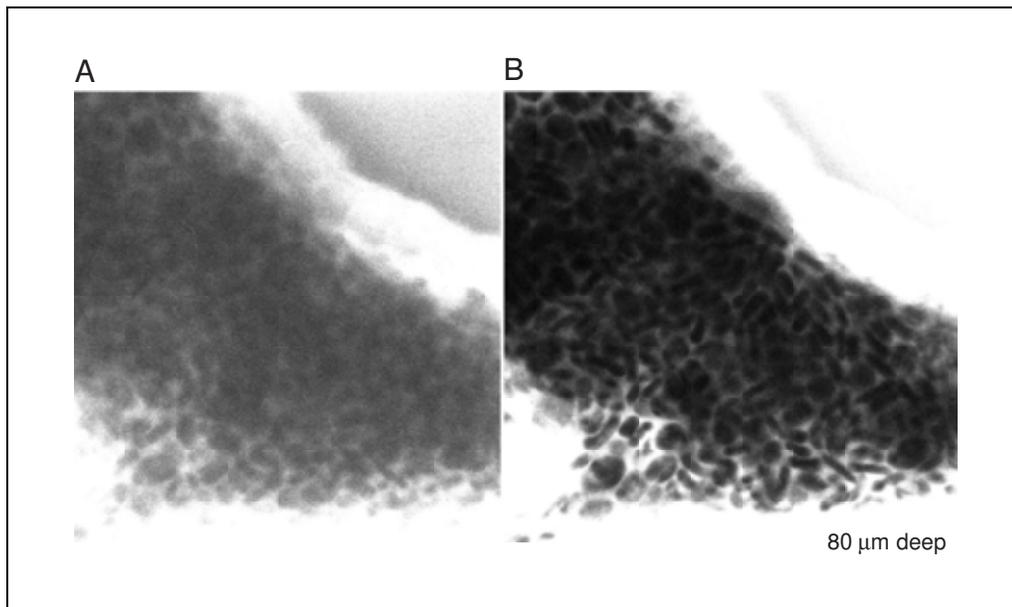


Figure 4.11.7 A shark choroid plexus stained with fluorescein. These images were collected 80 mm into the sample, where the image quality of the one-photon microscope is minimally acceptable. In contrast, using two-photon excitation at this same focal plane allows the collection of an image with excellent intensity contrast.

reasons behind this point are illustrated in Figure 4.11.6 (Denk and Svoboda, 1997).

In a confocal microscope, the excitation light (blue) is focused into the sample (a, in Fig. 4.11.6), and the fluorescence (green; represented by dotted lines) from that focal spot is captured by the objective lens, passes cleanly through the pinhole, and reaches the detector (b). This fluorescence light is the desired signal, but some of it can be scattered as it passes back through the sample (c). This scattered fluorescence does not pass through the pinhole and is therefore lost and not detected. These losses greatly reduce the detected fluorescence signal. As the excitation light passes through the sample, it may be absorbed (d) or scattered before it reaches the focus (e). If it is absorbed, it can generate fluorescence. Since this fluorescence does not arise from the focal spot, it does not pass through the pinhole, so it is not efficiently detected. However, a small portion of out-of-focus fluorescence can be scattered into the pinhole and then be detected. This fluorescence will create a background fog that will be roughly constant across the image, as shown in the examples below. This fog reduces the dynamic range of the image, thus reducing the image contrast. Likewise, the scattered excitation can generate fluorescence (e), and this fluorescence can also contribute to the background fog (f).

In the case of two-photon excitation, the excitation photons (red; represented by dashed

lines in Fig. 4.11.6) can also be scattered (g). However, the chance of two photons being scattered simultaneously to the same place is essentially zero, so that the background fog that plagues confocal microscopy in thick samples is not generated in two-photon excitation. Also, more of the excitation light reaches the focal plane (h and i) due to the two points discussed above—i.e., the reduced out-of-focus absorption and the decreased scattering of the redder two-photon excitation light. Importantly, the generated fluorescence (green) has an increased chance of being detected by the photomultiplier tube (PMT) even if scattered (j) because no pinhole is present to block it (k). This insensitivity to scattering effects and lack of out-of-focus absorption allow for the preservation of the full image contrast from deep within samples.

A comparison of confocal and two-photon microscopy imaging quality is shown in Figure 4.11.7 of a shark choroid plexus stained with fluorescein. These images were collected 80 μm into the sample, which is the maximal depth for sufficient image contrast from confocal microscopy for this sample. While the signal level of the brightest features can easily be matched between the two methods, the background fog seen in the confocal image greatly reduces the image contrast. The two-photon excitation image, on the other hand, shows excellent intensity contrast. However, because scattering of the fluorescence is significant in thick biologi-

cal samples, the use of descanned detection and an open pinhole is not enough to gain the advantages of two-photon excitation (Centonze and White, 1998). To gain the full advantage, a nondescanned detection scheme (Fig. 4.11.4, where the fluorescence does not pass back through the scanning system as it must in a confocal microscope) must be used to increase the fluorescence collection efficiency (Piston et al., 1994). Using the same shark sample shown in Figure 4.11.7, a comparison of imaging using descanned (pinhole-open) and nondescanned detection is shown in Figure 4.11.8. In each detection geometry, the same imaging optics (i.e., dichroic mirror, barrier filter, and PMT detector) were used. The sample was first imaged using descanned detection at approximately the largest depth (140 μm) that allowed some intensity contrast (Fig. 4.11.8A). Keeping all settings constant and switching to nondescanned detection, the image shown in Figure 4.11.8B was collected. This image is clearly saturated in many regions of the image, demonstrating the improved signal collection with this detection geometry. Because the excitation was identical in both cases, this 8-fold increase in signal is due solely to the collection of scattered fluorescence photons. To obtain a nonsaturated image, the PMT voltage was reduced from 1000 to 750 V (Fig. 4.11.8C). Clearly, this sample can now be scanned even more deeply using the nondescanned detection. In fact, the depth of penetration in this sample was not limited by the tissue but by the working distance of the objective lens.

Image Resolution

The resolution of two-photon excitation is no better than that achieved with a well-aligned confocal microscope. In fact, the use of longer excitation wavelengths (i.e., red or infrared, instead of ultraviolet or blue) results in a larger resolution spot for two-photon excitation (Sheppard and Gu, 1990). If a biological structure cannot be resolved in the confocal microscope, it will not be resolved in a two-photon excitation laser-scanning microscope either. This point is well understood by experts in the field, but it is often missed by prospective users in the biomedical research community.

Imaging Thick Samples

As mentioned above, there are three reasons why two-photon excitation is more effective in thick samples: lack of out-of-focus absorption allows more of the excitation light to reach the sample, the red exciting light is less scattered, and the effects of fluorescence scattering are less detrimental to two-photon microscopy than to confocal microscopy. When utilizing long-working-distance optics and a nondescanned detection mechanism, the depth of penetration and image quality is often limited by the ability to effectively label the tissue—i.e., with increasing depth it becomes increasingly difficult to introduce fluorescent labels into tissues. The expression of green fluorescent protein (GFP) in transgenic animals is likely to enhance the usefulness of two-photon excitation in vivo imaging. Transgenic animals promise enormous opportunity for the ability to fluorescently label specific organs and pro-

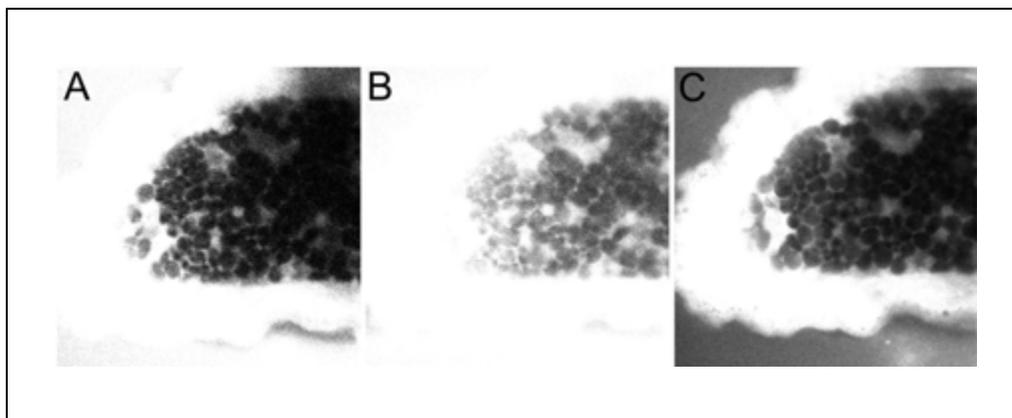


Figure 4.11.8 A comparison of descanned and nondescanned two-photon scanning depth. The sample was first imaged using descanned detection at approximately the longest depth (140 μm) that allowed achieving some intensity contrast (**A**). Keeping all settings constant and switching to nondescanned detection, the image shown in (**B**) was collected. This image is clearly saturated in many regions of the image demonstrating the improved signal collection with this collection geometry. In order to obtain a non-saturated image, the photomultiplier tube gain was changed from 1000 to 750 V (**C**).

teins of living animals for detection using two-photon excitation. Alternatively, tissue characteristics may also limit depth of the penetration. This is especially a concern in either heavily pigmented tissue, such as liver, or highly scattering tissue, such as skin.

Imaging Thin Samples

For imaging of thin samples, two-photon excitation might not offer significant advantages over conventional confocal microscopy. This is because of slightly increased photobleaching in the focal plane (the total photobleaching in a thick sample is still much reduced, though; see Fig. 4.11.3; Patterson and Piston, 2000). One example where two-photon excitation is beneficial, even for thin preparations, is the imaging of ultraviolet-excited fluorophores such as NADH (see Examples of Two-Photon Excitation Microscopy). In such experiments, ultraviolet light appears to be more harmful than the two-photon induced photobleaching. To determine if two-photon excitation would be beneficial, it is always worthwhile attempting the experiments on a confocal microscope first. Once it is known what the limitations of the confocal microscope are for the measurements, it can easily be determined whether the use of two-photon excitation would be advantageous for completing the experiments.

Absorption Spectrum

Two-photon absorption spectra can bear little resemblance to the corresponding single-photon spectra (Birge, 1986). Experience so far indicates that most fluorophores work pretty well with two-photon excitation at twice the wavelength of their one-photon absorption peak. For reasons beyond the scope of this unit, fluorophores with a nonsymmetric chemical structure tend to follow this rule more closely than symmetric ones. For example, fluorescent proteins (e.g., CFP, GFP, and YFP) have a nonsymmetric fluorophore and absorb well at twice their one-photon excitation. However, to take full advantage of two-photon excitation microscopy, these absorption spectra must be measured, which is considerably more difficult than measuring conventional one-photon absorption spectra. Only a few sources for this information exist (Birge, 1986; Xu et al., 1995, 1996; Albota et al., 1998; Neu et al., 2002), although, as this technique becomes more widespread, it is likely that two-photon absorption spectra will become more generally available.

Localized Photochemistry

Two-photon excitation also allows the initiation of photochemical reactions in the focal region. These chemistries involve ultraviolet light-induced reactions for which two-photon excitation can be substituted. For example, the uncaging of a dye, photochemically inducing a nonfluorescent molecule to become fluorescent, can be initiated in individual cells of a tissue using two-photon excitation. Alternatively, biological stimulators or suppressants can also be uncaged. Methods for two-photon-excited uncaging have not been fully developed, largely because the photoreactions can be quite slow (from milliseconds to seconds). Thus, the compound might diffuse over several micrometers within the sample between the time when it is excited and the time it becomes active. Nonetheless, this technique holds the promise of interesting biological applications, some of which will be discussed below.

Laser Sources

While the instrumentation requirements for two-photon excitation microscopy are largely indistinguishable from those for confocal microscopy, the laser excitation source is considerably different. Two types of ultrafast mode-locked laser systems are in general use today: Ti-sapphire lasers and Nd-YLF lasers. These systems do not require water cooling and plug in to regular electrical outlets, but they are considerably more expensive than the small air-cooled lasers used in confocal microscopy. The wavelength tunability of the Ti-sapphire laser (700 to 1100 nm) makes it more versatile than the single-wavelength Nd-YLF laser (1047 nm). Currently, computer-controlled Ti-sapphire lasers cover the wavelength range of 720 to 900 nm in an automated fashion. Further improvements in the ease of use and versatility of these systems are likely to continue in the near future.

Laser Power

The power necessary to excite a fluorescent sample has an optimal limit. Fluorescence intensity increases with increasing power, but the fluorophore eventually becomes saturated. Saturation occurs at laser powers that cause a significant proportion of the fluorescent molecules to exist in their excited rather than ground state (~1 mW at the sample for one-photon excitation, ~50 mW at the sample for two-photon excitation; Denk et al., 1990); therefore, more photons simply are unable to excite more molecules. Any additional excitation energy

beyond saturation contributes to increased photodamage and photobleaching. For each experimental setup, one needs to assess the damage imposed during beam scanning; it should be noted that trivial cell-viability tests (such as esterase activity or dye exclusion) do not always accurately reflect cellular photodamage. Often, a more rigorous functional test is more informative. For example, the viability of hamster embryos was confirmed by their continued development (Squirrell et al., 1999), and the viability of pancreatic islets was confirmed by their maintenance of normal glucose-stimulated NAD(P)H response (Bennett et al., 1996).

EXAMPLES OF TWO-PHOTON EXCITATION MICROSCOPY

This section will highlight some of the available literature to illustrate common situations where two-photon excitation is preferable to confocal imaging. These examples will focus on the benefits of two-photon excitation due to its reduced phototoxicity, increased tissue imaging depth, and ability to initiate localized photochemistry. The details of these experiments will be left to the specified references.

Two-photon excitation is generally less phototoxic than confocal microscopy. This is well demonstrated by time-lapse imaging of hamster embryo development (Squirrell et al., 1999). In this study, the embryo's development was monitored continuously for more than 10 hr using two-photon excitation of a vital mitochondrial dye. Conversely, normal embryo development ceased after only a few minutes of confocal laser exposure. It was thought that the two-photon excitation laser (1047 nm) greatly increased the embryo's viability. These researchers also used two-photon excitation microscopy to assess the effects of inorganic phosphate on hamster embryo development (Ludwig et al., 2001). In this study, the mitochondrial distribution of living hamster embryos cultured in varying amounts of inorganic phosphate was imaged at 6 hr of culture using two-photon excitation microscopy. The embryos were developed further prior to morphological assessment at 27 and 51 hr of culture. It was clear from these studies that the two-photon illumination was nonperturbing to the development of these embryos, while parallel confocal imaging damaged the embryos.

The nontoxic nature of two-photon excitation has allowed *in vivo* imaging of human skin (Masters et al., 1997). This work involved detailed spectroscopy of the autofluorescent signals from skin at various depths (0 to 50 and

100 to 15 μm) using excitation wavelengths ranging from 730 to 960 nm. When used in conjunction with reflected-light confocal microscopy, two-photon excitation microscopy nondestructively provides detailed white light and autofluorescent images of skin layers from the same region of skin (Masters and So, 1999).

Two-photon excitation is also used to avoid the phototoxic effects of ultraviolet irradiation. This feature is especially useful for the imaging of the naturally occurring reduced pyridine nucleotides—NAD(P)H—as an indicator of cellular respiration. NAD(P)H has a small absorption cross-section and a low quantum yield and absorbs in the ultraviolet. Thus, it is difficult to measure, and its imaging has the potential to cause considerable photodamage. NAD(P)H imaging has proven useful for studying the pathophysiology of cultured partially differentiated L6 myotube cells (Perriott et al., 2001). In cellular NAD(P)H images, the autofluorescence pattern reflects primarily NADH in mitochondria as spotted regions over a diffuse cytoplasmic signal. In differentiated cells, the fluorescence is evident as columns of mitochondria between muscle fiber striations. With increasing glucose concentration, an increase in fluorescence was readily apparent. Overall, this study showed that the kinetics of glucose utilization in real time can be defined in a single cell or averaged over several cells, and that there is homogeneity in glucose metabolism.

Quantitative two-photon imaging of NAD(P)H has also been done on individual β cells within the pancreatic islet (a quasi-spherical micro-organ consisting of ~1000 cells; Bennett et al., 1996). Furthermore, the spatial resolution of this technique allows the separation of both the cytoplasmic and mitochondrial NAD(P)H signals (Patterson et al., 2000). Figure 4.11.9 is a typical image of β cell NAD(P)H autofluorescence within an intact islet, displaying a signal from both the cytoplasm and mitochondria. The outlines of single cells are visible, as are the nuclei, both of which appear dark. The separation of cytoplasmic and mitochondrial signals allowed detailed examination of the metabolism of glucose and pyruvate in these regions of pancreatic islet β cells. Current models for glucose-stimulated insulin secretion (GSIS) suggest that metabolites further along the signal-transduction pathway should cause a similar cascade of signaling events and lead to insulin secretion. However, pyruvate potentiates GSIS but does not induce insulin secretion on its own. Using two-photon imaging of NAD(P)H and separation of cyto-

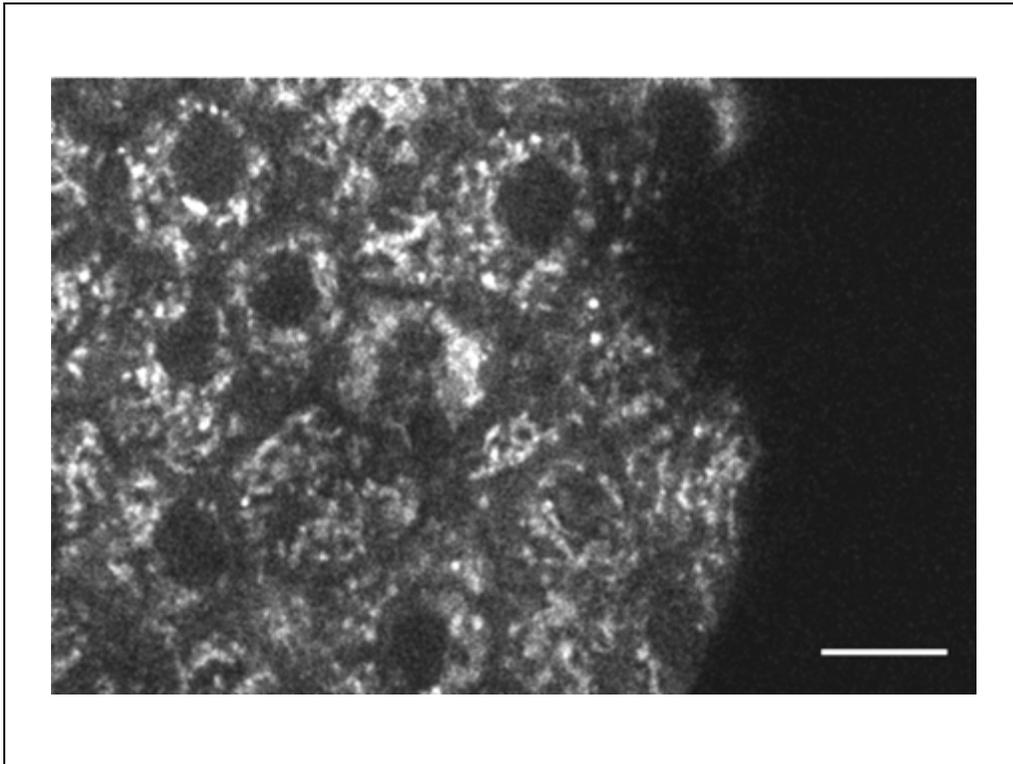


Figure 4.11.9 Optical section of NAD(P)H autofluorescence from an intact pancreatic islet. NAD(P)H signal arises from both the cytoplasm and mitochondria, the latter being brighter and somewhat punctate. Cell outlines and nuclei—where there is little or no NAD(P)H—appear dark. Bar, 10 μm .

plasmic and mitochondrial signals, it was shown that β cells metabolize pyruvate, although transiently (Rocheleau et al., 2002). This transient mitochondrial response suggests two separate models, which are currently under study: either mitochondrial pyruvate transport is inhibited during late pyruvate metabolism or the tricarboxylic cycle is inhibited during late pyruvate metabolism. In these studies, living pancreatic islets were repetitively scanned at sampling rates that are unobtainable by biochemical methods. Such repetitive imaging simply cannot be performed using confocal microscopy, owing to the limitations imposed by photobleaching and ultraviolet-induced photodamage.

Because two-photon microscopy uses mode-locked (pulsed) lasers, it is ideal for extension into fluorescence lifetime imaging (Piston et al., 1992; French et al., 1998). Imaging of the nanosecond fluorescence decay times gives information that is independent of fluorophore concentration. For instance, lifetime imaging can give an unambiguous value for fluorescence resonance energy transfer (FRET) efficiency between two probes (Harpur et al., 2001). Recently, two-photon-excited lifetime imaging microscopy of NAD(P)H has been

used to quantify the NAD(P)H concentration in different subcellular compartments. Free NADH levels in the nucleus regulate the corepressor CtBP, involved in cell-cycle regulation and transformation transcriptional pathways. Using two-photon NAD(P)H microscopy and lifetime imaging, it was shown that the free NADH levels in the nucleus closely correspond to the half-maximal concentration for CtBP binding (Zhang et al, 2002).

The technique of two-photon excitation is also useful with other established biophysical techniques, such as fluorescence correlation spectroscopy (FCS; Schwille et al., 1999) and fluorescence recovery after photobleaching (FRAP; *UNITS 13.5 & 21.1*; Brown et al., 1999). Both techniques generally utilize stationary one-photon (continuous-wave) lasers. FCS determines the occupation number and diffusion characteristics of fluorescent probes inside the focal volume of the stationary beam, and it has proven useful in molecule-interaction and diffusion studies. FRAP, through photobleaching of fluorescence in the focal area and observation of fluorescence recovery, has been used to study macroscopic diffusion of fluorescent molecules. These techniques have both been used extensively to examine the diffusion char-

acteristics of fluorescent probes on cultured cell membranes. Currently, the complexity of these two techniques has limited the utility of these techniques to *in vitro* systems and cell culture models. The well defined excitation volume in two-photon microscopy is useful for quantitative FCS and FRAP. Furthermore, these techniques are likely to show great utility in studying bimolecular dynamics in thick living tissues using two-photon rather than one-photon excitation.

Deep penetration with two-photon excitation allows *in vivo* imaging, although a number of complexities need to be overcome when imaging living animals. *In vivo* fluorescent imaging involves two-photon excitation imaging of a living animal through the skin, through surgical openings, or through coverslip windows placed onto the animal. When working with live animals, it is often more difficult to fluorescently label the sample. Specific labeling of neurons in living mice, using a Ca^{2+} indicator, has been done to monitor neural function using two-photon excitation (Svoboda et al., 1997). The expression of green fluorescent proteins (GFP) in transgenic animals to fluorescently label specific organs and proteins is likely to permit new applications of two-photon excitation *in vivo* imaging (Potter et al., 2001). Another complexity when working with living animals is that the samples can move during the imaging process. Currently, *in vivo* studies are done with anesthetized animals, and imaging rates are increased in order to limit the effect of this movement. In the future, it is likely that technological advances such as the miniaturization of two-photon microscopes for attachment directly to the animal (Helmchen et al., 2001) will allow *in vivo* imaging of freely moving animals.

The microcircuitry of neurons in brain slices has been mapped using two-photon imaging (Kozloski et al., 2001; Peterlin et al., 2001). These researchers used bulk loading of calcium indicator combined with two-photon imaging. They triggered a signal neuron and then mapped the calcium signal initiated in connecting (follower) neurons. It was determined that the neocortex is composed of precise circuits. The followers belonged to a few selective anatomical classes and their positions were determined between animals (Kozloski et al., 2001).

A final and potentially very powerful application of two-photon excitation microscopy is three-dimensionally resolved photorelease of caged compounds (uncaging). For example, techniques have been developed for quantita-

tive two-photon uncaging of calcium (Brown et al., 1999). As discussed previously, the photochemical reactions involved are generally quite slow (from milliseconds to seconds), which allows the compound to diffuse over several micrometers within the sample between the time when it is excited and the time it becomes active (Kiskin et al., 2002). Diffusion is not a problem when this technique is used to mark cells by uncaging membrane-impermeant fluorescent molecules. This technique has been successfully used to track the development of sea urchin embryo cell lineages (Piston et al., 1998). Faster-uncaging molecules have been successfully used in studies of uncaging stimulants using two-photon excitation to map neuron receptors (Denk, 1994; Matsuzaki et al., 2001). These studies utilized the three-dimensional nature of two-photon excitation to photochemically uncage stimulants in the imaging medium. When the stimulant was uncaged near the membrane, it stimulated receptors in close proximity, which was detected through a patch clamp of the cell. In this type of imaging, excitatory response, rather than photons, maps the image. In particular, a study utilizing uncaging of MNI-glutamate in conjunction with a whole-cell clamp for detection of signal was successfully used to map glutamate receptors on cultured hippocampal neurons and hippocampal CA1 pyramidal neurons in acute slice preparations (Matsuzaki et al., 2001). These researchers were able to obtain excellent lateral and axial FWHM diameters of 0.6 and 1.4 μm , respectively, in the slice preparations, indicating fast uncaging chemistries. These researchers determined that α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors are abundant in mushroom spines and that the distribution of these receptors is tightly correlated with spine geometry.

CONCLUSIONS

Two-photon excitation microscopy shows great utility for dynamic imaging of living cells in thick samples, such as intact tissue. This often makes possible experiments where conventional imaging cannot be performed. Using a mode-locked (pulsed) laser and photon crowding at the focal point, two-photon excitation only occurs at the focal plane. Localized excitation results in emission from only the focal region, providing sectioning ability without the use of a pinhole, and it reduces phototoxicity because photodamage is largely limited to the focal volume. Although two-photon

excitation microscopy does not generate images with higher resolution than confocal microscopy, it does allow for increased depth penetration into samples. This increased depth penetration is possible due in part to the open pinhole geometry of the two-photon microscope, lack of out-of-focus absorption of the excitation light, and decreased scattering of the excitation light. In order to take full advantage of the depth of penetration, it is necessary to use nondescanned detection geometries because of the dramatic increase in collection efficiency of scattered photons. Clearly, the advantages of two-photon excitation have allowed for experiments that would not be possible using confocal microscopy, and, as this technique gains popularity and the technology gets less expensive, it is expected that more and more exciting experiments will be achieved.

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Total Internal Reflection Fluorescence Microscopy for High-Resolution Imaging of Cell-Surface Events

UNIT 4.12

The wavelength of light imposes a physical limit of ~400 nm on the maximum resolution that can be achieved using light microscopy. This unit will describe the use of total internal reflection fluorescence microscopy (TIR-FM), or evanescent wave microscopy, an approach that partially overcomes this physical limit and permits one to selectively image just those fluorophores in the optical plane (along the z axis) within 50 nm of the cell surface. TIR-FM works by means of limiting the depth of penetration of the excitation light within this narrow region. This narrow excitatory plane not only provides a high signal-to-noise ratio but also minimizes the photodamage to the cell.

Basic through-the-objective TIR-FM setups are now commercially available from Olympus and Nikon, and these can be used for most generic applications. The Basic Protocol, however, outlines a procedure for setting up a TIR-FM microscope in the laboratory using a through-the-prism configuration, and the Alternate Protocol outlines a modification of this procedure for a through-the-objective configuration. These outlines are intended to serve as a starting point; the exact details of any home-made setup will ultimately be decided based on the exact application.

SETTING UP A THROUGH-THE-PRISM TIR-FM SYSTEM

A prism can be used to direct the excitation beam to the samples above the critical angle for total internal reflection. Based on the relative position of the prism, coverslip and the objective there are various ways to set up the prism based TIR setup. These variants are described in the Background Information and in Figure 4.12.3. This section describes the protocol used for setting up the basic prism based setup in which the prism is adjacent to the objective (Fig. 4.12.3C).

**BASIC
PROTOCOL**

Materials

- 40- to 100-nm fluorescent polystyrene beads (Molecular Probes)
- 0.05× phosphate-buffered saline (PBS; APPENDIX 2A)
- Immersion oil appropriate for objective (see Critical Parameters)
- Wide-field epifluorescence microscope, upright or inverted (UNIT 4.1) with appropriate excitation and emission filters
- Prism (see Background Information; Edmund Scientific)
- Laser (see Critical Parameters; Spectrum Physics, Coherent, Melles-Griot, and others), with single-mode laser optical fiber or system of mirrors and focusing lens
- Sykes-Moore chamber (Bellco Glass) or other coverslip mounting chamber
- Laser-safety goggles

Set up microscope, prism, and laser

1. Use a conventional wide-field fluorescence microscope (upright or inverted). Mount the prism either on the holder for the condenser or build a stable mounting that has the option of vertical motion.

The choice of prism shape (e.g., cubic, rectangular, equilateral, or trapezoidal) depends on the desired configuration of the setup—i.e., whether the prism is adjacent or opposite

Microscopy

4.12.1

Contributed by Jyoti K. Jaiswal and Sanford M. Simon

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to the objective. In this protocol, a through-the-prism system where the prism is adjacent to the objective is described (see Background Information and Fig. 4.12.3C).

The prism should have the freedom to move parallel to the optical axis with its surface in contact with the coverslip. For a microscope that focuses by moving the stage up and down, a micromanipulator that can hold a prism could be fixed to the microscope stage.

2. Direct the laser light to the prism by using either a single-mode laser fiber or a system of mirrors and focusing lens with adjustable angle mounts fixed to the table. Include a suitable shutter in the laser light path to permit switching between the epifluorescent illumination from the arc lamp and the TIR-FM illumination.

Establish area of TIR using fluorescent beads

3. Prepare a 1:1000 dilution of 40- to 100-nm fluorescent polystyrene beads in 0.05× PBS. Mount a coverslip on the microscope in the same manner as for the actual experiment (e.g., mounted using a Sykes-Moore chamber or any other coverslip mounting chamber). Add sufficient volume of solution to cover the entire coverslip.
4. Use the arc lamp illumination to focus on the fluorescent beads that are immotile (attached to the coverslip).
5. Place a small droplet of immersion oil directly on the prism. Carefully translate the prism vertically so that it touches and spreads the oil, but does not inhibit the lateral sliding motion.

Beading up of the oil around the edges of the prism should be prevented by using little oil and by wicking away the excess, as it could interfere with the illumination path.

6. Wearing the laser safety goggles, with the help of mirrors (and without any focusing lens in place), adjust the collimated laser beam position so that it is directly in line with the objective's optical axis (see Background Information).

The beam can be observed by the scattering of the laser light as it traverses the prism, oil, and oil-glass interface.

7. Insert the focusing lens and focus the beam roughly at the region of the interface in line with the objective. Using the focusing lens mount, adjust the lateral position of the lens (not the mirrors controlling the raw laser beam) so that the laser beam hits the interface between the coverslip and the aqueous solution directly in line with the objective. To guide this adjustment, look directly at the beam path for three closely aligned spots of scattered light, corresponding to where the focused beam first crosses the immersion oil layer, where it totally reflects off the sample surface, and where it exits by crossing the oil again.
8. Using the epifluorescence excitation, ensure that the immotile fluorescent beads are still in focus.

In epifluorescence, the motile beads will appear as a diffuse fluorescent blur. If TIR is achieved, the immotile beads (attached to the glass) should always be visible, but the motile fluorescent beads will appear to be constantly bouncing in and out of the evanescent field and hence in and out of the field of view. If this "bouncing" of beads is not clearly visible, then readjust the incident angle of the laser beam.

9. Once the conditions described under step 8 are obtained, use the focusing lens to center the area of TIR within the field of view.

The focusing lens can be moved forward or backward along the laser optical path to achieve the desired size of the total internal reflection area on the coverslip.

Examine specimen

10. Once the optics are correctly aligned for total internal reflection, translate the prism vertically to remove the sample with fluorescent beads and replace it with the experimental sample. Translate the prism back to the same position to make optical contact.

Although the total internal reflection region will not be in exactly the same spot because of irreproducibility in the prism height, it will be close enough to make final adjustments with the focusing lens while observing fluorescence from the new sample.

SETTING UP A THROUGH-THE-OBJECTIVE TIR-FM SYSTEM

An objective with a high numerical aperture (>1.45 NA, see Table 4.12.1) can be used to bring the excitation beam to the sample above the critical angle for total internal reflection. The same objective can be used to collect the resulting emission from the sample (Fig. 4.13.4A). This section will describe how this can be achieved. See Background Information for further details and Critical Parameters for choosing the proper objective for your purpose.

Additional Materials (also see Basic Protocol)

Optical mounts including mirrors, optical fiber couplers, and fiber optics or lenses for coupling the laser beam expander to the microscope port (Olympus, TILL Photonics)
Plano-convex lens of short radius of curvature *or* hemispherical or triangular prism and converging lens with focal length of several centimeters
High-numerical-aperture objective (see Critical Parameters)

1. Set up microscope and laser, and prepare bead suspension (see Basic Protocol).

For ease of switching back and forth between TIR-FM and epifluorescence illumination, the optics used to combine the arc lamp and TIR light sources should include a movable mirror that is easily reached and that can be used to switch between the two light paths. This can be achieved by making the laser beam perpendicular to the arc-lamp beam, and inserting a retractable mirror at an angle of 45° to both the beams at the place where the two meet. Depending on whether the mirror is inserted or not, one can choose between the arc lamp and the laser beam.

Steer the laser beam into the center of the objective

2. Place a triangular or hemispherical prism or a plano-convex lens (flat side down) on a coverslip and place this assembly on the microscope stage.
3. Add immersion oil between the objective and the coverslip and focus on the upper surface of the coverslip using the halogen lamp illumination.

Usually dust and defects on the coverslip can be easily seen, to assess the focus.

4. Allow the raw collimated laser beam to enter the standard epi-illumination port and field diaphragm along the optical axis. Steer the beam such that removing all the obstructions between the coverslip sample and the room ceiling produces a large area of laser illumination on the ceiling, roughly straight up.

Obtain and confirm TIR illumination

5. Reposition the beam so that it enters the center of the field diaphragm at an angle that is no longer parallel to the optical axis. Slowly increase the angle until the laser illumination on the ceiling, moving to an ever-lower position on the side wall, just disappears (at this angle, the beam is just blocked by the internal aperture of the objective). Reduce the entrance angle so that half the illuminated area is visible.

ALTERNATE PROTOCOL

6. In order to focus the raw beam at the back focal plane of the objective place the converging lens about 20 cm up-beam from the field diaphragm. This will cause the illuminated region on the wall to become smaller.
7. Move the converging lens longitudinally (along the axis of the laser beam) towards the field diaphragm.

The back-focal plane can be seen by removing the eyepiece. Some microscopes (e.g., Olympus 1 × 70) have a toggle switch near the eyepiece that permits visualizing the back focal plane without removing the eyepiece.

This will continuously minimize the illuminated region on the wall until the focal point of the lens falls exactly at the objective's back focal plane. At this position, the beam emerging from the objective lens is roughly collimated and would be propagating upward along the inner periphery of the objective.

8. Verify that total internal reflection is achieved by moving the coverslip horizontally to a new spot that is not under the prism.

Almost no light should emerge, except for some scattering, because of total internal reflection at the glass coverslip–air interface.

9. Replace the bare coverslip with an identical one containing a solution of fluorescent beads and again verify that no light emerges, even at the glass-water interface.

If the light does emerge, then change the angle of the incident beam using the steering mirrors just until the point when no light emerges.

10. Confirm that TIR excitation is indeed achieved by focusing the epifluorescence excitation on the fluorescent beads, then switching to TIR illumination.

When TIR is achieved, the diffuse background fluorescence from the beads will be lost. Instead there will be two populations of beads. A small number of immotile beads (attached to the glass) will always be visible and a larger population will bounce in and out of view. If this bouncing of beads is not apparent, adjust the incident angle of the laser beam until it is.

11. Fine tune the mirrors and converging lens to center the total internal reflection field.

The size of the total internal reflection fluorescence area on the sample is directly proportional to the laser-beam size at the field diaphragm. To change this size, replace the converging lens with another one of different focal length but always keep its focal point at the objective's back focal plane, which is at a fixed position up-beam from the microscope.

Examine specimen

12. Replace the fluorescent beads with the actual sample.

When the cells are in focus, the total internal reflection optics should be perfectly aligned without need for further adjustment.

SUPPORT PROTOCOL

MEASURING THE INCIDENT ANGLE OF THE EXCITATORY BEAM

While for the prism-based setup the angle of the incident beam can be measured directly. However, the following indirect method can be used to measure the angle of incidence for both through-the-prism and through-the-objective types of setup.

For materials, see Alternate Protocol.

1. Follow steps 1 to 9 of the Basic Protocol or 1 to 11 of the Alternate Protocol to obtain TIR.
2. Without disturbing the optical setup of the excitation beam, replace the coverslip containing the beads with a coverslip containing a hemispherical prism or a plano-convex lens (see Alternate Protocol, step 2).
3. Place a screen on the wall adjacent to the microscope to mark the position of the beam exiting the prism.
4. Carefully mark the position on the screen that corresponds to the vertical position of the coverslip on the stage.
5. Measure the horizontal positions of these two spots with relation to their points of exit from the coverslip.
6. Using the above values draw a triangle connecting these three points, and calculate the angle between the TIR beam and the base (abscissa) of the triangle.
7. Determine the angle of incidence of the excitatory beam by subtracting the calculated angle from 90° .

COMMENTARY

Background Information

The earliest recorded demonstration of the phenomena of total internal reflection was by John Tyndall on May 19, 1854. Tyndall, who is credited for explaining why the sky is blue, presented a demonstration entitled "On Some Phenomena Connected with the Motion of Liquids." His lecture covered the properties of thin streams of water. At the end, he presented a demonstration in which light, leaving a water tank through a hole in its side, would follow the water as it fell to the ground (Fig. 4.12.1). The light hit the interior sides of the water stream at an angle steep enough that it was completely reflected. No light was observed to leave the water stream and, instead, the water served as a light pipe (Pepper, 1860; Greenslade, 1997).

The fundamental principle of TIR-FM is based on one additional observation. Total internal reflection of the light means that there is no energy propagated on the opposite side of the interface. However, a standing field is generated at the interface, into the medium of lower refractive index on the side opposite the incident wave. This field, called the evanescent field or evanescent wave, decays exponentially with increasing distance from the interface (Fig. 4.12.2A). The characteristic distance for decay of the evanescent-wave intensity is a function of the incident angle of the light beam, its wavelength, and the difference in the refractive index of media on either side of the interface. For most biological applications, altering these parameters the field of illumination can be adjusted to any depth between 50 and 200

nm. Thus, the predominant excitation of fluorophores is within this distance from the interface.

In 1965, Hirschfeld developed this as a method for selectively illuminating a surface at a solid-liquid interface. However, it was not until 1981 that this approach was adopted for use in biology to image cell-substrate contact (Axelrod, 1981, 1983; Axelrod et al., 1981; Gingell, 1981). Although TIR-FM has been applied for a variety of biological applications over the past two decades, very recent technical advances in fast imaging and image analysis, as well as the availability of commercial TIR fluorescence microscopes, are opening up possibilities for wider and easier application of this technique (Axelrod, 1989, 2001). As a result, TIR-FM has been used for a variety of live-cell applications, including study of cell-surface contact, diffusion of proteins and lipids in the plasma membrane, submembrane actin dynamics, and vesicular exocytosis (Schmoranzner et al., 2000; Sund and Axelrod, 2000; Wagner and Tamm, 2000; Amann and Pollard, 2001; Steyer and Almers, 2001; Toomre and Manstein, 2001).

Theory of TIR-FM

When a beam of light encounters a medium with lower refractive index, it changes its path such that the resulting beam is refracted (bent away) from the path of the incident beam. For TIR-FM, the incident angle, θ , (as measured from the plane normal to the incident surface) has to be large enough for the beam to totally internally reflect back into the medium with the higher refractive index (Fig. 4.12.2B). This con-

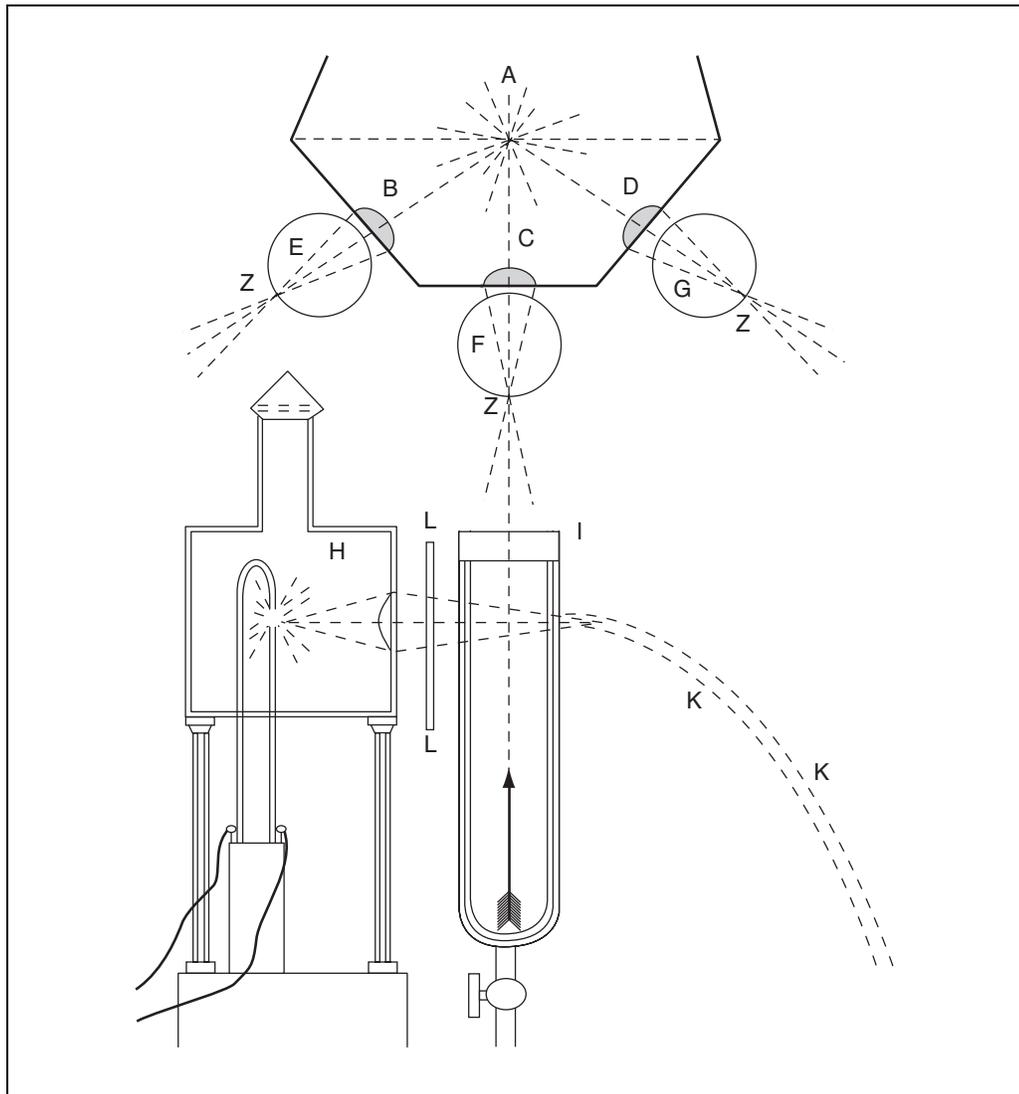


Figure 4.12.1 Illustration of the classic experiment (“The Liquid Vein”) demonstrating total internal reflection. The apparatus (viewed from the top in the upper part of the figure and from the side in the lower) consists of a Duboscq lantern with an electric arc light (A), three sides of which are furnished with lenses (E, F, and G). Outside the lantern are three cylinders of water, each with a circular glass window and orifices at (Z) from which the water and the rays of light pass out. In the lower diagram, a section is seen of one side of the Duboscq lantern (H) and one cylinder of water (I), with the water entering from below through the stopcock. The stream of illuminated water is labeled K, and L represents a bit of colored glass held between the lantern and the cylinder of water to impart color to the light. From Pepper (1860).

dition occurs above a critical angle. For a light beam that is traveling from a medium with high refractive index (n_1), such as oil, glass, or silica, into a medium with lower refractive index (n_2), such as water or air, the critical angle (θ_c) is:

$$\theta_c = \sin^{-1} \frac{n_2}{n_1}$$

Equation 4.12.1

For TIR to occur, the ratio n_2/n_1 must be <1 , and the value of q should be $> q_c$. For all values of $q < q_c$, the light beam propagates through the interface, leading to a wide-field illumination. Glass coverslips typically used to grow tissue culture cells have a refractive index (n_1) of 1.515, while the cytosol and the medium surrounding the cells have refractive indices (n_2) ranging from 1.33 to 1.38. To obtain total internal reflection at the higher end of the cellular refractive index (1.38), the angle of incidence must be larger than the critical angle of $[\sin^{-1}(1.38/1.515) = 65.64^\circ]$.

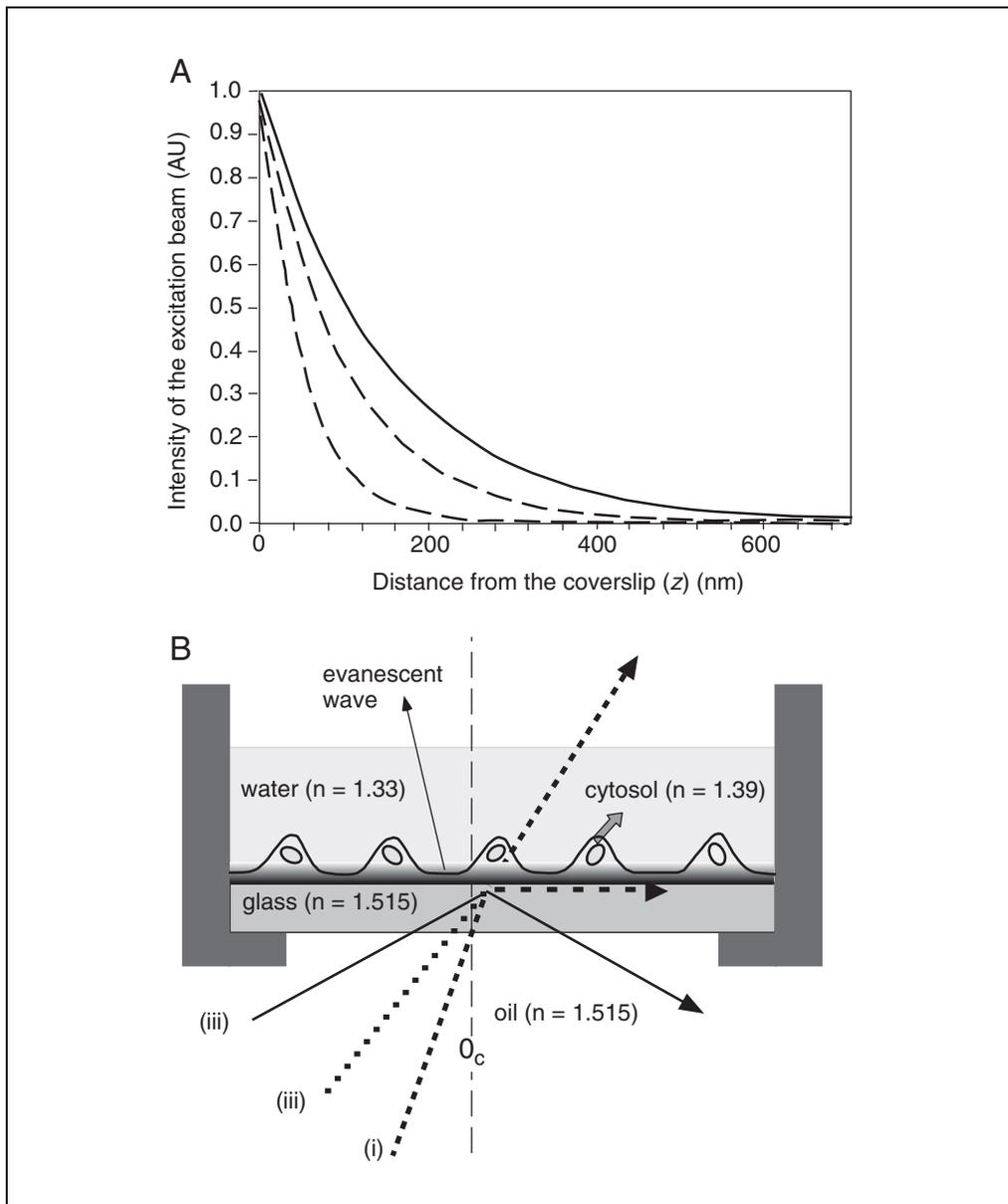


Figure 4.12.2 (A) Exponential decay of evanescent field. The intensity of the evanescent field in the aqueous medium is plotted over the distance from the coverslip/water interface for typical decay lengths (dotted line, $d = 50$ nm; dashed line, $d = 100$ nm; and solid line, $d = 150$ nm) used in most experiments. (B) Illustration of Snell's law, refraction, and total internal reflection. Beams that are (i) refracted, (ii) incident at the critical angle, and (iii) totally internally reflected are illustrated at a glass-water interface. The evanescent field is indicated as a gradient of gray at the interface that falls exponentially with distance from the interface.

If, instead of intact cells, permeabilized or fixed cells are used, the effective refractive index is that of aqueous buffer ($n_2 = 1.33$), and the critical incidence angle is reduced to 61.39° .

For values of $q > q_c$, the resulting electromagnetic evanescent field has the same frequency, wavelength, and polarity as the incident light. For an infinitely wide beam, the intensity of the evanescent wave (measured in units of energy/area/sec) decays exponentially with perpendicular distance from the interface between

the two media (Fig. 4.12.2A). The intensity (I) at any given point z is related to the intensity at the interface [$I(0)$] by:

$$I(z) = I(0)e^{-\frac{z}{d}}$$

Equation 4.12.2

where for a light of wavelength λ , which is incident at the interface at an angle θ , the depth of penetration (d) is given by:

$$d = \frac{\lambda}{4\pi} \left(n_1^2 \sin^2 \theta - n_2^2 \right)^{-\frac{1}{2}}$$

Equation 4.12.3

At $\theta > \theta_c$ evanescent wave intensity is equal to the intensity of the incident beam, but the intensity approaches zero as θ approaches 90° .

Methods for achieving total internal reflection

The necessary and sufficient criterion for obtaining total internal reflection is that the excitation light be introduced on the surface of the coverslip at an angle greater than the critical

angle. This can be achieved in a variety of different ways, and, as detailed below, each of these have their advantages and limitations.

Excitation through a prism: In the prism-type TIR-FM setup, the excitatory beam is coupled into the coverslip via a prism (Fig. 4.12.3A, B, and C). At the first interface between the air and the prism, the angle of the excitatory beam is narrow enough that most of it enters the prism with minimal refraction. The prism and the coverslip are in contact with the oil, whose refractive index matches that of the glass. Thus, the excitation beam passes through this interface with minimal reflection or refraction. However, changing the angle of incidence of the beam on the prism allows alteration of the angle at which the beam hits the interface of the coverslip and the cell. At a steep enough angle, the excitation beam is totally internally reflected at this interface, resulting in excitation

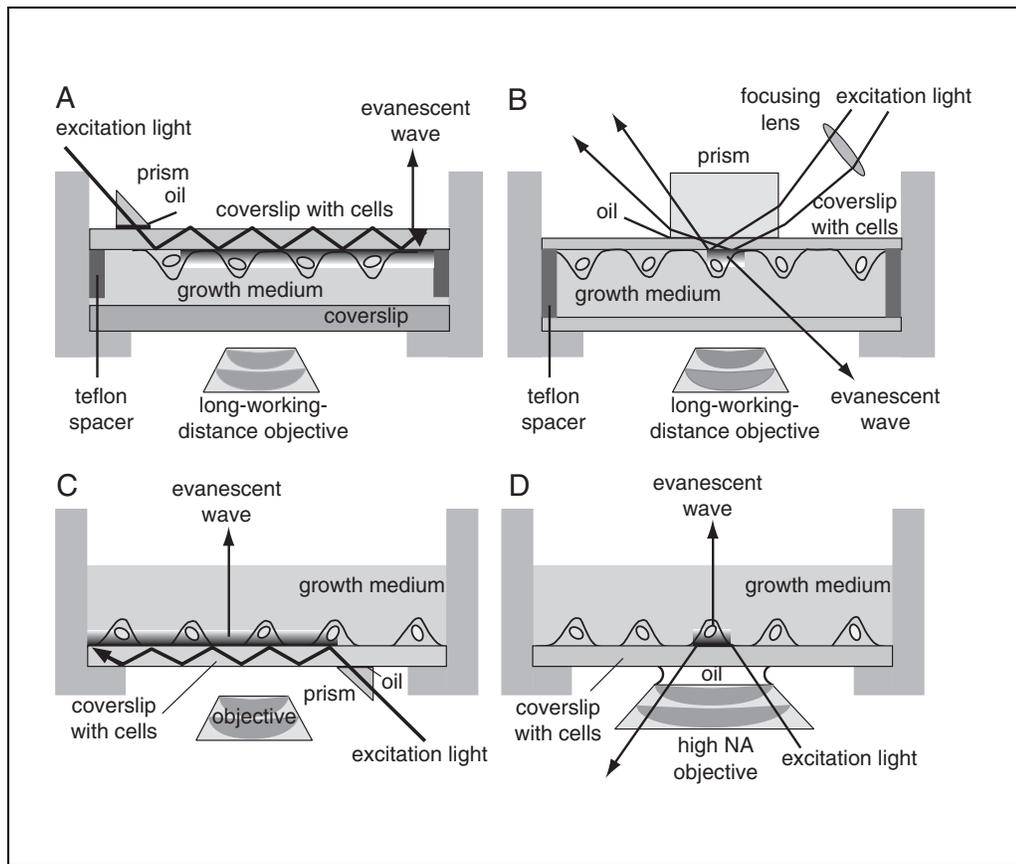


Figure 4.12.3 Possible arrangements for setting up total internal reflection fluorescence microscope. In (A) and (B), the excitation beam is passed through the prism opposite the objective. Unlike (A), a narrow field of TIR illumination is achieved in (B) with the use of a bigger prism that allows the internally reflected light to pass through the prism instead of being reflected back into the coverslip. In (C), the excitation beam passes through the prism adjacent to the objective, and in (D), excitation takes place through the objective.

of a narrow region of the cell near the coverslip. The resulting fluorescence from the cell is collected by an objective lens.

An advantage of the prism-based approach is that it allows separation of the excitation and emission light paths. This reduces the background fluorescence caused by scattering of the excitation light in the objective. This setup also reduces the need for elaborate optics necessary to prevent the bleed-through of the excitation light into the emission channel. However, there are many limitations to this approach. First, once the sample has been focused, the prism must be carefully positioned on the coverslip and maintained such that it does not lose the coupling with the coverslip. Second, the oil used to couple the prism to the coverslip must be kept very clean throughout an experiment; any dust will cause scattering of the excitation light. Third, during positioning of the prism on the surface, the oil must not be allowed to spread on the coverslip, since TIR will be lost from places where the oil spreads on the surface of the glass.

Depending on the position of the prism used to target the excitation light, with respect to the objective lens, there are two basic types of prism-based setup, as described in the following paragraphs.

Excitation through the prism opposite to the objective: As described in Figure 4.12.3A and B, in this setup the fluorescence emission is collected through an objective from the side opposite to that of the prism. Depending on the prism used, cells on the entire coverslip can be illuminated (Fig. 4.12.3A) or the excitation light can be limited to a small region under observation (Fig. 4.12.3B). The relatively large distance between sample and objective limits this application to an objective with a long working distance and a low NA (typically 0.75 or less). Thus, this technique is less sensitive and not suitable for low-light applications.

Excitation through the prism adjacent to the objective: In this setup, the prism is on the same side of the coverslip as the objective, while the specimen is on the opposite side (Fig. 4.12.3C). The specimen can be translated while the prism remains laterally fixed. The advantage of this approach is that it allows the use of objectives with higher NA than can be used with the prism on the opposite side of the objective. A disadvantage of this configuration is that oil- or glycerol-immersion objectives cannot be used. If oil/glycerol were used at the interface of the

prism and coverslip and any of it were to smear, it would lead to loss of total internal reflection. Thus, the choice of objectives is limited to air- or water-immersion objectives. This puts an upper limit to the numerical aperture of the objective, thus reducing the sensitivity of the detection. Depending upon how the coupling is done, this could lead to a fairly wide field of excitation which could be an advantage or disadvantage depending upon the experimental interest.

Excitation through the glass support setup: This approach, which is useful for large-scale evanescent wave excitation, is a slight variation on the excitation-through-the-prism approach. Here the excitation beam is directly coupled to a thin glass surface with the specimen on it. The laser has to be introduced at an angle such that, after refraction in the glass support, the light impinges upon the sample at an angle $> \theta_c$, thus generating an evanescent wave. This can be done by making an appropriate angle at the edge of the glass surface. This allows the use of any objective of choice; at the same time it does not restrict the accessibility to the sample. However, due to the larger field of evanescent illumination, it leads to photobleaching even outside the field of view. Also, if the coupling between the laser and the coverslip is not robust, it could lead to fluctuations or even a total loss of evanescent field.

Excitation through the objective: In the objective-type TIR-FM setup, the supercritical angle on the coverslip-cell interface is reached by focusing the excitatory beam on the periphery of the back focal plane of the objective (Fig. 4.12.4A). In this way, the beam is refracted by the objective and directed through the refractive index-matched media: the objective, the immersion liquid, and the coverslip. The objective thus functions both as the prism, delivering the exciting beam, and as the optics, collecting the emission. The main advantage is that objective-type TIR-FM employs high-numerical-aperture, short-working-distance objectives, resulting in collection of much higher fluorescence emission compared to the prism-type approach. This results from the relationship whereby light collection is approximately equal to the square of the NA; thus, a 1.65 NA objective used in the objective-type TIR will collect 90% more light than a 1.2 NA water objective required for the prism-type TIR. Moreover, such a setup makes the sample fully accessible for other manipulations such as microinjection, patch

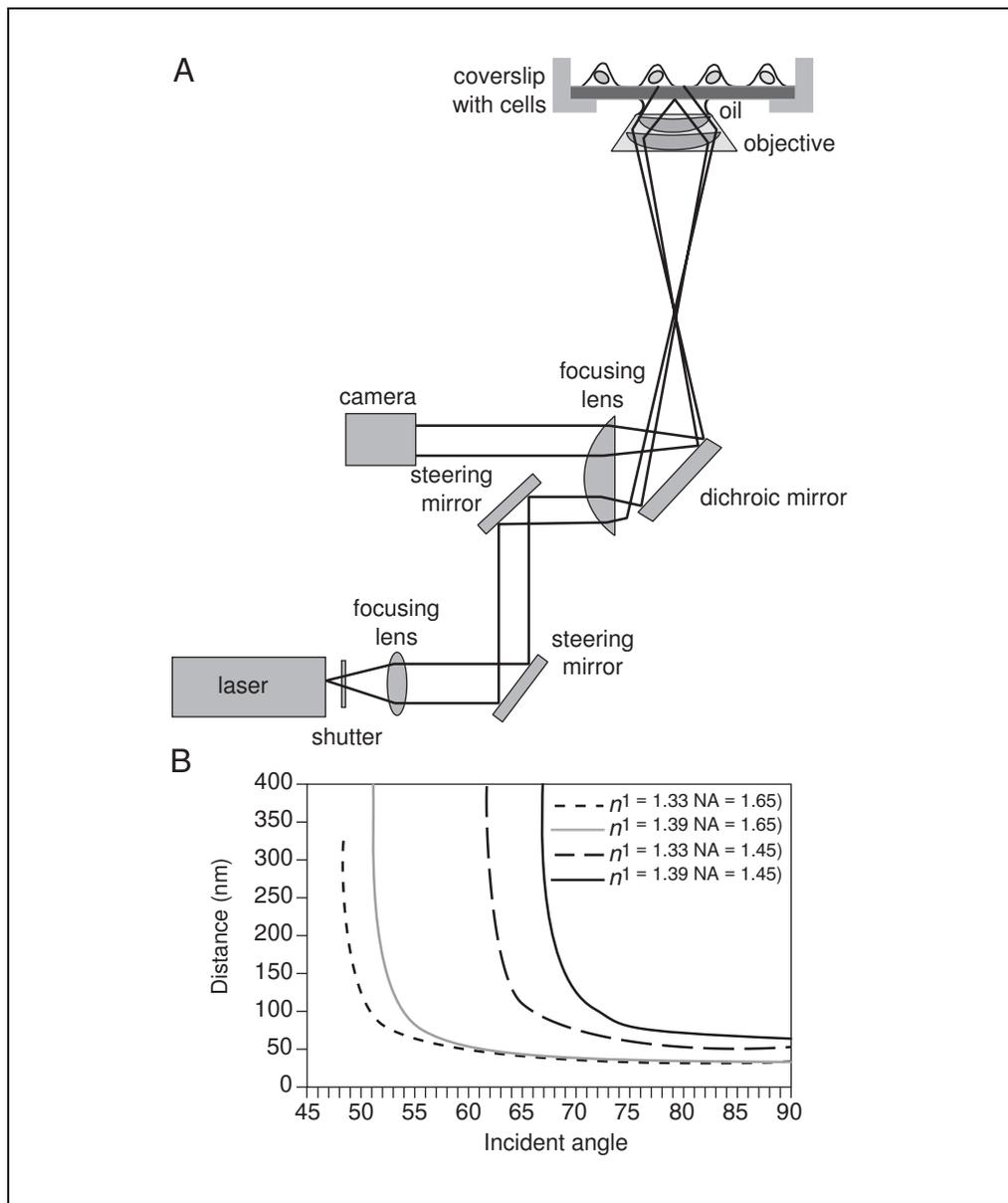


Figure 4.12.4 Through-the-objective TIR-FM setup. **(A)** Excitation and emission light path. **(B)** Relationship between incident angle of the beam and its evanescent depth. The spatial decay constant for the evanescent field is plotted for both the NA 1.45 and 1.65 objectives according to Equation 4.12.2 for refractive indices (n_1) of 1.33 and 1.39, typical of water and cytosol, respectively.

clamping, and atomic force microscopy. Another advantage of this approach is that it permits the use of both a laser-based and an arc lamp-based excitation light source for TIR. Finally this approach is easier and more reproducible in terms of setup: it does not require oil to couple the prism to the objective; it does not require a careful positioning of the prism on the coverslip; and from experiment to experiment, there is less variability in alignment of the excitatory beam. This approach has some limitations. First, since the excitation light has to

pass through the same optics as are used to collect the emission, dichroics and filters must be used to minimize inadvertent detection of the excitation light. Second, if the excitation is not properly aligned, it is possible to get scattering within the objective, which would lead to loss of TIR-FM.

Critical Parameters

Depending on which of the methods is used to achieve TIR, the optics used (prism or high-NA objective, or edged glass) to target the

excitation light need to be altered, but the rest of the setup is similar in all cases.

Epifluorescence microscope

Both an upright or an inverted epifluorescence microscope can be used for an TIRF microscope. For applications that require access to the sample for other manipulations such as microinjection, atomic force microscopy, patch clamping etc. use of inverted microscope with the objective-type setup (Fig. 4.12.3D) or with a “prism adjacent to the objective” setup (Fig 4.12.3C) is recommended. However, an upright microscope with long working distance objective can also be used in conjunction with “prism opposite to the objective” setup to provide a limited access to the sample.

Light source

In principle, it is possible to employ conventional tungsten halogen or mercury/xenon arc lamps to conduct TIR-FM experiments. However, using an arc lamp requires generation of a sharp-edged shadow image of an opaque circular disc at the back focal plane of the objective. Although the arc lamp provides the convenience of selecting any excitation wavelength from the UV to the visible spectrum just by changing the excitation filters, a majority of the investigations reported in the literature are performed using laser illumination. The reason for this is that laser light is brighter, coherent, polarized, and well collimated. Hence, it is easier to direct into the objective or prism using standard beam expanders, mirrors, and focusing lenses. Collimation of the light emitted by the arc lamp results in severe (>90%) reduction of intensity, making it difficult to use the commercial arc lamps as light sources for TIR-FM. Another advantage of the laser source is that it can be used to illuminate an area of a defined geometry that can be easily adjusted to accommodate experimental variations. However, a limitation of laser illumination is the unavoidable interference fringing on the specimen. These fringes can be somewhat reduced by meticulously cleaning the optical surfaces. Additionally, a field with a uniform concentration of fluorophore can be used to generate an image that can then be used to normalize for the spatial inhomogeneities in illumination. Alternatively, for experiments requiring highly uniform illumination, the fringing can be eliminated by using a commercially available laser fiber phase scrambler.

For most applications, a laser with a single-wavelength output of ≥ 20 mW should be adequate. However, for applications requiring short exposure time, e.g., FRAP (*UNITS 13.5 & 21.1*), lasers with output of 100 mW should be used. In either case, the laser should provide an option for regulating its output externally. If the laser source does not provide the means to regulate the power output, then an acousto-optic modulator (AOM) or a set of neutral-density filters should be introduced in the laser light path. Note that AOMs provide a continuous control of the intensity from zero to the maximum, but they tend to lose up to 50% of the input light. Hence, use of AOMs necessitates lasers with higher power.

For any of the types of TIR-FM setup described above, excitation light from a laser source can be introduced either through an optical fiber or as a free-standing beam. However, the exact optical arrangement is dependent upon the setup used to obtain TIR-FM. For through-the-prism setup, use of optical fiber minimizes the risk of accidental direct exposure to the laser beam. Depending on the position of the prism (with respect to the sample), the fiber has to be placed either above or below the microscope stage and positioned to target the beam into the prism at an appropriate angle for TIR. Similarly a fiber-based setup should be used for the through-the-glass-support-based setup. For the objective-based setup, the laser light could be introduced using a fiber in the same port as the epifluorescence light from the arc lamp; the two light sources can thus be coupled. There are commercially available adaptors from Olympus, Nikon, and TILL Photonics (see *SUPPLIERS APPENDIX*) that permit coupling the light sources for a variety of microscopes. Alternatively the free-standing laser light can also be steered into the microscope using mirrors and focused on the back focal plane of the objective using a set of focusing lens (Fig. 4.12.4A).

Objectives

As described in Equation 4.12.1, in order to collect maximum amount of light emitted by the sample (and hence obtain brighter images) it is necessary to use an objective with as large a numerical aperture (NA) as possible. Any long-working-distance objective can be used for a prism-based TIR-FM setup with the prism on the opposite side (Fig. 4.12.3A and B). However, for a setup with the prism on the same

side of the sample as the objective (Fig. 4.12.3C), the choice is an air objective (preferably with a high NA). The choice of objective for through-the-objective TIR-FM setup is crucial. This can be achieved reliably only with oil-immersion objectives having an NA of 1.45 or larger.

As can be deduced from Equation 4.12.1, for an objective lens with refractive index of 1.515 and a NA of 1.40, the maximum angle at which the light can be incident on the sample is [$\sin^{-1}(1.4/1.515) = 67.53^\circ$]. For viewing live cells using an objective with $n = 1.515$, the critical angle is 65.63° (Table 4.12.1; Fig. 4.12.4B). This leaves a very small region of the objective that can be used to obtain TIR. Because the refractive index of the cell is not uniform along all of its surface, and because even a small extent of scattering of excitation light could lead to a loss of total internal reflection, use of objectives with NA of 1.4 or lower is not practical for obtaining through-the-objective TIR. Development of objective lenses with NA of 1.45 by Olympus, Nikon, and Zeiss, and with NA of 1.65 by Olympus, has made it possible to reliably obtain TIR through the objective (Table 4.12.1).

The 1.65 NA objectives: In contrast to all other objectives that make use of glass with a refractive index of 1.515, the 1.65 NA Apo 100 \times objective by Olympus uses glass with a refractive index of 1.788. The higher refractive index of the glass, together with the high numerical aperture of the objective, has a few consequences for TIR microscopy. First, there is a relatively wide range of angles over which TIR can be achieved (Terakawa et al., 1997; Kawano et al., 2000; Table 4.12.1, Fig. 4.12.4B). Second, the higher refractive index makes the TIR field much less sensitive to variations in the refractive index of the cell, resulting in a uniform evanescent field all along the cell surface. At an angle of 63° (typical for this objective) the space constant ranges from 44 nm ($n_2 = 1.33$) to 48 nm ($n_2 = 1.37$). As the angle is varied for $n_2 = 1.33$, the space constant for this field ranges from 49 nm (for 60°) to 42 nm (for 66°). The main disadvantages of this objective are the requirement for a special high-refractive-index immersion liquid ($n = 1.78$) and high-refractive-index coverslips ($n = 1.788$). The currently available immersion liquid is volatile and slightly toxic, leaving a crystalline remnant on the objective after a few hours in a heated environment. The high-refrac-

tive-index cover slips supplied by Olympus (composed of Ohara glass LAH64, thickness 0.150 mm) are very expensive, fragile, and acid- and base-labile, and hence difficult to reuse. An acid-resistant alternative for this coverslip is the Schott-glass type SF11, available in 0.150 mm thickness for one-third the price of the Olympus coverslips from VA Optical (see *SUPPLIERS APPENDIX*). However, SF11 is slightly more autofluorescent than LAH64 and causes higher chromatic error. It is therefore only usable for bright and single-color fluorescent samples.

The 1.45 NA objectives: Most of the problems associated with the 1.65 NA objective are overcome by the recent introduction of 60 \times 1.45 NA objective (Olympus; Nikon) and the 100 \times 1.45 NA objective (Zeiss; Nikon), which use regular immersion oil and coverslips. The decay constant of the evanescent field for the 60 \times 1.45 NA objective is also larger ($d = 90$ to 200 nm) than for the 100 \times 1.65 NA objective ($d = 50$ to 100 nm). The range of incident angles for TIR is smaller for the 1.45 NA objectives compared to the 1.65 NA objective (Table 4.12.1). Also, since the 1.45 NA objective uses standard glass with a refractive index of 1.515, the decay distance is more sensitive to local variations in the refractive index of the cell (Table 4.12.1).

Immersion oil

For the objective-type TIR-FM setup it is crucial that the immersion oil being used is refractive index matched with the objective and the cover glass. While use of low autofluorescence immersion oil is desired, most commercially available oil do not have refractive indices suitable for use with the available high NA objectives. Thus the refractive index of the immersion oil should be carefully examined before its use in TIR-FM.

Filters and mirrors

Special excitation and emission filters and dichoric mirrors need to be used with the TIR setup. Due to the higher intensity of the laser light sources, instead of the conventional excitation filters (which are appropriate for blocking light from an arc lamp), excitation filters with higher optical density (>4) should be used. The emission filters must also be carefully chosen so that they are blocked for light outside of the desired wavelength range. Also, the higher laser intensity necessitates use of di-

Table 4.12.1 Total Reflection Angles and Maximum Angles for Objective Lenses

NA of objective	n_1	n_2	Critical angle (θ_c)	Maximum angle of incidence (θ)	Least depth of penetration, d (nm)
1.40	1.515	1.38	65.63° (NA = 1.38)	67.53° (NA = 1.40)	168
1.45	1.515	1.38	65.63° (NA = 1.38)	73.15° (NA = 1.45)	89
1.65	1.788	1.38	50.51° (NA = 1.38)	67.34° (NA = 1.65)	55

chroic mirrors with better antireflective coating, so as to minimize the reflection off the back surface of the mirror. These measures are necessary because leakage of even small amounts of scattered excitation light of different wavelength or of light reflected from the back surface of the dichroic mirror (hence traveling at an angle different from that of the primary beam) would decrease the signal-to-noise ratio.

Troubleshooting

Beam path

If total internal reflection cannot be achieved, make sure that the beam path is not obstructed and that it is still being targeted to the sample. Follow the steps in the Basic and Alternate Protocols to achieve TIR and confirm the existence of TIR using the fluorescent beads. An alternative approach to confirm the existence of TIR involves the use of two dyes, e.g., Fluorescein (green) and DiI (red). A procedure for using these dyes to test for the TIR is as follows.

1. Treat the surface of the cover glass for 10 min with a solution of DiI dissolved at 0.5 mg/ml in ethanol.
2. Rinse off the coverslip with water and add a 0.1 mg/ml solution of carboxyfluorescein.
3. Illuminate the sample with 488 nm epi-fluorescent light. This should lead to both red and green emission.
4. Now switch to TIR illumination. If the TIR setup is not optimal, fluorescence emission of both the dyes will still be visible. Adjust the angle of the incident beam to the point where most of the emission is red, while the green emission is minimal. By shifting the excitatory beam back and forth, one can identify the critical point at which the emission sharply shifts from green (epi) to red (TIR); this will be the point at which the beam is incident on the sample at the critical angle.

Image homogeneity

Often the TIR-FM image of cells that appear homogeneous in epi-illumination appear heterogeneous in TIR illumination. This heterogeneity could be due to dirt and defects on the surface of the coverslip or to the fact that the cells have not adhered well enough to the coverslip. Either of these factors would cause the cell to be illuminated nonuniformly all along the bottom surface of the cell. Since TIR excitation decays exponentially with distance from the coverslip, even slight undulations in the cell membrane due to improper adherence or due to dirt and defects on the coverslip could cause illumination artifacts. To ascertain whether the heterogeneity is due to the illumination artifact of TIR, label the cell membrane using a membrane-specific dye (e.g., DiI) and observe using TIR. If the cell is not attached uniformly to the glass surface, then the fluorescence will be nonuniform. This problem can be remedied by coating the surface of the coverslip with suitable matrix molecules, such as fibronectin or collagen, that will promote better adherence of cells to the coverslip surface. However, cells that do not adhere well to the coverslips might not be suitable for carrying out TIR-FM.

Anticipated Results

As with any form of optical sectioning microscopy, TIR-FM minimizes the out-of-focus fluorescence, and hence it has a high signal-to-noise ratio for the images. This is essential for imaging fluorescently tagged molecules that are present throughout the cell but that carry out their function only in a very narrow region near the cell membrane. For example, actin bundles present near the cell surface are those that are involved in cell adhesion and migration. While wide-field fluorescence imaging of cells expressing fluorescently tagged actin collects fluorescence from actin both near the cell sur-

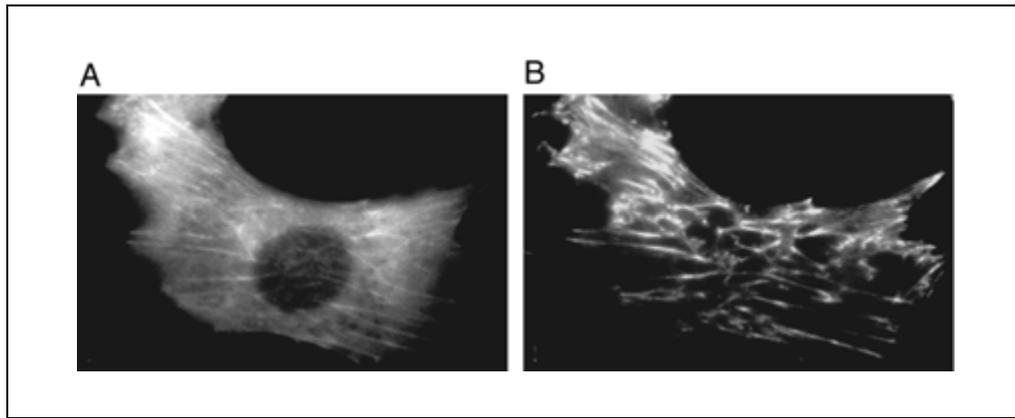


Figure 4.12.5 Imaging the distribution of GFP-labeled actin in live cells. Basal surface of normal rabbit kidney (NRK) cells stably expressing the actin-EGFP was imaged using (A) epifluorescence illumination and (B) TIR-FM illumination. While the fluorescence of G actin from the entire cell body makes it difficult to resolve the presence of the F actin bundles using epifluorescence, TIR-FM allows visualizing these bundles by illuminating only a narrow region of the cell adjacent to the membrane-coverslip interface.

face and deeper inside the cell, TIR-FM allows imaging of only the actin near the cell surface (Fig. 4.12.5). This allows visualization of changes in actin dynamics as the cells attach and migrate on the coverslip.

Time Considerations

The sample preparation procedure for TIR-FM is just the same as for conventional fluorescence imaging. Hence, once the microscope is set up for operating in the TIR-FM mode, the only additional time needed for carrying out TIR-FM imaging is that involved in ensuring that the TIR beam is properly aligned (<15 min).

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Fluorescence microscopy techniques for yeast cells are fairly similar to those used with mammalian tissue culture cells. As with mammalian cells, fluorescent dyes can be used to identify organelles, GFP fusion proteins can be used to track the localization and dynamics of a specific protein, and membrane trafficking pathways can be evaluated using pulse-chase methods with fluorescent markers. This unit is designed to present a range of fluorescent techniques commonly used for the baker's yeast *Saccharomyces cerevisiae*.

One major difference between fluorescent labeling in mammalian cells versus yeast cells is size. Mammalian cells can range from 10 to 100 μm , compared to ~ 3 to 5 μm for a yeast cell, making identification of some subcellular structures and clear colocalization determinations somewhat challenging. Using larger, diploid cells for imaging can sometimes help with this problem. In addition, newer confocal microscopes (UNIT 4.5) have been designed with more precise control over pinhole size and z -axis movements, allowing slices as thin as 0.4 μm , which is thin enough to image multiple planes inside a yeast cell. For reference, most publications involving yeast cell light microscopy use a range of confocal, deconvolution (McNally et al., 1999) and standard fluorescence microscopy and are presented at $\sim 1000\times$ magnification.

The focus of this unit is fluorescence labeling for localization of a specific protein as well as techniques for tracing the movement of molecules through the endocytic pathway. The protocols begin by describing a few of the basic techniques commonly used to label the vacuole (see Basic Protocol 1 and Alternate Protocol 1), which is the degradative compartment of a yeast cell (equivalent to the lysosome of mammalian cells) and is among the more easily identified organelles in yeast. Following this method are assays of the endocytic pathway, which generally leads to delivery to the vacuole, using fluorescent labels (see Basic Protocols 2 and 3, and Alternate Protocol 2). The unit also describes both live and fixed cell imaging techniques for specific proteins as well as techniques to label the filamentous actin cytoskeleton in yeast cells (see Basic Protocols 4 to 6, and Alternate Protocols 3 and 4). Finally, the authors have included a simple technique for identifying budding patterns in haploid and diploid yeast cells (see Basic Protocol 7).

This is by no means a comprehensive list of the methods available for fluorescent techniques in yeast cells. In this unit, the authors have tried to focus on methods that have been tested repeatedly in their laboratory to provide as much insight as possible. The internet is a great resource for additional and alternate yeast protocols that laboratories are willing to share. Entering "yeast protocol" in most search engine queries will provide numerous options. In addition, methods describing flow cytometry/cell sorting of fluorescently labeled yeast (Vida and Wendland, 2002), as well as vital fluorescent dyes to label mitochondria and other structures (Erdmann and Gould, 2002; Nunnari et al., 2002) are also available. The Molecular Probes catalog is another great resource for many useful reagents.

STRATEGIC PLANNING

For each of the protocols, the first step requires the ability to prepare growing yeast cells in medium. See UNIT 1.6 for a description of liquid culture growth protocols for yeast cells (also see UNIT 3.8). Unless otherwise stated, either rich or selective/minimal medium is acceptable. Measure the concentration of yeast cells by an OD_{600} reading in a spectrophotometer expressed as an OD_{600} per milliliter culture. For most of the protocols (i.e., unless specifically stated otherwise), it is acceptable to dilute cells back from a semisatu-

rated/saturated culture in the morning as long as the cells double in concentration at least once (after the dilution in the morning) before beginning the assay.

When pelleting yeast cells, it is important to centrifuge at low speeds to preserve the shape and structure of the yeast cells and organelles. For small volumes (≤ 1.5 ml), use a microcentrifuge and pellet cells at 300 to $800 \times g$ for 1 to 5 min. For larger volumes, use a clinical centrifuge and 15- to 50-ml tubes, but pellet cells for 5 to 10 min at 300 to $800 \times g$. If the cells are deformed in any way, decrease the centrifugation speeds. Also, cells in minimal/synthetic medium often do not pellet as well as cells in rich medium (e.g., YEPD). To improve recovery, it may be necessary to pellet the cells, remove ~80% to 90% of the medium, and then centrifuge again before removing the remaining medium. As noted in each protocol, safe centrifugation speeds are normally in the range of 300 to $800 \times g$ for live yeast cells.

Most of the methods prescribe the use of concanavalin A (con A)-coated coverslips (22 \times 22-mm) to help immobilize yeast cells. The production of con A-coated coverslips is described in Reagents and Solutions.

Yeast strains that are *ade2* mutants (grow as pink or red colonies) accumulate a fluorescent intermediate in the vacuole. If an *ADE2*⁺ strain cannot be used, it will be necessary to take special measures to reduce or prevent accumulation of this interfering signal. To do so, add adenine (from a concentrated stock) to a final concentration that is five times normal (i.e., 0.1 mg/ml) to the medium at all times (even to rich/YEPD medium), including the overnight growth medium.

BASIC PROTOCOL 1

LABELING THE VACUOLE IN LIVE CELLS WITH CDCFDA

5(6)-Carboxy-2'-7'-dichlorofluorescein diacetate (CDCFDA, Molecular Probes) is a vacuole-specific vital dye. Briefly, when incubated with cells, it passively diffuses through membranes because of its neutral state. When the dye reaches the vacuole, esterases cleave CDCFDA to form a charged, fluorescent molecule. The charge prevents subsequent diffusion of the fluorescent label from the vacuole. Cells can be viewed by fluorescence microscopy under a FITC filter.

Materials

Yeast cells (see Strategic Planning)
0.5 M sodium citrate, pH 4.0 (see recipe)
10 mM 5(6)-carboxy-2'-7'-dichlorofluorescein diacetate (CDCFDA; Molecular Probes) in anhydrous DMSO (store in the dark for 3 months to 1 year at -20°C)
PBS (pH 7.4)/2% (w/v) glucose (see recipe)
Shaking incubator set to appropriate growth temperature
Con A-coated coverslips (see recipe)
FITC filter
Additional reagents and equipment for yeast cell culture (UNIT 1.6) and fluorescence microscopy (UNIT 4.2)

1. Culture yeast cells overnight to log phase (~ 0.3 to $1.0 \text{ OD}_{600}/\text{ml}$; UNIT 1.6) and transfer 900 μl into a room temperature 1.5-ml microcentrifuge tube.
2. Add 100 μl of 0.5 M sodium citrate, pH 4.0, and 1 μl of 10 mM CDCFDA in anhydrous DMSO.
3. Incubate 15 min at growth temperature with shaking.
4. Centrifuge gently 3 to 5 min at $300 \times g$, room temperature. Aspirate supernatant.

5. Resuspend in 100 μ l PBS (pH 7.4)/2% (w/v) glucose.
6. Mount 2 to 3 ml onto con A-coated coverslips and observe using a FITC filter (UNIT 4.2).

LABELING ACIDIC ORGANELLES WITH QUINACRINE IN LIVE CELLS

6-Chloro-9(4-diethylamino-1-methylbutylamino)-2-methoxyacridine dihydrochloride (quinacrine) is a vital dye that can be used to label acidic compartments in cells (i.e., it gives more general information about the state and function of the vacuole). The unprotonated form can diffuse across cell membranes, allowing labeling of intact live cells. Upon encountering an acidic environment, the molecule becomes protonated, restricting its ability to diffuse across membranes and enhancing its fluorescent signal, making quinacrine an effective tool for specific labeling of acidic organelles. When the dye is used in excess, the quinacrine signal is quenched, so if the fluorescent signal is low, try decreasing the amount of quinacrine used as well as increasing it. The labeled cells are viewed by fluorescence microscopy under a FITC filter.

Additional Materials (also see Basic Protocol 1)

Rich medium (UNIT 1.6)

1 M HEPES, pH 7.6: adjust to pH 7.6 with KOH (store up to 1 year at room temperature)

20 μ M quinacrine (Sigma) in 1 M HEPES, pH 7.6 (store in the dark for 3 months to 1 year at -20°C)

100 mM HEPES (pH 7.6)/2% (w/v) glucose (see recipe), ice cold

1. Culture yeast cells overnight to log phase (0.3 to 1.0 $\text{OD}_{600}/\text{ml}$; UNIT 1.6). Transfer 1 ml cells to a 1.5-ml microcentrifuge tube and centrifuge 3 to 5 min at $300 \times g$, room temperature. Aspirate medium and cool pellets on ice (~ 2 to 5 min).
2. Resuspend in 0.9 ml rich medium. Add 100 μ l of 1 M HEPES, pH 7.6, and 10 μ l of 20 μ M quinacrine in 1 M HEPES, pH 7.6.
3. Incubate 5 min at growth temperature with shaking.
4. Gently pellet cells by centrifuging 3 to 5 min at $300 \times g$, room temperature. Remove medium and cool pellets on ice (~ 2 to 5 min).
5. Resuspend in 50 μ l ice-cold 100 mM HEPES (pH 7.6)/2% (w/v) glucose. Keep on ice.
6. Mount 2 to 3 ml onto con A-coated coverslips and observe using a FITC filter (UNIT 4.2).

The quinacrine signal is often faint, so be prepared to take images for up to 10 sec.

LABELING THE ENDOCYTIC PATHWAY IN LIVE CELLS WITH FM4-64

N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide (FM4-64; Molecular Probes), is an amphipathic fluorescent molecule that inserts into lipid membranes, fluorescing only when in this hydrophobic environment. Its charged polar head prevents passive diffusion through membranes, thus it labels only the exoplasmic leaflet of the bilayer. This property makes FM4-64 an excellent marker of the endocytic pathway. Briefly, cells are labeled with dye for a short pulse, then uninternalized dye, including that inserted into the plasma membrane, is washed away. The dye is then chased in live cells for 40 to 60 min, allowing it to reach a steady state localization. In wild-type cells, the FM4-64 accumulates at the vacuolar membrane. The total amount of cell-associated fluorescence represents the approximate rate of internalization over the pulse time, such that differences in intensity can reflect differences in internalization rates.

**ALTERNATE
PROTOCOL 1**

**BASIC
PROTOCOL 2**

Microscopy

4.13.3

If cells labeled with this method exhibit an altered fluorescent pattern, this suggests a block or kinetic delay in transit through the intermediate compartments of the endocytic pathway. Cells are viewed by fluorescence microscopy using a rhodamine/TRITC filter.

Materials

Yeast cells (see Strategic Planning)
Rich medium (UNIT 1.6) at growth temperature
FM4-64 (see recipe)

Heat block at assay temperature
Water bath with shaker set at assay temperature
Con A-coated slides (see recipe)
Rhodamine/TRITC filter

Additional reagents and equipment for yeast cell culture (UNIT 1.6) and fluorescence microscopy (UNIT 4.2)

1. Culture yeast cells overnight to log phase (~ 0.3 to 1.0 OD₆₀₀/ml UNIT 1.6). If cells are not already in rich medium, transfer them into rich medium at growth temperature by centrifuging 3 to 5 min at ~ 300 to $800 \times g$, room temperature, pouring off the supernatant, and resuspending in rich medium.
2. *Optional:* If the assay temperature is different than the growth temperature (e.g., when testing a temperature sensitive mutant at its restrictive temperature), shift cells to the assay temperature for 10 min to 1 hr.

From this point on, keep all medium and cells at the assay temperature as much as possible (use a heat block at assay temperature to hold microcentrifuge tubes while aspirating and washing).

3. Transfer 1 ml cells to a 1.5-ml microcentrifuge tube and centrifuge gently 0.5 to 5 min at $300 \times g$, room temperature. Aspirate medium and add 50 μ l FM4-64.

Using shorter centrifugation times to pellet the cells will reduce the time they are not at the assay temperature.

4. Incubate cells 15 to 20 min at growth temperature with gentle shaking.
5. Add 1 ml prewarmed medium to each tube, then centrifuge gently as described in step 3. Remove supernatant and add 1 ml prewarmed medium. Repeat this wash two more times (for a total of three), incubating cells in medium for 15 to 60 sec between centrifugations.

These wash steps are necessary to extract uninternalized dye from the exoplasmic leaflet of the plasma membrane. FM4-64 has an off rate of ~ 2 sec.

6. Incubate 30 to 60 min in shaking water bath at assay temperature.

Use longer chase times for lower assay temperatures.

7. Pellet cells gently as described in step 3 and aspirate medium. Gently resuspend pellets in 50 to 100 μ l medium. If testing a temperature sensitive (ts) allele, store samples on ice until mounted on coverslips.

Storage on ice will prevent further trafficking of the dye in the nonrestrictive environment, particularly in the case of ts alleles that recover at permissive temperature.

8. Mount 2 to 3 μ l onto con A-coated coverslips and observe immediately under a rhodamine/TRITC filter (UNIT 4.2).

INTERNALIZATION TIME-COURSE ASSAY USING FM4-64

In order to better evaluate the location of a block or decreases in the rate of endocytosis through endosomes, a time-course assay can be used. Briefly, cells are labeled on ice rather than at growth temperatures, washed quickly to remove uninternalized dye from the medium while leaving the plasma membrane labeling in place, and then allowed to internalize dye for 0 to 20 min (or longer) in a chase at assay temperatures. Cells are collected and kept cold until placed on coverslips for viewing. See Figure 4.13.1 for an example of wild-type endocytosis patterns over 0 to 20 min chase time.

Additional Materials (also see *Basic Protocol 2*)

FM4-64 (see recipe), ice cold

Rich medium with and without a carbon source (*UNIT 1.6*), ice cold

1. Culture yeast cells and incubate at restrictive temperatures (if appropriate) as described (see *Basic Protocol 2*, steps 1 and 2). Gently pellet four or more separate 1-ml aliquots of each strain in microcentrifuge tubes (see *Basic Protocol 2*, step 1), aspirate, and cool the pellets on ice. Resuspend each tube in 50 μ l FM4-64, prechilled on ice.

Use a 1:50 dilution of stock FM4-64 (see recipe).

2. Label the plasma membrane by incubating tubes 15 to 20 min in an ice bath with sufficient water to make a slush.
3. To one tube from each strain (zero time point), add 1 ml ice-cold rich medium without a carbon source and centrifuge 3 min at $\sim 300 \times g$, 4°C. Aspirate and repeat this wash once more. Resuspend pellets in 50 to 100 μ l rich medium with no carbon source. Keep these cells on ice at all times from this point forward.
4. Wash the remaining three (or more) tubes of each sample two times with cold rich medium containing a carbon source as described in step 3, but do not incubate between washes. Resuspend in 1 ml rich medium containing a carbon source.

Do not wash more than this, otherwise it is possible to lose too much plasma membrane labeling.

5. Place the tubes into a water bath at assay temperature with shaking. After 5 min, remove 1 tube for each sample and wash cells twice with ice-cold rich medium without a carbon source. Resuspend as in step 3 (keep as the 5 min time point).

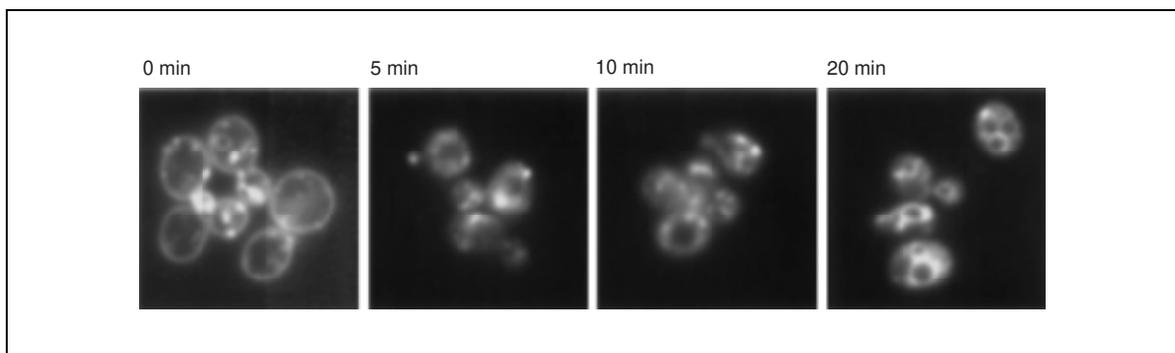


Figure 4.13.1 Wild-type behavior in an FM4-64 time-course assay. Cells were treated as described (see *Alternate Protocol 2*) with an assay temperature of 30°C and imaged using a Zeiss fluorescence microscope and 100 \times objective with a rhodamine/TRITC filter. Shown are wild-type images from time points 0, 5, 10, and 20 min. Note that at the zero time point, labeling is almost entirely on the plasma membrane, and by the 20 min time point, the label is mostly on the vacuolar membranes (one to five circular organelles that are $\sim 1/5$ to $1/2$ of the yeast cells).

Continue to incubate remaining tubes, collecting time points as described at 10, 15, and 20 min.

Longer time points can be collected as well by starting with more tubes for each sample in step 1 and continuing the assay. For lower assay temperatures, it may be necessary to collect chase time points for as long as 90 min.

6. Mount 2 to 3 μl onto con A-coated coverslips and observe with a rhodamine/TRITC filter (UNIT 4.2).

ENDOCYTIC RATE MEASUREMENT BY LUCIFER YELLOW UPTAKE IN LIVE CELLS

Lucifer yellow (LY) is a fluorescent fluid-phase dye that does not pass through membranes. It is taken into cells by endocytosis of bulk fluid-phase material from the extracellular space. It is delivered to the vacuole, where its accumulation is an indicator of general rates of internalization. This technique requires a few tricks, so on the first attempt be sure to follow the method and troubleshooting notes carefully. Note that it is unclear whether LY is completely stable for long-term storage. If the experiment doesn't work, try using a fresh batch of LY.

Materials

Yeast cells (see Strategic Planning)
Culture medium, preferably rich (UNIT 1.6)
Rich medium (UNIT 1.6)
40 mg/ml lucifer yellow (see recipe)
Phosphate/azide/fluoride buffer (see recipe), ice cold
16-G needle
Incubator with orbital shaker set at assay temperature
Con A-coated coverslips (see recipe)
FITC filter

Additional reagents and equipment for yeast cell culture (UNIT 1.6) and fluorescence microscopy (UNIT 4.2)

1. Culture yeast cells in culture medium (preferably rich) overnight to 1×10^7 cells/ml (~ 0.1 OD₆₀₀/ml depending on strain) at growth temperature (UNIT 1.6).

The overnight culture should be sufficiently dilute such that the density never exceeds this value—i.e., if the cells grow to a density greater than ~ 0.1 OD₆₀₀/ml, it is recommended that a new culture be started for this assay. The cells should not be diluted to a lower density and regrown until they have doubled.

For some strains, it is very important that the cells have never exceeded this density in liquid culture (see Critical Parameters); however, some labs have reported using cells that are ~ 0.5 OD₆₀₀/ml with success. The variability may be dependent on strain and LY batch/lot. The authors have found that SEY6210 does not work at all for LY-uptake experiments; however, the Research Genetics wild-type strain BY4741 has worked very well in the authors' experience.

2. Gently pellet 1 ml cells by centrifuging 2 min at 300 to 800 \times g, room temperature. Aspirate the medium.
3. Resuspend cells in 90 μl rich medium and add 10 μl of 40 mg/ml lucifer yellow. Mix well by pipetting carefully. Do not vortex.
4. Close the tube and poke a hole in the lid with a 16-G needle to allow gas exchange.

Gas exchange is very important.

5. Incubate tubes at growth or assay temperature with orbital mixing for 1.5 to 2 hr in the dark (e.g., covered with aluminum foil). Try to use an air incubator to avoid contamination of the sample through the hole in the lid.

It is important that cells be continuously aerated during this process through the hole in the lid. Also, mixing the tubes by snapping or flicking the tube periodically may help maintain proper aeration.

6. Add 1 ml ice-cold phosphate/azide/fluoride buffer and centrifuge as in step 2. Repeat this wash three times, resuspending the pellet between washes.

If cells are not forming tight pellets, remove only 80% to 90% of the medium from the pellet, then centrifuge again and remove the remaining medium for each wash.

7. Resuspend the final pellet in 30 μ l phosphate/azide/fluoride buffer and keep cells on ice.

8. Mount cells on con A–coated coverslips and view by fluorescence microscopy using a FITC filter (UNIT 4.2).

LY is stable for up to 4 hr in cells if kept cold, wet, and dark, so it is best to store cells in the ventilated tubes used for the assay on slushy ice that is kept covered. The signal may be faint, so be prepared to take images for up to 10 sec under the FITC filter.

Very bright vacuoles indicate cells that are dead or are about to die. This accumulation is due to a transporter in the vacuolar membrane that concentrates LY by a nonendocytic mechanism after the plasma membrane has become permeable.

LOCALIZATION OF PROTEINS IN LIVE YEAST CELLS WITH GFP-FUSION PROTEINS

BASIC PROTOCOL 4

A common technique for in vivo localization of a specific protein in any cell type is to create a chimera between the protein and the fluorescent protein GFP, or any of its variants. Described below is a method for viewing these fusion proteins in live yeast cells. This method was developed for use of fusion proteins with EGFP (Tsien, 1998), so keep in mind that use of different mutant forms of GFP may have slight differences in pretreatment of cells. In this assay, Tris buffer is added and incubated with cells briefly to achieve a slightly basic pH, which increases fluorescence of EGFP. In addition to this protocol, an alternate method (see Alternate Protocol 3) is described for viewing GFP fusion proteins in cells labeled with a rhodamine-phalloidin-labeled actin cytoskeleton.

Materials

Yeast cells expressing GFP-protein chimeras (APPENDIX 3A; also see Strategic Planning)

1 M Tris·Cl, pH 7.5 (APPENDIX 2A)

PBS (pH 7.4)/2% (w/v) glucose (see recipe)

Incubator set to appropriate growth temperature

Incubator set to assay temperature

Con A–coated coverslips (see recipe)

FITC filter

Additional reagents and equipment for yeast cell culture (UNIT 1.6) and fluorescence microscopy (UNIT 4.2)

1. Culture yeast cells expressing GFP-protein chimeras overnight to mid-log phase (\sim 0.3 to 0.8 OD₆₀₀/ml) at growth temperature (UNIT 1.6). If doing a temperature shift assay, incubate (i.e., shift) cells 15 to 60 min at assay temperature.

Use selective medium if the fusion protein is carried on a plasmid.

Microscopy

4.13.7

2. Add 1 M Tris·Cl, pH 7.5, to cultures to a final concentration of 10 mM.
3. Incubate cells ~5 min at assay temperature with shaking.
4. Centrifuge 1 ml cells in a 1.5-ml microcentrifuge tube 3 to 5 min at 300 to 800 × g, room temperature. Aspirate medium.
5. Resuspend cells in 50 to 200 μl PBS (pH 7.4)/2% (w/v) glucose.
6. Mount cells on con A–coated coverslips and view by fluorescence microscopy under a FITC filter (*UNIT 4.2*).

LABELING THE ACTIN CYTOSKELETON WITH RHODAMINE-PHALLOIDIN IN FIXED CELLS

The filamentous actin cytoskeletal network in yeast cells displays a dynamic polarized pattern throughout the cell cycle (Amberg, 1998). In cells about to bud and in small budded cells, filamentous actin cortical patches are polarized to the site of the newly emerging or emerged bud. Cytoplasmic filamentous actin cables are aligned along the mother-bud axis. As the yeast cell switches from polarized to isotropic growth, the actin patches and cables reorient throughout the mother and bud. At cytokinesis, the actin patches reaggregate to the mother-bud neck and the cables orient in both mother and bud towards the neck as well. Mushroom toxins, such as phalloidin, bind filamentous actin with high specificity. Attachment of fluorescent tags, such as rhodamine, to phalloidin allows the use of this toxin as a label in yeast cells for the filamentous actin cytoskeleton. Briefly, cells are fixed in mid-log phase to ensure a mixed population of early budded, late budded, and cytokinesing cells. The cells are then permeabilized and incubated with toxin. DAPI (Molecular Probes) is included in the toxin incubation to stain nucleic acids. This assists in determining cell cycle stage (see Lew and Reed, 1995). Cells are washed to remove unbound dye and viewed under fluorescence microscopy with rhodamine/TRITC and UV/DAPI filters. See Figure 4.13.2 for an example of F-actin patterns in early budded cells as well as those undergoing cytokinesis. In addition, see Figure 4.13.3 for the depolarized, chunky F-actin pattern in mutant cells.

Materials

- Yeast cells (see Strategic Planning)
- Rich or selective medium (*UNIT 1.6*)
- In-medium fixation cocktail (see recipe)
- Overnight fixation cocktail (see recipe)
- PBS, pH 7.4 (see recipe)
- 0.2% (v/v) Triton X-100 in SHA buffer (see recipe)
- PBS containing phalloidin and DAPI (see recipe)
- DABCO antifade (see recipe)
- Water bath with shaker set at assay temperature
- End-over-end rotator
- Con A–coated coverslips (see recipe)
- Rhodamine/TRITC and UV/DAPI filters
- Additional reagents and equipment for yeast cell culture (*UNIT 1.6*) and fluorescence microscopy (*UNIT 4.2*)

Prepare cells

1. Incubate 10 ml yeast cell cultures overnight to early- to mid-log phase (0.3 to 0.7 OD₆₀₀/ml) in rich or selective medium (*UNIT 1.6*).

Try to keep the final concentration (step 3) below 0.7 OD₆₀₀/ml.

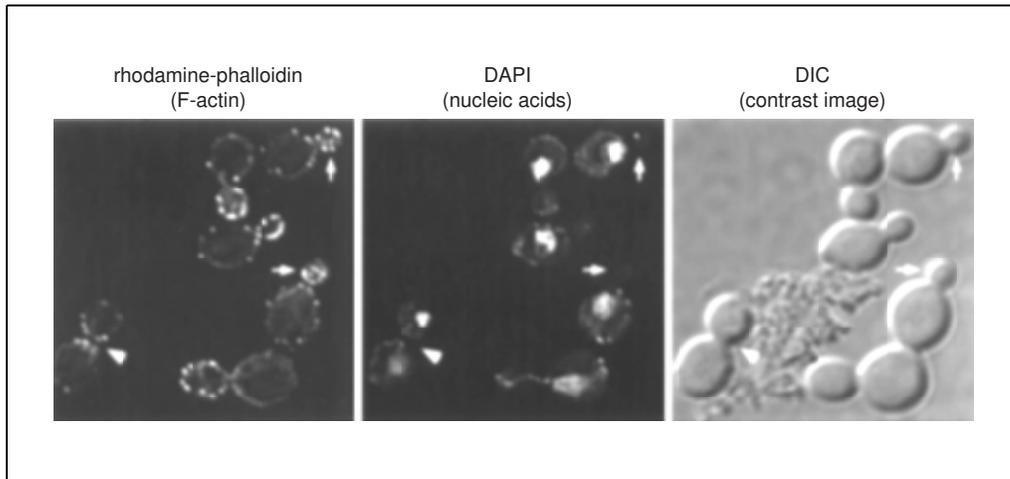


Figure 4.13.2 Filamentous actin in fixed wild-type yeast cells. Cells were treated as described (see Basic Protocol 5) with an assay temperature of 37°C and a temperature shift to 37°C 90 min before fixing. Cells were imaged using a Zeiss fluorescence microscope and 100× objective with a rhodamine/TRITC filter, DAPI optics, and differential interference contrast (DIC). The F-actin and nucleic acid images were deconvolved using a Deltavision deconvolution microscopy program. Arrows in all three panels show polarized F-actin patches in the growing bud of yeast cells in the early bud stage. The small number of patches in the mother cell are sometimes seen but indicate a slight depolarization, which may be due to incomplete recovery from the temperature shift. Arrow heads in all three panels delineate the mother-bud neck in cells undergoing cytokinesis. The large spots in the DAPI images are nuclei, and the smaller spots are nucleoids in mitochondria.

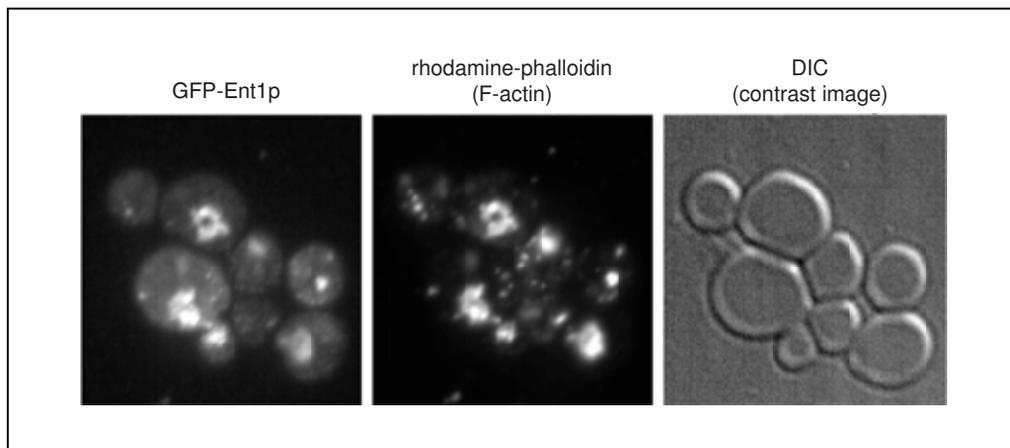


Figure 4.13.3 GFP-Ent1p and F-actin colocalization in mutant cells. Cells were treated as described (see Alternate Protocol 3) with an assay temperature of 30°C. Cells were imaged using a Zeiss fluorescence microscope and 100× objective with a FITC filter, a rhodamine/TRITC filter, and DIC. Note that the F-actin is both depolarized (not localized to growing small buds) and found in large clumps rather than small punctae. The GFP-Ent1p is localizing to these mislocalized F-actin clumps. Reprinted from Watson et al. (2001) with permission by the American Society for Cell Biology.

2. If the assay temperature is different than the growth temperature, shift the cells to the assay temperature when their concentration is in early-log phase (0.2 to 0.4 OD₆₀₀/ml), so that by the end of the shift (≥90 min) they will still be in the correct range.

Even wild-type yeast cells respond to heat shock by temporarily reorganizing their actin cytoskeleton to a depolarized state. A prolonged exposure (90 min) to the elevated temperature is required to allow cells to re-establish their normal actin polarity; therefore, cells must be shifted for ≥90 min prior to fixation.

Fix cells

3. Record the final concentration (OD₆₀₀/ml) of each sample. Then, in a ventilated hood, add 2.5 ml in-medium fixation cocktail to each 10 ml cell culture, adding directly to the culture (i.e., do not pellet cells).
- 4a. *For nonshifted cells:* Place cells back into a water bath with shaker and incubate 1 to 2 hr.
- 4b. *For temperature-shifted cells:* Keep cells in fix at the elevated temperature for at least the first 20 min out of a total 1 to 2 hr fixation period (see step 4a).

Once cells have begun to fix, temperature should no longer matter.

5. After at least 1 hr of fixation, prepare cells for further fixing overnight. Pellet cells (i.e., centrifuge 5 min at 1500 ×g, room temperature), aspirate, and resuspend in 1 ml overnight fixation cocktail per sample. Transfer to a 1.5-ml microcentrifuge tube.
6. Incubate overnight at room temperature, with agitation (end-over-end rotator), at room temperature.
7. Centrifuge 5 min at 1500 to 2000 ×g, room temperature. Wash once by resuspending in 1 ml water (use this resuspension to transfer cells to 1.5-ml microcentrifuge tubes if they are not in them already), then pelleting cells and aspirating. Wash twice with 1 ml PBS, pH 7.4.

Because cells are fixed, faster centrifugation (as denoted above) is okay.

Permeabilize cells

8. Resuspend cells in 1.0 ml of 0.2% (v/v) Triton X-100 in SHA buffer and incubate 10 to 15 min at room temperature.

There is no need to agitate.

9. Wash three times in 1 ml PBS to remove detergent. Resuspend cells to 10 OD₆₀₀/ml in PBS (using final OD₆₀₀/ml from step 3). Remove 100 μl (i.e., 1 OD₆₀₀) to a new tube, pellet cells and aspirate liquid.

It is important to wash out the detergent well. Remaining detergent will increase the level of background signal.

Label cells

10. Label cells by resuspending in 30 to 50 μl PBS containing rhodamine-phalloidin and DAPI. Incubate in the dark 1 hr at room temperature.

Alternative fluorescent labels conjugated to phalloidin are available and should work with this method.

It is also possible to label cells overnight at 4°C in the cold room. This works quite well. If this is done, wash at least two additional times.

11. Wash 3 times in 0.5 ml PBS.

To avoid losing the pellet in this step, remove the PBS from the pellet during each wash by pipetting rather than aspirating.

12. Resuspend final pellet in 30 to 50 ml DABCO antifade. Mount 2 to 3 ml of cells on con A-coated coverslips and view with rhodamine/TRITC and UV/DAPI filters (UNIT 4.2).

LABELING OF LIVE CELLS WITH RHODAMINE-PHALLOIDIN FOR COLOCALIZATION WITH GFP FUSION PROTEIN

**ALTERNATE
PROTOCOL 3**

Fixation is sometimes deleterious to GFP localization, in particular for some peripherally associated membrane proteins. The following method is used to label unfixed cells with rhodamine-phalloidin, which allows GFP localization to be maintained. Instead of fixing cells, they are simultaneously permeabilized and labeled with rhodamine-phalloidin. It is assumed that the actin cytoskeleton and associated proteins are immobilized during the process of labeling due to the ability of phalloidin to stabilize actin filaments (Cooper, 1987). With this method, GFP localization is often preserved; however, this should be confirmed by comparison to a control in the same experiment. This control should consist of nonpermeabilized live cells containing the same GFP-fusion protein but not stained with phalloidin (see Basic Protocol 4). See Figure 4.13.3 for an example of a GFP-tagged protein that colocalizes with F-actin in mutant cells.

Additional Materials (also see Basic Protocol 5)

Yeast cells expressing GFP-fusion constructs (APPENDIX 3A; also see Strategic Planning)

Permeabilization/staining solution (see recipe)

FITC and rhodamine/TRITC filters

Additional reagents and equipment for yeast cell culture (UNIT 1.6) and fluorescence microscopy (UNIT 4.2)

1. Culture yeast cells expressing GFP-fusion constructs overnight to early- to mid-log phase (~0.3 to 0.8 OD₆₀₀/ml; UNIT 1.6).

Use selective medium if the fusion protein is carried on a plasmid.

2. Harvest 1 to 5 ml cells (less if the OD₆₀₀ is at the upper value). Centrifuge 5 min at 300 to 600 × g, room temperature. Resuspend in 50 to 100 μl permeabilization/staining solution.
3. Incubate 10 min in the dark, then place on ice an additional 10 to 15 min, also in the dark.
4. Mount 2 to 3 μl cells on con A-coated coverslips and view under FITC and rhodamine/TRITC filters (UNIT 4.2).

For unknown reasons, many cells will exhibit either one fluorescent label or the other, so be patient in looking for cells with both labels. It is generally better to use the FITC channel to choose an area of cells, then switch to the rhodamine channel to check for phalloidin labeling.

Microscopy

4.13.11

**LOCALIZATION OF A PROTEIN IN FIXED YEAST CELLS WITH
IMMUNOFLUORESCENCE**

Methods for the use of immunofluorescence in yeast are very similar to methods for labeling mammalian cells, although the small size of yeast cells can make detailed localization slightly more difficult. However, many organelles have characteristic localization or available marker proteins so that double-labeling experiments with a specific protein can be effective. For choosing and pretesting antibodies for immunofluorescence, refer to *UNIT 4.3* on mammalian cell immunofluorescence. In brief, yeast cells are fixed, made into spheroplasts to remove the cell wall (which is necessary for antibody access to intracellular compartments), and permeabilized before they are attached to coverslips or slides using polylysine. The cells are then blocked, labeled with primary antibody, labeled again with fluorescently conjugated secondary antibodies, and viewed using a fluorescence microscope.

Materials

- Yeast cell cultures (see Strategic Planning)
- 1× and 5× fixation cocktail (see recipe)
- SHA buffer (see recipe)
- Spheroplasting solution (see recipe)
- 1% (v/v) Triton X-100 in SHA buffer
- 2 mg/ml poly-L-lysine (Sigma; store up to 3 months to 1 year at -20°C)
- WT buffer (see recipe)
- Primary and secondary antibodies
- Fixed or spheroplasted cells from a strain with the target antigen deleted (optional)
- 1 mg/ml DABCO antifade (see recipe)
- Nail polish

- Incubators set at growth temperature and 30°C
- Standard slides or slides coated with Teflon to form wells (Polysciences)
- Pipet tip connected to an aspirator
- Moisture chamber (see recipe)
- Weight (e.g., book)
- Fluorescence filters

- Additional reagents and equipment for yeast cell culture (*UNIT 1.6*) and fluorescence microscopy (*UNIT 4.2*)

NOTE: It is recommended that 1× AEBSF or similar protease inhibitors be added to all prepared solutions used in this protocol. This reduces loss of rare antigens due to activity remaining from vacuolar proteases.

Fix cells

1. Incubate 10 ml yeast cell cultures overnight to mid- to late-log phase (~0.5 to 1.0 OD₆₀₀/ml; *UNIT 1.6*).
2. Add 2.5 ml of 5× fixation cocktail to each 10 ml of culture. Shake for 1 to 2 hr at growth temperature.
3. Centrifuge 5 min at 300 to 800×g, room temperature. Pour off medium and resuspend in 1 ml of 1× fixation cocktail. Transfer to 1.5-ml microcentrifuge tubes. Fix cells for 12 to 24 hr with gentle mixing at room temperature.
4. Pellet cells as described in step 3 and aspirate the supernatant. Resuspend in a small amount of SHA buffer for storage at 4°C (up to 1 to 2 days) or continue directly to step 6 for immediate use.

Make spheroplasts and permeabilize cells

5. If cells have been stored, pellet as described in step 3. Aspirate the supernatant.
6. Resuspend in 1 ml spheroplasting solution. Incubate 30 min at 30°C (or lower temperature if assay/growth temperature was below 30°C, but increase time of incubation by 5 to 10 min for every 2°C less) flicking tubes every 10 min or so to mix.
7. Centrifuge 5 min at $300 \times g$, room temperature. Aspirate supernatant and resuspend in 0.5 ml of 1% (v/v) Triton X-100 in SHA buffer. Incubate ~10 min at room temperature.

As an alternative, 1% (w/v) SDS can also be used to permeabilize, but since it is a harsher detergent, it may damage cells.
8. Wash twice in 1 ml SHA buffer and resuspend in SHA buffer to ~1.2 OD₆₀₀ per 50 µl (~24 OD₆₀₀/ml).

Prepare for incubation on slides

9. Prepare slides by applying 10 to 20 µl of 2 mg/ml poly-L-lysine. Spread the solution with a pipet tip over the area of the slide where the cells should stick (usually a ~3- to 4-mm diameter circle). Incubate 15 min at room temperature

There are two types of slides that can be used. The first alternative is to use standard slides. After the cells settle, the area of interest will be visible on the slide. The second alternative is to use slides precoated with Teflon to form wells (e.g., Polysciences slides). This nicely contains the liquid on the slide during incubations and doesn't put pressure on the cells when the coverslips are applied, but it can lead to unflattened cells with multiple out-of-focus layers.

10. Aspirate polylysine by placing a pipet tip connected to an aspirator at one edge of the puddle to draw off the fluid. Wash three times by dripping water over the area and aspirating as with the polylysine.
11. Add 15 µl fixed spheroplasts (step 8) dropwise as in step 10 to each slide and let settle 15 to 30 min in a moisture chamber. Aspirate excess liquid as described above (step 10).

From now until mounting coverslips, be careful not to let the cells dry out on the slide and keep the slides inside the moisture chamber. Keep liquid on the cells at all times, aspirating just before adding a new solution.

12. Block cells by dropwise addition of 15 to 30 µl of WT buffer and incubation for at least 15 min at room temperature. Aspirate immediately before antibody treatment.
13. *Optional:* Incubate primary and secondary antibodies with fixed or spheroplasted cells from a strain with the target antigen deleted. Pellet the deletion cells out of the blocked antibody solutions (i.e., 5 min at $\geq 10,000 \times g$, room temperature) and use the supernatant (which will already be diluted) for steps 14 (primary) and/or 17 (secondary).

Try to use the same dilutions and incubation times as will be used in the assay (it is often convenient to set this up during step 12). This can help reduce background, if needed. In addition, microcentrifuging all antibodies (even those not preincubated with deletion cells) can help by removing precipitates. After diluting the antibodies in WT buffer (both primary and secondary), pellet precipitates by microcentrifuging 5 min at maximum speed, room temperature.

Incubate in primary antibody

14. Add 15 μ l primary antibody diluted in WT buffer to the blocked cells on the slide. Incubate 60 to 90 min at room temperature or overnight at 4°C in the moisture chamber.

The dilution concentration depends on the antibody but typically ranges from 1:10 to 1:500. A good rule of thumb is to use ten times the concentration used for successful immunoblotting.

15. Wash five times with 15 to 30 μ l WT buffer by dripping liquid over the cells and then aspirating for each wash.

Incubate in secondary antibody

16. Add 15 μ l secondary antibody diluted in WT buffer. Incubate 45 to 90 min in the dark at room temperature or overnight at 4°C in the moisture chamber.

Longer incubations will give higher backgrounds.

Signal can be increased by using a nonconjugated secondary antibody and a fluorescently conjugated tertiary antibody. For example, use primary antibody raised in a rabbit that recognizes the protein, followed by secondary antibody goat anti-rabbit IgG, then tertiary antibody FITC-conjugated donkey anti-goat IgG. For each antibody after the primary, follow the secondary antibody incubation times and wash conditions in this step.

17. Wash five times as described in step 15.

Mount the samples

18. Aspirate the liquid and add 3 to 5 μ l of 1 mg/ml DABCO antifade to the cells. Place the coverslip over the cells on the slide (or overturn the coverslip with the cells attached onto a slide). For gentle flattening of the cells for viewing, cover the entire slide (with coverslip already present) with a folded Kimwipe (to absorb excess mounting solution) and press down firmly and evenly with a weight (e.g., a book) for 1 to 2 min.

Paraphenylenediamine (PPD) is sometimes used for antifade, but it has been found to cleave some fluorophores off the antibody. DABCO does not cause this problem.

19. To seal the coverslip and prevent drying of cells, paint the edges of with nail polish. Let dry in the dark.

Be careful not to use too much or the polish will seep under the coverslips and obscure the cells.

20. View cells under the appropriate fluorescent filters, which depends on the fluorophore conjugated to the final antibody (UNIT 4.2). To store cells for viewing on another day, keep the already sealed slides at 4°C (or -20°C) in the dark (up to 2 weeks).

ALTERNATE PROTOCOL 4

LOCALIZATION IN SPHEROPLASTED THEN FIXED CELLS WITH IMMUNOFLUORESCENCE

Although the method described above (see Basic Protocol 6; i.e., fixation followed by spheroplasting cells) generally works well, this alternative method works better for labeling some proteins. The best method may depend on the antigen or antibody, and needs to be determined empirically; however, for proteins localized to cortical actin patches, the cells need to be fixed before spheroplasting to maintain polarized localization.

Materials

Yeast cell cultures (see Strategic Planning)

Softening medium: 0.1 M Tris·Cl, pH 9.4 (*APPENDIX 2A*)/10 mM DTT (freshly prepared)

Spheroplasting medium (see recipe)

37% (v/v) formaldehyde

SHA buffer (see recipe)

SHA buffer/1% LDAO (see recipe)

Incubator set to growth temperature

30°C incubator with orbital shaker

Additional reagents and equipment for yeast cell culture (*UNIT 1.6*) and permeabilizing and staining cells for proteins (see Basic Protocol 6, steps 9 to 20)

NOTE: It is recommended that 1× AEBSF or similar protease inhibitors be added to all solutions prepared for this protocol. This reduces loss of rare antigens due to activity remaining from vacuolar proteases.

1. Incubate 10 ml yeast cell cultures overnight to mid- to late-log phase (~0.5 to 1.0 OD₆₀₀/ml).
2. Centrifuge an amount of cells equivalent to an OD₆₀₀ of 10 for 5 min at 300 to 800 × g, room temperature. Aspirate and resuspend in 2 ml softening medium. Incubate 10 min at room temperature.
3. Pellet cells gently as described in step 2, aspirate, and resuspend in 2 ml spheroplasting medium. Mix by vortexing on low. Incubate 10 min with orbital shaking at 30°C.
4. To fix cells, add 37% formaldehyde to a final concentration of 4%. Incubate 1 hr at 30°C.
5. Pellet cells gently as described in step 2, aspirate, and resuspend in 1 ml SHA buffer. Transfer to a 1.5-ml microcentrifuge tube.
6. Pellet cells gently as described in step 2, aspirate, and resuspend in 0.5 ml SHA buffer/1% LDAO to permeabilize. Incubate 10 min at room temperature.
7. Wash cells twice with 1 ml SHA buffer, pelleting, aspirating, and resuspending each time.
8. Resuspend final pellet in 400 μl SHA buffer. Visualize proteins as described (see Basic Protocol 6, steps 9 to 20).

LABELING CELL WALL CHITIN DEPOSITION WITH CALCOFLUOR WHITE

Bud scars are enriched in the polysaccharide chitin and can be observed as rings on the cell walls of yeast. In haploid cells, the normal pattern of budding is axial (the next bud forms adjacent to the site of the previous bud), whereas in diploid cells it is bipolar (occurs from either end of the elongated cell). When cells are defective for processes such as endocytosis or for actin cytoskeletal structure, budding patterns often become random in the diploid cell, resulting in rounder, less football-shaped diploids. By labeling cells with calcofluor white (CfW), which binds irreversibly to chitin in the cell wall, improper budding can be seen as nonbipolar patterns of rings on diploid cells (e.g., bud scars are found at the equator). See Figure 4.13.4 for an example of normal and mutant diploid bud scars stained with calcofluor white.

BASIC PROTOCOL 7

Microscopy

4.13.15

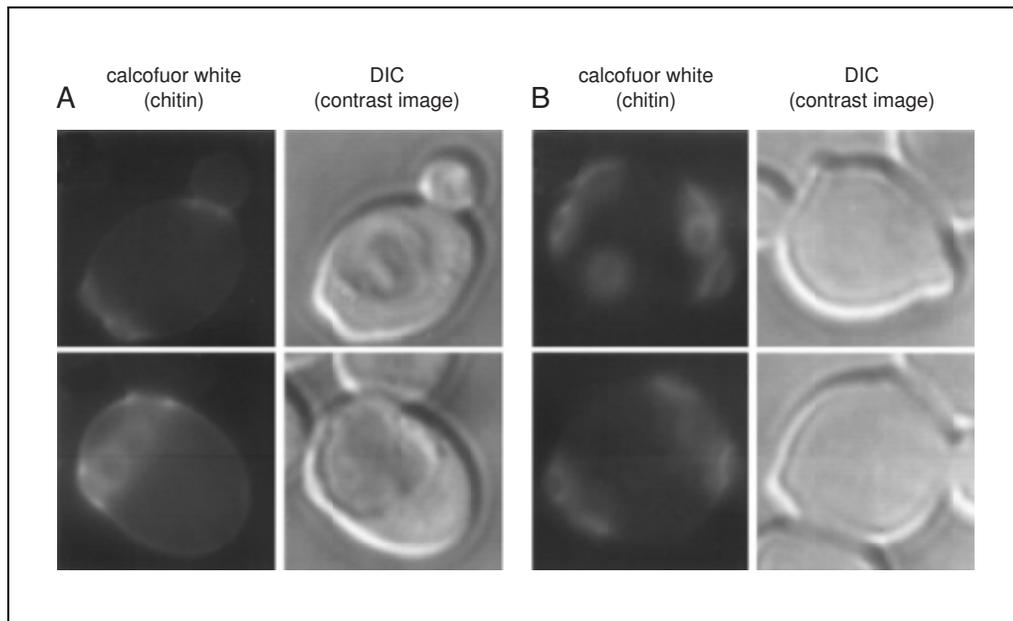


Figure 4.13.4 Bud scars in wild-type and mutant cells. Diploid cells were treated as described (see Basic Protocol 7). Cells were imaged using a Zeiss fluorescence microscope and 100 \times objective with DAPI optics and DIC. **(A)** Two cells exhibiting normal bud scars at each of the elongated ends of these diploid cells. **(B)** Two mutant cells, exhibiting randomly arranged bud scars. It is also clear that these diploid cells do not have a normal elongated shape.

Materials

Yeast cells (see Strategic Planning)
 1 mg/ml CfW (see recipe)
 Incubator set to growth temperature
 Con A–coated coverslips (see recipe)
 UV/DAPI filter

Additional reagents and equipment for yeast cell culture (*UNIT 1.6*) and fluorescence microscopy (*UNIT 4.2*)

- Culture yeast cells overnight to a high density (>1.0 OD₆₀₀/ml; *UNIT 1.6*).
This increases the population of cells that are old enough to have budded multiple times.
- Using an amount of cells equivalent to an OD₆₀₀ of 2 to 5, centrifuge 5 min at 300 to 800 \times g, room temperature. Aspirate medium.
- Resuspend cells to 5 OD₆₀₀/ml in 1 mg/ml CfW solution.
- Incubate 5 min at room temperature with gentle agitation.
- Centrifuge as described in step 2 and aspirate medium. Resuspend in 1 ml water, centrifuge again, and aspirate medium. Repeat this wash twice more.
- Resuspend final pellet in 100 to 500 μ l water.
Use more for a larger cell pellet.
- Mount cells on con A–coated coverslips and view under a UV/DAPI filter (*UNIT 4.2*).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

CfW, 1 mg/ml

Dissolve calcofluor white (CfW; Sigma) to 1 mg/ml in water. CfW takes a long time to dissolve; however, dissolution may be aided by the addition of 0.1 N sodium hydroxide dropwise with mixing until the solution clears. Store up to 3 months to 1 year in the dark at 4°C.

CfW is also known as Fluorescent Brightener 28.

Con A-coated slides

Prepare a 2 mg/ml stock solution of concanavalin A (con A; Sigma) in water (store up to 1 year at -20°C). Pipet 5 µl con A onto each 22 × 22-mm coverslip, spread with the side of a clean pipet tip, and allow to air dry in a dust-free room (if possible). Con A-coated coverslips can be made a few days in advance, but it is preferable to make them the same day of use. Store at room temperature.

Poly-L-lysine can also be used to immobilize yeast cells for microscopy (see Basic Protocol 6). Be sure to keep track of which side of the coverslip is coated.

DABCO antifade

Dissolve 1,4-diazabicyclo[2.2.2]octane (DABCO; Molecular Probes) in 90% glycerol to 1 mg/ml. Protect from light as much as possible during preparation. Store in the dark at -80°C for long term storage (i.e., up to 1 year), or store smaller aliquots at -20°C for short term use (i.e., up to 3 months).

PPD (paraphenylenediamine) is sometimes used for antifade, but it has been found to cleave some fluorophores off the antibody. DABCO does not cause this problem.

DAPI, 1 mg/ml

Dissolve sufficient 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes) in water to prepare a 1 mg/ml (1000×) solution. Store in the dark for up to 3 months to 1 year at -20°C.

Fixation cocktail, 5×

0.5 M potassium phosphate, pH 6.5 (see recipe)

20% (v/v) formaldehyde (from 37% stock)

Prepare fresh

All 37% (v/v) formaldehyde should be used within 3 months of opening.

FM4-64

Stock solution: Prepare a 1 mg/ml solution of FM4-64 (Molecular Probes) in DMSO. Store in small aliquots up to a few months at 20°C.

Working solution: Freshly dilute 1 mg/ml FM4-64 stock (see above) 1:50 for assay temperatures ≤30°C or 1:100 for assay temperatures >30°C in rich medium (UNIT 1.6). Once the dye is diluted in rich medium, prewarm to assay temperature.

IMPORTANT NOTE: *Do not prepare diluted FM4-64 more than 30 min prior to use.*

Repeated freeze thaws may decrease lifetime (see Critical Parameters).

HEPES/100 mM/ 2% (w/v) glucose

Dilute 1 M HEPES, adjusted to pH 7.6 with potassium hydroxide, 1:10 in water. Freshly add glucose from a concentrated stock (i.e., 50% w/v) to 2% (w/v). Store up to 24 hr at room temperature.

In-medium fixation cocktail

Combine 1.15 ml of 1 M potassium phosphate buffer, pH 6.5 (see recipe), and 1.35 ml of 37% (v/v) formaldehyde for every 10 ml cells to be fixed. Prepare fresh.

All 37% (v/v) formaldehyde should be used within 3 months of opening.

Lucifer yellow, 40 mg/ml

Dissolve the dilithium salt of lucifer yellow CH (LY; Molecular Probes or Sigma) to 40 mg/ml in water. Store at 4°C for short periods (weeks) or -20°C for long periods (months).

It is unclear whether LY is completely stable for long-term storage (see Critical Parameters). If the experiment doesn't work, try using a fresh batch of LY.

Moisture chamber

A moisture chamber is used for keeping the cells on the slides/coverlips from drying out during incubations; they should be kept moist and in the dark. One recommended method is to use a tissue culture dish (15 cm or 6 well) with a damp paper towel pressed into the lid (it shouldn't fall if it is damp, but not soaked, and pressed firmly) and aluminum foil covering the entire lid and base.

Overnight fixation cocktail

For every 10 ml needed, mix 7.9 ml water, 1.0 ml of 1 M potassium phosphate, pH 6.5 (see recipe), and 1.1 ml of 37% formaldehyde. Prepare fresh.

All 37% (v/v) formaldehyde should be used within 3 months of opening.

PBS, pH 7.4

10 mM sodium phosphate buffer, pH 7.4 (APPENDIX 2A)

150 mM NaCl

Filter, sterilize, and store up to 6 months at room temperature.

PBS containing phalloidin and DAPI

Dilute 200 U/ml rhodamine-phalloidin stock (see recipe) 1:10 and 1 mg/ml DAPI (see recipe) 1:1000 in PBS, pH 7.4 (see recipe). Make fresh within 1 hr of use and keep dark.

PBS (pH 7.4)/2% (w/v) glucose

Add glucose fresh from a concentrated stock (i.e., 50% w/v) to PBS, pH 7.4 (see recipe), at a final concentration of 2% (w/v). Prepare fresh.

Permeabilization solution

In PBS, pH 7.4 (see recipe) prepare:

1 M sorbitol

0.1% (w/v) saponin

0.1 mg/ml RNase

5 mM MgCl₂

Prepare fresh

Permeabilization/staining solution

Add 50 µl of 200 U/ml rhodamine-phalloidin stock (see recipe) per 1 ml permeabilization solution (see recipe). Make fresh within 1 hr of use and keep dark.

Phosphate/azide/fluoride buffer

50 mM potassium phosphate buffer, pH 7.5 (APPENDIX 2A)

10 mM NaN₃ (add fresh from concentrated stock)

10 mM NaF (add fresh from concentrated stock)

Prepare fresh

Some methods use sodium rather than potassium phosphate buffer.

Potassium phosphate, 0.04, 0.5, and 1 M (pH 6.5)

For a 1 M solution, mix 2 parts 1 M dibasic potassium phosphate (K_2HPO_4) with 1 part of 1 M monobasic potassium phosphate (KH_2PO_4). Store up to 1 year at room temperature. To prepare a 0.5 M solution, dilute 1:1 with water. For a 0.04 M solution, dilute 1:25 in water.

Rhodamine- or Texas red-phalloidin, 200 U/ml

Dissolve sufficient rhodamine- or Texas red-phalloidin in 1.5 ml methanol to prepare 200 U/ml as directed by manufacturer (see instructions on tube). Store in the dark for 3 to 6 months at $-20^{\circ}C$.

Alternative fluorescent labels conjugated to phalloidin are available and should also work.

SHA buffer

1 M sorbitol
0.1 M HEPES, pH 7.5 (adjust pH with NaOH)
5 mM sodium azide
Prepare fresh from stock solutions

SHA buffer/LDAO, 1% (w/v)

Freshly prepare 1% (w/v) lauryldimethylamine oxide from a concentrated (i.e., 10% w/v) stock solution in SHA buffer (see recipe). Prepare fresh.

Sodium citrate, 0.5 M (pH 4.0)

Prepare a 0.5 M solution of citric acid and adjust the pH to 4.0 with sodium hydroxide. Store up to 1 year at room temperature.

Spheroplasting medium

Prepare the following 10 to 50 ml (depending on the number of samples) synthetic growth medium (i.e., YNB/SD) containing amino acids and 2% glucose:

1 M sorbitol
0.04 M potassium phosphate buffer, pH 6.5 (see recipe)
10 μ g/ml zymolyase
Make fresh within 1 hr of use.

Spheroplasting solution

In SHA buffer (see recipe) prepare:
0.2% (v/v) β -mercaptoethanol
45 μ g/ml zymolyase (Seikagaku Corp.)
Make fresh within 1 hr of use.

WT buffer

50 mM HEPES, pH 7.5 (adjust pH with NaOH)
150 mM NaCl
0.1% (v/v) Tween-20
1 mM sodium azide
5% (w/v) IgG-free BSA
Prepare fresh if possible, but can be stored up to 2 days at $4^{\circ}C$ during the course of assay.

COMMENTARY

Background Information

Imaging in yeast cells is constantly improving due to advances in microscopy techniques, and also as a result of the development of new and better microscopes and organelle-specific labels (Kohlwein, 2000).

Elucidating pathways

Saccharomyces cerevisiae is best known for its genetic malleability, allowing easy creation and identification of mutant phenotypes. The membrane trafficking pathways in yeast cells have been extensively investigated and characterized over the last few decades, leading to identification of the molecular components of the secretory pathway (Novick et al., 1980), the endocytic pathway (Chvatchko et al., 1986; Raths et al., 1993; Munn and Riezman, 1994; Wendland et al., 1996), and the organelles involved in these two important transport pathways and movement between them (Wendland et al., 1998; D'Hondt et al., 2000; Antonny and Schekman, 2001). This unit provides a few of the methods that have been developed to easily, and sometimes quickly, identify defects in endocytic and vacuolar sorting pathways. In addition to these vital microscopic techniques are, of course, a complementary set of biochemical techniques to study many of these same molecular structures (Conibear and Stevens, 2002; Sipos and Fuller, 2002).

GFP-fusion proteins

The use of GFP-fusion proteins in both mammalian and yeast cells has exploded in the last decade or so. Roger Tsien provides a detailed and careful review of the many achievements since the discovery of this useful protein and its applications (Tsien, 1998). In genetically malleable yeast cells, creation and introduction of GFP-fusion proteins is fairly straightforward. Shuttle vectors are available that allow simple cloning of a specific gene directly into promoter- and GFP-containing vectors at polylinkers designed to allow in-frame insertions. In addition, GFP tags can be fused to the C terminus of a protein behind its endogenous promoter. Reviews on the creation and use of GFP-fusion proteins are also available (Rines et al., 2002; Tatchell and Robinson, 2002).

Labeling cells using rhodamine-phalloidin

Early work on yeast actin and tubulin led to the discovery of a distinct pattern in the distri-

bution and structure of these two cytoskeletal elements (Adams and Pringle, 1984). In *Saccharomyces cerevisiae*, the actin cytoskeleton can be recognized in two distinct forms, actin cortical patches and actin cables. Each structure corresponds to bundles of filamentous actin, but they are structurally distinct and believed to perform different functions. The stereotyped localization pattern of actin cytoskeletal elements during the cell cycle is required for normal polarized bud growth and cytokinesis. Early methods for visualization of filamentous actin in yeast cells using the mushroom toxin phalloidin are still very similar to those used today (Adams and Pringle, 1991), although the protocol described in this unit includes a permeabilization step that the authors have found increases the percentage of labeled cells dramatically.

Immunofluorescence

Immunofluorescence techniques have been applied in yeast cells with much success, and several suggestions and methodologies have been described previously (Pringle et al., 1989, 1991). The development of confocal microscopes that allow optical sections thinner than 0.4 μm have aided in the creation of better, more precise immunofluorescence images. In addition, colocalization images can be more definitive. The methods described here are only two of many possible immunofluorescence preparations available, although most methods used are simply variations on the two basic methods provided. Removal of the cell wall to allow antibody access to internal structures is an essential step to any immunofluorescence protocol using yeast, but because spheroplasting (cell wall removal) can be stressful to living yeast cells, many methods include this step after fixation is completed.

Labeling chitin in the yeast cell wall

Chitin is a minor component of the yeast cell wall in content, but it is essential for yeast cell survival. Its role in septum formation at the mother-bud neck in cytokinesis is believed to be this essential function (Cabib et al., 1982, 2001). Chitin is deposited in a ring at the site where a bud emerges. These chitin rings represent the major concentration of chitin in the cell wall, although minor amounts of chitin are distributed throughout to provide strength and rigidity. The action of three chitin synthases, Chs1p, Chs2p, and Chs3p, are required for

different functions in the synthesis and deposition of chitin during the cell cycle. Chs3p exhibits a unique form of regulation that requires the recycling endocytic pathway for delivery to sites of chitin synthesis (Ziman et al., 1996; Holthuis et al., 1998). In this assay, deposition of chitin in rings is visualized to examine cells for a polarized pattern of bud site selection. Loss of this polarized pattern correlates with defects in polarized cell growth in general and actin cytoskeletal defects (Casamayor and Snyder, 2002).

Critical Parameters

Labeling the vacuole in live cells with CDCFDA

CDCFDA must be made in anhydrous DMSO to prevent premature hydrolysis and maintain the neutral, diffusible form. For this assay (see Basic Protocol 1), the cells do not need to be at a precise concentration, but using cells in early- to mid-log phase at the time of assay and centrifuging as gently as possible will increase the likelihood of visualizing pretty yeast cells with vacuoles that are larger and fewer in number. Haploid yeast cells that are rounded rather than elongated or bent, with vacuoles visible as solid circles are desired.

Labeling acidic organelles with quinacrine in live cells

This is a fairly simple method (see Alternate Protocol 1), but the signal is often faint. As with labeling vacuoles (see Basic Protocol 1), it is preferable to have the yeast cells in early- to mid-log phase, but it is not required for the assay to work.

Labeling the endocytic pathway in live cells with FM4-64

FM4-64 is fairly stable in DMSO, but the authors have found that repeated freeze-thawing cycles will decrease its fluorescence. It is best to dissolve the compound in DMSO and aliquot small amounts for storage at -20°C . In addition, once the FM4-64 is diluted into medium, it will lose activity slowly, so do not prepare diluted FM4-64 too far in advance (see Reagents and Solutions). In this assay (see Basic Protocol 2), it is *very* important to treat the cells gently, because this will increase the likelihood of obtaining pretty yeast cells and vacuoles; therefore, pellet cells as gently as possible and resuspend them by pipetting or inverting tubes gently, never vortex. It is also

easier to measure pixel intensity of larger vacuolar membranes because there are fewer overlapping membranes through the yeast cell, so use cells that are closer to early- to mid-log if possible. Finally, it is important to wash the cells in medium thoroughly after the pulse incubation to make sure that all plasma membrane labeling is removed. Leftover FM4-64 on the plasma membrane may mask any dim phenotypes because the cells will have plenty of time during the chase to internalize this label.

Internalization time course with FM4-64

Follow many of the critical parameters described for labeling the endocytic pathway (see Basic Protocol 2) for this method (see Alternate Protocol 2) as well; however, it is important *not* to wash cells thoroughly after labeling. In this method, plasma membrane labeling is necessary since no internalization should have occurred during the label period on ice. Wash cells gently and briefly by spinning immediately after resuspension, keeping them cold. For better temperature control during the labeling and washing process, keep microcentrifuge tubes sitting in slushy ice. Finally, keep in mind that chase time assay temperatures $<30^{\circ}\text{C}$ will require longer time points than 20 min, and may take up to 60 to 90 min before cells exhibiting a kinetic delay (i.e., mutants) will show vacuolar labeling.

Endocytic rate measurement by LY uptake in live cells

Endocytic measurement by lucifer yellow (LY) uptake (see Basic Protocol 3) often requires strict adherence to several critical parameters, and in some strains lucifer yellow uptake will not work at all. In particular, for this fluid to be internalized successfully in many strains, the cells must be grown overnight from inoculation to very early- or early-log phase only. To achieve this most conveniently, it is often easier to inoculate a dilution series of the cells for overnight cultures, allowing use of whichever culture is at the best concentration when ready to begin the assay. Some other laboratories, however, have found that their strains can be at mid-log concentrations and still internalize lucifer yellow at high rates. Thus, after the described method has been found to work, it may be desirable to experiment with different growth concentrations to see if mid-log phase cells work for the strain in question.

While cells are incubated with the lucifer yellow label, it is *essential* that they be allowed proper gas exchange with the outside air. Closed microcentrifuge tubes do not allow this, so it is best to use a needle (~16 to 18 G) to poke a hole in the top of each of the sample tubes. Do not invert the tubes after this. It is also very important that the lucifer yellow be kept in the dark at all times. Cover the tubes with aluminum foil whenever possible and keep them in the dark on ice until they are mounted onto slides. Finally, lucifer yellow has been reported to work after storage at both 4° and -20°C. Once the assay is working with fresh stocks, it may be a good idea to try several different storage conditions to see which one works best for the particular lucifer yellow stock being used.

Localization of proteins in live yeast cells with GFP fusion proteins

Viewing GFP-fusion proteins can be very easy, or a terrible ordeal, but this depends much more on the fusion protein than on the GFP viewing method. The method described in this unit (see Basic Protocol 4) is fairly simple, and the only critical parameter is that if the GFP-fusion protein is not integrated into the genome of the yeast strain being used, selection for the plasmid carrying it must be maintained at all times. Also, if the medium is already neutral/slightly basic, it is not necessary to add the Tris buffer. The best way to determine this is a side-by-side comparison.

Labeling the actin cytoskeleton with rhodamine-phalloidin in fixed cells

There are several critical parameters for labeling the actin cytoskeleton properly, but most of them involve proper preparation of cells before fixation. In order to achieve a nice mix of cell cycle stages at the end of this assay (see Basic Protocol 5), it is vital that the cells be either in early- or mid-log phase. Even late-log phase will result in a much lower percentage of early budded and cytokinesing cells. To this end, if cells are diluted in the morning, make sure that they have doubled *more* than one time to get all of the cells back into the early- to mid-log stages. In addition, even in wild-type cells, heat shock (i.e., a temperature shift from the overnight growth temperature), will result in complete depolarization of the actin cytoskeleton (as does centrifugation at excessive speeds), followed by a repolarization. Repolarization is a 90-min process, so if cells are shifted, they need to be at the assay temperature for ≥90

min before fixation. During this shift, keep in mind that the concentration is still important because the cells will continue to grow during this shift, and often grow faster at the higher temperature. During the assay, it is important to wash the cells well between each major step (fixation to permeabilization, permeabilization to dye labeling, dye labeling to viewing) to prevent the accumulation of background fluorescence.

Labeling of live cells with rhodamine-phalloidin for colocalization with GFP fusion proteins

This is a pretty simple method (see Alternate Protocol 3) that, if it works, can be very informative. The main critical parameter is to prepare GFP-labeled only cells (see Basic Protocol 4) alongside this assay to make sure that permeabilization or treatment with the toxin does not affect the GFP pattern.

Localization of a protein in fixed yeast cells with immunofluorescence

Immunofluorescence techniques are all fairly similar and have a few standard critical parameters. The most important is to use a very specific primary antibody. Polyclonal antibodies are often used, but they need to be tested thoroughly for their specificity. This is usually done by immunoblot, which is described in *UNIT 6.2*. Washes between antibody incubations are necessary to remove nonspecifically bound antibodies, and it is also important to make sure the cells are kept moist at all times. In addition, it is important to make sure that the protocol instructions (see Basic Protocol 6) and incubation times are followed carefully. Finally, when doing colocalization, always test the fluorescent tags for bleed-through in both fluorescent channels/filter sets by preparing two extra sets of cells, each with one of the two labels being used.

Localization in spheroplasted, then fixed cells with immunofluorescence

The same critical parameters apply for this protocol (see Alternate Protocol 4) as for localization of proteins in fixed yeast cells with immunofluorescence (see Basic Protocol 6). If it is not known what localization pattern is expected, it is a good idea to try both this protocol and Basic Protocol 6, to determine which label works best, as well as to verify that the pattern of fluorescence is the same with both methods.

Labeling cell wall chitin deposition with calcofluor white

This method (see Basic Protocol 7) is fairly simple. To make sure that most cells will be old (i.e., have undergone multiple divisions), it is necessary to have a cell population that is saturated. This decreases the percentage of newly formed daughter cells that will have only the birth scar.

Troubleshooting

With all fluorescent methods, the first thing to try if there is little to no fluorescent signal is a fresh batch of the fluorescent compound. Also, *always* include a wild-type control in all assays in case the lack of signal is from a mutant strain. Finally, make sure that the correct filter sets for the compound being visualized are being used on the microscope (spectra for dyes are usually available from the suppliers).

Labeling the vacuole in live cells with CDCFDA

If a fresh batch of CDCFDA is being used and a good signal is still not achieved, try adjusting the concentration of CDCFDA in the incubation. Too much may be causing quenching of the signal, so try starting with a range from ~5 μM to ~20 μM to check for improvement in signal. Also, try adjusting the pH or concentration of sodium citrate in case the content of the medium is affecting the assay.

Labeling acidic organelles with quinacrine in live cells

If a fresh batch of quinacrine is being used and a good signal is still not achieved, try decreasing the concentration of quinacrine by setting up four tubes of the same strain and using final concentrations of 1, 1.5, 2, and 2.5 μM . If a better signal is obtained, it may be desirable to try a series of concentrations around the best signal until the optimum concentration for the strain is obtained. If the signal is still too low, try increasing the incubation time with the dye.

Labeling the endocytic pathway in live cells with FM4-64

If a fresh batch of FM4-64 is being used and a good signal is still not achieved, try using the FM4-64 at a higher concentration/lower dilution (i.e., 1:50 or 1:75 if 1:100 is currently being used). If a higher concentration of FM4-64 is already being used, try using a lower concentration (more dilute), because high concentra-

tions of FM4-64 can quench the signal. If the amount of fluorescent signal is high enough, but the wild-type yeast vacuoles are unusually fragmented, try using cells closer to early log phase. If the cells are not rounded and pretty, centrifuge at lower speeds and resuspend as gently as possible to improve their structure.

Internalization time course with FM4-64

If the fluorescence is too dim in the wild-type cells in this assay, troubleshooting will depend on which time point is giving the low signal. If the zero time point is missing plasma membrane labeling, most likely the washing is too thorough. Decrease the number, time of incubation, and/or volume of washes for all of the samples (so that later time points also maintain this PM label to have dye to internalize), and try to resuspend cells more gently. If the first time point after zero is too faint, but the zero time point and later times were okay, it may be that the assay temperature and strain being used require more time to internalize before the first time point is taken. If all of the time points after zero are too faint, these samples may be being washed more thoroughly than the zero time point. Try to make washes as close to equal as possible. Finally, if the final time point does not show mostly vacuolar staining in at least the wild-type cells, it is necessary to extend the assay to later time points until it does. Keep in mind that the number of samples to view under the microscope later increase rapidly with this assay, so rather than including additional time points, it may be desirable to spread the time points evenly over the extended time.

Endocytic rate measurement by LY uptake in live cells

If a fresh batch of lucifer yellow is being used, and a good signal is still not achieved, first try borrowing or getting a few different background strains and testing them out. As a positive control, the Research Genetics strain, BY4741 has worked most robustly and reliably for the authors. If none of the other wild-type strains give a visible/measurable signal, then start troubleshooting the method. If some of these strains give a signal, but the laboratory background strain does not, it may be that the strain will not work with this assay (for example, the authors have never seen lucifer yellow uptake in the wild-type strain SEY6210). To troubleshoot the method, start by making sure that all of the details of the protocol are being

followed, then try some of these suggested modifications to improve the signal.

1. Use one or two strains at several different starting concentrations and see if any one concentration works better.
2. Try altering the lucifer yellow concentration during the internalization incubation.
3. Try increasing the size of the gas exchange hole in the microcentrifuge tube, or increasing or decreasing the agitation rate during the dye incubation.

Keep in mind that this signal is often very low even when the assay works, so if vacuolar labeling is visible in the wild-type at all, this may be the maximal signal. Also remember that an extremely brightly stained vacuole, as noted in the method, is a sign of a dying yeast cell and is not due to endocytosis.

Localization of proteins in live yeast cells with GFP fusion proteins

If a good signal is not being achieved for the fusion protein, start by trying a GFP-fusion protein that is known to work (e.g., borrow from another laboratory or obtain one for which details on construction and strain are published so it will be known that nothing is being missed). If neither works, try changing to a different medium in case the experimental medium is too acidic or contains fluorescence quenchers. If the tester GFP-fusion protein works, then it is likely to be a problem with the fusion protein. First confirm that the fusion protein is stably produced by immunoblotting (*UNIT 6.2*). Also, many GFP-fusion proteins give a faint signal when a non-GFP version of the same protein is already available in the cell. Try integrating the GFP fusion in place of the genomic copy (or deleting the genomic copy and using the GFP fusion as the only copy on a plasmid). If this doesn't work, try using a high-copy-number vector, but keep in mind that increasing the levels of the fusion protein can harm the cell and/or result in altered localization due to high protein levels. Sometimes fusing GFP to the protein affects its folding/fluorescence, localization, or function, so it is possible to try creating an N-terminal fusion if previously a C-terminal fusion was created. Keep in mind that any fusion proteins used should be tested for functionality by rescue of mutant phenotypes wherever possible.

Labeling the actin cytoskeleton with rhodamine-phalloidin in fixed cells

If a fresh batch of rhodamine-phalloidin is being used and a good signal is still not

achieved, it may be that the cells are not being sufficiently permeabilized. Try using about half the total OD₆₀₀ amount of cells and make sure to use a full 1 ml of 0.2% Triton X-100 for the permeabilization step. If the actin is not properly polarized, the cells may be becoming distressed before or at the beginning of fixation. Be extra careful to treat cells gently until they have been in the fixation solution for at least 1 hr. If the cells are deformed or otherwise ugly in structure, the formaldehyde being used during fixation may be bad or the centrifugation used during preparation may be too fast. Try using fresh formaldehyde first. Finally, if a high background level of fluorescence is being observed, try adding extra washing steps after both the permeabilization (high background often comes from residual detergent when phalloidin is added) and phalloidin incubation steps. All of these washes make it easy to lose the pellet; remember that it is better to remove wash solutions with a pipet than an aspirator.

Labeling of live cells with rhodamine-phalloidin for colocalization with GFP fusion proteins

If a fresh batch of rhodamine-phalloidin is being used and a good signal for the actin is still not being achieved, try decreasing the total amount of cells taken and increasing the volume of permeabilization/staining solution to give each individual cell better access to both detergent and toxin label. If the GFP signal is too low in the actin-labeled cells, but not in the nonpermeabilized GFP-labeled-only cells, check the pH of the permeabilization solution and make sure that it is still around 7.4 (acidic pH decreases EGFP signal).

Localization of a protein in fixed yeast cells and in spheroplasted, then fixed cells with immunofluorescence

If the fluorescent signal is too low, try increasing the primary antibody concentration (it may be desirable to do a series of dilutions), or the time or temperature used when incubating with primary antibody. If doing colocalizations and both fluorescent signals are low, try adjusting the permeabilization or spheroplasting methods to give the antibodies better access to their targets. If the background signal is too high, try decreasing the secondary antibody incubation time or concentration, increasing the number of washes between antibody treatments, or changing the primary antibody.

Labeling cell wall chitin deposition with calcofluor white

Calcofluor white staining is usually very bright. If this signal is low, make sure the calcofluor white has dissolved into solution before use. If too much background is present, decrease the concentration of CfW. If not enough bud scars per cell are being achieved to assess budding patterns, try adjusting the growth conditions used in preparing the cells for the assay.

Anticipated Results

Labeling the vacuole in live cells with CDCFDA

CDCFDA evenly labels the entire lumen of any compartment exhibiting esterase activity. In wild-type cells one to five filled circles of fluorescence should be observed, taking up $\sim 1/3$ to $1/2$ of the total cell volume. Mutant cells with defective delivery of hydrolases to the vacuole could exhibit normal structure but severely decreased fluorescence or abnormal structure including fragmented vacuoles and/or a Class E compartment, an enlarged late endosome-like structure adjacent to the vacuole (Raymond et al., 1992).

Labeling acidic organelles with quinacrine in live cells

Quinacrine labels the entire lumen of acidic membrane-enclosed compartments. In wild-type cells one to five filled circles or fluorescence should be observed, taking up $\sim 1/3$ to $1/2$ of the total cell volume. Mutant cells may exhibit many of the defects described for CDCFDA.

Labeling the endocytic pathway in live cells with FM4-64

FM4-64 labels membranes only, and in wild-type cells should accumulate on the vacuolar membrane after the chase. In wild-type cells approximately one to five rings of fluorescence should be observed, each with a dark interior, taking up $\sim 1/3$ to $1/2$ of the total cell volume. Mutant cells with a defect in endocytosis could exhibit abnormal fluorescence patterns such as endosomal labeling (punctate spots around the vacuole or adjacent to the plasma membrane) as well as decreased total cell-associated fluorescence. This can be measured by a cell sorter or approximated by measuring pixel intensity of vacuolar membranes from microscopy images taken under the same exposure conditions.

Internalization time course with FM4-64

In an internalization time course, the zero time point should exhibit a single ring of fluorescence for each cell corresponding to the plasma membrane. If the zero time point cells show some endosomal label, they may be getting a little warmer during the labeling or washing steps than they should. Make sure the centrifugation is performed at 4°C . When looking through time points after zero, endosomal membrane labeling should be visible (several punctate spots near the plasma membrane), then late endosomal labeling (punctate spots farther in the interior of the cell), then finally vacuolar labeling (one to five rings of fluorescence, taking up $\sim 1/3$ to $1/2$ of the cell interior). See Figure 4.13.1 for an example of wild-type internalization at 30°C . Mutant cells defective for endocytosis could exhibit a delay in the progression of the dye through these stages, as well as decreased signal in the time points after zero (where all samples should have approximately equal label) due to removal of plasma membrane label in the postchase washes.

Endocytic rate measurement by lucifer yellow uptake in live cells

Lucifer yellow is a fluid-phase dye; therefore, in wild-type cells it should accumulate in approximately one to five filled circles of fluorescence, taking up $\sim 1/3$ to $1/2$ of the interior of the cell. Mutant cells defective for endocytosis should exhibit decreased or absent fluorescence signal.

Localization of proteins in live yeast cells with GFP fusion proteins

The anticipated localization pattern for this assay will depend completely on the GFP-fusion protein being observed.

Labeling the actin cytoskeleton with rhodamine-phalloidin in fixed cells

Filamentous actin will be found in two forms, cortical actin patches and extended actin cables. As described, these two structures exhibit a distinct pattern throughout the cell cycle. In cells with small buds under DIC, $\sim 90\%$ of the cortical actin patches should be in the small daughter bud. Actin cables should extend along the axis from the bud into the mother cell, perpendicular to the bud neck. In cells with large buds, if DAPI staining shows the nucleus near the mother-bud neck, the nucleus is about to divide and the actin cytoskeleton should be randomly distributed in both cells. If the DAPI staining shows two separate nuclei, one in each

cell, then the actin cortical patches should be at the mother-bud neck and cables should extend outward from this neck in both directions. Figure 4.13.2 shows an example of these wild-type patterns. Mutants may be defective for polarization, for formation of cables or actin patches, or may have defects in regulating actin dynamics—e.g., few large actin clumps rather than several small patches, which may be depolarized. See Figure 4.13.3.

Labeling of live cells with rhodamine-phalloidin for colocalization with GFP fusion protein

Filamentous actin should follow the same patterns described for Basic Protocol 5, but again, GFP-fusion protein patterns will depend on the fusion protein used. Keep in mind that the protein might localize to cortical patches that are distinct from cortical actin patches. Figure 4.13.3 shows an example of GFP-Ent1p, which does localize to actin patches, but in these mutant cells actin patches are chunky and depolarized.

Localization of a protein in fixed yeast cells and in spheroplasted then fixed cells with immunofluorescence

The anticipated localization pattern for these assays will depend completely on the protein being observed.

Labeling cell wall chitin deposition with calcofluor white

Calcofluor white binds to chitin deposits in the yeast cell wall. Because there are low levels of chitin throughout the cell wall, with heavier deposits at scars formed at previous bud sites, the CfW pattern is usually characterized by a barely visible outline of the yeast cell, with several small rings of bright fluorescence on the outside edges/surface of the cell. Each individual ring is usually $<1/10$ the size of the yeast cell, and in wild-type diploid cells, one to several rings will usually be found on each end of the elongated cell. The middle third of each diploid yeast cell should be dark (devoid of chitin rings) in wild-type cells. In cells defective for recycling endocytosis, chitin deposition is decreased and rings will be absent or barely visible. In mutant cells defective for polarized cell growth, including actin cytoskeletal defects, the chitin rings will be found randomly over the surface of the diploid cells, and the cells themselves are usually not elongated but instead are round. Examples of both wild-type and depolarized chitin deposition are shown in Figure 4.13.4.

Time Considerations

For all of the protocols, there is an initial time investment required to prepare the cells for the assay. Inoculation of a culture the day before the assay is required to obtain a liquid culture ready for staining. Most of these assays require log-phase cells which may take up to 5 hr the next day to prepare if cells are saturated in the morning and must be diluted to bring the cells back to log phase. Remember that not all assays can use the cells diluted in the morning.

Labeling the vacuole in live cells with CDCFDA

Preparation of CDCFDA-labeled cells should take <1 hr once cells are in log phase. For an experienced fluorescence microscopist, viewing time should take 10 to 15 min per sample.

Labeling acidic organelles with quinacrine in live cells

Preparation of quinacrine labeled cells should take <30 min once cells are in log phase. For an experienced fluorescence microscopist, viewing time should take 10 to 15 min per sample.

Labeling the endocytic pathway in live cells with FM4-64

The FM4-64 assay should take anywhere from 1 to 3 hr once cells are in log phase. Once cells are ready for viewing, they should be observed as soon as possible but preferably not more than 1 hr after preparation. For an experienced fluorescence microscopist, viewing time should take 10 to 20 min per sample.

Internalization time course with FM4-64

The FM4-64 time course should take anywhere from 1 to 2 hr once cells are in log phase. Prepare the solutions just before harvesting the cells, making sure that all media are completely prechilled. For an experienced fluorescence microscopist, viewing time should take 10 to 20 min per sample (remember that this is per strain multiplied by the number of time points).

Endocytic rate measurement by lucifer yellow uptake in live cells

The lucifer yellow assay should take ~ 3 hr once cells are in early log phase. For an experienced fluorescence microscopist, viewing time should take 10 to 20 min per sample.

Localization of proteins in live yeast cells with GFP-fusion proteins

Preparation of GFP-fusion protein cells for viewing is very simple. Other than additional time for a temperature shift, once cells are in log phase they can be ready for viewing in as little as 5 min. For an experienced fluorescence microscopist, viewing time should take 10 to 15 min per sample.

Labeling the actin cytoskeleton with rhodamine-phalloidin in fixed cells

Preparation of F-actin labeled cells could take as little as 4 to 5 hr once cells are in log phase, but it can also be stretched out over 3 days. For an experienced fluorescence microscopist, viewing time should take 10 to 20 min per sample.

Labeling of live cells with rhodamine-phalloidin for colocalization with GFP fusion protein

Preparation of colabeled F-actin and GFP cells should take <1 hr once cells are in log phase. For an experienced fluorescence microscopist, viewing time should take 15 to 25 min per sample because it may take more time to find cells with both labels.

Localization of a protein in fixed yeast cells with immunofluorescence

Preparation of yeast cells with immunofluorescence requires an overnight fixation step after cells are in log phase. Once cells are fixed, the preparation could take as little as 5 hr, but often requires additional overnight incubation with the primary antibody as well. It is best to plan well and plan ahead for this assay, reading the method thoroughly before beginning the preparation. For an experienced fluorescence microscopist, viewing time should take 10 to 15 min per sample.

Localization in spheroplasted, then fixed, cells with immunofluorescence

This method for preparation of yeast cells with immunofluorescence can be done in 1 long day (~7 hr of preparation once cells are in log phase) but is likely to require at least 2 days for proper preparation of cells. As with Basic Protocol 6, make sure to read the method thoroughly before beginning the assay. For an experienced fluorescence microscopist, viewing time should take 10 to 15 min per sample.

Labeling cell wall chitin deposition with calcofluor white

Preparation of calcofluor white labeled cells should take <15 min starting from a dense overnight culture. Prepare the CfW solution a few days prior to beginning the assay since it may take over 24 hr to dissolve in solution. For an experienced fluorescence microscopist, viewing time should take 10 to 15 min per sample.

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UNIT 4A Organelle Atlas: Appendix to Chapter 4

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Abstract

This atlas contains images of cellular organelles visualized using a wide variety of reagents and techniques and demonstrates the diversity of methods for exploring the cell.

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CHAPTER 5

Characterization of Cellular Proteins

INTRODUCTION

Proteins are the most diverse, versatile components of living organisms. They are widely variable in size, shape, charge, stability, solubility, and other physical-chemical properties. This diversity derives from their being composed of combinations of about 20 distinct amino acids arranged in any number and sequence. The polypeptide chain folds into a particular conformation which is largely determined by the sequence of amino acids. The virtually limitless number of possible conformations allows proteins to perform a variety of cellular functions. Proteins can act as catalysts, receptors, hormones, carriers, membrane channels, molecular motors, cytoskeletal elements, protective shells, regulators of gene expression, components of the translation and trafficking machineries, and so on. They are found in every subcellular compartment and some are secreted into the extracellular space, where they modify the surroundings of the cell or exert effects on other cells.

The structural and functional plasticity of proteins, and their critical roles in most cellular processes, make them prime subjects of interest to cell biologists. There are many excellent methodologies to characterize the structural features of purified proteins. These methodologies have been covered in several books. Most often, however, cell biologists are confronted with the task of characterizing specific proteins in mixtures with other proteins, as is the case for whole cells, subcellular fractions, cell extracts, and partially purified proteins. In many cases, the protein of interest is a minor component of these mixtures. Over the years, scientists have learned how to take advantage of the structural and functional diversity of proteins to devise methods that allow the characterization of specific proteins in complex mixtures. These methods consist of separating proteins according to their physical-chemical properties using techniques such as ultracentrifugal sedimentation on sucrose gradients or gel filtration, followed by detection of the proteins by some specific enzymatic activity or, more often, by their reactivity with specific antibody reagents. This type of approach is the focus of the protocols presented in this chapter.

The chapter begins with an overview of the physical state of proteins within cells (*UNIT 5.1*). The overview discusses the classification of proteins into globular, fibrous, and transmembrane proteins. This is followed by a description of the modes of association of proteins with membranes and the determinants of topology of transmembrane proteins. *UNIT 5.1* then discusses the factors that determine the physical heterogeneity of proteins, including size, charge, chemical modifications, and oligomeric or multimeric assembly. This leads to a description of some of the methods that are used to characterize the properties of proteins in complex mixtures.

Transmembrane proteins can assume a number of different topologies. For example, the amino and carboxy termini of the polypeptide chains can be exposed to either the luminal/extracellular or cytosolic aspects of intracellular organelles. The simplest integral membrane proteins have only one membrane-spanning segment. However, many polypeptide chains span the membrane two or more times. Although the topology of transmembrane proteins can, in many instances, be inferred from amino acid sequence

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**Characterization
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5.0.1

Supplement 6

information, experimental verification is always required. *UNIT 5.2* includes a set of protocols designed to assess the topologies of proteins within membranes. The first is based on the accessibility of proteins within intact cells or membrane-bound organelles to externally added proteases. The effects of proteolysis are then generally evaluated by one-dimensional polyacrylamide gel electrophoresis (*UNIT 6.1*), often in combination with immunoblotting (*UNIT 6.2*) or immunoprecipitation (*UNIT 7.2*). Another protocol in *UNIT 5.2* is based on the accessibility of protein epitopes to antibody reagents, using immunofluorescence microscopy as a means of detecting the antibody (also see *UNIT 4.3*). Methods for marking proteins with epitope tags are presented as a complement to the accessibility-based protocols. *UNIT 5.2* ends with a method to assess topology using reporter fusions in yeast cells.

Sizing of a protein in its native state can be accomplished by determining its hydrodynamic parameters, such as the sedimentation coefficient and Stokes' radius. Knowledge of these parameters in many cases allows calculation of the molecular masses of the native protein or protein complexes. *UNIT 5.3* contains a detailed description of one of the most commonly used methods for estimating the size of cellular proteins: sedimentation-velocity analysis on sucrose gradients. This method consists of analyzing the migration of proteins on a continuous gradient of sucrose upon application of a centrifugal field. After fractionation of the gradient, proteins are detected by measurement of an intrinsic activity or by polyacrylamide gel electrophoresis (*UNIT 6.1*) in combination with immunoblotting (*UNIT 6.2*) or immunoprecipitation (*UNIT 7.2*). *UNIT 5.3* also describes the use of equations to calculate the hydrodynamic parameters of proteins from sedimentation experiments.

UNIT 5.4 presents several methods for determining the nature of protein-membrane associations (i.e., distinguishing peripheral versus integral membrane proteins). Although most integral membrane proteins are associated with membranes via hydrophobic polypeptide segments, a subset are tethered to the membranes by glycosyl phosphatidylinositol (GPI) groups. This unit presents a method for examining membrane association through GPI groups, based on hydrolysis with phosphatidylinositol-specific phospholipase C (PI-PLC). GPI-anchored proteins and some transmembrane proteins are resistant to solubilization by certain nonionic detergents such as Triton X-100. For some, this is due to their association with plasma membrane domains enriched in glycosphingolipids and cholesterol. These domains are sometimes contained within invaginations of the plasma membrane known as caveolae. This unit provides a protocol for solubilization of Triton-insoluble plasma membrane proteins using detergents such as octylglucoside or CHAPS. Also included are procedures for isolation of caveolar membrane domains.

In addition to sedimentation-velocity analysis (*UNIT 5.3*), another commonly used method for estimating the size of cellular proteins in their native state is size exclusion chromatography (also known as gel filtration; *UNIT 5.5*). In this method, a solution of proteins (the "mobile phase") is passed over a column containing a matrix of porous particles (the "stationary phase"). Proteins partition according to their ability to penetrate the interior of the particles, such that larger proteins elute earlier than smaller ones. The size of the proteins can be estimated by comparison with the behavior of protein standards of known molecular size. The parameter that best characterizes the behavior of proteins on size exclusion chromatography is the Stokes radius, R_s . Knowledge of this parameter, and of parameters derived from sedimentation-velocity analysis (*UNIT 5.3*), allows calculation of the molecular weights of proteins.

Juan S. Bonifacino

Overview of the Physical State of Proteins Within Cells

The word protein comes from the Greek word *proteios*, meaning primary. And, indeed, proteins are of primary importance in the study of cell function. It is difficult to imagine a cellular function not linked with proteins. Almost all biochemical catalysis is carried out by protein enzymes. Proteins participate in gene regulation, transcription, and translation. Intracellular filaments give shape to a cell while extracellular proteins hold cells together to form organs. Proteins transport other molecules, such as oxygen, to tissues. Antibody molecules contribute to host defense against infections. Protein hormones relay information between cells. Moreover, protein machines, such as actin-myosin complexes, can perform useful work including cell movement. Thus, studying proteins is a prerequisite in understanding cell structure and function.

The physical characterization of proteins began well over 150 years ago with Mulder's characterization of the atomic composition of proteins. In the latter half of the nineteenth century Hoppe-Seyler (1864) crystallized he-

moglobin and Kühn (1876) purified trypsin. A variety of physical methods have been developed over the years to increase convenience and precision in the characterization and isolation of proteins. These include ultracentrifugation, chromatography, electrophoresis, and others. In many instances our understanding of cell proteins parallels the introduction and use of new techniques to examine their structure and function.

PROTEIN CLASSIFICATIONS

All proteins are constructed as a linear sequence(s) of various numbers and combinations of ~20 α -amino acids joined by peptide bonds to form structures from thousands to millions of daltons in size. Proteins are the most complex and heterogeneous molecules found in cells, where they account for >50% of the dry weight of cells and ~75% of tissues.

Proteins can be classified into three broad groups: globular, fibrous, and transmembrane (Fig. 5.1.1; Table 5.1.1). Globular proteins are, by definition, globe-shaped, although in prac-

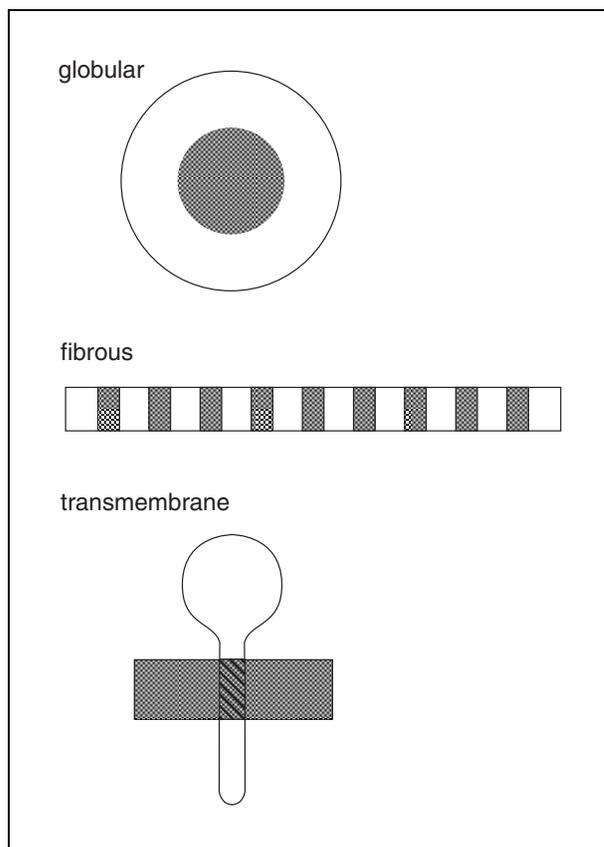


Figure 5.1.1 General classifications of proteins. In these schematic representations of globular, fibrous, and transmembrane proteins, hydrophobic regions are shaded. Note that the disposition of hydrophobic residues often reflects the protein class.

Table 5.1.1 Broad Classifications for Proteins^a

Type	Location/type	Examples
Globular	Intracellular	Hemoglobin, lactate dehydrogenase, cytochrome <i>c</i>
	Extracellular	Serum albumin, immunoglobulins, lysozyme
Fibrous	Intracellular	Intermediate filaments, tropomyosin, lamins
	Extracellular	Collagen, keratin, elastins
Transmembrane	Single pass	Insulin receptor, glycoporphin, HLAs ^b
	Multipass	Glucose transporter, rhodopsin, acetylcholine receptor

^aAdditional information regarding fibrous and transmembrane proteins can be found in Squire and Vibert (1987) and Petty (1993). Information concerning globular proteins can be found in numerous books on proteins and enzymes such as Schultz and Schirmer (1979).

^bHuman histocompatibility leukocyte antigens.

tice they can be spherical or ellipsoidal. Globular proteins are generally soluble in aqueous environments. Examples of globular proteins are hemoglobin, serum albumin, and most enzymes. Fibrous proteins are elongated linear molecules that are generally insoluble in water and resist applied stresses and strains. Collagen is a physically tough molecule of connective tissue. Just as collagen gives strength to connective tissues, intermediate filaments linked to desmosomes give strength to cells in tissues. The third general class of proteins, transmembrane proteins, contain a hydrophobic sequence buried within the membrane; these proteins are discussed more fully below (see Membrane Proteins).

These protein categories are not mutually exclusive. For example, the nominally fibrous intermediate filament proteins also have globular domains. Similarly, transmembrane proteins almost always possess globular domains. Thus, these definitions serve as a useful guide but should not be rigidly applied.

HYDROPATHY PATTERNS OFTEN REFLECT A PROTEIN'S CLASSIFICATION

A key physical feature of proteins is their hydrophathy pattern (i.e., the distribution of hydrophobic and hydrophilic amino acid residues). Indeed, hydrophobic interactions provide the primary net free energy required for protein folding. Figure 5.1.1 illustrates the disposition of hydrophobic amino acids in proteins. In an intact globular protein, hydrophobic

amino acids are generally shielded from the aqueous environment by coalescing at the center of the molecule, with the more hydrophilic residues exposed at its surface. However, the linear arrangement of hydrophobic residues fluctuates in an apparently random fashion. The α helices within globular proteins may express a hydrophobic face oriented toward the center of the protein. (Within these helices hydrophobic residues are nonrandomly positioned every three or four amino acids to yield a hydrophobic face.) For coiled-coil α helix-containing fibrous proteins, such as tropomyosin and α -keratin, hydrophobic residues at periodic intervals allow close van der Waals contact of the chains and potentiate assembly as hydrophobic residues are removed from the aqueous environment (Schulz and Schirmer, 1979; Parry, 1987). Secondly, regularly spaced charged groups can also contribute to the shape of fibrous proteins (Schulz and Schirmer, 1979; Parry, 1987). Transmembrane proteins provide a rather different physical arrangement of hydrophobic residues in which hydrophobic residues are collected primarily into a series of amino acids that is embedded within a cell membrane.

One important means of analyzing the hydrophathy of a sequenced protein is a hydrophathy plot (Kyte and Doolittle, 1982). In this method, each amino acid residue is assigned a hydrophathy value, an ad hoc measure that largely reflects its relative aqueous solubility; these values are plotted after being averaged. The successful interpretation of hydrophathy plots

depends on the parameters chosen for averaging. The parameters are the number of residues averaged (amino acid interval or “window”) and how many amino acids are skipped when calculating the next average (step size). Using this approach with a window of ~10 residues, it is often possible to find the positions of hydrophobic residues coalescing near the interior of globular proteins. The method is particularly useful in predicting transmembrane domains of proteins, generally with a window of ~20 amino acids. To detect the repetitious pattern of coiled-coil fibrous proteins, however, windows smaller than the repeat length would be required.

MEMBRANE PROTEINS

In addition to their presence in the extracellular and intracellular milieus, proteins are also found in association with biological membranes. Proteins constitute one-half to three-quarters of the dry weight of membranes. Membrane proteins perform a broad variety of functions including intermembrane and intercellular recognition, transmembrane signaling, most energy-harvesting processes, and biosynthesis in the endoplasmic reticulum (ER) and Golgi complex.

Membrane proteins have been traditionally characterized as integral (or intrinsic) or peripheral (or extrinsic) on the basis of operational criteria. Peripheral membrane proteins are associated with membrane surfaces and can be dislodged from membranes using hypotonic or hypertonic solutions, pH changes, or chela-

tion of divalent cations. Components of the erythrocyte membrane skeleton, for example, are peripheral membrane proteins. Although most peripheral proteins are removed by washing a sample with buffers, integral proteins cannot be removed by such treatments. To isolate integral membrane proteins, which are embedded within the lipid bilayer, one must use detergents that disrupt the bilayer and bind to the proteins, thus solubilizing them. In general, integral membrane proteins have a portion of their peptide sequence buried in the lipid bilayer whereas peripheral proteins do not. However, the discovery of glycosylphosphatidylinositol (GPI)-linked membrane proteins added to the ambiguity of the situation. GPI-linked proteins are globular proteins with no membrane-associated peptide sequence, yet they require harsh conditions for solubilization.

As the technology for studying membrane proteins improved, it became necessary to develop a more precise vocabulary to describe membrane proteins. Transmembrane integral membrane proteins have at least one stretch of amino acids spanning a membrane. Membrane proteins are classified as type I, II, III, or IV depending on the nature of their biosynthesis and topology in membranes (Spiess, 1995; Table 5.1.2 and Fig. 5.1.2). The biosynthetic insertion of these proteins in membranes is, in turn, dependent on the presence or absence of a cleavable signal peptide, the relative positions of the hydrophobic transmembrane domain and positively charged topogenic signals, and/or

Table 5.1.2 Definitions of Integral Transmembrane Proteins

Type	Definition	Examples
I	An N-terminal–cleavable signal peptide is removed at the luminal face yielding a luminal N terminus during biosynthesis. (Positive charges are found on C-terminal side of first long hydrophobic sequence after the signal peptide.)	LDL receptor, insulin receptor, glycoporphin A, thrombin receptor
II	An N-terminal–uncleaved signal peptide leads to a cytoplasmic N terminus. (Positive charges are generally found on N-terminal side of first long hydrophobic sequence.)	Transferrin receptor, sucrase/isomaltase, band 3
III	A long N-terminal hydrophobic sequence is followed by a sequence of positive charges. This leads to a luminal N terminus in the absence of a cleavable signal peptide.	β -Adrenergic receptor, cytochrome P450
IV	A short C terminus is present at the luminal side of membrane. A large N terminus is exposed at the cytoplasmic face.	Synaptobrevin, UBC6

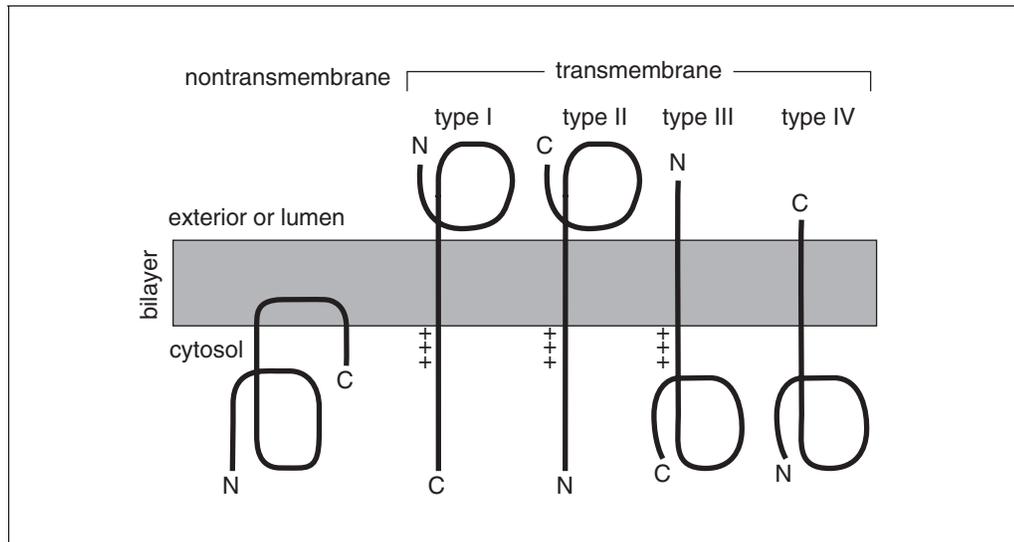


Figure 5.1.2 Membrane proteins containing hydrophobic anchors. A nontransmembrane or monotopic membrane protein is anchored to the membrane via a hydrophobic amino acid sequence. Transmembrane proteins are classified as types I, II, III, and IV (Table 5.1.2). The first transmembrane segment of a multispanning membrane protein can be inserted as in type I, II, or III proteins. This segment functions as a start-transfer peptide. Subsequent transmembrane segments will function as stop-transfer and start-transfer sequences, resulting in a multispanning membrane topology (see Fig. 5.2.1).

the mechanism of nascent protein delivery to the ER.

Type I membrane proteins are synthesized with an amino-terminal signal sequence that is inserted into the ER membrane. When the signal sequence is proteolytically removed in the ER lumen, a new luminal amino terminus is exposed. A series of positively charged residues at the C-terminal side of the first hydrophobic transmembrane domain following the signal sequence generally denotes the end of the first transmembrane domain (von Heijne and Gavel, 1988). Although a hydrophobic transmembrane domain followed by a positive sequence of amino acids is sufficient to act as a stop-transfer signal, this motif is not required for stop-transfer events and other, less well-understood regulatory mechanisms are also involved (Andrews and Johnson, 1996).

Membrane proteins types I, II, and III are delivered to the ER membrane via a signal recognition particle (SRP)-dependent mechanism. In contrast to type I proteins, type II and III membrane proteins do not have a cleavable N-terminal signal sequence. Instead, they have an internal hydrophobic signal that acts as both a signal sequence for ER delivery and a transmembrane domain in the mature protein. Type II proteins have a cytoplasmic amino terminus and a luminal (or extracellular) carboxyl terminus. In this case a positively charged sequence

of amino acids at the N-terminal side of the first hydrophobic sequence causes the amino terminus to be retained at the cytoplasmic face of the ER membrane. Thus the internal uncleaved signal peptide becomes the transmembrane domain of the mature protein.

Type III membrane proteins have the same overall topology as type I proteins, but they are inserted into membranes by a different mechanism. In type III proteins the first hydrophobic sequence of amino acids is immediately followed by a series of positively charged amino acids. Thus, the first hydrophobic sequence becomes the transmembrane domain of the protein, with the amino terminus at the luminal face of the membrane.

Type IV membrane proteins are characterized by a large, cytoplasmically exposed amino-terminal domain and a short carboxyl-terminal domain facing the lumen. Importantly, these proteins are delivered to the ER by an unknown SRP-independent mechanism.

In addition to the single-pass membrane proteins just described, integral membrane proteins can display zero, two, three, or more transmembrane domains. Some membrane proteins, such as cytochrome *b₅*, have protein segments buried in the hydrophobic core of membranes but do not cross the membrane. Membrane proteins with multiple membrane-spanning domains are classified as type I, II, or

III depending on the topogenic signals in the first transmembrane domain. For example, a multspan membrane protein with a cleavable signal sequence, luminal amino terminal, and a positively charged sequence following the first transmembrane domain from the amino terminal, such as the thrombin receptor, is a type I membrane protein. The remaining transmembrane domains are inserted into the bilayer depending on the orientation of the first transmembrane domain. Multispanning type II and III proteins are similarly defined according to the properties of their single-spanning counterparts.

In addition to hydrophobic protein sequences acting as membrane anchors, membrane proteins may also carry bilayer-associated hydrophobic lipid components. These hydrophobic lipid anchors define three broad groups of lipid-modified proteins: fatty acylated, isoprenoid-linked, and GPI-linked (Fig. 5.1.3). Several cytosolic transmembrane proteins have been identified that contain a covalently attached hydrophobic fatty acyl residue. For example, fatty acids, including palmitic, palmitoleic, *cis*-vaccenic, and cyclopropylene-

hexadecanoic, are covalently linked to the amino terminus and the amino-terminal glycercylcysteine of *E. coli* lipoprotein. Moreover, palmitate- and myristate-labeled transmembrane proteins have been observed in eukaryotic cells (e.g., Schlesinger et al., 1980).

In both isoprenoid-linked and GPI-linked proteins, globular proteins become membrane-bound due to the addition of a hydrophobic lipid moiety. Certain proteins containing conserved cysteine residues at or near the C-terminus are modified by prenylation, in which a farnesyl or geranylgeranyl isoprenoid tail is added (Zhang and Casey, 1996). This hydrophobic moiety promotes protein association with the cytoplasmic face of cell membranes. Notably, cytosolic G proteins and protein kinases that participate in signal transduction are prenylated.

GPI-linked proteins are a major class of membrane proteins (Cardoso de Almeida, 1992; Englund, 1993). In contrast to isoprenoid-modified proteins, GPI-linked proteins are attached to the luminal or extracellular face of membranes via a glycosylphosphatidylinositol anchor of variable structure (e.g.,

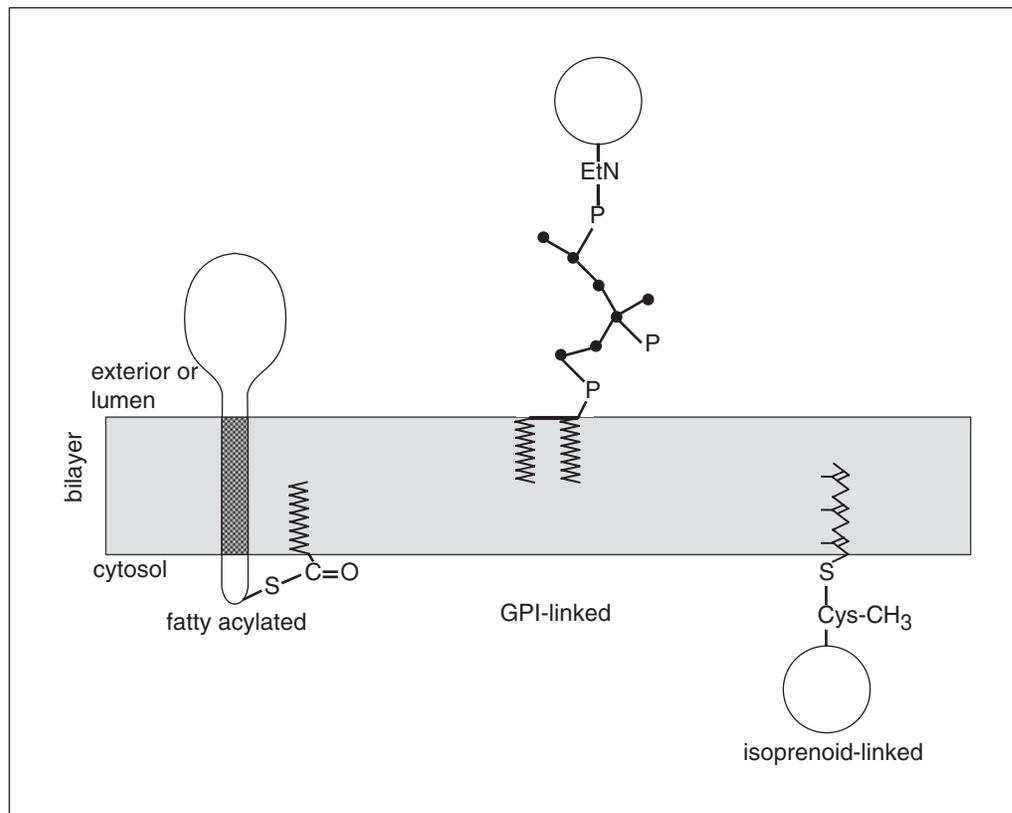


Figure 5.1.3 Membrane proteins containing lipid moieties. In the simplest case, fatty acids can be covalently attached to transmembrane proteins. Hydrophobic tails are also attached to proteins to form isoprenoid-linked proteins. A third class of lipid-attached proteins are the GPI-linked proteins. Hydrophobic regions are shaded.

Fig. 5.1.3). Well over 100 GPI-linked proteins have been identified in cells, where they perform numerous functions including acting as enzymes and receptors. The ability of GPI-linked proteins, which possess no transmembrane or cytosolic sequences, to elicit transmembrane signals seems paradoxical. However, studies have suggested that interactions with other proteins, including transmembrane integrins (Petty and Todd, 1996), contribute to transmembrane signaling of these proteins. Although GPI-linked proteins must collaborate with other membrane proteins to elicit signals, they do possess certain functional advantages. First, GPI-linked proteins (and isoprenoid-linked proteins as well) diffuse in membranes much faster than transmembrane proteins and thus relay information faster. Second, certain cells, such as leukocytes, can rapidly shed their GPI-linked proteins, thus altering their functional properties in seconds. Although the importance of lipid-linked membrane proteins has only recently been appreciated, the impact of these structures on our understanding of cell properties is growing rapidly.

ADDITIONAL FACTORS AFFECTING THE PHYSICAL HETEROGENEITY OF PROTEINS

Additional factors contributing to the physical-chemical heterogeneity of proteins are size, charge, chemical modifications, and assembly. A typical amino acid has a molecular mass of ~110 Da, and a small protein has a molecular mass of a few thousand daltons (e.g., for insulin, $M_r = 5733$). Large proteins have molecular masses of several hundred thousand daltons. When proteins are assembled to form large multiprotein complexes such as ribosomes, molecular masses are well into the millions. The diameters of these structures range from 4 Å for an individual amino acid to ~30 nm for a ribosome.

Electrostatic charge is of major importance in protein structure and function. Charged proteins are more soluble than uncharged proteins. The large number of positive charges on histones allow them to bind DNA. The spatial arrangement of charges on cytochrome *c* allows it to bind the complementary charges of its oxidase and reductase, thereby orienting the proteins prior to electron transfer. Similarly, the arrangement of charges on the apoprotein and receptor for low-density lipoprotein (LDL) allows for lock-and-key-like interactions (Petty, 1993). In addition to structural and binding considerations, electrostatic interactions play a

regulatory role. For example, the phosphorylation and dephosphorylation of insulin receptors alter electrostatic interactions between the active site and a regulatory loop of the kinase domain, thereby changing its three-dimensional shape (Hubbard et al., 1994). This changes the V_{max} of the kinase, thus triggering intracellular signals.

In addition to the types of physical heterogeneity listed above, >100 distinct chemical modifications of proteins have been observed. These include, for example, glycosylation, ubiquitin attachment, phosphorylation, acetylation, and hydroxylation (Table 5.1.3). Thus, proteins undergo extensive physical-chemical modification.

PROTEIN ASSEMBLIES

Proteins can be assembled in a variety of states in both aqueous media and within membranes. Protein assembly into complex supramolecular structures plays vital roles in enzyme regulation, cell skeleton formation, and transmembrane signaling. Both covalent bonds and noncovalent bonds participate in protein assembly. One frequently encountered covalent mechanism of protein assembly is the formation of disulfide bonds. These covalent linkages often form during protein maturation. They can link two separate proteins together or two portions of the same protein. For example, the two chains of insulin molecules are held together by disulfides, as are the two chains of its membrane receptor. However, disulfide bond formation is mostly limited to oxidative environments such as the ER lumen and the exterior face of the cell surface.

One of the best-known examples of noncovalent assembly is the formation of hemoglobin tetramers. Polymerization is another frequently encountered mechanism for protein assembly in cells. The globular protein actin polymerizes to form microfilaments in the absence of covalent bond formation. Intermediate filaments are formed by the polymerization of fibrous proteins. Under certain circumstances transmembrane proteins polymerize as well; bacteriorhodopsin, for example, forms two-dimensional pseudocrystals called purple membranes. Protein assemblies formed from various numbers of similar units are homodimers, homoooligomers, and homopolymers.

Assembly of protein structures from dissimilar subunits is more common than assembly from identical subunits. For example, heterodimers are formed from the α and β chains of integrins within cell membranes. Complex

Table 5.1.3 Common Physical-Chemical Modifications of Proteins^a

Modification	Example
Homodimerization	Transferrin receptor
Homooligomerization	<i>S. typhimurium</i> glutamine synthetase
Homopolymerization	Actin
Heterodimerization	Integrins
Heterooligomerization	Histones, proteasomes
Heteropolymerization	Ribosomes
Proteolytic cleavage	Signal peptide cleavage in ER
Prosthetic group addition	Heme addition to cytochromes and hemoglobin
Oxidation-reduction	Disulfide bond formation in ER
Glycosylation	Glycoprotein maturation
Phosphorylation	Regulation of protein function, such as the tyrosine kinase activity of insulin receptors
Acetylation	Blockage of N-termini of certain membrane proteins
Ubiquitination	Ubiquitin-dependent proteolysis via proteasomes, histones
Hydroxylation	Proline hydroxylation on collagen
Fatty acylation	Insulin receptors, <i>E. coli</i> lipoprotein
Isoprenylation	G proteins
GPI addition	Alkaline phosphatase, urokinase receptors

^aFor details, see Freedman and Hawkins (1980, 1985), Schlesinger et al. (1980), Englund (1993), and Zhang and Casey (1996).

heterooligomeric and heteropolymeric structures vary from relatively small structures such as histone octamers, which bind to DNA in the nucleus, to large particles such as ribosomes, found both in the cytosol and attached to nuclear and ER membranes. The signal recognition particle is a relatively small heterooligomeric structure, composed of one RNA subunit and six proteins, that potentiates the delivery of secretory and most membrane proteins to the ER membrane. Membrane-associated heterooligomeric structures have also been observed. One of the best examples of such structures is the components of the electron transport systems in chloroplasts and mitochondria (Petty, 1993). For example, the ubiquinone-cytochrome *c* reductase is composed of eleven different subunits. Thus, proteins can be assembled in a variety of manners within cells.

Although some protein assemblies, such as intermediate filaments, are static structures, many are dynamic structures which provide functional flexibility. For example, microfilaments can rapidly assemble and disassemble. In addition to the physical changes in assembly state, compositional dynamics is also observed. For example, interferon γ treatment alters the composition of proteasomes. Developmental changes in protein composition are also ob-

served. As an example, fetal and newborn forms of a component of cytochrome *c* reductase are expressed in humans. Thus, protein assemblies can be characterized by both physical and compositional dynamics.

ALTERING THE SOLUBILITY OF PROTEINS: PROTEIN EXTRACTION

The *in vitro* characterization of cellular proteins begins with their extraction from tissues or cells into a buffer. With the exception of globular secretory proteins, such as those found in plasma, proteins are generally not easily accessible for experimental manipulation. For example, many fibrous proteins are not soluble in aqueous buffers. Cellular proteins are entrapped within or on a cell and therefore must be extracted from the cell in a soluble form.

A variety of methods including osmotic lysis, enzyme digestion, homogenization using a blender or mortar and pestle, and disruption by French press and sonication have been employed to disrupt cells. For a cytosolic protein such as hemoglobin, no further extraction from the sample is necessary. However, many important cellular proteins, such as those associated with membranes, cytoskeletal components, and DNA, remain insoluble. To further solubilize cell proteins, both nonionic (e.g., Triton

X-100) and ionic (e.g., sodium dodecyl sulfate) detergents are often employed. Detergents are small amphipathic molecules that interact with both nonpolar and polar environments. Detergents disrupt membranes. They also bind to hydrophobic regions of proteins, such as their transmembrane domains, thereby replacing the unfavorable contacts between hydrophobic protein regions and water with the more favorable hydrophilic domains of the detergent. Thus, instead of the hydrophobic regions of the insoluble protein forming an aggregate in the bottom of a test tube, the protein becomes soluble and can be employed in most in vitro analyses.

In addition to detergents, several other solubilization strategies are useful for the extraction and in vitro characterization of proteins (Table 5.1.4). Chaotropic agents enhance the transfer of nonpolar molecules to aqueous environments by their disrupting influence on water structure. Chaotropic agents are generally large molecular ions such as thiocyanate (SCN^-), perchlorate (ClO_4^-), and trichloroacetate (CCl_3COO^-). Hydrophobic interactions are also reduced by exposure to organic solvents

and low salt concentrations. Electrostatic interactions are reduced by high salt conditions; this decreases the Debye-Hückel screening length and coulombic attraction. To disrupt hydrogen bonds, high concentrations of urea or guanidine are often employed. More vigorous methods of sample denaturation using very low pH or harsh detergents such as sodium dodecyl sulfate are also used to diminish intermolecular contacts.

Once proteins are extracted, their size can be characterized by ultracentrifugation on sucrose gradients (UNIT 5.3), gel filtration chromatography, SDS-PAGE (UNIT 6.1), and other methods (Table 5.1.5). The charge characteristics of proteins can be assessed using isoelectric focusing and ion-exchange chromatography. Specific interactions, such as antigen-antibody and biotin-avidin interactions, can also be employed in the characterization and isolation of proteins. These are useful in immunoblotting (UNIT 6.2) and affinity chromatography methods.

LIMITATIONS OF THE IN VITRO MANIPULATION OF PROTEINS

The very act of isolating proteins perturbs their physical environment. Although this is not

Table 5.1.4 Physical Bases of Common Protein Extraction and/or Elution Methods

Physical property perturbed	Agents
Hydrogen bonds	Urea or guanidine-HCl, pH changes
Ion pair interactions	High salt, pH changes
Hydrophobic interactions	Detergents, chaotropic agents, organic solvents, low salt

Table 5.1.5 Physical Bases of Common Protein Characterization and Isolation Methods

Physical property	Method	References to other units ^a
Solubility	Extraction with salts, detergents, and enzymes	Racker (1985)
Size	Ultracentrifugation on sucrose gradients	CPCB 5.3
	Gel filtration	CPMB 10.9
	SDS-PAGE	CPCB 6.1
Charge	Isoelectric focusing	CPMB 10.3
	Ion-exchange chromatography	CPMB 10.10 & 10.13
Biospecific interaction	Immunoblotting	CPCB 6.2
	Immunoaffinity chromatography	CPMB 10.11A
Hydrophobicity	Hydrophobic chromatography	CPMB 10.15
	Reversed-phase HPLC	CPMB 10.12

^aFor references in CPMB see Ausubel et al. (eds.) 1998.

often a major problem, a few cautionary notes should be made. The most primitive compartment of a cell, the cytosol, is a chemically reducing environment. Consequently, free sulfhydryl groups are observed in the cytosol; in fact, multiple cytosolic pathways help in preserving the proper redox conditions. On the other hand, the extracellular milieu and the luminal side of the ER are oxidative environments. The oxidizing condition within the ER is presumably due to the unidirectional transport of glutathione and cystine. Consequently, disulfides are frequently observed in the ER and extracellular environments. Thus, to prevent disulfide formation during manipulation, sulfhydryl blocking reagents such as iodoacetamide are included in extraction buffers (see *UNIT 7.2*). The cytosol is also a K^+ -rich and Ca^{2+} -poor solution. These parameters should be considered in designing physiologically relevant experiments.

The experimental manipulation of membrane proteins is decidedly more difficult. The exterior face exists in a high Na^+ and Ca^{2+} solution that is oxidative; just the opposite is true for the cytoplasmic face. Since no appropriate solvent exists for such isolated proteins, experimental questions can be directed at properties associated with just one face of the molecule. A second limitation common to all *in vitro* studies of transmembrane proteins is that they must be solubilized using detergents. In addition to solubilizing a transmembrane protein, detergents can also bind to hydrophobic regions in the globular domain(s) of the protein, thus affecting the properties under study. One means of countering this problem is to test several detergents in the hope of finding one that retains the full biological activity of the purified protein.

Protein solubilization can also lead to loss of physiologically relevant protein-protein interactions. This can occur by simple dilution or by disruption of noncovalent interactions among proteins. For example, hemoglobin exists as a supersaturated solution *in vivo* which cannot be duplicated *in vitro*. Furthermore, protein-protein associations are generally stronger in the restricted confines of membranes than after solubilization into a buffer. Thus, protein assemblies found in cells may disappear during solubilization. One means of countering these potential difficulties is to covalently cross-link protein assemblies prior to disruption and to solubilize proteins using mild detergents (e.g., Brij-58).

CONCLUSIONS

Structural motifs, especially stretches of hydrophobic amino acids, contribute to the shape of a protein and its classification as globular, fibrous, or transmembrane. Proteins are heterogeneous at many different levels including physical attributes, covalent modifications, and supramolecular assembly. The physical properties of proteins are used to characterize and isolate these molecules. For example, the size of a protein is examined by sedimentation on sucrose gradients (*UNIT 5.3*), gel filtration, and polyacrylamide gel electrophoresis (*UNIT 6.1*). Its charge is the key physical parameter in isoelectric focusing and ion-exchange chromatography. The units that follow contain detailed protocols describing the characterization of cellular proteins.

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INTERNET RESOURCES

<http://www.expasy.ch>

A user-friendly protein database including two-dimensional PAGE data and 3D protein structures.

<ftp://ftp.pdb.bnl.gov/>

Contains protein crystallography data.

Contributed by Howard R. Petty
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Determining the Topology of an Integral Membrane Protein

A variety of approaches have been developed for assigning the aqueous domains of integral membrane proteins to either side of a biological membrane. This unit describes three general methods that can be used to solve the topologies of eukaryotic membrane proteins whose primary sequences have been deduced from their corresponding DNA sequences: (1) testing of water-soluble domains for their accessibility to a reagent added to membranes exhibiting a defined orientation (see Basic Protocols 1 and 2), (2) epitope tagging (see Support Protocol), and (3) a genetic approach in which a reporter enzyme fused to various truncated fragments of a membrane protein acts as a sensor of sequences disposed intra- or extracytoplasmically (see Basic Protocol 3).

STRATEGIC PLANNING

Before initiating an experimental analysis, it is helpful to build a conceptual model of a protein's topology (see Fig. 5.1.2 for a representation of different types of integral membrane proteins). Inspection of the primary sequences of integral membrane proteins usually reveals a pattern of alternating stretches of hydrophilic (water-soluble) and hydrophobic (water-insoluble) amino acids. Very good computer-based approaches are used to illuminate these patterns (Kyte and Doolittle, 1982; Engelman et al., 1986). The results are presented as a graph, in which each amino acid is compared to the hydrophobicity of the amino acids immediately surrounding it in the linear sequence. When visual inspection of the graph reveals a hydrophobic stretch containing at least 18 to 20 amino acids, a segment is identified that is long enough to potentially span the membrane in an α -helical configuration (Deisenhofer et al., 1985). To build this conceptual model, the authors use MacVector software available from Oxford Molecular.

A representative hydropathy analysis of signal peptidase subunit SPC12 (Kalies and Hartmann, 1996) is shown in Figure 5.2.1A. Since this graph reveals an even number of putative transmembrane segments (two) for SPC12, the resulting model predicts that the N and C termini localize to the same side of the membrane. It should be noted that intra- and extracytoplasmic domains are not distinguishable using this analysis; thus, two opposing models for SPC12 topology are compatible with the analysis performed (see Fig. 5.2.1B). A hydropathy plot alone is, therefore, not a predictor of transmembrane orientation, nor is it assured that such a plot will identify all membrane-spanning segments. Experimental evidence is necessary to establish the orientations of integral membrane proteins within the lipid bilayer.

Protease Digestion

In this experimental approach (see Basic Protocol 1), a protease exhibiting a broad substrate specificity is used as a probe of membrane protein topology. When the enzyme is added to only one side of the target membrane or membrane vesicle, the size of the proteolytic fragment(s) generated reveals the accessible site(s) and hence the orientation across the membrane of the protein segment(s) containing this site or sites. A proteolytic fragment is detected using a specific antibody. Usually, a series of antipeptide antibodies are prepared, such that each hydrophilic domain is recognized by a specific antibody. The number of hydrophilic domains is usually greater than the number of hydrophobic domains by one. For example, SPC12 has two hydrophobic domains and three hydrophilic domains. Thus, a minimum of three antipeptide antibody preparations are needed to probe the hydrophilic domains of SPC12. Sometimes, however, an antipeptide antibody does

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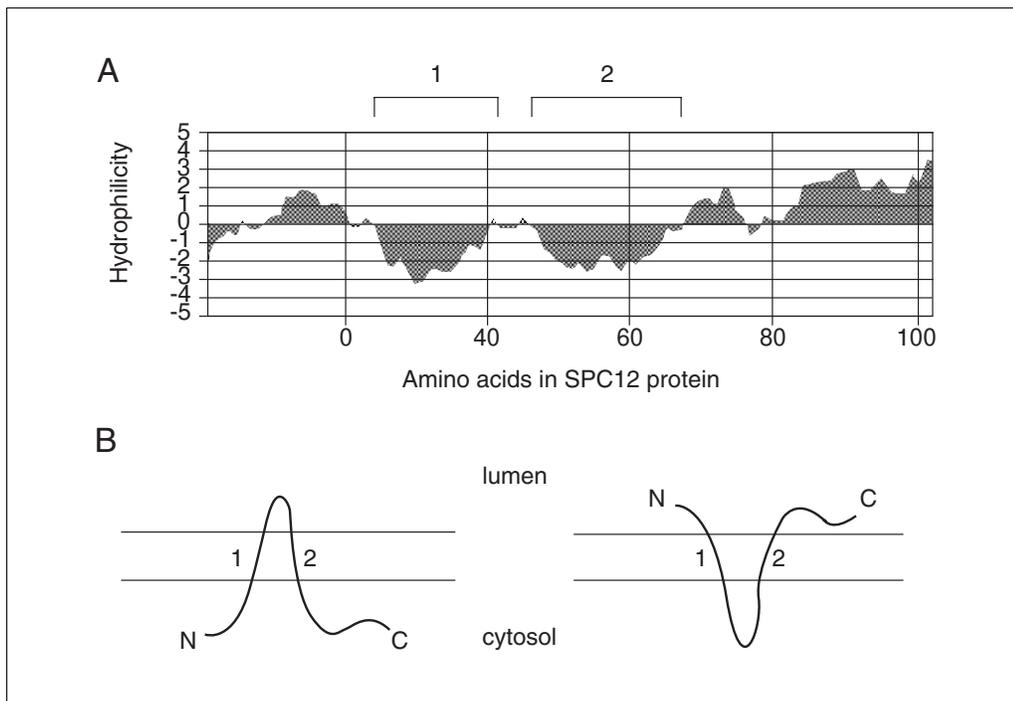


Figure 5.2.1 (A) Hydropathy analysis of SPC12 protein (Kalies and Hartmann, 1996). The data were obtained using the method of Kyte and Doolittle (1982). Hydrophilicity was examined using a window of seven amino acids. The positions of two potential transmembrane segments are shown. (B) Conceptualized topologies of SPC12. The data from (A) were used to construct two possible topologies. Hydrophobic segments 1 and 2 are shown spanning the membrane.

not recognize the corresponding peptide sequence in the intact protein; consequently, two or more anti-peptide antibody preparations directed against a single hydrophilic domain may be needed.

In addition to this series of anti-peptide antibodies, a control antibody is needed that recognizes a luminal protein of the closed membrane system to be analyzed. The orientation of a hydrophilic domain proteolyzed on addition of a protease can then be determined with confidence, providing the luminal protein control is resistant under this condition. A second control uses a mild detergent to solubilize the membranes. Protein sequences localized to both membrane surfaces and to the luminal space are exposed to the protease after detergent addition (Fig. 5.2.2 illustrates these points).

Unusually small hydrophilic domains may be resistant to proteolysis, even if they are exposed to the protease in the absence of detergent. Since addition of detergent often renders the entire protein susceptible, it is not always clear whether a domain that is resistant to proteolytic attack in the absence of detergent is localized to the lumen of the vesicle or just naturally resistant. As discussed below, the accessibility of such domains may be enhanced by using a foreign epitope (or multiple epitopes) inserted into the sequence of a small hydrophilic domain, thereby increasing its size (see Epitope Tagging and Reporter Gene Fusions).

The major advantage of using protease digestion to determine membrane protein topology is that proteins can be examined in their native form without any alterations to their primary sequences, such as those resulting from epitope additions and gene fusions (see Epitope Tagging and Reporter Gene Fusions). The picture derived from protease digestion should therefore reflect the true state of the protein in the cell, especially when the protein is examined in an *in vivo* expressed form (such as in whole cells or a specific cellular

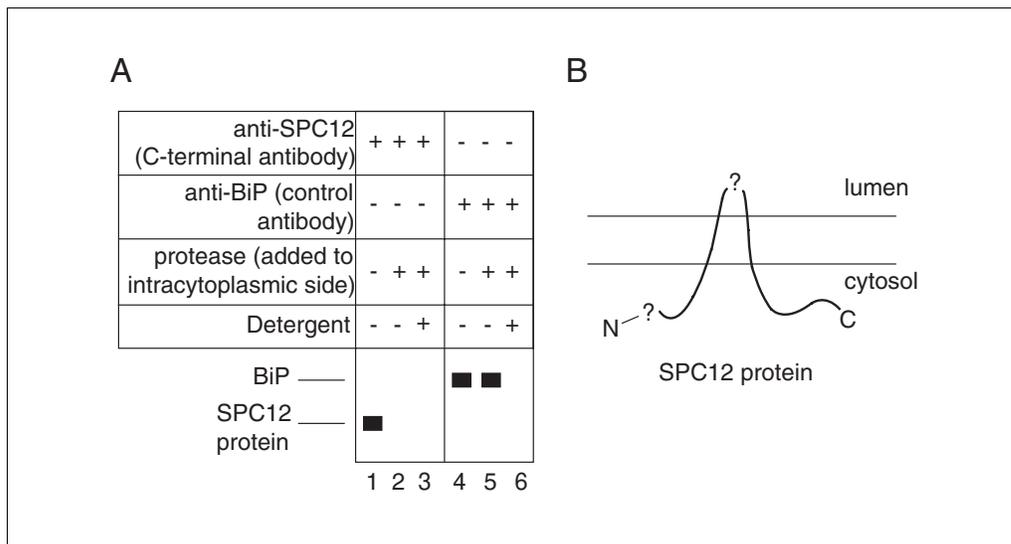


Figure 5.2.2 (A) Idealized immunoblot. The cartoon depicts an immunoblot of proteolyzed microsomes probed with an antibody directed against a C-terminal peptide of SPC12 or control antibodies directed against BiP, a luminal binding protein present in microsomal vesicles (Haas and Wabl, 1984; Bole et al., 1986). Addition or omission of a reagent from the mixture is indicated by + and –, respectively. The positions of BiP and SPC12 are indicated. For further discussion of (A), see Anticipated Results. (B) Deduced topology of SPC12's C terminus. The C terminus of SPC12 is presumed to localize to the cytosolic side of the membrane on the basis of the idealized results depicted in (A). The locations of the N terminus and presumed luminal domain of SPC12 cannot be assigned from these data and are therefore indicated with question marks.

organelle). Since membrane proteins are present both at the cell surface and at the surface of intracellular organelles, the literature should be reviewed to pick a procedure for enrichment of the desired membrane from a particular cell type. Examples of protease-digestion studies of membrane proteins residing within isolated organelles and organelle-derived vesicles are described by Graham et al. (1994), Kalies and Hartmann (1996), Kalish et al. (1995), and Li and Shore (1992). Regardless of the procedure chosen, it should produce membranes having a defined orientation, such that polypeptide domains exposed to the lumen, which corresponds to the extracytoplasmic side of the membrane for enriched organelle preparations and microsomal vesicles, are insensitive to the added protease in the absence of detergents.

A disadvantage of the protease digestion approach is that protein fragments must sometimes be identified to assess the topology of a particular region within a membrane protein. Since protein fragments are usually observed by SDS-PAGE (see Fig. 5.2.2) using the protease digestion protocol, small fragments (<2 kDa) may be difficult to detect.

While it is preferable to examine a protein in the membrane in which it resides normally, a procedure may not be available for enrichment of the desired membrane. As an alternative, an *in vitro* system of enriched microsomal membranes incubated cotranslationally with a membrane protein can be used (Walter and Blobel, 1983; Mize et al., 1986; see UNIT 11.4). This *in vitro* system is useful for examining proteins residing within the endoplasmic reticulum (ER) membrane and proteins transported from the ER to either the lysosome or another compartment of the secretory pathway (such as the cell surface).

Immunofluorescence Staining

An immunofluorescence staining technique is presented in this unit (see Basic Protocol 2) as a second experimental approach for determining membrane protein topology. In principle, this technique is similar to protease digestion, except that a fluorophore-con-

jugated secondary antibody is employed, instead of a protease, as a probe. A series of anti-peptide antibodies is prepared, each recognizing a specific hydrophilic domain of the membrane protein under study. The anti-peptide antibodies used for the protease digestion approach may be useful in the immunofluorescence staining approach provided that the antibodies recognize their corresponding epitopes in the membrane protein embedded within the lipid bilayer.

For the immunofluorescence staining protocol, the antibodies are added individually to the surface of an appropriate membrane, which must be closed and exhibit a defined orientation. When the antibody binds to an exposed hydrophilic domain, the antibody can be visualized using a fluorophore-conjugated secondary antibody that emits light detectable by confocal laser scanning microscopy or epifluorescence (see *UNIT 4.3* for a description of these microscopic techniques). Light detected from a bound antibody is evidence that the corresponding hydrophilic domain localizes to the exterior surface of the membrane examined. Protein domains exposed to the inside surface of the plasma membrane can also be probed after membrane permeabilization (Canfield and Levenson, 1993).

As with protease digestion, a major advantage of using the immunofluorescence staining approach is that a protein can be examined in its native form. A further advantage not available using protease digestion is that the orientation of a domain is determined using a microscope instead of detections of proteolytic fragments that may be very small. On the other hand, both immunofluorescence staining and protease digestion approaches suffer from the problem that particularly small hydrophilic domains may be inaccessible to the antibody or protease added. Thus, it may be possible to assign only a subset of the hydrophilic domains to a particular side of the membrane using either approach. Another potential problem with the immunofluorescence staining procedure is that the detergent used to permeabilize the membrane may expose an extracellular domain that is buried within a protein or protein complex. An extracellular domain inaccessible to the antibody in the absence of detergent could therefore be interpreted falsely to localize to the intracellular surface of the membrane.

Epitope Tagging

In determining membrane protein topology using epitope tagging (see Support Protocol), a foreign epitope is placed within the sequence of a membrane protein. The topology of the domain containing the inserted epitope can then be determined using protease digestion or immunofluorescence staining. The advantage of using a foreign epitope to assess topology is that a single antibody preparation available commercially is employed in lieu of a series of anti-peptide antibodies, which can be expensive, time-consuming to prepare, and may not always recognize the intact membrane protein. Furthermore, a small epitope is inserted into a protein's sequence as opposed to fusion of a longer reporter domain, such as in construction of gene fusions (see Reporter Gene Fusion). This small epitope is less likely to alter the native orientation of the protein under study. In addition, multiple epitopes can be inserted into a single hydrophilic domain. The addition of multiple epitopes increases the likelihood that small hydrophilic domains will be accessible to the protease or antibody added. A foreign epitope may, however, inhibit the function of the protein into which it is placed. Since it may be unclear whether epitope insertion disrupts amino acids critical for activity or alters a protein's topology, the results obtained are more reliable when the epitope-tagged protein is shown to be functional in cells or in an *in vitro* assay.

A general guideline for minimizing interference with the topology of the target protein is to place the epitope ≥ 15 amino acids from the nearby transmembrane segment(s). This avoids interfering with charged residues that may be important for determining the

orientation of a transmembrane segment (Hartmann et al., 1989). In addition, tagging the N- and C-terminal ends of some membrane proteins should be avoided, since their termini often contain compartment-localization signals (for example, see von Heijne, 1984; Nilsson et al., 1989; Swinkels et al., 1991). However, if it is determined that such signals do not exist at the termini of the target protein, the N and/or C termini are often good sites for epitope tagging, because placement of the epitope at either end does not interrupt the linear sequence of the target protein internally. If the hydrophilic domain to be probed is very small, it may be impossible to place a foreign epitope >15 amino acids from the upstream and/or downstream transmembrane segment. In such instances, one can still place the epitope within the hydrophilic domain; however, caution should be used when interpreting the topological picture derived from study of a very small domain tagged with a foreign epitope.

A variety of epitope tags are available (see Background Information); however, the HA epitope derived from the influenza hemagglutinin protein (Wilson et al., 1984) is recommended because it contains only two nonclustered charges and has been used previously in topological studies (e.g., Canfield and Levenson, 1993). Clusters of negatively charged amino acids have been shown to interfere with the integration of membrane proteins into the ER membrane, especially when the charge cluster is located near the luminal side of a transmembrane segment (Green and Walter, 1992).

Representative DNA fragments used to tag membrane proteins internally and at the N- and C-terminal ends are shown in Figure 5.2.3. These fragments can be synthesized (complementary strands must be prepared) and then inserted into naturally occurring restriction sites or into sites constructed by site-directed mutagenesis. The restriction sites used should be located in sequences corresponding to the protein's hydrophilic domains. To insert these DNA fragments, blunt-end restriction sites should be used when possible, or sites producing sticky ends can be made blunt-ended by standard molecular biological techniques. In addition, the DNA fragments shown in Figure 5.2.3 contain a blunt-end restriction site (*PvuII*). This restriction site provides a means to introduce a second fragment encoding the HA epitope. Such double-tagged proteins may allow the target protein to be better recognized by anti-HA antibodies than their corresponding single-tagged versions (Canfield et al., 1996). Repetitive insertion of DNA fragments encoding the HA epitope into each new *PvuII* site makes it possible to create proteins tagged multiple times within a single hydrophilic domain. It should be noted, however, that increasing the size of a particular domain significantly may render the protein nonfunctional.

Reporter Gene Fusions

A genetic approach for determining membrane protein topology is also available (see Basic Protocol 3). A series of gene fusions are constructed in which an enzyme (reporter moiety) is joined to various truncated fragments of an integral membrane protein (Fig. 5.2.4). The enzyme used for a reporter moiety is chosen because it exhibits activity only when placed on one side of the membrane. On expression of the fusions in vivo, measurements of enzyme activity indicate the orientation of the domains that contain the fusion joints (Fig. 5.2.5). This approach has been developed to examine proteins inserted into the membrane of the ER in the yeast *Saccharomyces cerevisiae* (Sengstag et al., 1990). However, numerous membrane proteins located throughout the secretory pathway and on the surfaces of cells contain signals for targeting to the ER membrane. These proteins are therefore amenable to topological analysis on integration within the ER membrane prior to trafficking to their ultimate destinations.

To avoid interfering with integration of the target protein, the joint connecting the membrane protein fragment and the reporter moiety should be placed ≥ 15 amino acids

A DNA fragments used for tagging a protein internally

I 5' - gln leu tyr pro tyr asp val pro asp tyr ala
3' - **CAG CTG** TAC CCA TAC GAC GTC CCA GAC TAC GCG -3'
3' - **GTC GAC** ATG GGT ATG CTG CAG GGT CTG ATG CGC -5'

II 5' - C gln leu tyr pro tyr asp val pro asp tyr ala
3' - G **CAG CTG** TAC CCA TAC GAC GTC CCA GAC TAC GCG CC -3'
3' - G **GTC GAC** ATG GGT ATG CTG CAG GGT CTG ATG CGC GG -5'

III 5' - CC gln leu tyr pro tyr asp val pro asp tyr ala
3' - GG **CAG CTG** TAC CCA TAC GAC GTC CCA GAC TAC GCG C -3'
3' - GG **GTC GAC** ATG GGT ATG CTG CAG GGT CTG ATG CGC G -5'

B DNA fragments used for tagging a protein at its N terminus

I 5' - ATG met gln leu tyr pro tyr asp val pro asp tyr ala
3' - TAC **CAG CTG** TAC CCA TAC GAC GTC CCA GAC TAC GCG -3'
3' - TAC **GTC GAC** ATG GGT ATG CTG CAG GGT CTG ATG CGC -5'

II 5' - ATG met gln leu tyr pro tyr asp val pro asp tyr ala
3' - TAC **CAG CTG** TAC CCA TAC GAC GTC CCA GAC TAC GCG C -3'
3' - TAC **GTC GAC** ATG GGT ATG CTG CAG GGT CTG ATG CGC G -5'

III 5' - ATG met gln leu tyr pro tyr asp val pro asp tyr ala
3' - TAC **CAG CTG** TAC CCA TAC GAC GTC CCA GAC TAC GCG CC -3'
3' - TAC **GTC GAC** ATG GGT ATG CTG CAG GGT CTG ATG CGC GG -5'

C DNA fragments used for tagging a protein at its C terminus

I 5' - gln leu tyr pro tyr asp val pro asp tyr ala
3' - **CAG CTG** TAC CCA TAC GAC GTC CCA GAC TAC GCG TAA -3'
3' - **GTC GAC** ATG GGT ATG CTG CAG GGT CTG ATG CGC ATT -5'

II 5' - C gln leu tyr pro tyr asp val pro asp tyr ala
3' - G **CAG CTG** TAC CCA TAC GAC GTC CCA GAC TAC GCG TAA -3'
3' - G **GTC GAC** ATG GGT ATG CTG CAG GGT CTG ATG CGC ATT -5'

III 5' - CC gln leu tyr pro tyr asp val pro asp tyr ala
3' - GG **CAG CTG** TAC CCA TAC GAC GTC CCA GAC TAC GCG TAA -3'
3' - GG **GTC GAC** ATG GGT ATG CTG CAG GGT CTG ATG CGC ATT -5'

Figure 5.2.3 Examples of DNA fragments used for tagging proteins with the HA epitope. Use of fragments I, II, and III allow in-frame fusions to any reading frame internally (**A**), at the N terminus (**B**), or at the C terminus (**C**). If the translation initiation and termination codons endogenous to the gene of interest are used, the ATG codons shown in (B) and TAA codons shown in (C) can be eliminated from the fragments depicted here. *Pvu*II (bold) and *Aat*II (underlined) restriction sites are indicated.

from the upstream transmembrane segment. When probing a hydrophilic domain containing <15 amino acids, it is acceptable to place the fusion joint after the first 3 to 5 amino acids of the following hydrophobic stretch, if one exists, in order to avoid disrupting charged residues that may be critical for orienting the upstream transmembrane segment (an example of this placement within a downstream transmembrane segment is shown in Figure 5.2.6). If, on the other hand, the small domain being probed lies at the protein's C terminus, the fusion joint should be placed at the C-terminal end of the protein.

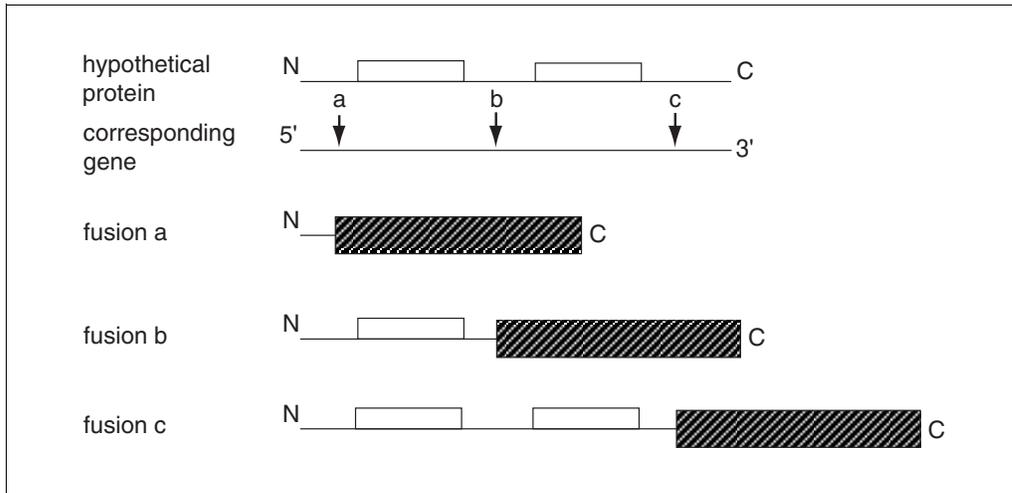


Figure 5.2.4 Placement of fusion joints in an integral membrane protein. Putative transmembrane segments are indicated by open boxes. The positions of the fusion joints to be constructed in the corresponding gene are indicated by arrows and denoted a, b, and c. A series of fusions of the invertase fragment-histidinol dehydrogenase (inv-HD) sequence (striped box) to N-terminal fragments of the hypothetical protein are indicated as fusions a, b, and c.

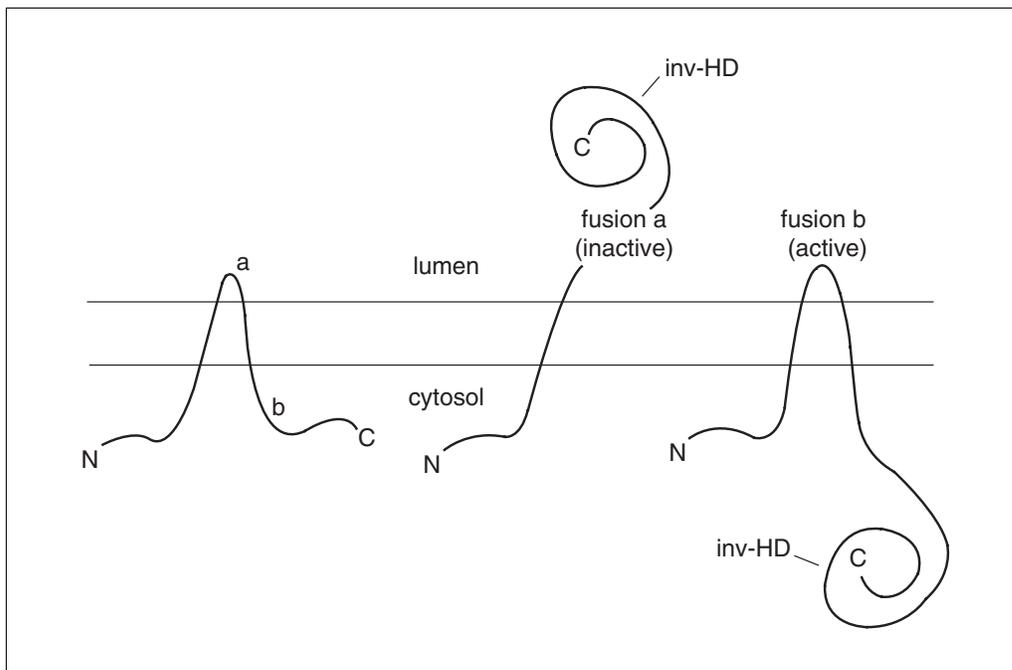


Figure 5.2.5 Fusion approach to determining membrane protein topology. The fusion of inv-HD at positions a and b in a hypothetical protein are indicated. Fusion a lacks HD activity due to the translocation of HD to the luminal side of the membrane. Fusion b exhibits HD activity due to the presence of HD in the cytosol.

Selecting a Method

A number of factors govern the selection of a protocol best suited for assessing the topology of a particular protein. Since protease digestion and immunofluorescence staining are used to probe a full-length protein, as opposed to truncated fragments, results obtained from these approaches may be more reliable, although problems associated with accessibility to the protease or antibody used can occur. Use of a foreign epitope to assess membrane protein topology can also yield very reliable results, provided the inserted epitope does not inhibit function of the target protein. Because of its simplicity, however, the reporter fusion approach is often employed as a way to get a general picture. As this approach involves fusion of a large foreign domain to N-terminal fragments, the topological assignments should be augmented with measurements of at least a few hydrophilic regions in the intact functional protein to determine whether different approaches yield the same topological picture. By complementing the reporter fusion analysis with one of the first two approaches, the derived map should provide a reasonably accurate assessment of the protein's topology. An example of this complementary approach is described by Lai et al. (1996).

PROTEASE DIGESTION

This protocol uses ER-derived vesicles from canine pancreas. These microsomes are purified according to the method of Walter and Blobel (1983) and exhibit a cytoplasmic-side-out orientation. Digestion of a hydrophilic domain present on the surface of microsomal vesicles therefore indicates that the exposed region is disposed to the cytosolic side of the membrane. This protocol uses immunoblotting to examine unlabeled proteins present in these membranes, although radiolabeled proteins integrated *in vitro* can also

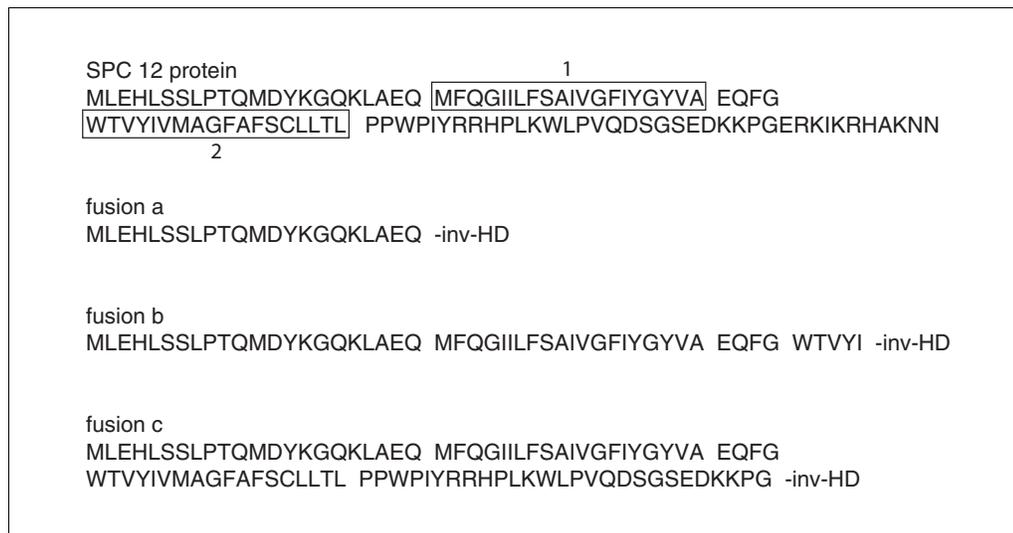


Figure 5.2.6 Positioning of fusion joints in SPC12 protein. The inv-HD reporter was placed at three positions in the SPC12 protein to discern the orientation of the three hydrophilic domains flanking hydrophobic segments 1 and 2 (open boxes). Note that the fusion joint of fusion c is placed >15 amino acids from the upstream hydrophobic segment. However, since the hydrophilic domain located between hydrophobic segments 1 and 2 consists of only 4 amino acids (EQFG), it is not possible to place the fusion joint more than 15 amino acids from hydrophobic segment 1 without capturing most of hydrophobic segment 2 in fusion b. Therefore, the fusion joint is positioned such that only 5 amino acids of hydrophobic segment 2 are present in fusion b. In placing the fusion joint at this position, the charged residues immediately following hydrophobic segment 1 are left undisturbed. Fusion a lacks a putative transmembrane segment and will remain cytosolic regardless of the topology of the N terminus of intact SPC12 protein. One exception to this is that, if the polypeptide chain contains an N-terminal signal sequence (von Heijne, 1984), fusion a would probably be transported to the ER lumen.

be studied (Walter and Blobel, 1983; Mize et al., 1986; see *UNIT 11.4* for analysis of protein translocation into canine microsomes). The protocol can also be adapted to microsomes prepared from metabolically labeled cells (*UNIT 7.1*; Bonnerot et al., 1994). As a control to determine whether the target protein is inherently sensitive to the protease used, proteolyzed membranes are compared to membranes solubilized in a mild detergent, such as Triton X-100, prior to protease addition.

In this protocol, proteinase K is employed as a representative protease due to its broad substrate specificity. In addition, proteinase K is active under the buffer conditions described (as are many other proteases), and proteinase K retains activity in the presence of Triton X-100. The buffer also contains sucrose to help maintain the integrity of membrane vesicles. Another control, which tests for the presence of intact membranes, examines a luminal protein native to the membrane vesicles used. Provided the membranes are intact, the luminal protein control should be resistant to proteinase K in the absence of detergent. Examples of ER proteins used for control purposes are TRAP β (Kalies and Hartmann, 1996) and BiP (Mullins et al., 1995).

Materials

- Canine pancreatic microsomal membranes (see *UNIT 11.4*)
- Magnesium/sucrose/BSA (MSB) buffer (see recipe)
- 20% (w/v) Triton X-100
- 10 mg/ml proteinase K (see recipe)
- 100% (w/v) trichloroacetic acid (TCA) (see recipe)
- 2 \times SDS sample buffer (*APPENDIX 2A*)
- Antibodies directed against a series of peptides corresponding to specific hydrophilic regions of the target protein
- Control antibodies directed against a luminal protein or a luminal domain of a membrane protein in the membrane system to be analyzed
- Additional reagents and equipment for preparing canine pancreatic microsomes (*UNIT 11.4*), separating proteins by SDS-PAGE (*UNIT 6.1*), and detecting proteins by immunoblotting (*UNIT 6.2*)

Prepare microsomal membranes

1. Purify microsomal membranes according to the method described in *UNIT 11.4*.

Microsomes are stored at -80°C at a final concentration of 50 A_{280} units per ml. One equivalent is defined as 1 μl of this mixture. When stored under these conditions, microsomes are stable ≥ 1 year.

*Be sure that inhibitors of the protease to be used are omitted from the original membrane preparation (such as PMSF in the case of proteinase K; see *APPENDIX 1B*). If protease inhibitor is present, wash the membranes by centrifuging 20 min at $\sim 100,000 \times g$, 4°C . Suspend the pellet in MSB buffer at the same concentration by pipetting up and down in a plastic tip.*
2. Slowly thaw $\sim 50 \mu\text{l}$ of microsomes by placing a tube containing the microsomes in ice.

This amount of membranes is needed for examinations that use one antipeptide antibody and a control antibody. An additional 50- μl aliquot is needed for each additional examination.
3. Prepare ten samples, each containing 5 μl (5 equivalents) rough microsomes diluted with MSB buffer to a final volume of 20 μl in microcentrifuge tubes.

Steps 3 to 8 should be performed using tubes kept in ice. Polypropylene microcentrifuge tubes have been found to work satisfactorily.

4. Add 1 μl of 20% (w/v) Triton X-100 to five of the tubes and mix by pipetting the solution up and down.

These detergent-treated aliquots serve as controls to demonstrate that the target protein is inherently sensitive to the protease used. The five tubes lacking Triton X-100 are sample tubes and the five tubes containing Triton X-100 are control tubes.

Perform protease digestion

5. Add 10 mg/ml proteinase K to four of the sample tubes and four of the control tubes to yield final concentrations of 1, 20, 100, and 500 $\mu\text{g/ml}$.

The amount of protease is varied to find the concentration which allows good digestion of the target protein yet is low enough that the protease does not destroy the membrane structure (thus making the membrane permeable to the protease added). Protease should be omitted from the fifth tube in the sample and control sets.

6. Mix samples by pipetting the solutions up and down a few times and incubate 30 min.
7. Stop proteolysis by adding 100% (w/v) TCA to a final concentration of 15%.

Analyze protein digests

8. Incubate all ten tubes 15 min on ice and collect TCA pellets by microcentrifuging 5 min at $\sim 10,000$ rpm ($8,000 \times g$), room temperature.
9. Remove supernatant and suspend the pellets in 10 μl of 2 \times SDS sample buffer and 10 μl H_2O by vortex mixing at room temperature.

Be sure that all of the pellet has been suspended. The mixtures should be blue because of bromphenol blue, which is present in the sample buffer. If the tubes are yellow (because of high acidity), 1- μl aliquots of 1 M Tris base can be added successively and then mixed until the mixture turns blue.

10. Load 10 μl from each tube into sample wells of a SDS-PAGE gel.

The lanes should be organized as follows. The first five lanes contain one-half of the mixture from the five sample tubes, and the next five lanes contain one-half of the mixture from the five control tubes. The next five lanes contain one-half of the mixture from the five sample tubes, and the final five lanes contain one-half of the mixture from the five control tubes.

A 12.5% polyacrylamide gel can be used to identify protein fragments of ~ 2 to 30 kDa, whereas a 7% acrylamide gel can be used to identify protein fragments of 30 to 100 kDa.

11. Analyze samples using SDS-PAGE (UNIT 6.1).
12. Examine the separated protein fragments by immunoblotting (UNIT 6.2), using one of the anti-peptide antibodies and the control antibody directed against a luminal protein or a luminal domain of a membrane protein in the membrane system to be analyzed.

The blot should be cut in half. The gel portion containing the first ten lanes is to be immunoblotted with an antibody directed against the target protein. The gel portion containing the second group of ten lanes should be subjected to immunoblotting using an antibody directed against the luminal protein control. The data should be interpreted by following examples described in Figure 5.2A and Anticipated Results.

13. Repeat steps 3 to 12 using each of the anti-peptide antibodies and the control antibody.

IMMUNOFLUORESCENCE STAINING

This protocol illustrates use of an immunofluorescence technique to probe the topology of membrane proteins. The protocol is designed specifically to examine proteins confined to the plasma membranes of cells lacking an outer cell wall, such as cultured mammalian cells. This protocol has been optimized for using human embryonic kidney (HEK) 293 cells, although other cultured cell lines can be used with only minor modifications (noted below). These cells can be grown on glass coverslips and permeabilized with low concentrations of a mild detergent (Nonidet P-40 is used here), which allows antibodies access to the cytoplasmic compartment. The permeabilized cells and a second nonpermeabilized cell preparation are incubated with an antipeptide antibody followed by incubation with a fluorophore-conjugated secondary antibody. Cells are then examined by fluorescence microscopy (*UNIT 4.2*). As a control, cells expressing a plasma membrane protein of known topology should be examined using antibodies directed to a cytosolic domain and an extracytoplasmic domain.

This approach can employ primary antibodies directed against peptides corresponding to the membrane protein under study, such as the antipeptide antibodies described in Basic Protocol 1. However, antipeptide antibodies are found often not to recognize their corresponding epitopes within a native membrane protein (Carrasco et al., 1986). The procedure described here therefore uses a membrane protein that has been tagged with the HA epitope (see Support Protocol for methods regarding epitope tagging). Use of the HA epitope can minimize cost and time associated with the preparation of antipeptide antibodies. The primary and secondary antibodies used in this protocol are the anti-HA epitope mouse monoclonal antibody 12CA5 and a rhodamine-conjugated rabbit anti-mouse IgG, respectively, but other combinations of antibodies can be used as well (Canfield and Levenson, 1993).

Materials

HEK 293 cells (ATCC #CRL 1573)
4% (w/v) paraformaldehyde (see recipe)
Nonidet P-40/goat serum/BSA (NGB) solution (see recipe)
Anti-HA mouse monoclonal antibody 12CA5 (Boehringer Mannheim)
Rhodamine-conjugated rabbit anti-mouse immunoglobulin G (IgG)
DMEM/FBS/HEPES (DFH) solution (see recipe)
Fluoromount G mounting medium (Fisher)

6-well tissue culture plates
Glass coverslips, 22-mm diameter

Additional reagents and equipment for immunofluorescence staining of fixed mammalian cells (*UNIT 4.3*), epifluorescence (*UNIT 4.2*) or confocal laser microscopy, and growing cultured mammalian cells (*UNIT 1.1*)

1. Seed HEK 293 cells stably expressing the polypeptide of interest on glass coverslips and grow 2 to 3 days at 37°C in 6-well plates before processing for immunofluorescence.

Alternatively, transient transfections can be performed on cells grown on glass coverslips in 6-well plates (UNIT 1.1). Cells should be handled using typical aseptic technique required for growing cells in culture (UNIT 1.3).

The glass coverslips should be cleaned thoroughly using a strong detergent and washed extensively with distilled water.

Duplicate cultures are needed in order to provide sets to be examined in the absence and presence of permeabilizing agents and in the absence or presence of the polypeptide of

interest. A single 6-well plate thus provides a triplicate analysis of cells to be treated with permeabilizing agents and containing the polypeptide of interest and a triplicate analysis of cells treated with permeabilizing agents and lacking the polypeptide of interest. A distinct plate provides a triplicate analysis for the set not treated with permeabilizing agents and either containing or not containing the polypeptide of interest (see below).

All steps except the antibody incubations can easily be carried out in 6-well tissue culture plates, using volumes of 2 to 3 ml per well for incubations and washes.

Methods for HA tagging, and testing for function of tagged proteins in transiently transfected HEK 293 cells are described in the Support Protocol.

2. For the permeabilized set, aspirate culture medium and wash coverslips once in PBS. Replace PBS with 4% paraformaldehyde (2 to 3 ml) and fix 10 min at room temperature.
3. Aspirate fixing reagent and rinse cells three times with PBS. Permeabilize and block cells in NGB solution (2 to 3 ml) for 15 min at room temperature.
4. Dilute monoclonal antibody 12CA5 to 1 $\mu\text{g/ml}$ in NGB. Centrifuge antibody dilution 2 min at $8000 \times g$ to remove aggregates.

Although this antibody is supplied at a specified concentration, different batches appear to have different characteristics, and it may be necessary for individual users to optimize their antibody dilutions.

5. Place coverslips cell-side-up on Parafilm and immediately add primary antibody (100 μl per coverslip). Incubate 1 hr at room temperature in a covered, humidified chamber.

A small petri dish or any small covered container will serve as a humidified chamber.

For more tightly adherent cells, the volume of antibody used can be reduced further as follows. Pipet antibody (50 μl) directly onto Parafilm. Place coverslips on droplet, cell-side-down.

6. Return coverslips to NGB (2 to 3 ml) in the 6-well plate. Wash three times with PBS (10 min each wash) and once with NGB.
7. Incubate cells 1 hr at room temperature in NGB solution (2 to 3 ml) containing a rhodamine-conjugated rabbit anti-mouse IgG.

The secondary antibody should be diluted according to the supplier's recommendations. Optimization of antibody dilution may be necessary.

8. Return coverslips to NGB solution and wash three times with PBS.
9. Dip coverslips in distilled water and mount on slides using Fluoromount-G (15 μl per slide).
10. For the nonpermeabilized set, replace growth medium with DFH solution and chill 15 min to 4°C .

These cells provide the set that is nonpermeable in the presence of primary antibody. These steps can most conveniently be performed concurrently with processing of permeabilized cells.

11. Incubate with monoclonal antibody 12CA5 (2 $\mu\text{g/ml}$) for 1 hr in a humidified chamber at 4°C as described in step 5.

Note that a higher concentration of antibody is used for this low-temperature incubation.

12. Wash three times with cold DFH and once with cold PBS.

13. Fix, permeabilize, and incubate “nonpermeabilized” cells with secondary antibody, as described in steps 2 to 3 and 7 to 9.

The nonpermeabilized set is permeabilized immediately before addition of secondary antibody. This step serves not only to bind the secondary antibody to the primary antibody but also to determine whether the secondary antibody binds nonspecifically to either surface of the plasma membrane.

14. Examine permeabilized and nonpermeabilized cells using epifluorescence or confocal laser scanning microscopy.

Fluorescence staining of only the permeabilized set is suggestive of an antibody bound to a cytosolic domain, whereas fluorescence appearing in both the permeabilized and nonpermeabilized sets reveals a domain placed at the extracytoplasmic side of the plasma membrane. A range from 5% to 25% of the cells on a particular coverslip may be stained well.

The control cells lacking the polypeptide of interest are needed because one difficulty associated with the use of 12CA5 is that it is not absolutely specific for the HA epitope. Cross-reactivity is seen both on immunoblots and by immunofluorescence in nontransfected or nontagged cells. The resulting background may decrease the sensitivity with which the transfected, tagged polypeptide can be visualized. If the background signal is too high to detect the protein of interest, even after diluting the primary and secondary antibodies further than that described above, try other anti-HA antibody preparations such as those available from BAbCO. Note, however, that some of these antibodies may not recognize HA tags placed internally within the protein sequence.

EPITOPE TAGGING

In this protocol, a series of molecular biological manipulations are used to place a foreign epitope into a hydrophilic region of a membrane protein. Integration of the expressed protein leads to placement of the epitope on one side of the membrane. The orientation of the tagged domain can be assessed by monitoring the accessibility of the epitope to a protease (see Basic Protocol 1) or to its cognate antibody (see Basic Protocol 2).

The HA epitope, YPYDVPDYA, is recommended for use in this protocol because it contains only two charged amino acids. As shown in Figure 5.2.3, a set of three DNA fragments (differing by addition of 0, 1, or 2 base pairs at one or both ends) can be used to place the HA epitope internally within the sequence of a protein, N-terminally, or C-terminally. The fragments are inserted into a blunt-end restriction site occurring naturally within the protein’s gene or into a restriction site constructed by site-directed mutagenesis (see APPENDIX 3). Depending on the reading frame of the site into which the epitope is inserted, only one of the three DNA fragments is needed to permit in-frame fusion between the membrane protein sequence and the epitope at its N and/or C termini. These fragments also contain the blunt-end *PvuII* restriction site. This site provides a convenient place to insert a second fragment encoding the HA epitope, which is used if the single tag is found not to present the membrane protein adequately to the added antibody. Alternatively, DNA fragments containing a blunt-end restriction site other than *PvuII*, such as *EcoRV*, can be synthesized if this *PvuII* site is not unique on the plasmid containing the gene of interest. The DNA sequence encoding the HA epitope depicted in Figure 5.2.3 also contains an *AatII* restriction site that can be used to verify insertion of the fragment into the plasmid used. It is usually necessary to verify orientation of the inserted fragment by DNA sequencing. This is not the only way to introduce an epitope into a membrane protein. A DNA sequence encoding the epitope can be introduced by site-directed mutagenesis.

Having introduced a DNA fragment encoding the HA epitope into a cloned gene, the tagged protein should be tested for function. If the tagged protein exhibits normal functional properties, then it is likely that the epitope introduced has not altered the overall

SUPPORT PROTOCOL

Characterization of Cellular Proteins

5.2.13

structure of the protein. To test for function, many types of assays can be performed. The type of assay used is dependent on the particular protein under study. For example, the HA-tagged rodent Na,K-ATPase α subunit can be tested for its ability to confer ouabain resistance to HEK 293 cells transfected with the tagged DNA construct (see Basic Protocol 2; Canfield et al., 1996). A tagged protein can be introduced into yeast cells, testing for complementation of a mutant lacking that protein or an evolutionarily conserved protein (Kurihara and Silver, 1993). Alternatively, a tagged protein may be expressed to high levels, enriched, then tested for activity using an in vitro assay.

Materials

TE buffer (APPENDIX 2A)

Plasmid DNA encoding the protein of interest

E. coli cells to be transformed

Additional reagents and equipment for synthesizing oligonucleotides (APPENDIX 3), ligating DNA (APPENDIX 3), transforming *E. coli* (APPENDIX 3), isolating plasmid DNA from *E. coli* (APPENDIX 3), identifying plasmids by restriction endonuclease digestion (APPENDIX 3), and sequencing oligonucleotides (APPENDIX 3)

1. Determine whether fragment I, II, or III (Fig. 5.2.3) is needed to produce an in-frame fusion between the N- and/or C-terminal ends of the HA epitope and the target protein.
2. Synthesize the appropriate DNA fragment(s) identified from step 1.
Complementary oligonucleotides should be synthesized.
3. Mix two complementary DNA strands together, each to a final concentration of 1 A_{260} unit per 50 μ l in TE buffer.
Oligonucleotides are usually synthesized with their 5' ends unphosphorylated. The oligonucleotides used should be left unphosphorylated to prevent insertion of multiple oligonucleotides into the linearized plasmid.
4. Anneal the strands by heating the solution 5 min at 70°C and then letting the solution cool in air at room temperature.
5. Mix 5 μ l of the oligonucleotide solution with 0.1 μ g of linearized plasmid DNA encoding the protein of interest, ligate the mixture in 10 μ l of ligation buffer, and transform *E. coli* with the ligation mixture (APPENDIX 3).

The DNA should be linearized at a restriction site contained within a sequence corresponding to the hydrophilic domain to be probed. If such a restriction site does not exist, one can be introduced by site-directed mutagenesis (as illustrated below, blunt-end sites are preferable). If possible, the epitope should be placed ≥ 15 amino acids from the nearby transmembrane segment(s). If the restriction site used produces sticky ends, the site should be made blunt-ended using standard molecular biological manipulations. To minimize religation of the plasmid without the DNA fragment to be inserted, the ligation mixture should be treated with the restriction enzyme used to produce the blunt end, provided a restriction enzyme producing blunt ends was used.

The above-described ratio of fragment and linear plasmid is acceptable in many instances. However, sometimes this ratio may need to be varied in order to achieve efficient ligation of the fragment to the plasmid. Try increasing the concentration of oligonucleotides from 1 A_{260} unit to 2, 5, and 10 A_{260} units per 50 μ l in TE buffer (step 3).

*Any of the *E. coli* strains commonly used for plasmid transformations is suitable, such as HB101 and MC1061.*

6. Isolate plasmids from individual *E. coli* transformants and identify plasmids containing the insert using restriction analysis (APPENDIX 3).

If a fragment shown in Figure 5.2.3 is synthesized, restriction enzymes *AatII* and *PvuII* are useful for identifying the desired construct.

- Determine the DNA sequence of the fragment inserted and residues surrounding this fragment to ensure correct orientation and in-frame fusion.
- Express the epitope-tagged protein in an appropriate cell and test for its function. Examine its topology using Basic Protocols 1 or 2.

If the tagged protein is not recognized by antibodies directed against the HA epitope, double-HA-tagged proteins can be constructed by inserting the appropriate DNA fragment (Fig. 5.2.3) into the *PvuII* site of the construct tagged with only one HA epitope. Successive insertions into each new *PvuII* site will produce proteins tagged with increasing numbers of HA epitopes.

Plasmid DNA can be stored indefinitely in TE buffer at -20°C and should be introduced into the cell examined prior to use.

REPORTER GENE FUSIONS

In this protocol, a series of gene fusions is constructed in which a reporter moiety is fused to various truncated fragments of a target membrane protein. To construct these fusions, DNA fragments encoding a series of N-terminal fragments of a membrane protein should be synthesized by the polymerase chain reaction (PCR; see APPENDIX 3). For each PCR amplification, two primers are required: one corresponding to a sequence upstream of the promoter of the relevant gene and one corresponding to sequences at the desired fusion joint. Only one upstream primer is required, whereas a downstream primer corresponding to each of the fusion joints is usually needed. For construction of PCR fragments, the upstream primer contains a *Bam*HI site or a site compatible with the *Bam*HI sticky end, such as *Bgl*II or *Bcl*I. The downstream primer contains a *Xho*I site or a site compatible with *Xho*I, such as *Sal*I. Compatible sites are needed when the DNA fragment to be inserted contains an internal *Bam*HI or *Xho*I site. To ensure that the reading frame is maintained across the fusion joint, the *Xho*I site in the downstream primer is placed in the following reading frame: C TCG AG (where TCG encodes an in-frame serine).

A vector that can be used for expression of fusions in yeast is pA189invHD (Green and Walter, 1992; Fig. 5.2.7). pA189invHD is used for construction of gene fusions encoding the C-terminal moiety histidinol dehydrogenase (HD). HD is a cytoplasmic enzyme that is enzymatically inactive when fused to a luminal domain of a membrane protein inserted into the ER membrane. This shuttle vector contains the 2 μ m DNA fragment for replication in yeast and the *ColE1* replication origin for replication in *E. coli*. pA189invHD can be selected in yeast cells containing a mutation in the *URA3* gene due to the presence of plasmid-borne *URA3*. The vector can also be maintained in *E. coli*, as it confers ampicillin resistance. pA189invHD contains a single *Bam*HI site and a single *Xho*I site. Restriction of pA189invHD with *Bam*HI and *Xho*I generates two DNA fragments (13 kb and 0.8 kb). The larger of these fragments contains the selectable markers and origins of replication described above. The smaller fragment encodes a portion of arginine permease, whose topology was analyzed previously using this vector system (Green and Walter, 1992). The fragment encoding arginine permease is therefore replaced with PCR-amplified fragments encoding N-terminal truncations of the membrane protein to be examined. For examining the topology of a protein without its native promoter, the yeast *ADH1* promoter (Bennetzen and Hall, 1982) is included on pA189invHD. When using the *ADH1* promoter, the upstream primer should contain a *Bam*HI site in front of the initiation codon for the membrane protein. For efficient expression from the *ADH1* promoter in pA189invHD, it is important that no ATG codon is present between the *Bam*HI site and the initiation codon.

BASIC PROTOCOL 3

Materials

Reporter plasmid (Fig. 5.2.7): pA189invHD (available from Neil Green, Vanderbilt University)

S. cerevisiae strain FC2-12B (*MAT α trp1-1 leu2-1 ura3-52 his4-401 HOL1-1 can1-1*; available from Neil Green, Vanderbilt University)

SD +HIS agar plates (see recipe)

SD +HOL agar plates (see recipe)

Thermocycler

Additional reagents and equipment for the polymerase chain reaction (PCR; APPENDIX 3), agarose gel electrophoresis (APPENDIX 3), restriction endonuclease digestion (APPENDIX 3), and transformation of *E. coli* and *S. cerevisiae* (APPENDIX 3)

Construct gene fusions

1. Amplify by PCR a series of DNA fragments encoding truncations of a membrane protein, using appropriate primers.

The DNA primers used in the PCR amplification can be synthesized by one of a number of companies offering services in oligonucleotide synthesis. The upstream primer should

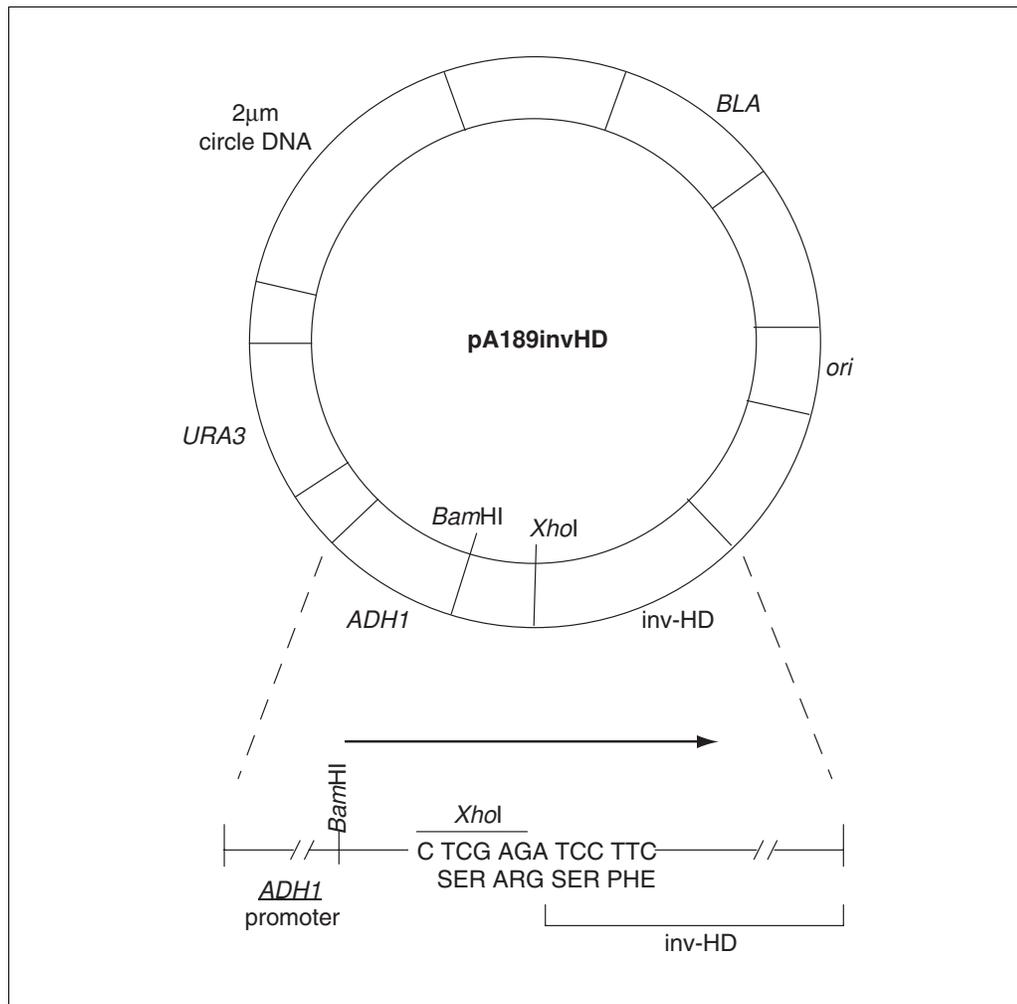


Figure 5.2.7 Gene fusion vector pA189invHD. The direction of transcription is indicated by an arrow. Abbreviations: *BLA*, β -lactamase gene; *ADH1*, alcohol dehydrogenase gene promoter; *URA3*, orotidine-5'-phosphate decarboxylase gene; *ori*, ColE1 replication origin; *inv-HD*, fusion of invertase fragment-histidinol dehydrogenase genes.

contain a 5' *Bam*HI site (or compatible site) followed by at least 20 nucleotides complementary to the DNA sequence to be amplified. The downstream primer should contain a 5' *Xho*I site (or compatible site) followed by at least 20 nucleotides from the reverse strand of the DNA sequence to be amplified. The *Bam*HI site should be placed immediately before the initiation codon if the *ADH1* promoter of *pA189invHD* is to be used or before the promoter of the gene to be studied. The *Xho*I site should correspond to a site in a hydrophilic domain of the membrane protein studied. It is also recommended that the primers contain 2 to 3 nucleotides at the 5' end of the restriction site for efficient cutting by the restriction enzyme after PCR amplification.

2. Purify the amplified fragments by agarose gel electrophoresis and digest with *Bam*HI and *Xho*I.

Overnight restrictions are sometimes needed for efficient cutting of XhoI sites located near the ends of DNA fragments.

3. Digest the reporter plasmid *pA189invHD* with *Bam*HI and *Xho*I.

4. Purify the larger, 13-kb DNA fragment produced from the restriction digestion by agarose gel electrophoresis (see APPENDIX 3). Ligate the 13-kb fragment to each of the fragments produced in step 2.

The smaller fragment (0.8 kb) encodes a part of arginine permease and should be discarded.

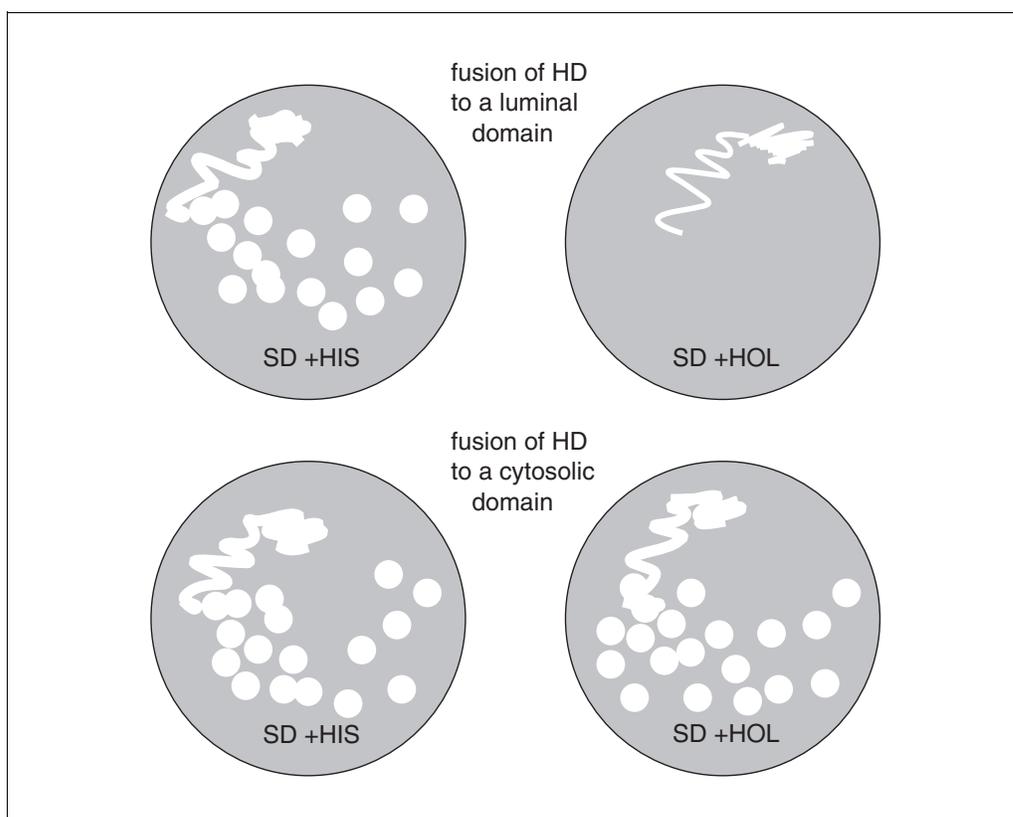


Figure 5.2.8 Cell growth assay for determining membrane protein topology. Cells of yeast strain FC2-12B bearing a fusion protein that fuses the HD moiety to a luminal domain of an integral membrane protein or bearing a fusion protein that fuses the HD moiety to a cytosolic domain of an integral membrane protein are streaked for single colonies on SD +HIS agar plates and SD +HOL agar plates, then incubated 4 to 5 days at 30°C. When large colonies appear on SD +HIS agar plates and little growth is detected on SD +HOL agar plates, the reporter is interpreted to be fused to a luminal domain of the membrane protein under study. On the other hand, when large colonies appear on both the SD +HIS and SD +HOL agar plates, the reporter is interpreted to be fused to a cytosolic domain.

5. Transform a standard Amp^s *E. coli* strain and isolate the desired plasmid construct.

A variety of E. coli strains can be used, such as strains HB101 and MC1061.

Perform genetic assay

6. Transform the construct isolated from *E. coli* into *S. cerevisiae* strain FC2-12B and select transformants on SD +HIS agar plates.

Yeast cells should be handled using aseptic technique.

Strain FC2-12B contains a ura3 mutation that permits selection for pA189invHD-derived plasmids. Transformants should appear as individual colonies after 4 days on agar plates incubated at 30°C.

7. Test transformed yeast cells for growth by streaking cells on SD +HOL agar plates and SD +HIS agar plates.

Individual colonies should appear after 4 to 5 days at 30°C. An example of the results expected is shown in Figure 5.2.8. As shown in this figure, fusion of HD to a luminal or cytosolic domain does not affect cell growth on SD +HIS agar plates. However, fusion of HD to a luminal domain inhibits growth on SD +HOL agar plates. Fusion of HD to a cytosolic domain does not inhibit cell growth on either type of agar plate.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

DMEM/FBS/HEPES (DFH) solution

10% (v/v) fetal bovine serum

20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4

Prepare in supplemented Dulbecco's modified Eagle medium (DMEM; APPENDIX 2A)

Prepare fresh

The fetal bovine serum should not be heat-inactivated.

Magnesium/sucrose/BSA (MSB) buffer

150 mM potassium acetate

5 mM magnesium acetate

50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.6

200 mM sucrose

1 mM dithiothreitol (APPENDIX 2A)

Prepare fresh

Nonidet P-40/goat serum/BSA (NGB) solution

0.05% (v/v) Nonidet P-40 (Igepal AC-630)

5% (v/v) goat serum

1% (w/v) BSA

Prepare in PBS (APPENDIX 2A), pH 7.4

Prepare fresh

Paraformaldehyde, 4%

Heat 900 ml water to 55° to 60°C on a stirring hot plate in a fume hood. Add 40 g paraformaldehyde powder and stir 30 min. If powder has not dissolved, add a few NaOH pellets one at a time (waiting a few minutes between pellets) until the paraformaldehyde dissolves. Add 100 ml of 10× PBS (APPENDIX 2A), filter, cool to room temperature, and adjust to pH 7.4 with HCl. Store up to 1 week at 4°C.

CAUTION: The fume hood is used because paraformaldehyde fumes are toxic. The "Prill" form of paraformaldehyde from EM Sciences is safest to use, as it pours without creating a cloud.

Proteinase K, 10 mg/ml

10 mg proteinase K (lyophilized powder, ~80% protein, 10 to 20 U/mg protein; Sigma)

1 ml 50 mM HEPES, pH 7.6

Prepare fresh on ice

No activation of the proteinase K is necessary; the purchased enzyme is ready to use.

SD +HIS agar plates

0.7 g/liter yeast extract (without amino acids; Difco)

20 g/liter glucose

20 g/liter agar

0.1 mg/ml L-tryptophan

0.1 mg/ml L-leucine

0.1 mg/ml L-histidine

Mix the ingredients together before autoclaving. Pour the autoclaved solution into 100 × 15-mm petri dishes. Store plates <1 month at 4°C.

SD +HOL agar plates

0.7 g/liter yeast extract (without amino acids; Difco)

20 g/liter glucose

20 g/liter agar

0.1 mg/ml L-tryptophan

0.1 mg/ml L-leucine

0.1 mg/ml L-histidinol dihydrochloride (Sigma)

Filter-sterilize the histidinol solution and add to the remainder after autoclaving. Store plates <1 month at 4°C.

TCA, 100%

250 g solid trichloroacetic acid (TCA)

141 ml water

Store <6 months at 4°C

COMMENTARY**Background Information**

Integral membrane proteins can often be distinguished from peripherally bound proteins in that the latter are extractable from the membrane under conditions of high pH (Steck and Yu, 1973; Fujiki et al., 1982; Russel and Model, 1982). Resistance of integral membrane proteins to extraction is attributed to the presence of transmembrane segments which help anchor proteins to the lipid bilayer. Visual and computer-based inspections of the primary sequences of integral membrane proteins usually reveal one or more hydrophobic amino acid stretches interspersed among hydrophilic stretches in an alternating pattern. This pattern suggests a topology in which neighboring hydrophilic domains are positioned on opposite sides of a membrane through integration of hydrophobic transmembrane regions. These topological predictions have been generally confirmed through an emerging number of crystal structures of integral membrane pro-

teins (Deisenhofer et al., 1985; Xia et al., 1997). Despite the availability of methods for X-ray diffraction analyses of membrane proteins, topological studies still occupy an important place in protein structural studies. This is due to the fact that structures of membrane proteins are much less easily obtained than those of water-soluble proteins because of difficulties in obtaining crystals. As most integral membrane proteins require a specific topology for function, determining the orientations of hydrophilic domains may provide significant insight into the proteins' roles in vivo.

Protease digestion

Protease digestion is one of the oldest methods for determining membrane protein topology. This approach relies on the principle that the lipid bilayer is generally impermeable to proteases. Therefore, addition of a protease to the outside surface of a membrane vesicle results in degradation of protein sequences lo-

cated on the exterior surface, thereby revealing the luminal content (Gerber et al., 1977). It is best to examine a protein in its native membrane environment. The protease digestion protocol described in this unit (see Basic Protocol 1) is useful for examining proteins residing within isolated ER membrane vesicles by immunoblotting, although this procedure can be adapted easily to the study of proteins present in the plasma membrane and in other membrane-bound organelles. The primary difference is in the enrichment procedure used to isolate the desired membrane preparation (Li and Shore, 1992; Graham et al., 1994; Kalish et al., 1995).

Alternatively, one may examine the topologies of proteins integrated into microsomal vesicles *in vitro*. Such proteins are found to assume a specific topology that can be probed by the addition of a protease (Mize et al., 1986). *In vitro*-expressed proteins can, however, suffer the problem of being integrated inefficiently (Skach et al., 1994), and *in vitro* systems may not offer a functional assay assuring that the membrane protein has achieved its native structure. However, the membrane integration machinery in the ER is believed to perform accurately *in vitro*, at least with native membrane proteins (see UNIT 11.4).

In order to gain a complete picture of a protein's topology using the protease digestion method, each hydrophilic domain must be inherently sensitive to the protease used. That is, accurate pictures are obtained only when each domain is digestible either at the surface of the vesicle or in detergent-solubilized solutions. To ensure that the hydrophilic domain being probed can be proteolytically cleaved, it should contain a site recognized by the protease used. Proteases exhibiting a broad substrate specificity, such as proteinase K and trypsin, should therefore be used.

For particularly small domains, accessibility to a protease may be a problem. Furthermore, since digested regions are probed with antibodies directed against a series of peptides corresponding to various hydrophilic regions within the membrane protein, there is no guarantee that all of the antibody preparations will recognize the intact protein (Seckler et al., 1986). Despite these potential problems, the protease digestion approach is a proven method, particularly as it has been used to probe large hydrophilic domains.

Immunofluorescence staining

Immunofluorescence staining has been used widely to analyze the topology of proteins localized to the plasma membrane (Canfield and Levenson, 1993; Canfield et al., 1996). This method (see Basic Protocol 2) has the advantage that domains present on either side of the membrane can be visualized with a microscope, as opposed to detection of proteolytic fragments which may be small and difficult to find by SDS-PAGE. Compared to the protease digestion approach, immunofluorescence staining has not been used generally to study membrane protein topology in organelle-based systems. It should be possible, however, to identify hydrophilic domains located at the surface of peroxisomes in mammalian cells by taking advantage of the fact that the plasma membrane, but not the peroxisomal membrane, is permeabilized with the detergent digitonin (Swinkels et al., 1991). Since both plasma and peroxisomal membranes are permeabilized after addition of Triton X-100, use of these detergent combinations should permit a topological analysis of peroxisomal proteins.

Epitope tagging

Preparation of antipeptide antibodies needed for Basic Protocols 1 and 2 is costly and time-consuming. These problems are exacerbated by the fact that many antipeptide antibodies fail to recognize their corresponding epitope within the intact membrane protein. An alternative approach, epitope tagging, is therefore presented in this unit (see Support Protocol). A DNA fragment encoding a foreign epitope recognized by a commercially available antibody is introduced into the gene encoding a membrane protein. A tagged domain can then be identified using protease digestion (see Basic Protocol 1) or immunofluorescence staining (see Basic Protocol 2; Canfield and Levenson, 1993; Skach et al., 1994; Canfield et al., 1996).

There are, however, disadvantages that may result from the addition of an epitope. The epitope may render the membrane protein into which it has been inserted nonfunctional (Canfield et al., 1996). In such cases, it is not always clear whether the topology has been disrupted or whether the epitope has been inserted into a site critical for activity. In either event, results derived from studies of a nonfunctional protein are unreliable in the absence of supportive topological data. Even if the tagged protein is functional, the possibility remains that the foreign

epitope has disrupted the topology of a region unimportant to the protein's activity under the conditions measured.

Although the HA epitope has been used successfully for determining membrane protein topology (Canfield and Levenson, 1993), it contains two charged amino acids (YPYDVPDYA; Wilson et al., 1984). A number of studies have shown that charged residues located near membrane-spanning sequences play a role in determining transmembrane orientation in eukaryotic cells. Indeed, introduction of clusters of negatively charged amino acids proximal to transmembrane segments can inhibit their integration (Green and Walter, 1992). However, the HA epitope contains fewer charged amino acids than FLAG (DYKDDDDK; Hopp et al., 1988), c-myc (EQKLISEEDL; Evan et al., 1985), and VSV-G (YTDIEMNRLGK; Kreis, 1986) epitopes and is therefore recommended for use. The AU1 epitope (DTYRYI) derived from the papillomavirus major capsid protein also contains only two charged amino acids (Lim et al., 1990); however, it has not yet been utilized for topological analysis to the extent that the HA epitope has.

To avoid interfering further with integration of the target protein, the foreign epitope should be placed ≥ 15 amino acids from the neighboring transmembrane segment(s) without disrupting membrane targeting signals which are commonly present at the N- and/or C-terminal ends of the polypeptide chain. Another concern is that addition of artificial sequences into a membrane protein may affect its stability or localization to a compartment within the cell. Indeed, retention of mutant proteins in the secretory pathway is well documented (Gething et al., 1986; Kreis and Lodish, 1986). Loss of stability or missorting may be evidenced through failure to find the tagged protein in its normal cellular location.

Reporter gene fusions

Since the mid-1980s, a genetic approach has been used to solve membrane protein topologies (see Basic Protocol 3). The reporter gene fusion method was developed originally to study the topology of plasma membrane proteins in bacterial systems (Manoil and Beckwith, 1986; Froshauer et al., 1988) and then adapted for the study of proteins integrated into the ER membrane in the yeast *Saccharomyces cerevisiae* (Sengstag et al., 1990). More recently, the reporter enzyme β -galactosidase has been used for topological studies in *Caenor-*

habditis elegans (Doan et al., 1996; Lai et al., 1996). Reporter fusion approaches developed for analyses in yeast and *C. elegans* examine membrane proteins integrated into the ER and either residing within the ER or transported to a compartment of the secretory pathway or to the lysosome. Due to conservation of the ER membrane integration machinery in diverse eukaryotic organisms (Hartmann et al., 1994), cross-species analyses of eukaryotic proteins using the simple yeast system are probably valid. Recently, a gene fusion system has been used to study the topology of a protein localized to the peroxisomal membrane (Elgersma et al., 1997), and it is likely that over the next few years the reporter gene fusion method will be extended to other organelles and eukaryotic cell types.

The cytoplasmic enzyme histidinol dehydrogenase (HD) is well suited for use as a reporter of membrane protein topology in yeast cells (Sengstag et al., 1990). HD is encoded by the *HIS4* gene in *Saccharomyces cerevisiae* (Donahue et al., 1982). When expressed on the cytosolic side of the membrane, HD converts histidinol to histidine, an activity that permits yeast that are *his4⁻* to grow on agar plates supplemented with histidinol instead of histidine. On the extracytoplasmic side, however, HD is enzymatically inactive *in vivo*, presumably due to its sequestration from the histidinol substrate, glycosylation of the HD moiety, and/or degradation of HD within the ER lumen (Green and Walter, 1992; Mullins et al., 1995).

To construct gene fusions in pA189invHD, the 800-bp *Bam*HI-*Xho*I fragment can be replaced with a DNA fragment encoding an N-terminal truncation of the target membrane protein. This leads to the production of fusions between the upstream membrane protein fragment and the C-terminal inv-HD sequence. In pA189invHD, the *inv* sequence (an internal fragment of the yeast invertase protein; Taussig and Carlson, 1983) serves as a spacer that separates HD from the membrane-spanning segments of the upstream membrane protein (Green and Walter, 1992).

The main advantage of the reporter gene fusion approach described here is its simplicity. The cell growth assay provides a quick and reliable means of assessing the location of a hydrophilic domain within a membrane protein examined in yeast. Furthermore, a fusion joint can be constructed anywhere along the primary sequence using standard molecular biological protocols. This means that all hydrophilic domains within a multispreading protein, includ-

ing small domains, can, in principle, be examined by constructing a series of gene fusions. However, such fusions eliminate from the construct all sequences in the membrane protein located downstream of the fusion joint. This approach therefore makes the assumption that membrane proteins integrate into the bilayer in a linear fashion, from the N to the C terminus. This idea is in accord with the model that membrane proteins contain alternating signal and stop transfer signals positioned linearly along the primary sequence (Friedlander and Blobel, 1985). While this assumption may be accurate in some instances, the fusion approach cannot identify transmembrane domains that insert into the membrane with the help of C-terminal sequences (Finer-Moore and Stroud, 1984).

In light of current models suggesting that multiple transmembrane domains accumulate within the translocation channel before their entry into the lipid bilayer (Hegde and Lingappa, 1997), caution should be used when interpreting results obtained using only the reporter fusion approach. For this reason, combining the gene fusion approach with one of the other approaches described in this unit will not only yield a more reliable topological picture but may also provide information about the mechanism of integration of the protein examined.

Critical Parameters

Protease digestion

Several factors are important for interpreting membrane protein topology using the protease digestion method. Determining the location of a hydrophilic domain that is at the side of the membrane to which the protease has not been added can be difficult, if the domain in question is small. This is due to the fact that digestion of those portions exposed to the protease may leave a fragment too small to detect using SDS-PAGE. Thus, the failure to detect a protein fragment may be due to a detection problem, not to the susceptibility of the domain to the protease added (see Anticipated Results). On the other hand, a domain exposed to the protease may be resistant or inaccessible if the domain is small. Therefore, it is critical to interpret carefully results regarding the disposition of a small domain. To assign a small hydrophilic domain to the correct side of the membrane, it is best to examine the topology over the entire protein, then determine whether the results obtained are consistent with the deduced assignment of the domain(s) in ques-

tion. One way to enhance the chance that a small domain is susceptible is to use a protease that has a broad substrate specificity, such as proteinase K or trypsin. The use of a control protein or protein domain that faces the luminal side of the membrane system to be probed is essential to the analysis, since the membranes examined must be closed and impermeable to the protease added in the absence of detergent.

Immunofluorescence staining

There are several technical features of the immunofluorescence staining protocol that are critical to a successful outcome. The use of very clean coverslips is necessary. Coverslips should be washed in a strong detergent and rinsed thoroughly to remove all traces of the detergent. The adherence of tissue culture cells to the coverslips can be a problem sometimes. The protocol described here works well for HEK 293 cells, but when other cells are used, different types of coverslips can be tried. For example, Lab-Tek Chamber Slides (Nunc) provide a surface to which many cell types attach well. When using HEK 293 cells, it is observed that cells which are about 70% confluent yield the best staining results. Using the procedures outlined here, it takes about 3 days following seeding on coverslips to achieve this confluency; however, different times may be necessary in different experiments. In addition, these conditions may need to be varied for cell types other than HEK 293 cells.

The most important control is to examine cells lacking the protein of interest. This control detects the background staining that may appear in the cells under study. If the cells containing the protein of interest give a much more intense staining than cells lacking the protein, then the results should be reasonably reliable. The protocol listed here calls for a membrane protein control to be analyzed. This control is very useful, provided that antibodies directed against this control protein are available. If not, then it is essential that at least one hydrophilic domain located on the intracytoplasmic side of the membrane be probed, as such a domain controls for the possible disruption of the membrane prior to addition of the primary antibody.

Epitope tagging

Epitope tagging offers an inexpensive shortcut to analyzing membrane protein topology using either the protease digestion or the immunofluorescence staining protocol. However, there is one issue that is important in achieving the reliability necessary. The results are more

certain to be valid if the epitope does not inhibit function of the target protein. It is therefore important to examine the function of the epitope-tagged protein using an *in vivo* or *in vitro* assay. To improve the chance that the epitope does not alter the topology of a protein (and thus its function), an epitope lacking clusters of charged amino acids, such as the HA epitope, should be used. Furthermore, the epitope should be placed >15 amino acids away from the upstream and/or downstream transmembrane segments, in order to avoid disrupting the content of charged amino acids already existing near the transmembrane segments. If it is not possible to obey this 15 amino acid restriction (because the domain probed is small) then place the epitope as far away as possible from the transmembrane segment(s).

Reporter gene fusions

When membrane protein topology is assessed using an *in vivo* assay (such as the one described here), the reporter gene fusion approach is the quickest way to determine membrane protein topology. However, the fusions constructed usually render the target protein nonfunctional. Topological assignments are therefore best served by using this method to gain only a general picture of a protein's topology and then complementing the data with at least a partial analysis using one of the above-listed approaches. Since a functional assay is not always practical, there are some issues to consider when constructing the fusions in order to minimize the chance that the reporter affects the topology of the protein being probed. The fusion joint should be constructed >15 amino acids away from the upstream transmembrane segment. For small hydrophilic domains, the fusion joint can be placed after the first few amino acids of the downstream transmembrane segment if one exists (see Fig. 5.2.6). Another factor that is important is the amino-terminal sequence of the reporter itself. When the reporter contains clusters of charged amino acids at its amino terminus, the reporter may inhibit the integration of the upstream transmembrane segments. Indeed, the His4p reporter appears to suffer this problem (Green and Walter, 1992). For this reason, the reporter construct described in this protocol contains a spacer sequence consisting of a fragment of invertase (Fig. 5.2.7).

Troubleshooting

Refer to Table 5.2.1 for a troubleshooting guide.

Anticipated Results

A typical result expected from the protease digestion method (see Basic Protocol 1) is shown in Figure 5.2.2A. Note that no apparent fragment is detected from proteolysis of the representative protein SPC12 in the absence of detergent (lane 2). If a fragment had been detected, the result would have suggested that the C terminus localizes to the luminal side of the membrane. However, such a fragment could have been quite small (as few as 20 to 30 amino acids) if the C-terminal hydrophilic domain contained only a few amino acids. A clue as to the expected size of a particular fragment can be obtained by examining a hydropathy plot of the protein. Data similar to those shown in Figure 5.2.2A are expected from the analysis of epitope-tagged proteins (see Support Protocol), except that instead of antipeptide antibodies, antibodies directed against the epitope employed should be used. Results expected from immunofluorescence analyses (see Basic Protocol 2) of epitope-tagged proteins have been reported (Canfield and Levenson, 1993; Canfield et al., 1996). Results expected from reporter fusion analyses (see Basic Protocol 3) in yeast (Sengstag et al., 1990) and *C. elegans* (Doan et al., 1996; Lai et al., 1996) have also been reported.

Time Considerations

The time frames given below are for procedures that work well in the first few attempts. Longer times will, of course, be needed when significant troubleshooting is required.

Protease digestion

Antipeptide antibodies directed against each hydrophilic domain of the target protein can take 3 months or longer to prepare. This time can be spent working out the conditions for preparing enriched membranes containing the protein of interest. If control antibodies are available, they can be used to verify the integrity of the membranes being prepared. It usually takes no more than 2 to 3 days to obtain most membrane preparations. The protease treatments and SDS-PAGE analyses also take 2 to 3 days (see Basic Protocol 1).

Immunofluorescence staining

As with the protease digestion technique, most of the time is spent preparing a series of antipeptide antibodies. This time is lessened if commercially available epitope tags are used (see Support Protocol). When examining cells grown in tissue culture medium (see Basic

Table 5.2.1 Troubleshooting Guide for Problems Encountered in Determining Membrane Protein Topology

Problem	Possible cause	Solution
<i>Protease digestion</i>		
Target protein is not degraded in detergent.	Protein is resistant to protease used.	Try another protease.
	Protein is shielded by other proteins.	Treat membrane with buffer containing 500 mM potassium acetate and add 500 mM potassium acetate to the detergent control. To release ribosomes from ER microsomes, treat with MSB containing 500 mM potassium acetate, 0.2 mM GTP, 1 mM puromycin.
	Protein is part of a protease-resistant complex.	Preincubate membranes 10 min at 40°-60°C. Monitor membrane integrity using a control membrane protein.
The target protein is digested in the detergent but not in intact vesicles.	The protein may lack a cytoplasmic domain or is shielded.	Treat membranes with 500 mM potassium acetate as described above before adding the protease.
Poor proteolysis of target and control proteins is found.	Protease inhibitors or nonfunctional protease is present.	Ensure inhibitors are absent from membrane preparation by washing membranes by high-speed centrifugation (see Basic Protocol 1, step 1). Be sure protease is active by examining proteolyzed and unproteolyzed membranes using SDS-PAGE (UNIT 6.1).
Degradation of a luminal protein control occurs even without addition of detergent.	Too much protease is used.	Titrate protease to lower concentrations than those indicated in Basic Protocol 1.
	The membrane is leaky.	Change the cell fractionation protocol (Chapter 3).
<i>Immunofluorescence staining</i>		
The antibody used does not recognize the target protein in the permeabilized and nonpermeabilized sets.	The antibody used binds the target protein inefficiently in the context of the membrane.	Test whether antibody binds the protein by immunoblotting (UNIT 6.2) or immunoprecipitation (UNIT 7.2) from detergent-solubilized membranes. If the hydrophilic domain being probed is small, try inserting one or more epitopes into the domain (see Support Protocol) and repeat analysis.
	The nonpermeabilized cells were leaky at the time primary antibody was added.	Be sure that detergent was not added to DFH solution. Examine a control protein with known topology.
Hydrophilic domains presumed to be located on opposite sides of the membrane are detected in both the permeabilized and nonpermeabilized sets.	The concentrations of primary and/or secondary antibodies are too high.	Test whether dilution of the antibodies decreases the background signal. Try using a different primary antibody preparation.
Control cells lacking the polypeptide of interest are stained strongly by the secondary antibody.		
<i>Epitope tagging</i>		
The tagged protein is not functional.	The protein is degraded, mislocalized, or out of frame.	Test whether protein is present in its native membrane by immunoblotting (UNIT 6.2) or immunoprecipitation (UNIT 7.2). Sequence the gene segment encoding the epitope and flanking amino acids.

continued

Table 5.2.1 Troubleshooting Guide for Problems Encountered in Determining Membrane Protein Topology, continued

Problem	Possible cause	Solution
	The epitope inhibits the active site or topology of the protein.	Place the epitope at a different site in the protein domain, including sites further away from the transmembrane stretches. Alternatively, proceed with topology assays, but corroborate results using a different method.
The antibody does not recognize an epitope-tagged protein even though the protein is functional.	The epitope is inaccessible.	Introduce multiple epitopes into the site.
Reporter gene fusions		
Fusions to hydrophilic domains on both sides of a putative transmembrane segment possess HD enzymatic activity.	The L-histidinol reagent is contaminated with histidine.	Test whether strain FC2-12B grows on SC +HOL agar plates. If it does, try a different batch of histidinol or a different supplier.
	The hydrophobic segment identified does not actually span the membrane, or the transmembrane segment inserts into the bilayer aided by downstream sequences not contained in the fusion protein.	Reassess whether the hydrophobic segment is a transmembrane segment. Monitor topology of the region in question using a different approach.
None of the fusions possess enzymatic activity.	The fusions are not made.	Sequence fusion joints to ensure reading frame is maintained. Ensure that no ATG codons are present between the promoter and the initiation codon. Use the strong <i>ADHI</i> promoter on pA189invHD.
Fusions expected to be inactive are active.	The fusion joint interferes with integration of the upstream sequences.	Position the joint further away from the upstream transmembrane segment. Corroborate results using a different method.

Protocol 2), it takes about 3 days to grow cells on coverslips before the immunofluorescence analysis. The analysis takes about 1 day, which includes fixing the cells, incubating with primary and secondary antibodies, and examining the cells by microscopy.

Epitope tagging

The time required to perform the molecular biological manipulations required to introduce a foreign epitope into each hydrophilic domain of a membrane protein (see Support Protocol) depends on the number of distinct hydrophilic domains present in the protein and whether single, double, or multiple tags are introduced into each domain. A person skilled in the use of molecular biological techniques could introduce a set of single and double tags into a type I integral membrane protein (Fig. 5.1.2) in <1 month, test the function of the various tagged

proteins in vivo or in vitro in a few weeks, and localize the epitopes with respect to the membrane using protease digestion (see Basic Protocol 1) or immunofluorescence staining (see Basic Protocol 2) in ~1 week using either technique. Thus, in ~2 months, one could tag such a protein and examine its topology. Longer periods (up to 6 months) may be required to examine proteins containing several transmembrane segments.

Reporter gene fusions

Using the described systematic approach for constructing gene fusions (see Basic Protocol 3), a series of constructs can be made in <1 month by a person experienced in using molecular biological techniques. Analyses of the fusions require ~1 week using the yeast system or a few weeks using a higher eukaryotic system.

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Analysis of the Association of Proteins with Membranes

UNIT 5.4

This unit contains several protocols for analyzing the association of proteins with membranes. These include complementary protocols for determining if a given protein is an integral or peripheral membrane protein—i.e., alkaline carbonate extraction (see Basic Protocol 1), urea extraction (see Alternate Protocol 1), high-salt extraction (see Alternate Protocol 2), and Triton X-114 phase separation (see Alternate Protocol 3).

In addition, protocols are included for assessing whether a protein is associated with membranes via a C-terminal glycosylphospholipid anchor, termed glycosyl phosphatidylinositol (GPI). Here, a phosphoinositol (PI)-specific phospholipase is used to enzymatically release the hydrophobic “greasy foot” from this class of membrane proteins (see Basic Protocol 2). Some membrane components are selectively resistant to solubilization by Triton X-100 at 4°C; these proteins can be solubilized using other detergents (see Basic Protocol 3).

Finally, there are two protocols for purifying caveolae-derived plasma membrane domains from cultured cells (see Basic Protocol 4). One uses Triton X-100; the other is a detergent-free method (see Alternate Protocol 4).

The unit also contains protocols for Triton X-114 precondensation (see Support Protocol 1) and preparation of a protease inhibitor stock solution (see Support Protocol 2) to be used in the corresponding basic protocols.

ALKALINE CARBONATE EXTRACTION

This method developed by Fujiki et al. (1982a) uses alkaline pH to disrupt membranes. Under these conditions, membrane vesicles are converted to open membrane sheets that can be recovered with an ultracentrifugation step. The soluble contents of the vesicles are released into the supernatant fraction. Proteins peripherally associated with the membrane are also found in the supernatant. Only integral membrane proteins remain associated with the membrane under those conditions. This method can be used on membrane proteins from all intracellular membranes. Examples include but are not limited to membranes isolated from the endoplasmic reticulum (Fujiki et al., 1982a,b), peroxisomes (Fujiki et al., 1982a,b), mitochondria (Fujiki et al., 1982a,b; Scherer et al., 1992), Golgi (Scherer et al., 1996) and plasma membrane (James et al., 1989).

The identical procedure can be performed with cells radiolabeled as described in *UNIT 7.1*. If an immunoprecipitation of the respective fraction is desired rather than a direct application to SDS-PAGE, the samples should be treated as described in steps 1 to 4; however, instead of adding an equal volume of 2× SDS-PAGE sample buffer in step 5a, the samples should be treated with nonionic detergents as in steps 5b and 6b (also see *UNIT 7.2*)

Materials

- Cells of interest
- Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice-cold
- 100 mM NaCl
- 100 mM sodium carbonate, pH 11.5, ice cold
- 2× SDS-PAGE sample buffer (*APPENDIX 2A*)
- Triton X-100

**BASIC
PROTOCOL 1**

**Characterization
of Cellular
Proteins**

5.4.1

N-octyl glucoside
Tris·Cl, pH 7.5 (APPENDIX 2A)
NaCl

2-ml Dounce homogenizer
Beckman TL-100 centrifuge with TLA 100.2 rotor
26-G needles and 1-ml syringes
95°C water bath

Additional reagents and equipment for SDS-PAGE (UNIT 6.1), immunoblotting (UNIT 6.2), and immunoprecipitation (UNIT 7.2)

- 1a. *For adherent cells:* Wash $\sim 1 \times 10^7$ cells three times with ice-cold 20 ml PBS, then once with 100 mM NaCl.
- 1b. *For nonadherent cells:* Wash $\sim 1 \times 10^7$ cells as in step 1a, but isolate cells between washes by gentle microcentrifugation (2 min at a maximum of $1000 \times g$, 4°C).
2. Scrape (adherent) or resuspend (nonadherent) cells into 1 ml of ice-cold 100 mM sodium carbonate, pH 11.5, and homogenize with five strokes in a 2-ml Dounce homogenizer.
3. Incubate homogenate 30 min at 4°C, then centrifuge 60 min at 150,000 to 200,000 $\times g$ (100,000 rpm in a TLA 100.2 rotor), 4°C.
4. Transfer the supernatant to a fresh tube and resuspend pellet in 1.0 ml of 100 mM sodium carbonate, pH 11.5.

To facilitate the resuspension of the pellet, the pellet fraction can be homogenized in the Dounce homogenizer.

For SDS-PAGE and/or immunoblotting

- 5a. Immediately mix pellet and supernatant fractions with an equal volume of 2 \times SDS-PAGE sample buffer. Shear genomic DNA in pellet sample by passing through a 26-G needle several times.

If necessary, samples can be neutralized by adding 5 to 10 μ l of 1 M Tris·Cl, pH 7.0. The shearing will decrease the sample's viscosity.

- 6a. Heat samples at 95°C for 5 min.

The samples can now be used directly for SDS-PAGE (UNIT 6.1) and immunoblot analysis (UNIT 6.2). Load equal volumes of both supernatant and pellet fraction. Alternatively, samples can be stored frozen at -20° or -80°C for weeks.

For immunoprecipitation

- 5b. Add Triton X-100 to a final concentration of 1% (w/v), *N*-octyl glucoside to a final concentration of 60 mM, Tris·Cl, pH 7.5, to a final concentration of 50 mM, and NaCl to a final concentration of 300 mM.

The pH of the final solution should not exceed 8.0. If necessary, add more Tris·Cl, pH 7.5, to lower the pH.

- 6b. Incubate sample on ice for 5 min, then microcentrifuge 10 min at 15,000 $\times g$, 4°C, to remove insoluble debris. Immunoprecipitate using a standard reaction (UNIT 7.2).

UREA EXTRACTION

This procedure is identical to the alkaline carbonate extraction (see Basic Protocol 1), except that 100 mM sodium carbonate, pH 11.5, is replaced with a solution containing 2 M urea and 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5. Moderately high concentrations of urea (2 M) are commonly used to extract peripheral and soluble proteins from vesicles (Schook et al., 1979). Urea diminishes the hydrophobic interactions and thereby removes these proteins from the membrane. It is important to note that cyanate is formed spontaneously from urea. The cyanate ion can react with both amino and sulfhydryl groups of amino acids causing such problems as irreversible inactivation of enzymes and altered retention times on reversed-phase HPLC after Edman degradation of proteins. Therefore, solutions should be prepared fresh from high-quality urea, or the solution should be deionized prior to use. Store urea-containing solutions up to 1 week at 4°C.

HIGH-SALT EXTRACTION

At high concentrations, salt exerts effects on proteins that depend both on the concentration and the nature of the salt. In this context, NaCl and KCl are primarily used to disrupt polar interactions between peripheral membrane proteins and integral membrane proteins. Once again this procedure is identical to the alkaline carbonate extraction (see Basic Protocol 1), except in this case 100 mM sodium carbonate is replaced with a solution containing 10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)/0.5 M NaCl. KCl may be used as an alternative to NaCl. Salt solutions can be stored at room temperature, but must be chilled well prior to use.

TRITON X-114 PHASE SEPARATION

A solution of the nonionic detergent Triton X-114 is homogeneous at 0°C but separates into an aqueous phase and a detergent phase above 20°C. The extent of this detergent-phase separation increases with temperature. When membrane fractions are solubilized with a Triton X-114 solution on ice and then placed at 37°C, hydrophilic proteins are found in the aqueous phase, whereas integral membrane proteins are recovered in the detergent phase. Integral membrane proteins can thus be separated from soluble and peripheral proteins.

Materials

- Cells of interest
- Cell lysis buffer (see recipe)
- Tris/NaCl/EDTA buffer (see recipe)
- TNET-OG buffer (see recipe)
- 2× SDS-PAGE sample buffer (*APPENDIX 2A*)
- Additional reagents and equipment for immunoprecipitation (*UNIT 7.2*), SDS-PAGE (*UNIT 6.1*), and immunoblotting (*UNIT 6.2*)

1. Add 1 ml ice-cold cell lysis buffer to 2–5 × 10⁶ cells and lyse cells by incubating 45 to 60 min at 4°C.
2. Transfer solution to a microcentrifuge tube and microcentrifuge 15 min at 10,000 × *g*, 4°C.
3. Transfer supernatant to a fresh microcentrifuge tube and incubate at 37°C for 3 min.
4. Centrifuge solution 1 min at 10,000 × *g*, room temperature. Transfer the upper (aqueous) phase, which should represent ~95% of the total volume, to a fresh microcentrifuge tube. Retain the detergent phase for step 6.

**ALTERNATE
PROTOCOL 1**

**ALTERNATE
PROTOCOL 2**

**ALTERNATE
PROTOCOL 3**

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5.4.3

5. Re-extract the aqueous phase from step 4 by adding 100 μ l cell lysis buffer and repeating steps 3 and 4. Discard the lower detergent phase from this step.
6. Re-extract the detergent phase from step 4 by adding 10 vol Tris/NaCl/EDTA buffer. Repeat steps 3 and 4 then discard the upper, aqueous phase.
- 7a. *For aqueous phase:* Use the aqueous phase (from step 5) directly for immunoprecipitation reactions (UNIT 7.2).
- 7b. *For detergent phase:* Dilute the detergent phase (from step 6) to 1 ml with TNET-OG buffer and use for immunoprecipitation (UNIT 7.2).
- 7c. *For direct analysis of the extracted samples by SDS-PAGE:* Mix 5% of both aqueous (approx. 50 μ l) and detergent phases (2.5 μ l) with 50 μ l 2 \times SDS-PAGE sample buffer.

If not to be analyzed immediately, samples can be stored at -20° or -80° C for 1 to 2 weeks.

**SUPPORT
PROTOCOL 1**

TRITON X-114 PRECONDENSATION

The purpose of this support protocol is to prepare a precondensed stock solution of Triton X-114 (Bordier, 1981). Precondensation is required to remove certain impurities that may interfere with the phase separation properties of Triton X-114.

Materials

Triton X-114
 Tris-buffered saline (TBS): 10 mM Tris-Cl, pH 7.4 (APPENDIX 2A)/150 mM NaCl
 Sodium dodecyl sulfate (SDS)
 Triton X-100

1. Add 20 g Triton X-114 to 980 ml Tris-buffered saline.
2. Allow to dissolve at 4 $^{\circ}$ C for ~1 hr with stirring or until solution is clear, then incubate at 30 $^{\circ}$ C overnight with stirring.
3. Discard the large aqueous phase (upper phase) and replace with fresh Tris-buffered saline. Once again dissolve at 4 $^{\circ}$ C and incubate overnight at 30 $^{\circ}$ C.
4. Repeat step 3 two more times.

The last detergent phase (lower phase) is the stock solution to use for experiments.

5. *Optional:* Determine the concentration of the last detergent phase by measuring the absorbance of a 1:1000 dilution in the presence of 1% SDS at 275 nm and compare to the absorbance of a set of standard solutions of Triton X-100 at 275 nm to determine the final concentration.

The extinction coefficient of Triton X-100 is virtually identical to that of Triton X-114.

There is actually no real need to determine the exact concentration of the final stock as it varies between 9.5% and 10.5% Triton X-114 and can simply be used at a dilution of 1:10. See recipe for cell lysis buffer in Reagents and Solutions.

6. Store the final stock solution up to 1 week at 4 $^{\circ}$ C.

PREPARATION OF 100× PROTEASE INHIBITORS STOCK SOLUTION

Protease inhibitors are used to prevent proteolysis during extraction. This stock mixture inhibits most common proteases (also see *APPENDIX 1B*).

Materials

Antipain
Pepstatin A
Leupeptin
Dimethylsulfoxide (DMSO)

1. Make separate 1000× solutions of antipain, pepstatin A, and leupeptin by dissolving 5 mg of each in 500 μ l DMSO.
2. Combine 50 μ l of each 1000× solution and add 350 μ l DMSO.
3. Divide this combined 100× stock solution into small aliquots (i.e., 100 μ l) and store at -20°C .
4. Use 10 μ l/ml for a final concentration of 10 $\mu\text{g/ml}$ of each protease inhibitor. Do not exceed 10 μ l/ml (final DMSO concentration should be $<1\%$).

PI-PLC CLEAVAGE OF GPI-LINKED PROTEINS

This procedure is used to detect glycosyl phosphatidylinositol (GPI)-linked proteins by converting them from a hydrophobic to a hydrophilic state. The procedure consists of the enzymatic removal of the GPI anchor using phosphatidylinositol-specific phospholipase C (PI-PLC). The removal of the GPI anchor causes the protein to shift from the detergent-rich phase to the detergent-poor phase of Triton X-114 extracts.

Materials

Cells of interest
Tris/NaCl/EDTA buffer (see recipe)
PI-PLC incubation buffer (see recipe)
1000 U/ml PI-PLC (phosphatidylinositol-specific phospholipase C; Boehringer Mannheim)
Phenyl-Sepharose 4B (Pharmacia Biotech)
Tris-buffered saline (TBS): 20 mM Tris-Cl, pH 8.0 (*APPENDIX 2A*)/150 mM NaCl
12.5 mg/ml (100×) sodium deoxycholate
Trichloroacetic acid
2× SDS-PAGE sample buffer (*APPENDIX 2A*)
1 M Tris-Cl, pH 8.0 (*APPENDIX 2A*)
1% (w/v) SDS/200 mM Tris-Cl, pH 8.0 (see *APPENDIX 2A* for Tris-Cl)
TNET buffer (see recipe)

Eppendorf vortex mixer
Boiling water bath

Additional reagents and equipment for Triton X-114 phase separation (see Alternate Protocol 3), SDS-PAGE (*UNIT 6.1*), and immunoprecipitation (*UNIT 7.2*).

1. Using $\sim 1 \times 10^7$ cells, follow the Triton X-114 phase separation protocol (see Alternate Protocol 3) with the following variations:
 - a. In step 3 of Alternate Protocol 3, incubate samples at 37°C for 20 min instead of 3 min.
 - b. In step 6 of Alternate Protocol 3, re-extract samples twice against fresh Tris/NaCl/EDTA buffer, keeping the detergent phases.

2. Dilute the detergent-rich phases 10-fold with PI-PLC incubation buffer and add PI-PLC to a concentration of 4 to 8 U/ml.
3. Mix continuously on a vortex mixer at 37°C for 1 hr.
4. Repartition samples by microcentrifuging 1 min at 10,000 × g, room temperature. Collect the upper (aqueous) phases.
5. Prepare a 50% slurry of phenyl-Sepharose 4B in Tris-buffered saline. Add 200 μl of the slurry per ml aqueous phase and incubate samples overnight at 4°C to remove trace amounts of hydrophobic membrane proteins.
6. Microcentrifuge samples 10 sec at ≤2000 × g, 4°C, and transfer supernatants to fresh microcentrifuge tubes. Repeat to clear all beads.
7. Precipitate by adding sodium deoxycholate (as 100× stock) to 125 μg/ml and trichloroacetic acid to 6% (w/v). Incubate on ice for 10 min.
8. Microcentrifuge samples 4 min at 10,000 × g, 4°C, and remove supernatant.
- 9a. *For SDS-PAGE:* Resuspend each pellet in an equal volume of 2× SDS-PAGE sample buffer and neutralize by adding small amounts (~5 μl) of 1 M Tris·Cl, pH 8.0, and use for SDS-PAGE (UNIT 6.1).
- 9b. *For immunoprecipitation:* Resuspend each pellet in 100 μl of 1% (w/v) SDS/200 mM Tris·Cl pH 8.0. Boil 5 min, then dilute samples in 10 vol TNET buffer. Proceed with immunoprecipitation (UNIT 7.2).

**BASIC
PROTOCOL 3**

**DETERGENT SOLUBILIZATION OF TRITON X-100 INSOLUBLE
INTEGRAL MEMBRANE AND GPI-LINKED PROTEINS**

Several caveolar membrane components are selectively resistant to solubilization by Triton X-100 at 4°C, which is the most commonly used protocol for the solubilization of integral membrane proteins. The unusual Triton insolubility of these caveolar domains has been attributed to their high glycosphingolipid content, as glycosphingolipids are intrinsically Triton-insoluble. They can, however, be solubilized in other detergents, such as octylglucoside or CHAPS. Solubilization can also be performed using SDS under experimental conditions that permit denaturation of protein or protein complexes.

Materials

Cells of interest

Phosphate-buffered saline (PBS; APPENDIX 2A)

Detergent solution—one of the following:

60 mM *N*-octylglucoside in either TBS (20mM Tris·Cl, pH 8.0/150 mM NaCl)

or MES-buffered saline (25 mM MES, pH 6.5/150 mM NaCl)

30 mM CHAPS in either TBS (20mM Tris·Cl, pH 8.0/150 mM NaCl) or

MES-buffered saline (25 mM MES, pH 6.5/150 mM NaCl)

Wash buffer containing Triton X-100 (UNIT 7.2)

Additional reagents and equipment for immunoprecipitation (UNIT 7.2)

1. Wash 1×10^6 to 1×10^7 cells twice in PBS (see Basic Protocol 1, steps 1a and 1b).
2. Add 1 ml of 60 mM *N*-octylglucoside or 30 mM CHAPS in TBS or MES-buffered saline. Vortex, then incubate at 4°C for 30 min to 1 hr.

60 mM N-octylglucoside is most frequently used for solubilizing caveolae because it is the most efficient detergent for this purpose.
3. Centrifuge 10 min at 15,000 × g, 4°C, to remove insoluble debris.
4. Use the supernatant for a standard immunoprecipitation reaction (UNIT 7.2).

5. Wash immunoprecipitates with Triton X-100-containing wash buffer as described in *UNIT 7.2*.

This alternative wash buffer is used to minimize the amount of N-octylglucoside needed, as that detergent is rather expensive. After washing, the packed beads can be stored frozen for weeks.

TRITON-BASED PURIFICATION OF CAVEOLAE-DERIVED MEMBRANES

Caveolae are vesicular organelles attached to the plasma membrane that have a characteristic lipid and protein composition (Sargiacomo et al., 1993; Lisanti et al., 1994). Caveolae membranes are highly enriched in glycosphingolipids and cholesterol, making them extremely light and therefore buoyant in sucrose density gradients. This unique lipid composition also confers resistance to solubilization by nonionic detergents such as Triton X-100 and NP-40 at low temperatures. For example, when intact cells were fixed in paraformaldehyde, extracted with Triton X-100, and then examined by electron microscopy, the insoluble membranes that remained were found to be caveolae (Moldovan et al., 1995). These special properties allow the isolation of caveolae from other cell organelles as described below and in Alternate Protocol 4.

Materials

MDCK (Madin-Darby canine kidney) cells (or virtually any other nontransformed cell line)

DMEM medium containing 5% FBS (*UNIT 1.2*) with 100 IU/ml penicillin and 50 µg/ml streptomycin

Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice-cold

Lysis buffer for caveolae (see recipe)

5%, 30%, and 80% sucrose solutions (see recipe)

MES-buffered saline: 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)/150 mM NaCl

75-cm³ tissue culture flasks

150-mm tissue culture dishes

Cell scrapers

15-ml tubes

Dounce homogenizer

Beckman L8 ultracentrifuge with SW 41 rotor and corresponding ultraclear centrifuge tubes

Additional reagents and equipment for cell culture and trypsinization of cells (*UNIT 1.1*), preparation of serum-containing tissue culture medium (*UNIT 1.2*), and preparation of sucrose gradients (*UNIT 5.3*)

1. Split MDCK cells by trypsinization (*UNIT 1.1*) from one 75-cm² flask into three 75-cm² flasks, and grow to confluence in DMEM/5% FBS. Split cells from the flasks into six 150-mm dishes. Let cells grow for at least 2 days (to confluence) before harvesting.

Omit any antifungal agents such as Nystatin or Fungizone, as these are cholesterol-binding antibiotics that will disrupt caveolae architecture.

2. Rinse the cells in each 150-mm dish three times, each time with 20 ml ice-cold PBS. Thoroughly remove PBS and add 1 ml of ice-cold lysis buffer for caveolae. Collect cells with a cell scraper and place in a 15-ml tube on ice. Rinse dish with an additional 1 ml of lysis buffer for caveolae to collect remaining cells and pool with the first 1 ml in the tube.

Use each plate for a separate gradient.

3. Homogenize with 8 to 10 strokes of a Dounce homogenizer. Adjust to 40% (w/v) sucrose by adding an equal volume (2 ml) of ice-cold 80% sucrose. Vortex briefly until homogeneous.
4. Place homogenate at the bottom of an ultraclear Beckman ultracentrifuge tube and form a linear gradient atop it, using 4 ml each of 5% sucrose and 30% sucrose, at a flow rate of not more than 0.5 ml per min (UNIT 5.3). Adjust the weight of each gradient to within 0.1 g.
5. Centrifuge 12 to 16 hr (overnight) at $200,000 \times g$ (39,000 rpm in an SW 41 rotor), 4°C.
6. Collect twelve 1-ml fractions from the top (UNIT 5.3). Snap freeze in liquid nitrogen and store frozen at -80°C.

Fraction 13 is the insoluble pellet.

Subsequent analysis of the total fractions can be done without pelleting the membranes.

7. *Alternatively:* Harvest the opaque band (migrating at ~10% to 20% w/v sucrose), dilute with MES-buffered saline, and microcentrifuge 60 min at $15,000 \times g$, 4°C. Snap freeze the pellet in liquid nitrogen and store at -80°C.

A 150-mm dish of MDCK cells representing 9 to 11 mg protein yields ~4 to 6 μg of caveolae-enriched membrane domains—i.e., ~0.05 % of the initial homogenate. Triton X-100 extraction solubilizes ~85% of the protein (8.5 mg), the majority of which remains in the 40% (w/v) sucrose layer, while ~1.5 mg forms an insoluble pellet below the 40% sucrose.

Organelle-specific membrane marker assays can be performed (Sargiacomo et al., 1993; Lisanti et al., 1994). These assay systems are not affected by the presence or absence of 1% Triton X-100 in the initial homogenate.

The purified caveolar fractions can be combined.

8. Store frozen at -80°C for months, until further use in immunoblotting or immunoprecipitation.

ALTERNATE PROTOCOL 4

DETERGENT-FREE PURIFICATION OF CAVEOLAE-DERIVED MEMBRANES

Recently, it has been suggested that the inclusion of detergent in the initial homogenization step results in the loss of resident prenylated caveolin-associated proteins, such as G_{βγ} subunits. To preserve these interactions, a detergent-free method for the purification of caveolin-rich membrane domains has been developed. This slightly modified scheme replaces the detergent Triton X-100 with sodium carbonate. Sodium carbonate extraction is routinely used to determine if proteins are firmly attached to membranes and caveolin is not solubilized by sodium carbonate. Using this modified scheme, endogenous caveolin and recombinant caveolin have been shown to be recovered almost quantitatively in fractions 5 and 6 while excluding most cellular proteins. In addition, caveolin has been shown to be separated from the GPI-linked plasma membrane marker, carbonic anhydrase IV. This is consistent with recent observations that GPI-linked proteins are not concentrated directly within caveolae but may reside in close proximity to the “neck regions” of caveolae within intact cells. This method is adapted from Song et al. (1996).

Materials

MDCK (Madin-Darby canine kidney) cells or any other cell
 Phosphate-buffered saline (PBS; APPENDIX 2A), ice cold
 500 mM sodium carbonate, pH 11, ice cold

MES-buffered saline: 25 mM MES, pH 6.5/0.15 M NaCl
MES-buffered saline plus 1% (w/v) Triton X-100 and PMSF
5%, 35%, and 90% (w/v) sucrose (see recipe)
3× SDS-PAGE sample buffer (see recipe)

Cell scrapers

Polytron tissue grinder (Kinematica GmbH, Brinkmann Instruments)

Branson Sonifier 250 (Branson Ultrasonic)

Beckman ultracentrifuge with SW 41 rotor and corresponding (14 × 89–mm)
ultraclear tubes

Additional reagents and equipment for immunoprecipitation (UNIT 7.2) and
SDS-PAGE (UNIT 6.1)

1. Culture cells to produce six 150-mm plates at confluence (see Basic Protocol 4, step 1). Wash cells three times with ice-cold PBS (see Basic Protocol 4, step 2).
2. Add 1 ml ice-cold 500 mM sodium carbonate, pH 11.0 to one plate of cells, scrape the cells into the liquid and transfer to the second plate. Continue in this manner for all six plates. Repeat with a second 1-ml aliquot of sodium carbonate and combine.
3. Place cells on ice. Disrupt the cells using a Polytron tissue grinder at maximum setting for 30 sec.
4. Sonicate cells constantly for 30 sec at setting 2 on the Branson Sonifier 250. Next, sonicate cells with 30-sec bursts at a setting of 4 or 5. Look at the lysate. If it is relatively clear and free of clumps, then it is ready to use. If not, sonicate an additional 30 sec at setting 2.
5. Add the 2 ml of cell lysate to 2 ml of 90% (w/v) sucrose in a 14 × 89–mm Beckman centrifuge ultraclear tube. Vortex until homogeneous.

The volume of tube is a little more than 12 ml.

After mixing with the cell lysate the final concentration of sucrose is 45% (w/v).

6. Atop the 45% layer, add 4 ml of 35% sucrose, then 4 ml of 5% sucrose, to generate a discontinuous sucrose gradient.

Be careful when adding successive layers so as to not disrupt the layer beneath.

7. Centrifuge 12 to 16 hr (overnight) at 200,000 × g (39,000 rpm in an SW 41 rotor), 4°C.
8. Collect twelve 1-ml fractions from the top (UNIT 5.3). Snap freeze all fractions in liquid nitrogen and store frozen at –80°C.

Fraction 13 is the insoluble pellet.

A light-scattering band confined to the 5%/35% sucrose interface contains caveolin, a protein concentrated in caveolae, but excludes most other cellular proteins. Analyze total fractions (do not pellet).

The purified caveolar fractions can be stored frozen at –80°C for months until further use in immunoblotting (UNIT 6.2) or immunoprecipitation (UNIT 7.2).

9. Mix with an equal volume of 3× SDS-PAGE sample buffer that contains 100 mM NaOH to disrupt SDS-resistant caveolin complexes. Analyze by SDS-PAGE (UNIT 6.1).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Cell lysis buffer

1% (w/v) Triton X-114 (from precondensed stock; see Support Protocol 1)
10 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
150 mM NaCl
1 mM EDTA
Store up to 1 month at 4°C
10 µl/ml 100× protease inhibitor stock solution (see Support Protocol 2; final concentration 10 µg/ml each of antipain, pepstatin, and leupeptin), added immediately before use

Lysis buffer for caveolae

1% (w/v) Triton X-100
25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)
150 mM NaCl
Store up to 1 month at 4°C
Add 100 µl of saturated ice-cold ethanol solution of PMSF per 10 ml of buffer immediately before use.

PI-PLC incubation buffer

100 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
50 mM NaCl
1 mM EDTA
0.5 M methyl- α -D-mannopyranoside
Prepare fresh

SDS sample buffer with NaOH, 3×

150 mM Tris·Cl, pH 6.8
300 mM dithiothreitol
6% (w/v) SDS
0.3% (w/v) bromophenol blue
30% (w/v) glycerol
30 mM NaOH
Add 1 M NaOH to a final concentration of 100 mM just before use
Store up to several months at -20°C

Sucrose solutions for Basic Protocol 4 (80%, 30%, and 5% w/v)

80% (w/v): Prepare an 80% (w/v) solution of sucrose in MES-buffered saline [25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5/150 mM NaCl, without PMSF or Triton X-100].

30% (w/v): Dilute 37.5 ml of 80% (w/v) sucrose to 100 ml with MES-buffered saline.

5% (w/v): Dilute 6.25 ml of 80% (w/v) sucrose to 100 ml with MES-buffered saline.

Prepare fresh each time

Sucrose solutions for Alternate Protocol 4 (90%, 35%, and 5% w/v)

90% (w/v): Prepare 90% (w/v) sucrose in MES-buffered saline (final concentration of MES is 10 mM, pH 6.5, and that of NaCl is 150 mM; the rest of the volume is water). Since 90% (w/v) sucrose is hard to dissolve, it is generally heated. First, heat a beaker of water until it boils. Next, place the 90% sucrose mixture, contained in a smaller uncapped beaker/bottle, in the boiling water. Stir the sucrose until it completely dissolves. Store 90% (w/v) sucrose at room temperature for up to one

year (it crystallizes at colder temperatures). Prechill only the amount to be used, just before use.

35% (w/v): Dilute 90% (w/v) sucrose to 35% (w/v) in MES-buffered saline containing 250 mM sodium carbonate, pH 11.0.

5% (w/v): Dilute 90% (w/v) sucrose to 5% (w/v) in MES-buffered saline containing 250 mM sodium carbonate, pH 11.0.

Store the diluted sucrose solutions up to 6 months at 4°C.

A stock solution of 500 mM sodium carbonate is made by simply dissolving sodium carbonate in water. The pH of the solution is about 11. The pH does not need to be adjusted. This solution can be stored at 4°C up to one year.

TNET buffer

1% (w/v) Triton X-100

150 mM NaCl

5 mM EDTA

25 mM Tris·Cl, pH 8.0 (APPENDIX 2A)

Store up to 1 month at 4°C

10 µl/ml 100× protease inhibitor stock solution (see Support Protocol 2), added immediately before use

TNET-OG buffer

1% (w/v) Triton X-100

150 mM NaCl

5 mM EDTA

25 mM Tris·Cl, pH 8.0 (APPENDIX 2A)

60 mM *N*-octylglucoside

Store up to 1 month at 4°C

10 µl/ml 100× protease inhibitor stock solution (see Support Protocol 2), added immediately before use

Tris/NaCl/EDTA buffer

10 mM Tris·Cl, pH 7.4 (APPENDIX 2A)

150 mM NaCl

1 mM EDTA

Store up to 1 month at 4°C

10 µl/ml 100× protease inhibitor stock solution (see Support Protocol 2), added immediately before use

COMMENTARY

Background information

From a practical point of view, it is essential to isolate membranes that preserve their *in vivo* physical properties in order to identify proteins associated with specific membrane domains. The membrane isolation method that uses high pH sodium carbonate buffer efficiently removes peripherally associated proteins (>90%), releases <10% of the integral membrane proteins in caveolae, and preserves the original membrane properties. Thus this method is useful for preparing membranes for further isolation of specific domains. Integral proteins are also not removed using high salt buffer (1 M or 3 M

NaCl or 0.6 M KCl) or urea, treatments which efficiently remove peripheral proteins. Extraction with urea and high salt are alternative methods for isolating membranes to identify integral membrane components.

Caveolae

Caveolae are vesicular organelles attached to the plasma membrane, with a diameter of ~50 to 100 nm. The principal protein components of caveolae are members of the caveolin family of proteins, termed caveolins 1, 2, and 3. Caveolins 1 and 2 are most abundantly expressed in adipocytes, endothelial cells, and fibroblastic cell types, while the expression of

caveolin 3 is muscle-specific. Caveolins interact directly with a number of caveolae-associated signaling molecules, such as H-Ras, heterotrimeric G-proteins, epidermal growth factor receptor, protein kinase C, Src-family tyrosine kinases, and nitric oxide synthase (NOS) isoforms.

In addition to specific proteins, caveolae are enriched in certain lipids such as glycosphingolipids and cholesterol. Using liposomal systems, it has been well established that lipid microdomains consisting of glycosphingolipids and cholesterol can be formed when a certain ratio of glycosphingolipids, cholesterol, and phospholipids are mixed, even in the absence of protein components. It has been proposed that glycosphingolipids can be tightly packed with cholesterol because of their saturated fatty acid composition. These lipids are exclusively localized in the outer leaflet of the lipid bilayer. Phospholipids with saturated fatty acids and cholesterol constitute the inner leaflet of the microdomains. Caveolin associates with these microdomains by direct interaction with cholesterol and glycosphingolipids.

Caveolae purification

Several methods have been developed for purifying caveolae (Chang et al., 1994; Lisanti et al., 1994; Sargiacomo et al., 1993; Schnitzer et al., 1995a,b; Smart et al., 1995; Song et al., 1996). Initially, these methods took advantage of the natural detergent-insolubility of caveolae membranes. More recently, detergent-free methods have become available that are dependent on the intrinsic light buoyant density of caveolae membranes (Smart et al., 1995; Song et al., 1996). Purification of caveolae by all of these methods reveals the dramatic enrichment of caveolins and signaling molecules relative to plasma membrane. Signaling molecules appear to form a tight complex with caveolin 1.

Caveolae have also been shown to be detergent-resistant structures *in vivo*. For example, when intact cells were fixed with paraformaldehyde, extracted with cold Triton X-100, and then examined by electron microscopy, the insoluble membranes that remained were found to be caveolae (Moldovan et al., 1995). In support of these morphological findings, caveolins are Triton X-100-insoluble in caveolae (Sargiacomo et al., 1993; Lisanti et al., 1994), but Triton X-100 soluble in the Golgi (Smart et al., 1994). This compartment-specific Triton X-100 insolubility is most likely due to the association of caveolin with cholesterol and sphingolipids, which form a Triton X-100-in-

soluble microenvironment, termed a liquid-ordered phase.

Biochemical isolation of caveolae membrane domains takes advantage of the unique temperature dependence of solubilization with Triton X-100. Caveolae microdomains are solubilized by Triton X-100 at temperatures $>8^{\circ}\text{C}$, but not at 4°C . Therefore, caveolae microdomains can be isolated biochemically from cultured cells or tissues as insoluble material after solubilizing the cells with Triton X-100 at 4°C . Since caveolae membrane domains are light because of their lipid composition, caveolae microdomains are purified more than 2000-fold with a single sucrose gradient ultracentrifugation. Caveolae microdomains are recovered as a fine dense band at the border of 5% (w/v) and 35% (w/v) sucrose after ultracentrifugation at $200,000 \times g$ overnight. Caveolin-1 is used as a marker protein for caveolae organelles. The amount of caveolae protein isolated by this procedure is typically $5 \mu\text{g}$ out of 10 mg starting material (equivalent to six 150-mm culture dishes). Caveolae structure is still preserved in caveolae isolated by this procedure. As octylglucoside possesses a similar structure to glycosphingolipids, this nonionic detergent is used for solubilization of caveolae microdomains. Although urea, as well as high salt, is frequently used for solubilization of membrane-bound proteins, these methodologies do not solubilize caveolae microdomains.

Although the use of Triton X-100 for caveolae isolation is straightforward and well established, not all caveolae protein components are recovered in the caveolae fraction by this procedure. For example, the H-Ras protein was initially proposed not to be a caveolar component. Triton X-100 is able to dissociate H-Ras and other prenylated proteins from caveolae microdomains. The same is true for $\text{G}\beta\gamma$ subunits. The sodium carbonate method has been developed to alleviate this problem. Because of its high pH, sodium carbonate solubilizes proteins peripherally associated with the membrane. In contrast, integral membrane proteins are not solubilized by this method. This method also allows purification of caveolae microdomains by more than 2000-fold. Caveolin-1 is again recovered within caveolae microdomains. The purity of caveolae microdomains obtained with this methodology is therefore comparable to that with the Triton X-100 method. The caveolae purification approach with sodium carbonate is termed the “detergent-free caveolae purification method.” With this method, the H-Ras protein has been reproduc-

ibly recovered in caveolae microdomains. As caveolae residents are more broadly purified with detergent-free approaches, the authors recommend the detergent-free method as the first methodology to be employed for caveolae purification. Another potential problem in using Triton X-100 for caveolae isolation is a possible contamination of artificial lipid membrane domains for which lipid composition is similar to that of caveolae. Solubilized lipids released from membranes treated with Triton X-100 may form artificial lipid domains like caveolae, since glycosphingolipids have a high affinity for association with cholesterol. The detergent-free method has an advantage over the Triton X-100 method in this regard. The authors recommend using both of these methodologies to localize the protein of one's interest in caveolae.

Caveolae-related domains or pre-caveolae or both?

A number of investigators have purified "caveolae" from cells and tissues that lack apparent expression of caveolin (Kubler et al., 1996; Parolini et al., 1996; Brown and London, 1997; Simons and Ikonen, 1997). These domains have been termed detergent-insoluble glycolipid membrane complexes (DIGs), Triton-insoluble complexes (TICs), detergent-resistant membranes (DRMs), and low-density membranes (LDMs). The authors suggest that the term caveolae-related domains (CRDs) be used to describe these structures. Like caveolae, these microdomains are dramatically enriched in cholesterol, sphingolipids, and lipid-modified signaling molecules (Brown and London, 1997; Simons and Ikonen, 1997). The existence of "caveolae-related domains" or CRDs that do not contain caveolin has caused considerable confusion (Simons and Ikonen, 1997). However, this was at a time when only one caveolin gene was known to exist, i.e., caveolin (now termed caveolin-1). It is now known that there are other caveolin genes, such as caveolin-2 and -3. In addition, it has been shown that caveolin-1 and caveolae are down-regulated in response to cell transformation, while caveolin-2 levels remain constant (Scherer et al., 1997). As a consequence, many commonly used cell lines lack caveolin-1 protein expression and visible caveolae, because they are immortalized or transformed. Also, other detergent-insoluble membrane proteins have recently been cloned, and one or more of them may represent functional homologs of the caveolins (Bickel et al., 1997; Puertollano et al., 1997; Simons and Ikonen, 1997).

CRDs can also be produced *in vitro* simply by mixing cholesterol, sphingolipids, and phospholipids in the appropriate ratio (Brown and London, 1997). Their Triton insolubility is a physical property of their molecular organization that produces a liquid-ordered membrane domain (rather than fluid or liquid-crystalline). Caveolin-1 is found associated with glycosphingolipids *in vivo* as shown using chemical cross-linking with a radiolabeled glycosphingolipid GM1 (Fra et al., 1995a). It binds cholesterol directly (Murata et al., 1995) and requires a high local concentration of cholesterol (>30%) to insert into model lipid membranes (Murata et al., 1995; Li et al., 1996a). These findings suggest that a true functional relationship exists between CRDs and mature caveolae. For example, during the biogenesis of mature caveolae, CRDs would need to exist as precursors for proper insertion of caveolins into membranes. Thus, in cells that express caveolins, these CRDs may represent "pre-caveolae" that simply lack caveolins. In support of this reductionist model, recombinant expression of caveolin-1 in cells that lack morphologically detectable caveolae is sufficient to drive the formation of mature invaginated caveolae (Fra et al., 1995b; Li et al., 1996b; Engelman et al., 1997). This indicates that cells normally make the ingredients that are necessary for the formation of mature caveolae, and insertion of caveolin proteins may only be a late phase in this process.

GPI-anchored proteins and their association with caveolae

The GPI anchor contains two fatty acyl groups and several sugars, including glucosamine, mannose, and inositol (Fig. 5.4.1). Various experiments have shown that the phospholipid anchor is both necessary and sufficient for binding these cell-surface proteins to the plasma membrane. The enzyme PI-specific phospholipase C cleaves the phosphate-glycerol bond in phospholipids as well as in glycosyl phosphatidylinositol anchors. Treatment of cells with phospholipase C releases glycosyl phosphatidylinositol-anchored proteins such as the Thy-1 protein and alkaline phosphatase from the cell surface. Experimentally, the PI-PLC mediated cleavage can be performed in intact cells, extracted membranes, and purified proteins reconstituted in liposomes.

GPI-linked proteins can be associated with CRDs and caveolae in the outer leaflets of lipid bilayers by using their saturated fatty acid moiety directly inserted into the unique lipid environment.

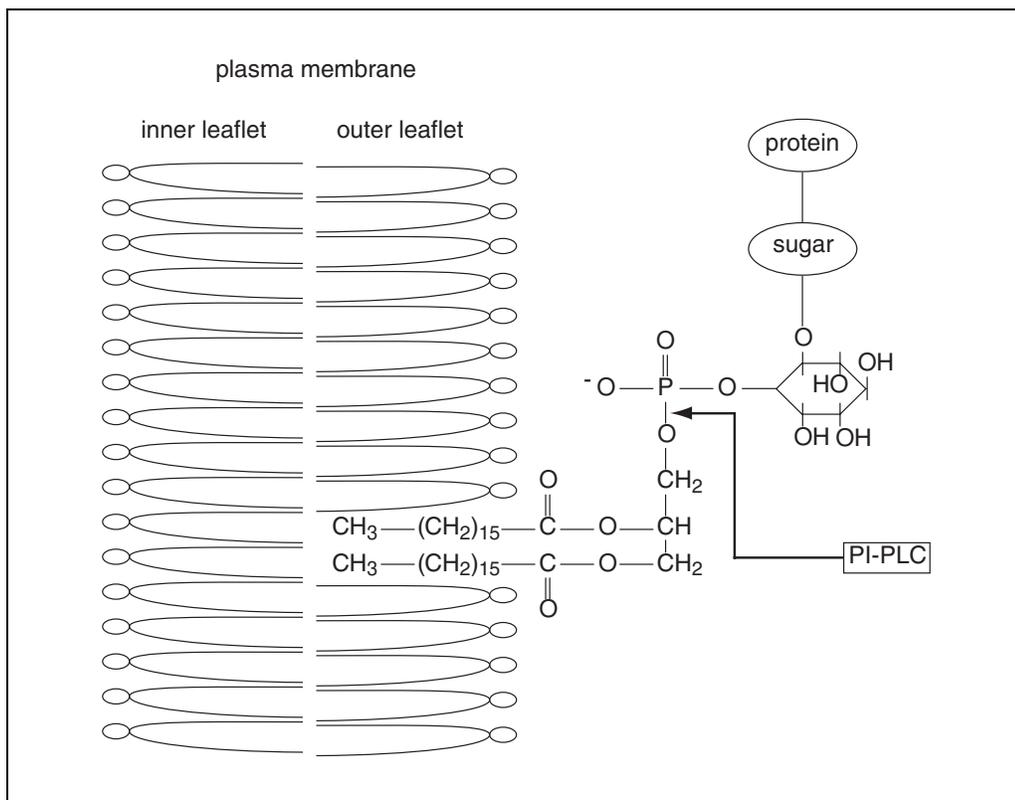


Figure 5.4.1 The glycosyl phosphoinositol (GPI) anchor. PI-PLC, phosphatidylinositol-specific phospholipase C.

It has been debated whether GPI-anchored proteins are constitutively associated with caveolae or are only associated with caveolae upon cross-linking with antibody probes. In the case of the tissue factor (TF) receptor system, this GPI-anchored protein is only associated with caveolae upon binding its natural ligand (Sevinsky et al., 1996). Thus, antibody probes may mimic natural endogenous ligands and target clustered GPI-anchored proteins to caveolae.

GPI-anchored molecules are recovered associated with membrane fractions that are resistant to solubilization to 1% Triton X-100 or 1% Triton X-114 at 4°C. It is, therefore, reasonable to speculate that GPI molecules are also associated with membrane domains with basic biochemical characteristics similar to those of caveolae, irrespective of the argument whether GPI molecules are in fact localized in caveolae. This assumption is further strengthened by the fact that the membrane fraction which contains GPI molecules is solubilized by 60 mM *N*-octylglucoside in a highly efficient manner. Of note, 20 mM CHAPS is less effective in its solubilization. At 37°C, however, 1% Triton X-100 solubilizes GPI-containing membrane with a similar efficiency to that of *N*-octylglu-

coside. At this temperature, 1% Triton X 114 solubilizes the membrane with 60% efficiency compared to Triton X-100. Since Triton X-114 partitions GPI molecules to the aqueous phase with PI-PLC treatment, Triton X 114 phase separation is used for the detection of GPI molecules in membranes. This method also makes it possible to identify membranes which have a similar biochemical property to that of caveolae.

Critical Parameters

Whether a given protein is an integral or peripheral membrane protein can be determined by alkaline carbonate extraction, urea extraction, high-salt extraction, and Triton X-114 phase separation protocols. The following parameters should be kept in mind while performing experiments.

In order to avoid protein degradation, experiments should be performed at 4°C. Solutions should also be chilled before use. For Triton X-114 phase separation, samples should be incubated at 37°C after solubilization, then centrifuged at room temperature.

In order to achieve optimal solubilization, the authors recommend that solubilization be performed from 30 min to 1 hr. For Triton

X-114 phase separation, samples should be kept at 37°C for 3 min after solubilization. This 3-min incubation should be strictly observed for achieving optimal phase separation and avoiding protein degradation.

Membrane proteins have ionic as well as hydrophobic interactions, and for this reason 150 mM NaCl is included in solubilization solutions for Triton X-114 phase separation protocol. As the salt concentration is already high, 100 mM NaCl is sufficient for the alkaline carbonate protocol. In urea and high-salt protocols, no additional NaCl is necessary.

The efficiency of solubilization is directly influenced by protein-salt extraction methods, and 1 ml of the Triton X-114-containing lysis buffer per $2\text{--}5 \times 10^7$ cells should be strictly observed for Triton X-114 phase separation protocols (see Basic Protocol 4, step 1).

In Basic Protocols 1 and 3, the criterion for solubilization is retention in the supernatant after centrifugation of solutions for 1 hr at $105,000 \times g$. The authors recommend centrifugation at $150,000$ to $200,000 \times g$ for 1 hr in order to recover completely solubilized proteins.

In order to avoid cyanate formation from urea, it is important to prepare urea solution just before use. For Triton X-114 stock solution, precondensation is required to remove impurities. Protease inhibitors can be kept as 100 \times condensed solutions in freezer.

PI-PLC cleavage releases GPI-linked proteins from membranes. In the method described here (Basic Protocol 2), PI-PLC cleavage should be performed at 37°C in Triton X-114-containing solution for at least 1 hr.

Proteins in caveolae or CRD, including GPI-anchored proteins, are solubilized in 60 mM *N*-octylglucoside or 30 mM CHAPS at 4°C. The following parameters should be kept in mind while performing these experiments.

Detergent solubility is strictly dependent upon the temperature. Temperature should be kept below 4°C in order to assess the Triton X-100 insolubility of the protein in CRD. All the experiments should be performed on ice. At 8°C, most of the proteins in CRD can be solubilized even by buffers containing 1% Triton X-100 as the detergent. This should be kept in mind especially when purifying caveolae using sucrose density gradient centrifugation. Cell extraction with Triton X-100 should also be performed at 4°C. It is recommended that the experiment be performed in the cold room, with the temperature kept below 4°C. Solutions should also be kept at 4°C before use.

Perform solubilization for 30 min to 1 hr in order to avoid protein degradation by proteases and insufficient solubilization.

The authors recommend detergent solubilization of proteins in CRD at concentrations of 60 mM *N*-octylglucoside or 30 mM CHAPS. These concentrations were determined by the critical micellar concentration (CMC) of these detergents. In order to achieve complete solubilization of proteins localized in CRDs, it is important to use detergents at twice the concentration of CMC value.

Add protease inhibitors to the solubilization buffer to a final 1 \times concentration in order to prevent protease-mediated degradation of proteins during solubilization. This is especially important when whole animal tissues are used as a source of protein for extraction. The protease inhibitors (Support Protocol 2) should be used in TNET-OG buffer, TNET buffer, and cell lysis buffer. 1 mM PMSF alone is sufficient for lysis for caveolae.

NaCl at 150 mM should be added to the solubilization buffer in order to maintain proteins under physiological conditions.

Caveolins are the principal protein component of caveolae. It is therefore of essence to probe the blot of fractionated samples with anti-caveolin antibodies, which are available commercially from Transduction Laboratories (see *SUPPLIERS APPENDIX*) to track the position of caveolae-derived membranes in these sucrose density gradients.

Anticipated Results

Integral membrane proteins can be separated from soluble and peripherally associated membrane proteins using alkaline carbonate extraction, urea extraction, or high-salt extraction protocols. In the Triton X-114 phase separation protocol, the aqueous phase contains peripherally associated membrane proteins and the detergent phase contains integral membrane proteins.

Proteins within CRDs can be separated from non-CRD proteins, as long as 1% Triton-X 100 is used at temperatures below 4°C. More than 99.95% of the CRD protein can be separated from non-CRD proteins following ultracentrifugation on a sucrose gradient.

Time Considerations

For alkaline carbonate extraction, urea extraction, and high-salt extraction protocols, it takes 1 hr for cell preparation and solubilization, 1 hr for centrifugation, 1 hr for SDS-PAGE, and 24 hr for immunoblotting. For Tri-

ton X-114 phase separation, it takes 3 hr for cell preparation and solubilization, 1 hr for SDS-PAGE, and 24 hr for immunoblotting.

For purification of caveolae, detergent solubilization takes 1 hr and ultracentrifugation requires 24 hr. SDS-PAGE takes one hr and immunoblotting takes another 24 hr. Results will be available within 2 days.

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Identification of Proteins in Complex Mixtures Using Liquid Chromatography and Mass Spectrometry

UNIT 5.6

Most cellular processes are executed and regulated by multiple proteins that act as part of a complex mixture. The identification of protein interactions in complex mixtures can provide fundamental information about the cellular processes being studied. Attempts to study protein interactions have traditionally focused on the isolation and identification of individual proteins. Understanding the role of a protein in a complex mixture, however, may depend not only on identifying an individual protein but also on identifying other proteins in the mixture and the effect of their association on the cellular system. Protein mixtures can be composed of proteins acting as tightly bound complexes, or they may include loosely bound proteins whose activity depends on self-assembly into a larger protein structure. Additionally, protein mixtures may include proteins that interact in a transient association, including protein-modifying enzymes such as proteases and kinases. This unit describes the current state of technologies available for directly identifying proteins in complex mixtures using liquid chromatography (LC) and mass spectrometry (MS). Two basic approaches are typically used. In one, proteins are separated by polyacrylamide gel electrophoresis (PAGE), isolated, digested, and analyzed by MS. In the second, LC and MS are directly coupled, allowing identification of proteins in complex mixtures without prior separation. For a detailed discussion of these approaches, see Background Information.

This unit focuses on the direct LC-MS methods. Samples are prepared from mixtures of proteins by proteolytic digestion without separation (see Basic Protocol 1). The sample is loaded onto a microcapillary column (see Basic Protocol 2) and analyzed on-line using a combined method of LC and electrospray-ionization tandem mass spectrometry (ESI-MS/MS; see Basic Protocol 3). Additional protocols are given for data analysis (see Basic Protocol 4) and pulling and packing the microcapillary column (see Support Protocol). Finally, to allow MS analysis of proteins separated by PAGE, a protocol is given for preparing digested proteins in slices from one- or two-dimensional polyacrylamide gels (see Alternate Protocol).

NOTE: To avoid contamination of the proteins, wear gloves while working with the protein sample and avoid exposing the sample to dust.

SAMPLE PREPARATION FOR DIRECT ANALYSIS OF PEPTIDES IN MIXTURES

**BASIC
PROTOCOL 1**

This protocol describes the preparation of samples to be analyzed directly in a one- or two-dimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS) experiment, and it is based on the method described by Link et al. (1999). The protocol is described for a sample containing 1 mg of protein, although samples with as little as 100 μg can be routinely analyzed. The insoluble fraction is treated with cyanogen bromide to digest proteins into long peptides which can be further digested by Lys-C and trypsin. Formic acid is used to increase solubility.

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of Cellular
Proteins**

5.6.1

Materials

Total cell lysates, insoluble fractions or membrane proteins, soluble fractions, or unknown sample
Cyanogen bromide (e.g., Aldrich), for insoluble fractions or membrane proteins only
96% (v/v) formic acid (e.g., Aldrich), for insoluble fractions or membrane proteins only
Ammonium bicarbonate (e.g., J.T. Baker), for insoluble fractions only
100 mM Tris, pH 8.5/8 M urea (e.g., Sigma)
1 M Tris (2-carboxyethyl)phosphine hydrochloride (TCEP; e.g., Pierce)/100 mM ammonium bicarbonate
500 mM iodoacetamide
Endoproteinase Lys-C (Roche Diagnostics)
100 mM CaCl₂ (e.g., Mallinckrodt Baker; *APPENDIX 2A*)
Porozyme bulk immobilized trypsin beads (Applied Biosystems)
88% (v/v) formic acid (e.g., Aldrich)

Additional reagents and equipment for trichloroacetic acid (TCA) precipitation (*UNIT 7.1*)

CAUTION: Cyanogen bromide is highly toxic and volatile; carry out all work with this compound in a well-ventilated fume hood. TCA is extremely caustic. Protect eyes and avoid contact with skin when preparing and handling TCA solutions

Prepare sample fractions (total cell lysates or unknown samples only)

1. Place a total cell lysate or unknown sample containing ~1 to 5 mg protein in a 1.5-ml microcentrifuge tube and separate the soluble and insoluble fractions by microcentrifuging 30 min at 4000 rpm. Transfer the soluble fraction to a clean 1.5-ml microcentrifuge tube and proceed to step 2. Keep the insoluble fraction in the original microcentrifuge tube and pretreat with cyanogen bromide (steps 3 to 5).
2. Precipitate a volume of the soluble fraction equivalent to 500 µg protein using TCA (*UNIT 7.1*). Proceed to step 6.

Starting amounts can be 100 µg to 2 mg. Adjust reagents accordingly.

Pretreat with cyanogen bromide (insoluble fractions or membrane proteins only)

3. Prepare a solution of 500 mg/ml cyanogen bromide in 96% formic acid in a chemical fume hood.
4. Add 100 µl of the cyanogen bromide solution to the insoluble pellet and incubate overnight at room temperature in the dark.
5. Add 100 µl water and carefully adjust the pH to 8 to 8.5 using solid ammonium bicarbonate. Reduce volume almost to dryness in a Speedvac evaporator. Proceed to step 6.

Reduce and carboxyamidate proteins

6. Resuspend sample (step 2 or step 5) in 30 µl of 100 mM Tris·Cl, pH 8.5/8 M urea.
7. Add 0.15 µl of 1 M TCEP (5 mM final concentration) and incubate 10 min at room temperature.
8. Add 1.2 µl of 500 mM iodoacetamide (20 mM final concentration) and incubate 20 min in the dark.

Perform enzyme digestion

9. Add 5 μl of 1.0 $\mu\text{g}/\mu\text{l}$ endoproteinase Lys-C and incubate 4 to 8 hr at 37°C in the dark.

A 1:100 ratio of enzyme to sample concentration is used.

10. Add 82.5 μl water to dilute solution to 2 M.
11. Add 2.4 μl of 100 mM CaCl_2 (2 mM final concentration) and 10 μl Porozyme bulk immobilized trypsin beads. Incubate with shaking 12 hr at 37°C in the dark.
12. Quench by adding 5.8 μl of 88% formic acid and microcentrifuge the samples 10 min at 14,000 rpm (maximum speed). Transfer supernatant to a clean 1.5-ml microcentrifuge tube and discard the pellet.

The sample is now ready to be loaded onto the LC column (see Basic Protocol 2). It should be loaded within a few hours, or can be stored indefinitely at -80°C .

SAMPLE PREPARATION BY IN-GEL DIGESTION OF SILVER- OR COOMASSIE-STAINED SPOTS FOLLOWING PAGE

ALTERNATE PROTOCOL

This protocol describes the technique used to isolate peptides from a protein purified by one- or two-dimensional PAGE, and is based on the method by Jensen et al. (1999). Gel slices of widely varying acrylamide content ($\leq 18\%$) and size can be processed in this way. A gel spot of interest is excised from the polyacrylamide gel and subjected to destaining. The gel piece is then dried and rehydrated with a reducing and alkylating procedure. The alkylated proteins are digested to peptides in-gel, extracted, lyophilized, and resuspended in 5% formic acid. Samples are then ready for analysis.

Materials

- Coomassie- or silver-stained (*UNIT 6.6*) one- or two-dimensional acrylamide gel (*UNITS 6.1 & 6.4*) containing proteins of interest
- 30 mM potassium ferricyanide
- 100 mM sodium thiosulfate
- Acetonitrile (e.g., Fisher), HPLC grade
- 25 and 100 mM ammonium bicarbonate (J.T. Baker)
- 10 mM Tris(2-carboxyethyl)phosphine (TCEP; e.g., Pierce)/100 mM ammonium bicarbonate
- Iodoacetamide/ammonium bicarbonate solution (see recipe)
- Trypsin digestion solution (see recipe), 4°C
- 50 mM ammonium bicarbonate/5 mM CaCl_2 (e.g., Mallinckrodt Baker)
- 5% (v/v) formic acid (e.g., Aldrich)
- Glass plate, cleaned with methanol or ethanol
- Scalpel or razor blades, cleaned with methanol or ethanol
- Gel-loading pipet tips

Excise protein band

1. Wash a Coomassie- or silver-stained polyacrylamide gel containing proteins of interest two times with water for 10 min each.
2. Transfer gel to a glass plate and use a scalpel or razor blade cleaned with methanol or ethanol to excise the band of interest. Cut as close to the protein as possible to reduce the amount of background gel. Excise a second gel piece of approximately the same size from a nonprotein region to use as a control.

The gel should be kept moist with water while the bands are excised.

Characterization of Cellular Proteins

5.6.3

- Cut the gel piece into 1×1 -mm squares (gel thickness will vary). If the gel stain is dark, place two to three 1×1 -mm squares into a 1.5-ml microcentrifuge tube. If it is faint, use up to ten pieces.

Volumes in subsequent steps are based on a starting gel volume of $\sim 100 \mu\text{l}$ and should be scaled as appropriate.

- Microcentrifuge 10 sec at 14,000 rpm (maximum speed) so that the gel pieces settle to the bottom of the tube.

Destain gel

- For silver stain only:* Prepare a fresh 1:1 (v/v) mixture of 30 mM potassium ferricyanide/100 mM sodium thiosulfate. Cover silver-stained gel cubes with 100 μl of this solution and incubate 5 min at room temperature. Discard liquid and wash gel pieces three times with 100 μl water to quench the reaction. Discard water and proceed to step 6.
- Wash gel pieces (silver-stained from step 5 or Coomassie-stained from step 4) with 2 vol (200 μl) water for 15 min. Shake well or vortex lightly.
- Remove water with a gel-loading pipet tip, being very careful not to remove any portion of the gel.
- Add 100 μl water followed by 100 μl acetonitrile and wash gel pieces 15 min.
- Remove liquid and add 100 μl acetonitrile to cover the gel pieces. Tap tube with fingers to mix.
- Allow the pieces to shrink (they will turn sticky white) and then remove the acetonitrile.
- Rehydrate with 100 μl of 100 mM ammonium bicarbonate for 5 min.
- Add 100 μl acetonitrile and incubate 15 min. If a strong blue color persists, incubate again for 15 min with constant vortexing.
- Remove liquid and dry ~ 15 min in a Speedvac evaporator. If blue color persists, repeat wash starting with step 9.

Reduce and alkylate proteins

- Rehydrate gel pieces with 100 μl of 10 mM TCEP/100 mM ammonium bicarbonate to cover the gel. Tap tube with fingers to mix and incubate 20 to 30 min at room temperature.
- Remove liquid and quickly add 100 μl freshly prepared iodoacetamide/ammonium bicarbonate solution. Incubate 30 min at room temperature in the dark.
- Remove liquid and add 100 μl acetonitrile to cover gel pieces.
- Allow the pieces to shrink (again, they will turn sticky white) and remove acetonitrile.
- Add 100 μl of 100 mM ammonium bicarbonate and rehydrate 5 min.
- Add 100 μl acetonitrile and incubate 15 min.

All the Coomassie stain should be removed at this point. If a blue color still persists, steps 9 to 19 should be repeated.

- Remove liquid and dry down completely in the Speedvac evaporator (≥ 30 min).

The actual drying time will depend on the original volume of gel.

Perform in-gel digestion

21. Add 50 μl prechilled (4°C) trypsin digestion solution to just cover the gel pieces and incubate 45 min on ice. Check gel pieces every 15 min and add more digestion solution if they are not covered.
22. Remove supernatant and add 50 μl of 50 mM ammonium bicarbonate/5 mM CaCl_2 . Incubate overnight at 37°C.

Extract peptides

23. Microcentrifuge tubes for ~30 sec at maximum speed, transfer buffer to a 1.5-ml microcentrifuge tube, and keep gel pieces.
24. Add 100 μl of 25 mM ammonium bicarbonate to cover the gel pieces and incubate 15 min at room temperature.

The volume should not exceed 100 μl .

25. Add 100 μl acetonitrile and incubate 15 min at room temperature while vortexing. Transfer supernatant to the 1.5-ml tube in step 23 and keep gel pieces.
26. Add 100 μl of 5% formic acid to the gel pieces and incubate 15 min at room temperature.
27. Add 100 μl acetonitrile and incubate 15 min at room temperature while vortexing. Transfer supernatant to the 1.5-ml tube in step 23 and keep gel pieces.
28. Repeat steps 26 to 27, but discard gel pieces.
29. Dry sample in the Speedvac evaporator for ~2 hr.
30. Resuspend peptides in 10 to 30 μl of 5% formic acid.

The peptides can be stored indefinitely at -80°C before being analyzed (see Basic Protocol 3).

LOADING A PROTEIN SAMPLE FOR MICROCAPILLARY COLUMN LIQUID CHROMATOGRAPHY

The digested protein sample is loaded onto the column in a method similar to column packing (see Support Protocol).

Materials

Digested protein sample (see Basic Protocol 1 or Alternate Protocol)
Pre-equilibrated microcapillary column (see Support Protocol)
High-pressure bomb designed for pneumatically loading microcapillaries (see Dongré et al., 1997) connected to helium gas tank

1. Place microcentrifuge tube containing acidified peptides in the center of a high-pressure bomb connected to a helium gas tank.
2. Close the bomb lid and tighten the screws.
3. Insert a pre-equilibrated microcapillary column into the bomb (point up) until the flat end of the column is immersed in the sample and is touching the bottom of the tube. Tighten the screw that holds the microcapillary.
4. Turn the helium gas tank on at 300 to 700 psi to pressurize the bomb and thus load the sample.

**BASIC
PROTOCOL 2**

**Characterization
of Cellular
Proteins**

5.6.5

Loading progress can be monitored by measuring the solvent displaced from the column. The volume of the eluent solvent should be equal to the volume of the sample being loaded.

CAUTION: The bomb is under high pressure. Make sure a safe and pressure-tested device is used.

5. After loading is complete, slowly depressurize the bomb to avoid disrupting the packing material, and securely grasp the column while opening the bomb.

Sample is now loaded onto the strong cation exchange portion of the column and is ready for analysis (see Basic Protocol 3). The loaded column should be used immediately. If not, the column should be stored tip down in buffer A (see Basic Protocol 3) and used as soon as possible. Column tips are very fragile.

SUPPORT PROTOCOL

PREPARING MICROCAPILLARY COLUMNS FOR LIQUID CHROMATOGRAPHY

Microcapillary columns (Kennedy and Jorgenson, 1989) for one- or two-dimensional LC-MS/MS are created from fused-silica microcapillaries by using a laser puller. The laser puller is critical for creating capillary columns, and attempts to manually pull the capillaries can give unsatisfactory and irreproducible results. The pulled capillary (column) is then packed using a protocol that is similar for single and biphasic columns.

Additional materials (also see Basic Protocol 2)

Methanol (e.g., Fisher), HPLC grade
C₁₈ packing material
Partisphere strong cation exchange (SCX; Whatman; for biphasic columns only)
Buffer A: 5% (v/v) acetonitrile/0.1% (v/v) formic acid
Laser puller (model P-2000; Sutter Instruments)
Ceramic scribe
100 × 365- μ m (i.d. × o.d.) fused-silica capillary (J&W Scientific)
Alcohol burner
PEEK microcross splitter (Upchurch)
Quaternary high-performance liquid chromatography (HPLC) pump

Pull microcapillary column

1. Set the program of a laser puller for three cycles as follows:

Heat	270°C
Filament	0
Velocity	30
Delay	128 msec
Pull	0.

These values are specifically for a 100 × 365- μ m microcapillary in the Sutter Instruments laser puller. They are unitless (except the delay) and are preset in this instrument. If another puller is used, the values will have to be determined empirically.

2. Use a ceramic scribe to cut a 50- to 54-cm length of 100 × 365- μ m fused-silica capillary from the bulk. Use an alcohol burner to burn off the coating covering ~3 cm in the center part of the microcapillary. Clean the capillary using a methanol-soaked lint-free tissue.
3. Place the microcapillary in the puller, run the program and form two pulled columns. Store the pulled columns at room temperature, free of dust, with special care to protect tip.

The column tip will have an internal diameter of ~2 to 5 μm and each column will be ~25 to 27 cm long.

Pack column

4. Measure ~500 μg C_{18} packing material into a 1.5-ml microcentrifuge tube and suspend it in 1 ml methanol.
5. Place the microcentrifuge tube in the center of a high-pressure bomb connected to a helium gas tank. Position the lid and make sure all screws are secure.
6. Insert the column (step 3) into the bomb (point up) until the flat end of the column is immersed in the packing material. Gently tap the end of the column against the bottom of the tube and then raise it ~0.5 cm from the bottom. Tighten the screw that holds the microcapillary.
7. Turn the helium on at 300 to 700 psi and fill column to a length of 7 to 10 cm.

Packing material should be seen moving up the microcapillary. As loading progresses, methanol accumulating at the tip of the column can be removed with a lint-free tissue. If the packing material slows too much before the desired packing length is achieved, the capillary can be loosened and tapped gently on the bottom of the tube to reactivate the process.

CAUTION: The bomb is under high pressure. Make sure a safe and pressure-tested device is used.

8. Slowly depressurize the bomb to avoid disrupting the packing material and securely grasp the column while opening the bomb.
- 9a. *For a one-dimensional reversed-phase column:* Remove the column from the bomb and store in methanol up to 1 week at 4°C until pre-equilibration.
- 9b. *For a two-dimensional (biphasic) reversed-phase column:* Remove the column from the bomb and replace the microcentrifuge tube with a second tube containing 500 μg Partisphere SCX in 1 ml methanol. Repeat column packing as described (steps 6 to 8). Store packed column in methanol up to 1 week at 4°C until pre-equilibration.

Because the SCX resin remains suspended in the methanol slurry, tapping the column on the bottom of the tube is not always necessary.

Pre-equilibrate column

10. Place the column in a PEEK microcross splitter.

The splitter is used to interface the microcapillary column with the mass spectrometer.

11. Place a quaternary HPLC pump in-line with the splitter and equilibrate the microcapillary column for 30 min using buffer A at a flow rate of 100 $\mu\text{l}/\text{min}$.
12. Store equilibrated column up to 24 hr at 4°C or room temperature.

MULTIDIMENSIONAL PROTEIN IDENTIFICATION TECHNOLOGY FOR ANALYZING COMPLEX MIXTURES

Combining liquid chromatography (LC) with electrospray ionization–tandem mass spectrometry (ESI-MS/MS) allows on-line analysis of complex protein mixtures. This protocol describes separation by multidimensional protein identification technology (MudPIT). In this technique, a pulled microcapillary column is packed with two independent chromatography phases. In the first dimension, peptides are separated by strong cation exchange and are eluted into the second phase by a step gradient of increasing salt concentration. The second phase consists of reversed-phase material, which separates the

**BASIC
PROTOCOL 3**

**Characterization
of Cellular
Proteins**

5.6.7

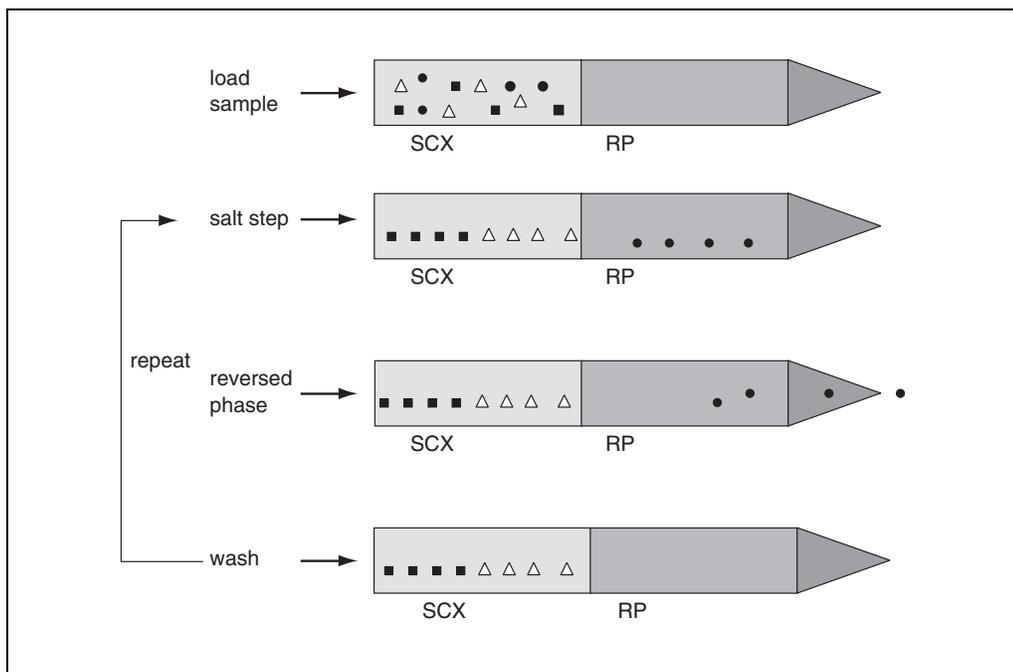


Figure 5.6.1 Integrated two-dimensional liquid chromatography. Peptides are separated in the first dimension by strong cation exchange (SCX) followed by reversed-phase (RP) separation and elution into the mass spectrometer. Triangles, circles, and squares represent different peptides.

peptides on the basis of hydrophobicity before they are eluted directly into the mass spectrometer (Fig. 5.6.1). The method described is for a six-step multidimensional separation. For highly complex or concentrated samples, twelve-step programs are frequently used and simply require smaller increments in the proportion of buffer C used in step 5. If one-dimensional separation is used, the salt steps in the chromatography are eliminated. For two-dimensional LC, a quaternary HPLC pump is required to deliver the three different buffers necessary for two-dimensional separations.

Materials

Microcapillary column loaded with protein sample (see Basic Protocol 2)

Buffer A: 5% (v/v) acetonitrile/0.1% (v/v) formic acid

Buffer B: 80% (v/v) acetonitrile/0.1% (v/v) formic acid

Buffer C: 500 mM ammonium acetate (e.g., Sigma)/5% (v/v) acetonitrile/0.1% (v/v) formic acid

PEEK microcross splitter (Upchurch)

LCQ ion trap mass spectrometer (ThermoFinnigan) including custom platform for nanoelectrospray, or equivalent tandem mass spectrometer

XYZ translational positioner (Newport)

HPLC system, including quaternary HPLC pump (e.g., Agilent, ThermoFinnigan, Waters; for two-dimensional LC only) and nitrogen gas source

Set up HPLC and MS system

1. Place a microcapillary column loaded with a protein sample in a PEEK microcross splitter and mount it on the custom platform of an LCQ ion trap mass spectrometer.

The platform replaces the original electrospray source and is described in Yates et al. (2000). Plans for the platform may be available from the author by a material transfer agreement.

2. Align the column with the mass spectrometer orifice using an XYZ translational positioner.

The column should be directly in line with the mass spectrometer at a distance of 2 to 3 mm.

3. Set up an HPLC system, including a quaternary HPLC pump for two-dimensional LC. Split the flow from the HPLC with the PEEK microcross splitter so that effective flow rate from the HPLC is 0.15 to 0.25 $\mu\text{l}/\text{min}$ and spray voltage is 2.4 kV. Set the nitrogen gas (sheath gas) flow rate to 0.2 liter/min.

For one-dimensional LC, a binary HPLC pump can be used.

Perform chromatography

4. Run a 100-min gradient of 0% to 100% buffer B in buffer A. Hold 10 min at 100% buffer B.
5. Run the following 110-min program.
 - a. 3 min with 100% buffer A
 - b. 2 min with 10% buffer C in buffer A
 - c. 5 min with 100% buffer A
 - d. 10-min gradient from 0% to 15% buffer B in buffer A
 - e. 90-min gradient from 15% to 55% buffer B in buffer A.

Repeat this program four more times, but increase the concentration of buffer C (substep b) in each repetition until 80% is obtained (e.g., 10%, 25%, 50%, and 80%).

6. Run the following 130-min program.
 - a. 5 min with 100% buffer A
 - b. 20 min with 80% buffer C
 - c. 5 min with 100% buffer A
 - d. 10-min gradient from 0% to 10% buffer B in buffer A
 - e. 90-min gradient from 10% to 45% buffer B in buffer A.

Set up and run mass spectrometer

7. Set the LCQ ion trap mass spectrometer to acquire a full MS scan over the m/z range of 400 to 1400, with an electron multiplier voltage of 1000 V.

The LCQ is operated via an Instrument Methods file in the Sequence Setup window of Xcalibur.

8. Select for the top three ions from the MS scan to be analyzed automatically in a tandem MS/MS experiment.

Dynamic exclusion is enabled with a repeat count of 1, a duration of 0.5 min, and a 30- to 45-sec exclusion duration window.

9. Set relative collision energy to 35% with a 30-msec activation time.
10. Run sample and collect data.

As the peptide mixture elutes into the mass spectrometer, data are acquired in a data-dependent manner.

The great quantity of data generated in a two-dimensional LC-MS/MS experiment is most efficiently analyzed by algorithms that search the experimental data against known sequences in DNA or protein databases (see Internet Resources). The most effective algorithms for searching databases with MS/MS data require no interpretation of the raw data. In these algorithms, the data are directly correlated with protein or DNA data contained in the database. Several algorithms exist to analyze MS/MS data. The SEQUEST algorithm (Thermo Finnigan) can run directly on the LCQ ion trap mass spectrometer. Alternatively, Web-based search tools are available (see Internet Resources).

SEQUEST matches a peptide sequence in the database with MS/MS spectra by first identifying those peptides with matching molecular weight. The specificity of the enzyme used to digest the mixture significantly reduces the number of possible match candidates. From this list of candidates, predicted fragment ions for each of the peptides are generated and the similarity of the reconstructed spectra to the experimental data is determined. The cross-correlation value is a measure of the quality of the match between the sequence and the spectrum. The difference between the correlation score for the top-scoring match candidate and the candidate ranked second is a measure of the quality of the match versus other possible sequences.

In general, spectra of modified peptides can be searched through available databases. The mass increment of the modification is added to the mass of the unmodified amino acid and this new mass is used in the database search. For example, to search for phosphorylated residues, the program is told to look for mass changes of 80 to Ser, Thr, and Tyr residues. This search is usually done in a differential manner by checking to see if each occurrence of Ser, Thr, or Tyr is modified. To search different types of modifications, the increase in molecular weight expected for the residue is input into the program. Some types of modifications (e.g., methionine oxidation, phosphorylation) may not occur at all possible locations, and thus the possibility of modification must be considered separately for each occurrence of the amino acid.

Database searching can be performed with peptides obtained from organisms without completed genome sequences if a genome sequence is available for a related organism. For example, to analyze peptides from a rat protein, one would create a database of all mammalian protein sequences for the search. Databases are large text files of sequences; consequently the sequences available for related organisms (e.g., human, mouse, and rat) are combined into a single file for the search.

REAGENTS AND SOLUTIONS

Use HPLC-grade water and solvents in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Iodoacetamide/ammonium bicarbonate solution

100 μ l 1 M ammonium bicarbonate (J.T. Baker; 100 mM final)
900 μ l H₂O
10 mg iodoacetamide (Sigma; 55 mM final)
Always make fresh

Trypsin digestion solution

5 μ l 1 M CaCl₂ (e.g., Mallinckrodt Baker; APPENDIX 2A; 5 mM final)
50 μ l 1 M ammonium bicarbonate (50 mM final)
945 μ l H₂O
Store up to 1 week at 4°C
Just before use, add 2.5 μ l of 0.5 μ g/ μ l trypsin (Promega) per 100 μ l total volume
(12.5 ng/ μ l final)

COMMENTARY

Background Information

The development of methods to separate complex mixtures of proteins using two-dimensional gel electrophoresis led to large-scale studies of global protein expression, which is now referred to as proteomics. Using two-dimensional electrophoresis, hundreds of proteins could be separated and visualized, but identification of the individual proteins using Edman sequencing methods was very difficult and time consuming (Aebersold, 1986; Matsudaira, 1987). Advances in techniques to sequence proteins using mass spectrometry (MS; Hunt, 1986) soon replaced the slower and less-sensitive chemical degradation methods.

MS techniques have been most frequently coupled with two-dimensional polyacrylamide gel electrophoresis (PAGE; Jensen et al., 1999b). Proteins separated by two-dimensional PAGE are identified by MS after a sequential analysis of tryptic digests of the individual gel spots. As powerful as this integrated method is, it is limited in its sensitivity and its ability to analyze specific classes of proteins (Gygi et al., 2000; Santoni et al., 2000), and it does not have a broad dynamic range. Two-dimensional PAGE is also a time-consuming and labor-intensive technique that is inherently limited for high-throughput analysis because it requires individual proteins to be excised and extracted from the gel prior to mass analysis.

As an alternative to gel-based methods, liquid chromatographic techniques have now been coupled directly with the mass spectrometer, enabling identification of proteins in complex mixtures through chromatographic separation based on chemical properties followed by sepa-

ration based on mass-to-charge ratios (m/z) and subsequent structural characterization (Eng et al., 1994; McCormack et al., 1997; Gatlin et al., 1998; Link et al., 1999; Washburn et al., 2001; Wolters et al., 2001; MacCoss et al., 2002).

Mass spectrometry

MS has proven to be a valuable technique for protein mixture analysis (Yates, 1998a; Washburn and Yates, 2000; Yates et al., 2000; Peng and Gygi, 2001). The design of a mass spectrometer makes it particularly well suited for this purpose. All mass spectrometers are composed of three fundamental parts (Fig. 5.6.2). The first segment is the ionization source where the sample molecules are converted into gas phase ions and are introduced into the mass spectrometer. The ions enter the second segment, the mass analyzer, where they are separated on the basis of their m/z values before passing into the third segment, the ion detector. Until the mid-1980s, MS was most commonly used for the characterization of small molecules because there was no way to convert larger biological molecules to gas phase ions. The advent of MS as a technique for the analysis of biological molecules was greatly expedited by the development of important ionization techniques that allowed the transfer of large, biologically important molecules into the gas phase with little or no fragmentation (Fenn et al., 1989). For protein analysis, the two most common ionization techniques are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI).

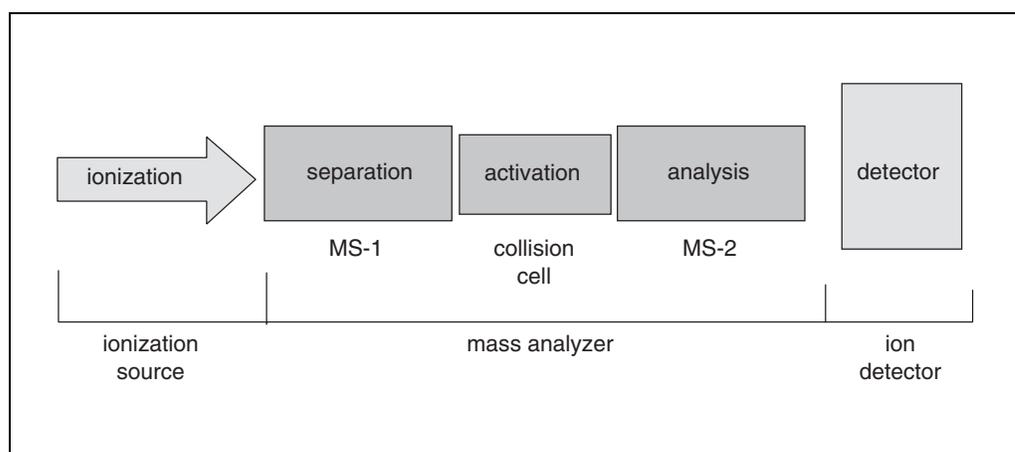


Figure 5.6.2 All mass spectrometers are composed of three basic parts: an ionization source, a mass analyzer, and an ion detector. The mass analyzer depicted in this figure is from a tandem mass spectrometer. MS-1 and MS-2 indicate the tandem mass spectrometers.

Matrix-assisted laser desorption/ionization

In the MALDI technique, analyte is co-crystallized with an acidified matrix and deposited on a polished stainless steel plate. The matrix is a small molecule that absorbs energy from a pulsed laser of specific wavelength. The energy absorbed from the laser is thermally dissipated, resulting in rapid dissociation of the matrix and analyte from the plate surface. The matrix and analyte are both transferred to the gas phase as ions. MALDI is typically coupled with a time-of-flight (TOF) mass analyzer. TOF analyzers separate ions based on the time it takes them to travel the length of the flight tube. Analytes are introduced into the TOF tube when the MALDI plate containing the co-crystallized analyte and matrix is placed in the vacuum chamber of the TOF instrument. High voltage is applied to the MALDI plate, generating a strong electric field between the plate and the entrance to the TOF analyzer. Ions of smaller mass are accelerated toward the flight tube entrance faster than those of larger mass and, once inside the TOF tube, smaller ions traverse the flight path and reach the detector more quickly. The detector records the time and intensity of ions and, once flight time is correlated with mass, generates a spectrum of observed m/z values. Because the charge of most ions generated in MALDI is 1, the spectrum is usually a straightforward account of ion masses.

Electrospray ionization

In the ESI technique, ionization is achieved when an electric field is applied to a flowing analyte solution. The applied potential imparts a charge to the liquid, creating small droplets of analyte-containing solvent that move toward the entrance to the mass spectrometer. As the solvent particles move into the mass spectrometer, they are repeatedly desolvated and fragmented into smaller particles in a process that results in multiply charged ions.

Unlike MALDI, ESI is performed at atmospheric pressure and can easily be coupled to separation methods such as high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) to allow continuous infusion of samples. By utilizing microbore capillaries and extremely low flow rates for introduction of the sample, detection limits at the femtomole level are possible with excellent signal-to-noise ratios (Gatlin et al., 1998). ESI is most frequently coupled with a triple quadrupole, quadrupole-time-of-flight (QTOF), or ion trap mass spectrometer. Triple quadrupole mass analyzers are comprised of three sets of four parallel

metal rods. When direct current and radio frequency voltage is applied to these rods, an electric field is created, which directs the trajectory of ions as they progress down the axis between the rods. If an ion meets the m/z requirements determined by the voltage to the quadrupole, it will pass through the quadrupole; all other ions will be rejected. In the triple quadrupole instrument, electric fields are generated in the first and third set of quadrupoles, whereas radio frequency voltages are applied to the second quadrupole. The second quadrupole serves as a collision cell where ions collide with atoms of neutral gases to form peptide fragments (Hunt et al., 1986). In the QTOF instrument, the third quadrupole is replaced by a TOF mass analyzer, resulting in a hybrid instrument that has the high mass accuracy of a TOF instrument coupled with the resolution of a quadrupole instrument.

An ion trap instrument operates by collecting and storing ions coming from the source in a trap, scanning the collection of ions for their m/z values, and sequentially ejecting ions from the trap for MS/MS analysis. Ions are maintained in the trap by the application of direct current and radio frequency voltages; they are ejected from the trap by stepping the radio frequency. For the analysis of proteins and peptides, as well as other biological molecules, the mass analyzer portion of the mass spectrometer typically includes two mass analyzers separated by an ion-activation device (Fig. 5.6.2). In this tandem arrangement of mass analyzers (MS/MS), a mixture of tryptic peptides is introduced into the first mass analyzer where they are separated by their m/z values. After separation, selected ions are advanced to the ion-activation device. While numerous ion-activation techniques are available, the one most commonly used is collision-induced dissociation (CID). In CID, slow-moving peptide ions collide with inert gas molecules with sufficient kinetic energy to cause peptide bonds to break. The charged fragments from this dissociation are advanced to the second mass spectrometer where they are separated on the basis of their m/z values before progressing to the ion detector (Fig. 5.6.3). These low-energy collisions result in fragmentation primarily at the amide bond of the peptide, creating a ladder of sequencing ions that differ by one amino acid. By assembling the collection of ions and determining the mass difference between fragments, the amino acid sequence of the peptide can be determined (Hunt et al., 1986).

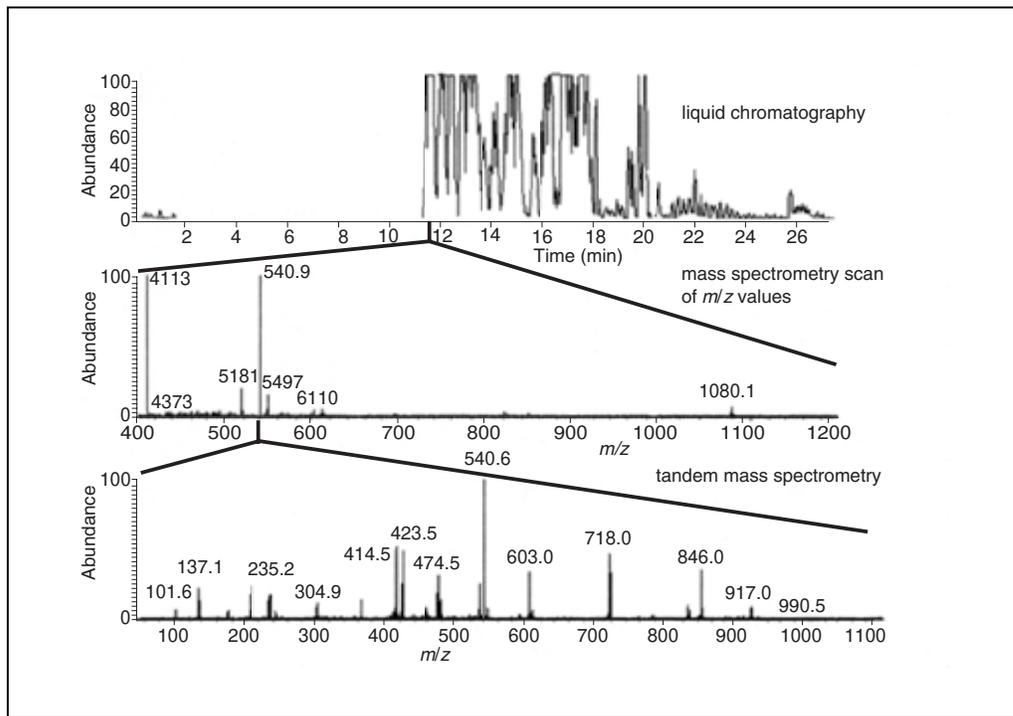


Figure 5.6.3 In the LC-MS/MS technique, peptides are first separated in a liquid chromatographic step. Fractions from the chromatography are subjected to MS and selected ions from the MS experiment are further fragmented in an MS/MS experiment.

Protein mixture analysis: MALDI versus ESI

Even using MS techniques, high-throughput analysis of protein mixtures was not feasible until large databases of protein and DNA sequence information became available and search algorithms were developed that could correlate MS data to the information in the databases (Eng et al., 1994; Yates, 1998b). The information generated by both MALDI-TOF-MS and ESI-MS/MS experiments has been used to identify proteins in complex mixtures.

The high accuracy of peptide mass measurement using a MALDI-TOF instrument allows elucidation of the amino acid composition of the peptide, and computerized search algorithms allow matching of the observed data with proteins in a database. In this method, a collection of peptides generated from tryptic digestion of a mixture of proteins is analyzed with the MALDI-TOF-MS. The masses of the individual peptides can be used to search against databases of known proteins to identify theoretical peptides of matching masses and their respective protein sources. Because mass alone is not always unique for peptide identification, it is typical that a number of peptides from a protein would be needed for identification.

Unlike MALDI, the peptide fragmentation data collected from the ESI-MS/MS experi-

ment are specific for a unique amino acid sequence. Because fragmentation patterns of peptides in a CID experiment are predictable, theoretical fragmentation patterns can be generated from sequences in a database, and the observed spectra can be searched against these (see Basic Protocol 4). Because the spectral data are sequence specific, only a single peptide spectrum is needed for protein identification. The ESI-MS/MS method requires no pre-interpretation of spectra prior to a database search. Algorithms automatically search uninterpreted MS/MS spectra against protein and DNA databases (Fig. 5.6.4). Sensitivity for peptide detection using either MALDI or ESI is 0.5 to 10 fmol.

Sample preparation: PAGE versus LC

When analyzing samples of highly complex mixtures, sample complexity must usually be reduced before the sample is introduced into the mass spectrometer. As discussed above, one method of achieving this is through separation of the proteins by gel electrophoresis. For protein mixtures of low complexity, one-dimensional SDS-PAGE (UNIT 6.1) has been used. Using this technique, proteins can be separated on the basis of molecular weight, but the degree of resolution is modest. For more complex mixtures, a two-dimensional SDS-PAGE sepa-

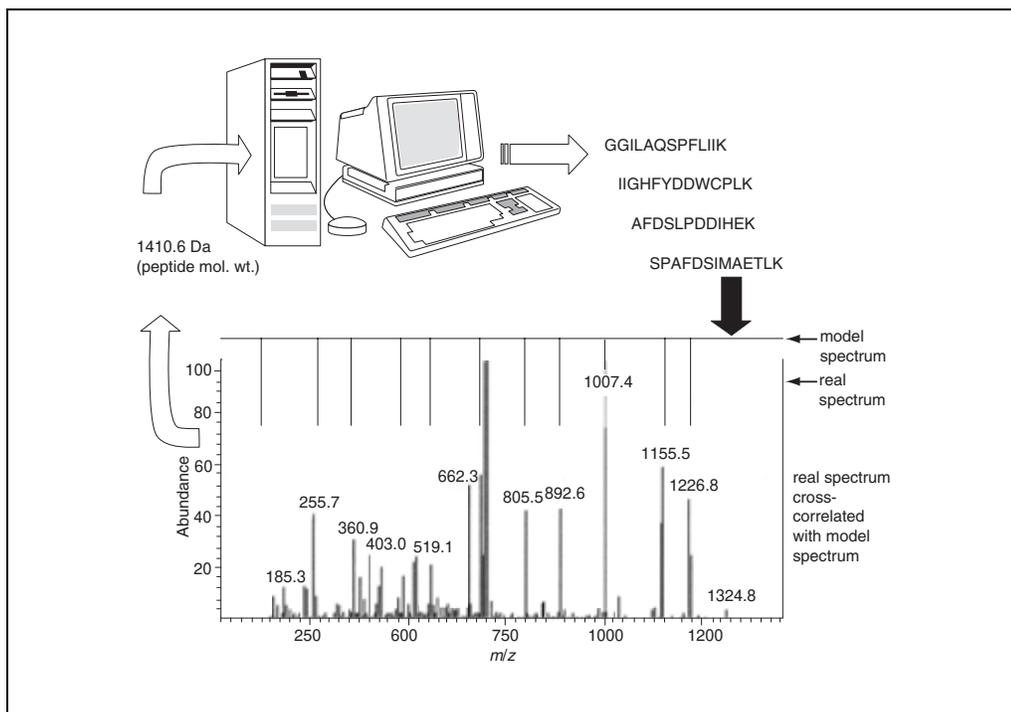


Figure 5.6.4 Search algorithms employed for LC-MS/MS data interpretation search uninterpreted MS/MS spectra against protein and DNA databases. Results are scored on the cross-correlation fit between a theoretical (or model) spectrum and the tandem mass spectra obtained from the experiment (real spectrum).

ration method (UNIT 6.4) has been widely adopted. In this technique, proteins are separated on the basis of isoelectric point in the first dimension and by molecular weight in the second. The resolution capability of two-dimensional SDS-PAGE is excellent, and it is theoretically capable of detecting up to 10,000 protein spots. Nevertheless, the method lacks sufficient dynamic range to identify low-abundance proteins in complex mixtures and has been shown to be incompatible with certain classes of proteins (Gygi et al., 2000; Santoni et al., 2000). To mitigate some of the problems of identifying minor protein constituents from a two-dimensional gel, an enrichment step is possible prior to electrophoresis. However, enrichment techniques require some knowledge of biological or chemical activity and are thus often not possible when working with unknown proteins.

Alternatively, mixtures of proteins can be proteolytically digested without separation and directly analyzed by microcolumn-LC-MS/MS. By combining liquid chromatography with ESI-MS/MS, on-line analysis of complex mixtures is possible. In this method, a pulled microcapillary column is packed with a reversed-phase packing material and molecules are separated on the basis of their molecular weight in the reversed-phase chromatography

step and then separated according to their m/z value in the mass spectrometer. After the mixture is loaded on the chromatography column, no further sample handling is necessary. When even greater peptide separation is desired, multidimensional protein identification technology (MudPIT) can be employed (Link et al., 1999; Washburn and Yates, 2000; Washburn et al., 2001; Wolters et al., 2001). In this technique, peptides are first separated by strong cation exchange, and are eluted onto a second phase by a step gradient of increasing salt concentration. The second phase consists of reversed-phase material that separates on the basis of hydrophobicity. Peptides are then eluted directly onto the MS. Methods for direct analysis of protein mixtures have been applied to a number of systems and have been proven useful over a large dynamic range and for all classes of proteins, including membrane proteins.

Critical Parameters

The success of protein mixture analysis by MS is largely dependent on the success of the methods used to isolate and purify the proteins to be analyzed. For gel-based methods, analysis is usually performed on peptides obtained by enzymatic digestion after separation of the proteins on a gel. Ideally, each band excised from the gel represents a single protein. Prefractiona-

tion of complex samples prior to gel electrophoresis might be required to obtain clean separation. Sample handling steps should be minimized to avoid unnecessary sample loss and to reduce the risk of contamination of the sample with keratin, which can easily obscure the sample protein in the mass spectrum. It is therefore critical to wear gloves when working with the proteins and to avoid exposure to dust. Care must also be taken to eliminate contaminants from glassware and plasticware. Proteins purified on a two-dimensional gel can be extracted from the gel and digested with trypsin prior to analysis on the mass spectrometer, or they can be digested in the gel prior to extraction. The protocol for in-gel digestion of proteins described here (Basic Protocol 1) is a widely used and very effective method. The authors have been able to reduce the time required for this protocol, however, by reducing or eliminating some of the wash steps.

For direct analysis of proteins using LC-MS/MS, low-flow rate LC (200 to 300 nl/min) is necessary to achieve sensitivity at the femtomole scale. Flow rates should not, however, drop below 100 nl/min, or the flow may stop completely. Optimal flow rates can be achieved using a standard LC setup equipped with a precolumn splitting device. Successful chromatography is also dependent on producing, packing, and loading a high-quality microcapillary column. Microcapillaries are most reproducibly constructed using a commercial laser puller, but in some labs they are produced manually by weighting the end of the capillary while heating the center. Microcapillary columns should have an internal diameter of 50 to 100 μm , with a tip internal diameter of $\sim 5 \mu\text{m}$. Microcapillaries with smaller diameters can clog easily; larger diameters result in less-efficient electrospray. Frequently, spray problems that arise from larger column diameters can be offset by increasing the voltage.

Columns should be packed with packing material that is 3 to 10 μm in size. A number of different packing materials are suitable for proteomics applications including Aqua (Phenomex), Betabasic (Thermo Hypersil-Keystone), Targa (Bodman Industries), and Polaris (Metachem). Zorbax (Hewlett-Packard) has also been used but requires the use of an ion-pairing solvent (i.e., 0.02% heptafluorobutyric acid added to HPLC solvents), which may cause signal suppression in the mass spectrometer.

Troubleshooting

For two-dimensional LC-MS/MS analysis, the production of the microcapillary column is critical for success. If the column is not properly pulled, then packing material will not load smoothly and chromatography will be poor. If no solvent flow is seen at the tip of the column during column loading, attempts can be made to slightly open the tip by gently scoring it with a ceramic scribe. If the solvent flow does not improve after a few attempts to score the column tip, it should be abandoned, as it usually proves problematic throughout the rest of the experiment. If the column becomes plugged during loading of the sample, it is frequently a sign that the sample has not been properly centrifuged prior to loading to ensure that particulate in the sample does not enter the column. Often, clogged tips can be opened by immersing the column in hot water. If the column tip breaks after the sample has been loaded, it is possible to pack another microcapillary column with 4 cm of the reversed-phase material and attach the original column with a PEEK connector.

It is important to use clean, fresh solvents for chromatography on the microcapillary column because impure HPLC solvents can result in high background noise. Finally, the mass spectrometer needs to be cleaned and tuned approximately every 10 to 15 days, depending on use. The loss of calibration can usually be detected by a blank MS/MS spectrum or a marked decrease in the size of the raw file in the data output after acquisition. If this happens, the instrument needs to be recalibrated using commercial standards.

Anticipated Results

Identification of proteins by LC-MS/MS or two-dimensional electrophoresis followed by MS/MS is made by the identification of peptides generated by enzymatic digestion of the protein. The fraction of the intact protein sequence represented by the identified peptides is referred to as sequence coverage. Reliable identification of a protein is possible with as little as 5% to 10% coverage. Experimental results depend on the complexity of the sample, the enzyme used to digest the sample, and the source of the sample. In a recent publication, MacCoss et al. (2002) showed sequence coverage for five proteins (Cdc2, Cdc13, Cig 1, Crystallin α A chain, and Crystallin α B chain). Trypsin digestion resulted in sequence coverage ranging from 28.9% to 74.5%. Combined results from digestion with three separate en-

zymes increased the sequence coverage to 57.8% to 94.9%.

Using these methods, all post-translational modifications—including phosphorylation, methylation, and acylation—can be identified. Attachment of carbohydrates and lipids is possible but more complex as these modifications are more labile.

Time Considerations

The time required for LC-MS/MS analysis is dependent on the complexity of the sample and the method used to analyze it. For trypsin-digested, gel-purified proteins, a one-dimensional column with a 20- to 50-min gradient is sufficient, whereas a one-dimensional separation of simple mixtures will usually require a 2- to 3-hr gradient. The time required for a multidimensional protein identification technology (MuDPIT) run is determined by the number of salt bumps incorporated in the experiment. A six-step run lasts ~12 hr and a twelve-step run lasts ~24 hr. Additionally, pulling columns will take less than 5 min and column packing will take 15 to 90 min depending on how many phases are being loaded. Sample loading will take anywhere from 15 min to 5 hr depending on the volume and viscosity of the sample and on the size of the column being loaded.

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Internet Resources

ncbi.nlm.nih.gov/Ftp/index.html

National Center for Biotechnology Information's FTP site, from which genome databases can be downloaded.

genome-www.stanford.edu

Web site for Stanford Genomic Resources, including genome databases.

www.tigr.org

Web site for The Institute for Genome Research, including genome databases.

prospector.ucsf.edu

www.matrixscience.com

prowl.rockefeller.edu/PROWL/pepfragch.html

Sites for Web-based search tools for analyzing MS/MS data.

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CHAPTER 6

Electrophoresis and Immunoblotting

INTRODUCTION

The development of powerful new technologies is a major driving force of scientific progress. A good example of this is the role that electrophoretic techniques have played in the evolution of modern cell biology. Electrophoresis and related applications have contributed greatly to the understanding of the molecular bases of cell structure and function. The combination of high resolution, ease of use, speed, low cost, and versatility of electrophoretic techniques is unmatched by any other method used to separate proteins. It is for this reason that electrophoresis is an indispensable tool in any cell biology laboratory and that papers describing basic techniques of protein electrophoresis (e.g., Laemmli, 1970, and O'Farrell, 1975, to name just a couple) are among the most cited articles in this field. Laemmli's technique of discontinuous gel electrophoresis in the presence of SDS, for example, continues to be widely used and referenced almost 30 years after publication. Thus, no book of techniques in cell biology would be complete without a detailed description of electrophoretic techniques.

Chapter 6 begins with *UNIT 6.1*, which is a collection of state-of-the-art protocols for analyzing proteins by one-dimensional electrophoresis under denaturing conditions on polyacrylamide gels. Sodium dodecyl sulfate (SDS), in combination with a reducing agent and heat, is most often used as a denaturant. This type of electrophoresis is thus referred to as SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Denaturation of the proteins prior to electrophoresis allows for enhanced resolution and discrimination of proteins on the basis of molecular size rather than charge or shape. Utilization of a discontinuous system (i.e., the apposition of "stacking" and "separating" gels) results in concentration of dilute samples and enhanced band sharpness. *UNIT 6.1* presents an overview of electricity and electrophoresis, followed by detailed protocols for SDS-PAGE using either Laemmli's buffers and gel system or modifications of this system (i.e., use of Tris-tricine buffers, higher concentrations of buffers, gradient gels, single-concentration gels, and minigels). The unit also explains how to calculate the apparent molecular weights of proteins from SDS-PAGE data.

The next unit in the chapter, *UNIT 6.2*, describes protocols for immunoblotting (also referred to as western blotting). In this technique, proteins separated by any of the electrophoretic techniques described in *UNIT 6.1* are electrophoretically transferred ("electroblotted") onto a membrane. The membrane, which thus becomes a replica of the polyacrylamide gel, is subsequently probed with antibodies to specific proteins. The primary antibodies can be revealed by an additional incubation with ¹²⁵I-labeled secondary antibodies or protein A, followed by autoradiography (*UNIT 6.3*). In recent years, however, the use of radioiodinated antibodies has been progressively replaced by nonradioactive detection with antibodies coupled to enzymes such as alkaline phosphatase or horseradish peroxidase (see *UNIT 16.5*). The enzymes act on substrates which are converted to colored, luminescent, or fluorescent products. Nonradioactive methods are just as sensitive as radioactive methods, with the added advantage that they do not require the special precautions associated with the use of radioactivity. Nonradioactive detection is nowadays the method of choice for visualizing immunoblotted proteins. A disadvantage of nonradioactive methods is that they have a narrower linear range of detection, which can be a problem in experiments that require accurate quantitation of protein levels.

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Electrophoresis
and
Immunoblotting

6.0.1

Supplement 15

Radiolabeled proteins separated by electrophoresis or proteins detected by immunoblotting with radioiodinated antibodies or protein A can be visualized by autoradiography, as described in *UNIT 6.3*. In this technique, ionizing radiation emanating from the radionuclides impresses a photographic film. The technique can be made more sensitive by the use of intensifying screens or scintillating compounds, which emit light upon radiation absorption, which then impresses the film. The unit contains several protocols for autoradiographic detection of various radionuclides, including methods for enhancing the signal with intensifying screens or by fluorography. Also included in *UNIT 6.3* are discussions of the quantification of film images by densitometry and the direct detection and quantification of radioactive samples in gels by phosphor imaging.

The resolution of electrophoretic techniques can be enormously enhanced by combining two different electrophoretic procedures performed successively in perpendicular directions (i.e., two-dimensional gel electrophoresis). The most common type of two-dimensional gel electrophoresis is based on separation of proteins by isoelectric focusing on a tube gel (first dimension) followed by SDS-PAGE on a slab gel (second dimension). The two processes separate proteins on the basis of charge and size, respectively, allowing resolution of up to several thousand proteins on a single two-dimensional gel. *UNIT 6.4* describes several methods for separating proteins by two-dimensional isoelectric focusing/SDS-PAGE. In addition, this unit presents a protocol for two-dimensional nonreducing/reducing electrophoresis in which proteins are separated by SDS-PAGE under nonreducing conditions in the first dimension and under reducing conditions in the second dimension. This type of two-dimensional gel electrophoresis allows analysis of intersubunit disulfide bonds in multiprotein complexes and, in some cases, of intrasubunit disulfide bonds. Both types of two-dimensional gel electrophoresis can be used for either analytical or preparative purposes.

Another useful method for electrophoretic separation of cellular proteins is one-dimensional electrophoresis under nondenaturing conditions. Two protocols describing variations of this method are included in *UNIT 6.5*. What distinguishes this method from those described in *UNIT 6.1* and *UNIT 6.4* is that protein samples are not exposed to denaturing agents (i.e., SDS or urea) either prior to or during electrophoresis. Thus, proteins migrate according to their native properties, such as size, shape, and charge. This allows analysis of the oligomeric state of proteins, conformational changes, charge heterogeneity, and post-translational modifications that affect conformation or charge while having minimal effects on the molecular weights of the proteins. In many cases, this method preserves the intrinsic function of the proteins, which allows their detection with specific activity or binding assays. The first protocol describes continuous electrophoresis on nondenaturing polyacrylamide gels. This system involves electrophoresis on a single separating gel and uses the same buffer in the chambers and the gel. The second protocol, discontinuous electrophoresis on nondenaturing polyacrylamide gels, is a variation of SDS-PAGE in which SDS and reducing agents are omitted from all the solutions. Determination of the migration of proteins on parallel gels made up of different concentrations of acrylamide and bisacrylamide allows calculation of their molecular weights using Ferguson plots.

Proteins separated by electrophoresis can be visualized by direct staining of the gels. Four procedures for staining proteins in gels based on different principles are presented in *UNIT 6.6*. These procedures involve staining with Coomassie blue, silver, SYPRO ruby or zinc ions. The unit describes the basic protocols and provides guidelines for the selection of a specific protocol.

The separation of proteins by polyacrylamide gel electrophoresis is limited to proteins with molecular weights less than ~300,000. The electrophoretic separation of larger proteins or multiprotein complexes requires the use of other matrix materials. A suitable material is agarose, which is most commonly used for the separation of DNA. *UNIT 6.7* presents protocols for the electrophoretic separation of proteins on agarose gels. In

addition to permitting the separation of very large proteins, these protocols allow the analysis of multimerization or aggregation. Although these processes can also be analyzed by sedimentation on sucrose gradients (*UNIT 6.3*) or gel filtration (*UNIT 6.4*), agarose gel electrophoresis is more convenient for analysis of large numbers of samples.

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One-Dimensional SDS Gel Electrophoresis of Proteins

Electrophoresis is used to separate complex mixtures of proteins, (e.g., from cells, subcellular fractions, column fractions, or immunoprecipitates) to investigate subunit compositions, and to verify homogeneity of protein samples. It can also serve to purify proteins for use in further applications. In polyacrylamide gel electrophoresis, proteins migrate in response to an electrical field through pores in the gel matrix; pore size decreases with higher acrylamide concentrations. The combination of gel pore size and protein charge, size, and shape determines the migration rate of the protein.

The standard Laemmli method (see Basic Protocol 1) is used for discontinuous gel electrophoresis under denaturing conditions, that is, in the presence of sodium dodecyl sulfate (SDS). The standard method for full-size gels (e.g., 14 × 14 cm) can be adapted for the minigel format (e.g., 7.3 × 8.3 cm; see Basic Protocol 2). Minigels provide rapid separation but give lower resolution.

Several alternate protocols are provided for specific applications. The first two alternate protocols cover electrophoresis of peptides and small proteins, separations that require modification of standard buffers: either a Tris-tricine buffer system (see Alternate Protocol 1), or modified Tris buffer in the absence of urea (see Alternate Protocol 2). Continuous SDS-PAGE is a simplified method in which the same buffer is used for both gel and electrode solutions and the stacking gel is omitted (see Alternate Protocol 3). Other protocols cover the preparation and electrophoresis of various types of gels: ultrathin gels (see Alternate Protocol 4), multiple single-concentration gels (see Support Protocol 1), gradient gels (see Alternate Protocol 5), multiple gradient gels (see Support Protocol 2), and multiple gradient minigels (see Support Protocol 3). Proteins separated on gels can be subsequently analyzed by immunoblotting (UNIT 6.2), autoradiography, or phosphor imaging (UNIT 6.3), or staining with protein dyes.

CAUTION: Before any protocols are used, it is extremely important to read the following section about electricity and electrophoresis.

ELECTRICITY AND ELECTROPHORESIS

Many researchers are poorly informed concerning the electrical parameters of running a gel. It is important to note that the voltages and currents used during electrophoresis are dangerous and potentially lethal. Thus, safety should be an overriding concern. A working knowledge of electricity is an asset in determining what conditions to use and in troubleshooting the electrophoretic separation, if necessary. For example, an unusually high or low voltage for a given current (milliampere) might indicate an improperly made buffer or an electrical leak in the chamber.

Safety Considerations

1. Never remove or insert high-voltage leads unless the power supply voltage is turned down to zero and the power supply is turned off. Always grasp high-voltage leads one at a time with one hand only. Never insert or remove high-voltage leads with both hands. This can shunt potentially lethal electricity through the chest and heart should electrical contact be made between a hand and a bare wire. On older or homemade instruments, the banana plugs may not be shielded and can still be connected to the

power supply at the same time they make contact with a hand. Carefully inspect all cables and connections and replace frayed or exposed wires immediately.

2. Always start with the power supply turned off. Have the power supply controls turned all the way down to zero. Then hook up the gel apparatus: generally, connect the red high-voltage lead to the red outlet and the black high-voltage lead to the black outlet. Turn the power supply on with the controls set at zero and the high-voltage leads connected. Then, turn up the voltage, current, or power to the desired level. Reverse the process when the power supply is turned off: i.e., to disconnect the gel, turn the power supply down to zero, wait for the meters to read zero, turn off the power supply, and then disconnect the gel apparatus one lead at a time.

CAUTION: If the gel is first disconnected and then the power supply turned off, a considerable amount of electrical charge is stored internally. The charge will stay in the power supply over a long time. This will discharge through the outlets even though the power supply is turned off and can deliver an electrical shock.

Ohm's Law and Electrophoresis

Understanding how a gel apparatus is connected to the power supply requires a basic understanding of Ohm's law: voltage = current \times resistance, or $V = IR$. A gel can be viewed as a resistor and the power supply as the voltage and current source. Most power supplies deliver constant current or constant voltage. Some will also deliver constant power: power = voltage \times current, or $VI = I^2R$. The discussion below focuses on constant current because this is the most common mode in vertical SDS-PAGE.

Most modern commercial equipment is color-coded so that the red or positive terminal of the power supply can simply be connected to the red lead of the gel apparatus, which goes to the lower buffer chamber. The black lead is connected to the black or negative terminal and goes to the upper buffer chamber. This configuration is designed to work with vertical slab gel electrophoreses in which negatively charged proteins or nucleic acids move to the positive electrode in the lower buffer chamber (an anionic system).

When a single gel is attached to a power supply, the negative charges flow from the negative cathode (black) terminal into the upper buffer chamber, through the gel, and into the lower buffer chamber. The lower buffer chamber is connected to the positive anode (red) terminal to complete the circuit. Thus, negatively charged molecules, such as SDS-coated proteins and nucleic acids, move from the negative cathode attached to the upper buffer chamber toward the positive anode attached to the lower chamber. SDS-PAGE is an anionic system because of the negatively charged SDS.

Occasionally, proteins are separated in cationic systems. In these gels, the proteins are positively charged because of the very low pH of the gel buffers (e.g., acetic acid/urea gels for histone separations) or the presence of a cationic detergent (e.g., cetyltrimethylammonium bromide, CTAB). Proteins move toward the negative electrode (cathode) in cationic gel systems, and the polarity is reversed compared to SDS-PAGE: the red lead from the lower buffer chamber is attached to the black outlet of the power supply, and the black lead from the upper buffer chamber is attached to the red outlet of the power supply.

Most SDS-PAGE separations are performed under constant current (consult instructions from the manufacturer to set the power supply for constant current operation). The resistance of the gel will increase during SDS-PAGE in the standard Laemmli system. If the current is constant, then the voltage will increase during the run as the resistance goes up.

Power supplies usually have more than one pair of outlets. The pairs are connected in parallel with one another internally. If more than one gel is connected directly to the outlets

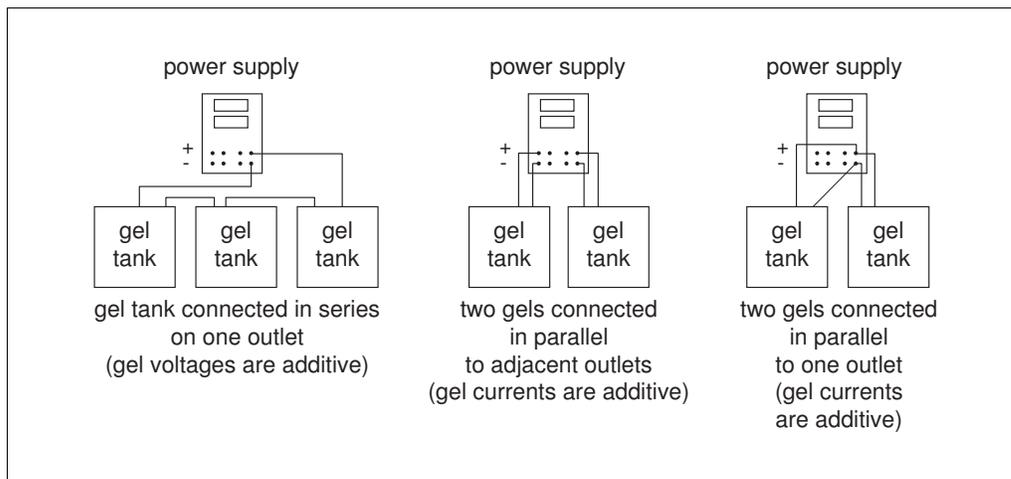


Figure 6.1.1 Series and parallel connections of gel tanks to power supply.

of a power supply, then these gels are connected in parallel. In a parallel circuit, the voltage is the same across each gel. In other words, if the power supply reads 100 V, then each gel has 100 V across its electrodes. The total current, however, is the sum of the individual currents going through each gel. Therefore, under constant current it is necessary to increase the current for each additional gel that is connected to the power supply. Two identical gels require double the current to achieve the same starting voltages and electrophoresis separation times.

Multiple gel apparatuses can also be connected to one pair of outlets on a power supply. This is useful with older power supplies that have a limited number of outlets. When connecting several gel units to one outlet, make certain the connections between the units are shielded and protected from moisture. The gels can be connected in parallel or in series (Fig. 6.1.1). In the case of two or more gels running off the same outlet in series, the current is the same for every gel. If 10 mA is displayed by the power supply meter, for example, each gel in series will experience 10 mA. The voltage, however, is additive for each gel. If one gel at a constant 10 mA produces 100 V, then two identical gels in series will produce 200 V (100 V each) and so on. Thus, the voltage can limit the number of units connected in series on low-voltage power supplies.

Gel thickness affects the above relationships. A 1.5-mm gel can be thought of as consisting of two 0.75-mm-thick gels run in parallel. Because currents are additive in parallel circuits, a 0.75-mm gel will require half the current of the 1.5-mm gel to achieve the same starting voltage and separation time. If a gel thickness is doubled, then the current must also be doubled. There are limits to the amount of current that can be applied. Thicker gels require more current, generating more heat that must be dissipated. Unless temperature control is available in the gel unit, a thick gel should be run more slowly than a thin gel.

NOTE: Milli-Q-purified water or equivalent should be used throughout the protocols.

**DENATURING (SDS) DISCONTINUOUS GEL ELECTROPHORESIS:
LAEMMLI GEL METHOD**

One-dimensional gel electrophoresis under denaturing conditions (i.e., in the presence of 0.1% SDS) separates proteins based on molecular size as they move through a polyacrylamide gel matrix toward the anode. The polyacrylamide gel is cast as a separating gel (sometimes called resolving or running gel) topped by a stacking gel and secured in an electrophoresis apparatus. After sample proteins are solubilized by boiling in the presence of SDS, an aliquot of the protein solution is applied to a gel lane, and the individual proteins are separated electrophoretically. 2-Mercaptoethanol (2-ME) or dithiothreitol (DTT) is added during solubilization to reduce disulfide bonds.

This protocol is designed for a vertical slab gel with a maximum size of 0.75 mm × 14 cm × 14 cm. For thicker gels, or minigels (see Basic Protocol 2 and Support Protocol 3), the volumes of stacking and separating gels and the operating current must be adjusted. Additional protocols describe the preparation of ultrathin gels (see Alternate Protocol 4) and gradient gels (see Alternate Protocol 5), as well as the use of gel casters to make multiple gels, both single-concentration gels (see Support Protocol 1) and gradient gels (see Support Protocol 2).

Materials

- Separating and stacking gel solutions (Table 6.1.1)
- H₂O-saturated isobutyl alcohol
- 1× Tris·Cl/SDS, pH 8.8 (dilute 4× Tris·Cl/SDS, pH 8.8; Table 6.1.1)
- Protein sample to be analyzed
- 2× and 1× SDS sample buffer (see recipe)
- Protein molecular-weight-standards mixture (Table 6.1.2)
- 6× SDS sample buffer (see recipe; optional)
- 1× SDS electrophoresis buffer (see recipe)

- Electrophoresis apparatus: Protean II 16-cm cell (Bio-Rad) or SE 600/400 16-cm unit (Amersham Pharmacia Biotech) with clamps, glass plates, casting stand, and buffer chambers
- 0.75-mm spacers
- 0.45-μm filters (used in stock solution preparation)
- 25-ml Erlenmeyer side-arm flask
- Vacuum pump with cold trap
- 0.75-mm Teflon comb with 1, 3, 5, 10, 15, or 20 teeth
- 25- or 100-μl syringe with flat-tipped needle
- Constant-current power supply (see introduction)

Pour the separating gel

1. Assemble the glass-plate sandwich of the electrophoresis apparatus according to manufacturer's instructions using two clean glass plates and two 0.75-mm spacers.

If needed, clean the glass plates in liquid Alconox or RBS-35 (Pierce). These aqueous-based solutions are compatible with silver and Coomassie blue staining procedures.

2. Lock the sandwich to the casting stand.
3. Prepare the separating gel solution as directed in Table 6.1.1, degassing using a rubber-stoppered 25-ml Erlenmeyer side-arm flask connected with vacuum tubing to a vacuum pump with a cold trap. After adding the specified amount of 10% ammonium persulfate and TEMED to the degassed solution, stir gently to mix.

Table 6.1.1 was prepared as a convenient summary to aid in the preparation of separating and stacking gels. The stacking gel is the same regardless of the separating gel used.

The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. Generally, use 5% gels for SDS-denatured proteins of 60 to 200 kDa, 10% gels for SDS-denatured proteins of 16 to 70 kDa, and 15% gels for SDS-denatured proteins of 12 to 45 kDa (Table 6.1.1).

- Using a Pasteur pipet, apply the separating gel solution to the sandwich along an edge of one of the spacers until the height of the solution between the glass plates is ~11 cm.

Use the solution immediately; otherwise it will polymerize in the flask.

Sample volumes <10 μ l do not require a stacking gel. In this case, cast the resolving gel as you normally would, but extend the resolving gel into the comb to form the well. The proteins are then separated under the same conditions as used when a stacking gel is present. Although this protocol works well with single-concentration gels, a gradient gel is recommended for maximum resolution (see Alternate Protocol 5).

- Using another Pasteur pipet, slowly cover the top of the gel with a layer (~1 cm thick) of H₂O-saturated isobutyl alcohol, by gently layering the isobutyl alcohol against the edge of one and then the other of the spacers.

Be careful not to disturb the gel surface. The overlay provides a barrier to oxygen, which inhibits polymerization, and allows a flat interface to form during gel formation.

The H₂O-saturated isobutyl alcohol is prepared by shaking isobutyl alcohol and H₂O in a separatory funnel. The aqueous (lower) phase is removed. This procedure is repeated several times. The final upper phase is H₂O-saturated isobutyl alcohol.

- Allow the gel to polymerize 30 to 60 min at room temperature.

A sharp optical discontinuity at the overlay/gel interface will be visible on polymerization. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED (N, N, N', N'-tetramethylethylenediamine), or both. Ammonium persulfate solution should be made fresh before use. Ammonium persulfate should "crackle" when added to the water. If not, fresh ammonium persulfate should be purchased. Purchase TEMED in small bottles so, if necessary, a new previously unopened source can be tried.

Pour the stacking gel

- Pour off the layer of H₂O-saturated isobutyl alcohol and rinse with 1× Tris-Cl/SDS, pH 8.8.

Residual isobutyl alcohol can reduce resolution of the protein bands; therefore, it must be completely removed. The isobutyl alcohol overlay should not be left on the gel longer than 2 hr.

- Prepare the stacking gel solution as directed in Table 6.1.1.

Use the solution immediately to keep it from polymerizing in the flask.

- Using a Pasteur pipet, slowly allow the stacking gel solution to trickle into the center of the sandwich along an edge of one of the spacers until the height of the solution in the sandwich is ~1 cm from the top of the plates.

Be careful not to introduce air bubbles into the stacking gel.

- Insert a 0.75-mm Teflon comb into the layer of stacking gel solution. If necessary, add additional stacking gel to fill the spaces in the comb completely.

Again, be careful not to trap air bubbles in the tooth edges of the comb; they will cause small circular depressions in the well after polymerization that will lead to distortion in the protein bands during separation.

- Allow the stacking gel solution to polymerize 30 to 45 min at room temperature.

A sharp optical discontinuity will be visible around wells on polymerization.

Table 6.1.1 Recipes for Polyacrylamide Separating and Stacking Gels^a**SEPARATING GEL**

Stock solution ^b	Final acrylamide concentration in separating gel (%) ^c									
	5	6	7	7.5	8	9	10	12	13	15
30% acrylamide/ 0.8% bisacrylamide	2.50	3.00	3.50	3.75	4.00	4.50	5.00	6.00	6.50	7.50
4× Tris·Cl/SDS, pH 8.8	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H ₂ O	8.75	8.25	7.75	7.50	7.25	6.75	6.25	5.25	4.75	3.75
10% (w/v) ammonium persulfate ^d	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Preparation of separating gel

In a 25-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution, 4× Tris·Cl/SDS, pH 8.8 (see reagents, below), and H₂O. Degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

STACKING GEL (3.9% acrylamide)

In a 25-ml side-arm flask, mix 0.65 ml of 30% acrylamide/0.8% bisacrylamide, 1.25 ml of 4× Tris·Cl/SDS, pH 6.8 (see reagents, below), and 3.05 ml H₂O. Degas under vacuum 10 to 15 min. Add 25 µl of 10% ammonium persulfate and 5 µl TEMED. Swirl gently to mix. Use immediately. Failure to form a firm gel usually indicates a problem with the persulfate, TEMED, or both.

REAGENTS USED IN GELS**30% acrylamide/0.8% bisacrylamide**

Mix 30.0 g acrylamide and 0.8 g *N,N'*-methylenebisacrylamide with H₂O in a total volume of 100 ml. Filter the solution through a 0.45-µm filter and store at 4°C in the dark. The 2× crystallized grades of acrylamide and bisacrylamide are recommended. Discard after 30 days, as acrylamide gradually hydrolyzes to acrylic acid and ammonia.

CAUTION: *Acrylamide monomer is neurotoxic. A mask should be worn when weighing acrylamide powder. Gloves should be worn while handling the solution, and the solution should not be pipetted by mouth.*

4× Tris·Cl/SDS, pH 6.8 (0.5 M Tris·Cl containing 0.4% SDS)

Dissolve 6.05 g Tris base in 40 ml H₂O. Adjust to pH 6.8 with 1 N HCl. Add H₂O to 100 ml total volume. Filter the solution through a 0.45-µm filter, add 0.4 g SDS, and store at 4°C up to 1 month.

4× Tris·Cl/SDS, pH 8.8 (1.5 M Tris·Cl containing 0.4% SDS)

Dissolve 91 g Tris base in 300 ml H₂O. Adjust to pH 8.8 with 1 N HCl. Add H₂O to 500 ml total volume. Filter the solution through a 0.45-µm filter, add 2 g SDS, and store at 4°C up to 1 month.

^aThe recipes produce 15 ml of separating gel and 5 ml of stacking gel, which are adequate for a gel of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the SDS (denaturing) discontinuous buffer system of Laemmli (1970).

^bAll reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

^cUnits of numbers in table body are milliliters. The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. See annotation to step 3, Basic Protocol 1.

^dBest to prepare fresh.

Prepare the sample and load the gel

12. Dilute a portion of the protein sample to be analyzed 1:1 (v/v) with 2× SDS sample buffer and heat 3 to 5 min at 100°C in a sealed screw-cap microcentrifuge tube. If the sample is a precipitated protein pellet, dissolve the protein in 50 to 100 µl of 1× SDS sample buffer and boil 3 to 5 min at 100°C. Dissolve protein-molecular-weight standards mixture in 1× SDS sample buffer according to supplier's instructions; use these standards as a control (Table 6.1.2).

For dilute protein solutions, consider adding 5:1 protein solution/6× SDS sample buffer to increase the amount of protein loaded. Proteins can also be concentrated by precipitation in acetone, ethanol, or trichloroacetic acid (TCA), but losses will occur.

For a 0.8-cm-wide well, 25 to 50 µg total protein in <20 µl is recommended for a complex mixture when staining with Coomassie blue, and 1 to 10 µg total protein is needed for samples containing one or a few proteins. If silver staining is used, 10- to 100-fold less protein can be applied (0.01 to 5 µg in <20 µl depending on sample complexity).

To achieve the highest resolution possible, the following precautions are recommended. Prior to adding the sample buffer, keep samples at 0°C. Add the SDS sample buffer (room temperature) directly to the 0°C sample (still on ice) in a screw-top microcentrifuge tube. Cap the tube to prevent evaporation, vortex, and transfer directly to a 100°C water bath for 3 to 5 min. Let immunoprecipitates dissolve for 1 hr at 56°C in 1× SDS sample buffer prior to boiling. DO NOT leave the sample in SDS sample buffer at room temperature without first heating to 100°C to inactivate proteases (see Critical Parameters and Troubleshooting). Endogenous proteases are very active in SDS sample buffer and will cause severe degradation of the sample proteins after even a few minutes at room temperature. To test for possible proteases, mix the sample with SDS sample buffer without heating and leave at room temperature for 1 to 3 hr. A loss of high-molecular-weight bands

Table 6.1.2 Molecular Weights of Protein Standards for Polyacrylamide Gel Electrophoresis^a

Protein	Molecular weight
Cytochrome <i>c</i>	11,700
α-Lactalbumin	14,200
Lysozyme (hen egg white)	14,300
Myoglobin (sperm whale)	16,800
β-Lactoglobulin	18,400
Trypsin inhibitor (soybean)	20,100
Trypsinogen, PMSF treated	24,000
Carbonic anhydrase (bovine erythrocytes)	29,000
Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)	36,000
Lactate dehydrogenase (porcine heart)	36,000
Aldolase	40,000
Ovalbumin	45,000
Catalase	57,000
Bovine serum albumin	66,000
Phosphorylase <i>b</i> (rabbit muscle)	97,400
β-Galactosidase	116,000
RNA polymerase, <i>E. coli</i>	160,000
Myosin, heavy chain (rabbit muscle)	205,000

^aProtein standards are commercially available in kits (e.g., Amersham Pharmacia Biotech, Life Technologies, Bio-Rad, or Sigma).

and a general smearing of the banding pattern indicate a protease problem. Once heated, the samples can sit at room temperature for the time it takes to load samples.

- Carefully remove the Teflon comb without tearing the edges of the polyacrylamide wells. After the comb is removed, rinse wells with 1× SDS electrophoresis buffer.

The rinse removes unpolymerized monomer; otherwise, the monomer will continue to polymerize after the comb is removed, creating uneven wells that will interfere with sample loading and subsequent separation.

- Using a Pasteur pipet, fill the wells with 1× SDS electrophoresis buffer.

If well walls are not upright, they can be manipulated with a flat-tipped needle attached to a syringe.

- Attach gel sandwich to upper buffer chamber following manufacturer's instructions.

- Fill lower buffer chamber with the recommended amount of 1× SDS electrophoresis buffer.

- Place sandwich attached to upper buffer chamber into lower buffer chamber.

- Partially fill the upper buffer chamber with 1× SDS electrophoresis buffer so that the sample wells of the stacking gel are filled with buffer.

Monitor the upper buffer chamber for leaks and if necessary, reassemble the unit. A slow leak in the upper buffer chamber may cause arcing around the upper electrode and damage the upper buffer chamber.

- Using a 25- or 100- μ l syringe with a flat-tipped needle, load the protein sample(s) into one or more wells by carefully applying the sample as a thin layer at the bottom of the wells. Load control wells with molecular weight standards. Add an equal volume of 1× SDS sample buffer to any empty wells to prevent spreading of adjoining lanes.

Preparing the samples at approximately the same concentration and loading an equal volume to each well will ensure that all lanes are the same width and that the proteins run evenly. If unequal volumes of sample buffer are added to wells, the lane with the larger volume will spread during electrophoresis and constrict the adjacent lanes, causing distortions.

The samples will layer on the bottom of the wells because the glycerol added to the sample buffer gives the solution a greater density than the electrophoresis buffer. The bromphenol blue in the sample buffer makes sample application easy to follow visually.

- Fill the remainder of the upper buffer chamber with additional 1× SDS electrophoresis buffer so that the upper platinum electrode is completely covered. Do this slowly so that samples are not swept into adjacent wells.

Run the gel

- Connect the power supply to the cell and run at 10 mA of constant current for a slab gel 0.75 mm thick, until the bromphenol blue tracking dye enters the separating gel. Then increase the current to 15 mA.

For a standard 16-cm gel sandwich, 4 mA per 0.75-mm-thick gel will run ~15 hr (i.e., overnight); 15 mA per 0.75-mm gel will take 4 to 5 hr. To run two gels or a 1.5-mm-thick gel, simply double the current. When running a 1.5-mm gel at 30 mA, the temperature must be controlled (10° to 20°C) with a circulating constant-temperature water bath to prevent "smiling" (curvature in the migratory band). Temperatures <5°C should not be used because SDS in the running buffer will precipitate. If the level of buffer in the upper chamber decreases, a leak has occurred.

22. After the bromphenol blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.

Refer to Safety Considerations under Electricity and Electrophoresis.

Disassemble the gel

23. Discard electrode buffer and remove the upper buffer chamber with the attached gel sandwich.
24. Orient the gel so that the order of the sample wells is known, remove the sandwich from the upper buffer chamber, and lay the sandwich on a sheet of absorbent paper or paper towels.
25. Carefully slide one of the spacers halfway from the edge of the sandwich along its entire length. Use the exposed spacer as a lever to pry open the glass plate, exposing the gel.
26. Carefully remove the gel from the lower plate. Cut a small triangle off one corner of the gel so the lane orientation is not lost during staining and drying. Proceed with protein detection.

The gel can be stained with Coomassie blue or silver (APPENDIX 3), or proteins can be electroeluted, electroblotted onto a polyvinylidene difluoride (PVDF) membrane for subsequent staining or sequence analysis, or transferred to a membrane for immunoblotting UNIT 6.2. If the proteins are radiolabeled, they can be detected by autoradiography (UNIT 6.3).

ELECTROPHORESIS IN TRIS-TRICINE BUFFER SYSTEMS

Separation of peptides and proteins under 10 to 15 kDa is not possible in the traditional Laemmli discontinuous gel system (Basic Protocol 1). This is due to the comigration of SDS and smaller proteins, obscuring the resolution. Two approaches to obtain the separation of small proteins and peptides in the range of 5 to 20 kDa are presented: the following Tris-tricine method and a system using increased buffer concentrations (see Alternate Protocol 2). The Tris-tricine system uses a modified buffer to separate the SDS and peptides, thus improving resolution. Several precast gels are available for use with the tricine formulations (Table 6.1.3).

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Table 6.1.3 Vertical Format Precast Gel Compatibility

Gel type and compatibility	Gel supplier			
	Bio-Rad	ISS/Daiichi	Jule	Novex
<i>SDS-PAGE gel type offered</i>				
Peptide (tricine)	×	×	×	×
Single concentration	×	×	×	×
Gradient	×	×	×	×
Minigel size	×	×	×	×
Standard gel size		×	×	
<i>Compatibility of gel with equipment manufactured by</i>				
Amersham Pharmacia Biotech		×	×	×
Bio-Rad	×	×	×	
Life Technologies	×	×	×	×
Novex		×		×
ISS/Daiichi		×	×	

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Additional Materials (also see Basic Protocol 1)

Separating and stacking gel solutions (Table 6.1.4)
2× tricine sample buffer (see recipe)
Peptide molecular-weight-standards mixture (Table 6.1.5)
Cathode buffer (see recipe)
Anode buffer (see recipe)
Coomassie blue G-250 staining solution (see recipe)
10% (v/v) acetic acid

1. Prepare and pour the separating and stacking gels (see Basic Protocol 1, steps 1 to 11), using Table 6.1.4 in place of Table 6.1.1.
2. Prepare the sample (see Basic Protocol 1, step 12), but make the following changes for tricine gels. Substitute 2× tricine sample buffer for the 2× SDS sample buffer. Dilute an aliquot of the protein or peptide sample to be analyzed 1:1 (v/v) with 2× tricine sample buffer. Treat the sample at 40°C for 30 to 60 min prior to loading.

If proteolytic activity is a problem, heating samples to 100°C for 3 to 5 min before loading the wells may be required (see Basic Protocol 1, annotation to step 12). Use the peptide molecular-weight-standards mixture for peptide separations (Table 6.1.5).
3. Load the gel and set up the electrophoresis apparatus (see Basic Protocol 1, steps 13 to 20) with the following alterations. Remove comb and, using the tricine-containing

Table 6.1.4 Recipes for Tricine Peptide Separating and Stacking Gels^a

SEPARATING AND STACKING GELS

Stock solution ^b	Separating gel	Stacking gel
30% acrylamide/0.8% bisacrylamide	9.80 ml	1.62 ml
Tris·Cl/SDS, pH 8.45	10.00 ml	3.10 ml
H ₂ O	7.03 ml	7.78 ml
Glycerol	4.00 g (3.17 ml)	—
10% (w/v) ammonium persulfate ^c	50 μl	25 μl
TEMED	10 μl	5 μl

Prepare separating and stacking gel solutions separately.

In a 50-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (Table 6.1.1), Tris·Cl/SDS, pH 8.45 (see reagents, below), and H₂O. Add glycerol to separating gel only. Degas under vacuum 10 to 15 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix, use immediately. Failure to form a firm gel usually indicates a problem with the persulfate, TEMED, or both.

ADDITIONAL REAGENTS USED IN GELS

Tris·Cl/SDS, pH 8.45 (3.0 M Tris·Cl containing 0.3% SDS)

Dissolve 182 g Tris base in 300 ml H₂O. Adjust to pH 8.45 with 1 N HCl. Add H₂O to 500 ml total volume. Filter the solution through a 0.45-μm filter, add 1.5 g SDS, and store at 4°C up to 1 month.

^aThe recipes produce 30 ml of separating gel and 12.5 ml of stacking gel, which are adequate for two gels of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the Tris-tricine buffer system of Schagger and von Jagow (1987).

^bAll reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

^cBest to prepare fresh.

Table 6.1.5 Molecular Weights of Peptide Standards for Polyacrylamide Gel Electrophoresis^a

Peptide	Molecular weight (Da)
Myoglobin (polypeptide backbone)	16,950
Myoglobin 1-131	14,440
Myoglobin 56-153	10,600
Myoglobin 56-131	8,160
Myoglobin 1-55	6,210
Glucagon	3,480
Myoglobin 132-153	2,510

^aPeptide standards are commercially available from Sigma.

cathode buffer, or water, rinse once and fill wells. Fill the lower buffer chamber with anode buffer, assemble the unit, and attach the upper buffer chamber. Fill the upper buffer chamber with cathode buffer and load the samples.

4. Connect the power supply to the cell and run 1 hr at 30 V (constant voltage) followed by 4 to 5 hr at 150 V (constant voltage). Use heat exchanger to keep the electrophoresis chamber at room temperature.
5. After the tracking dye has reached the bottom of the separating gel, disconnect the power supply.

Refer to Safety Considerations in the introduction.

Coomassie blue G-250 is used as a tracking dye instead of bromphenol blue because it moves ahead of the smallest peptides.

6. Disassemble the gel (see Basic Protocol 1, steps 23 to 26). Stain proteins in the gel for 1 to 2 hr in Coomassie blue G-250 staining solution. Follow by destaining with 10% acetic acid, changing the solution every 30 min until background is clear (3 to 5 changes). For higher sensitivity, use silver staining as a recommended alternative.

Prolonged staining and destaining will result in the loss of resolution of the smaller proteins (<10 kDa). Proteins diffuse within the gel and out of the gel, resulting in a loss of staining intensity and resolution.

NONUREA PEPTIDE SEPARATIONS WITH TRIS BUFFERS

A simple modification of the traditional Laemmli buffer system presented in Basic Protocol 1, in which the increased concentration of buffers provides better separation between the stacked peptides and the SDS micelles, permits reasonable separation of peptides as small as 5 kDa.

Additional Materials (also see Basic Protocol 1)

- Separating and stacking gel solutions (Table 6.1.6)
- 2× SDS electrophoresis buffer (see recipe)
- 2× Tris·Cl/SDS, pH 8.8 (dilute 4× Tris·Cl/SDS, pH 8.8; Table 6.1.1)

1. Prepare and pour the separating gel (see Basic Protocol 1, steps 1 to 6) using Table 6.1.6 in place of Table 6.1.1.
2. Prepare and pour the stacking gel (see Basic Protocol 1, steps 7 to 11), using 2× Tris·Cl/SDS, pH 8.8, rather than 1× Tris·Cl/SDS buffer, for rinsing the gel after removing the isobutyl alcohol overlay.

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3. Prepare the sample and load the gel (see Basic Protocol 1, steps 12 to 20) and substitute 2× SDS electrophoresis buffer for the 1× SDS electrophoresis buffer.

Table 6.1.5 lists the standards for small protein separations.

4. Run the gel (see Basic Protocol 1, steps 21 and 22).

Note that the separations will take ~25% longer than those using Basic Protocol 1. The increased buffer concentrations lead to faster transit through the stacking gel but lower mobility in the resolving gel.

5. Disassemble the gel (see Basic Protocol 1, steps 23 to 26).

Proteins in the gel may now be stained.

Table 6.1.6 Recipes for Modified Laemmli Peptide Separating and Stacking Gels^a

SEPARATING AND STACKING GELS

Stock solution ^b	Separating gel	Stacking gel
30% acrylamide/0.8% bisacrylamide	10.00 ml	0.65 ml
8× Tris·Cl, pH 8.8	3.75 ml	—
4× Tris·Cl, pH 6.8	—	1.25 ml
10% (w/v) SDS	0.15 ml	50 μl
H ₂ O	1.00 ml	3.00 ml
10% (w/v) ammonium persulfate ^c	50 μl	25 μl
TEMED	10 μl	5 μl

Prepare separating and stacking gel solutions separately

In a 25-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 8× Tris·Cl, pH 8.8 (separating gel) or 4× Tris·Cl, pH 6.8 (stacking gel), 10% SDS (see reagents, below), and H₂O. Degas under vacuum 10 to 15 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix; use immediately. Failure to form a firm gel usually indicates a problem with the persulfate, TEMED, or both.

ADDITIONAL REAGENTS USED IN GELS

4× Tris·Cl, pH 6.8 (0.5 M Tris·Cl)

Dissolve 6.05 g Tris base in 40 ml H₂O. Adjust to pH 6.8 with 1 N HCl. Add H₂O to 100 ml total volume. Filter the solution through a 0.45-μm filter and store up to 1 month at 4°C.

8× Tris·Cl, pH 8.8 (3.0 M Tris·Cl)

Dissolve 182 g Tris base in 300 ml H₂O. Adjust to pH 8.8 with 1 N HCl. Add H₂O to 500 ml total volume. Filter the solution through a 0.45-μm filter and store up to 1 month at 4°C.

10% (w/v) SDS

Mix 1 g SDS in 10 ml of H₂O. Use immediately.

^aThe recipes produce 15 ml of separating gel and 5 ml of stacking gel, which are adequate for one gel of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the modified Laemmli peptide separation system of Okajima et al. (1993).

^bAll reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

^cBest to prepare fresh.

CONTINUOUS SDS-PAGE

With continuous SDS-PAGE, the same buffer is used for both the gel and electrode solutions. Although continuous gels lack the resolution of the discontinuous systems, they are extremely versatile, less prone to mobility artifacts, and much easier to prepare. The stacking gel is omitted.

Additional Materials (also see *Basic Protocol 1*)

- Separating gel solution (Table 6.1.7)
- 2× and 1× phosphate/SDS sample buffer (see recipe)
- 1× phosphate/SDS electrophoresis buffer (see recipe)

1. Prepare and pour a single separating gel (see *Basic Protocol 1*, steps 1 to 4), except use solutions in Table 6.1.7 in place of those in Table 6.1.1 and fill the gel sandwich to the top. Omit the stacking gel. Insert the comb (see *Basic Protocol 1*, step 10) and allow the gel to polymerize 30 to 60 min at room temperature.
2. Mix the protein sample 1:1 with 2× phosphate/SDS sample buffer and heat to 100°C for 2 min.

*For large sample volumes or samples suspended in high ionic strength buffers (>50 mM), dialyze against 1× sample buffer prior to electrophoresis. Note that the precautions about proteases (see *Basic Protocol 1*, step 12) apply here.*

Table 6.1.7 Recipes for Separating Gels for Continuous SDS-PAGE^a**SEPARATING GEL**

Stock solution ^b	Final acrylamide concentration in separating gel (%) ^c										
	5	6	7	8	9	10	11	12	13	14	15
30% acrylamide/ 0.8% bisacrylamide	2.5	3.00	3.50	4.00	4.50	5.00	5.50	6.00	6.50	7.00	7.50
4× phosphate/SDS, pH 7.2	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H ₂ O	8.75	8.25	7.75	7.25	6.75	6.25	5.75	5.25	4.75	4.25	3.75
10% (w/v) ammonium persulfate ^d	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Preparation of separating gel

In a 25-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× phosphate/SDS, pH 7.2, and H₂O. Degas under vacuum about 5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

ADDITIONAL REAGENTS USED IN GELS**4× phosphate/SDS, pH 7.2** (0.4 M sodium phosphate/0.4% SDS)

Mix 46.8 g NaH₂PO₄·H₂O, 231.6 g Na₂HPO₄·7 H₂O, and 12 g SDS in 3 liters H₂O.

Store at 4°C for up to 3 months.

^aThe recipes produce 15 ml of separating gel, which is adequate for one gel of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the original continuous phosphate buffer system of Weber et al. (1972). The stacking gel is omitted.

^bAll reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

^cUnits of numbers in table body are milliliters. The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. See *Basic Protocol 1*, annotation to step 3.

^dBest to prepare fresh.

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3. Assemble the electrophoresis apparatus and load the sample (see Basic Protocol 1, steps 13 to 20), using the phosphate/SDS electrophoresis buffer and loading empty wells with 1× phosphate/SDS sample buffer.
4. Connect the power supply and start the run with 15 mA per 0.75-mm-thick gel until the tracking dye has entered the gel. Continue electrophoresis at 30 mA for 3 hr (5% gel), 5 hr (10% gel), 8 hr (15% gel), or until the dye reaches the bottom of the gel.

Use temperature control if available to maintain the gel at 15° to 20°C. SDS will precipitate below 15°C in this system.

5. Disassemble the gel (see Basic Protocol 1, steps 23 to 26).

Refer to Safety Considerations in introduction. Proteins in the gel may now be stained.

CASTING AND RUNNING ULTRATHIN GELS

Ultrathin gels provide superb resolution but are difficult to handle. In this application, gels are cast on Gel Bond, a Mylar support material. Silver staining is recommended for the best resolution. Combs and spacers for gels <0.5 mm thick are not readily available for most protein electrophoresis units. However, by adapting combs and spacers used for DNA sequencing, casting gels from 0.2 to 0.5 mm thick is straightforward.

Additional Materials (also see Basic Protocol 1)

- 95% (v/v) ethanol
- Gel Bond (FMC BioProducts) cut to a size slightly smaller than the gel plate dimensions
- Glue stick
- Ink roller (available from art supply stores)
- Combs and spacers (0.19 to 0.5 mm; sequencing gel spacers and combs can be cut to fit)

1. Wash gel plates with water-based laboratory detergent followed by successive rinses with hot tap water, deionized water, and finally 95% ethanol. Allow to air dry.

Gel plates must be extremely clean for casting thin gels.

Gloves are needed throughout these procedures to prevent contamination by proteins on the surface of skin.

2. Apply a streak of adhesive from a glue stick to the bottom edge of the glass plate. Quickly position the Gel Bond with the hydrophobic side down (a drop of water will bead up on the hydrophobic surface). Apply pressure with Kimwipe tissue to attach the Gel Bond firmly to the plate. Finally, pull the top portion of the Gel Bond back, place a few drops of water underneath, and roll flat with an ink roller.

Make sure the Gel Bond does not extend beyond the edges of the upper and lower sealing surface of the plate. This will cause it to buckle on sealing. Reposition the Gel Bond if needed to prevent it from extending beyond the glass plate. Material may also be trimmed to fit flush with the plate edge.

3. Assemble the gel cassette according to the manufacturer's instructions (also see Basic Protocol 1, steps 1 and 2). Just prior to assembly, blow air over the surface of both the Gel Bond and the opposing glass surface to remove any particulate material (e.g., dust).

Sequencing gel spacers can be easily adapted. First, cut the spacers slightly longer than the length of the gel plate. Position a spacer along each edge of the glass plate and assemble the gel sandwich, clamping in place. With a razor blade, trim the excess spacer at top and bottom to get a reusable spacer exactly the size of the plate.

4. Prepare and pour the separating and stacking gels (see Basic Protocol 1, steps 3 to 9). In place of the Teflon comb, insert a square well sequencing comb cut to fit within the gel sandwich. Allow the stacking gel to polymerize 30 to 45 min at room temperature.

Less solution is needed for ultrathin gels. For example, a 0.5-mm-thick gel requires 33% less gel solution than a 0.75-mm gel.

5. Prepare the sample and load the gel (see Basic Protocol 1, steps 12 to 20).

When preparing protein samples for ultrathin gels, 3 to 4 μ l at 5 μ g protein/ μ l is required for Coomassie blue R-250 staining, whereas 10-fold less is needed for silver staining.

6. Run the gel (see Basic Protocol 1, steps 21 and 22), except conduct the electrophoresis at 7 mA/gel (0.25-mm-thick gels) or 14 mA/gel (0.5-mm-thick gels) for 4 to 5 hr.

7. When the separation is complete, disassemble the unit and remove the gel (see Basic Protocol 1, steps 23 to 26) with the Gel Bond still attached. With a gloved hand, wash away the adhesive material from the back of the Gel Bond under a stream of water before proceeding to protein detection.

Either Coomassie blue or silver staining may be used, but silver staining produces particularly fine resolution with thin Gel Bond-backed gels. Compared to staining thicker (>0.75 mm) gels, thin (<0.75 mm) gels stain and destain more quickly. Although the optimum staining times must be empirically determined, all steps in Coomassie blue and silver staining procedures are generally reduced by half.

CASTING MULTIPLE SINGLE-CONCENTRATION GELS

Casting multiple gels at one time has several advantages. All the gels are identical, so sample separation is not affected by gel-to-gel variation. Furthermore, casting ten gels is only slightly more difficult than casting two gels. Once cast, gels can be stored for several days in a refrigerator.

Additional Materials (also see Basic Protocol 1)

Separating and stacking gels for single-concentration gels (Table 6.1.8)

H₂O-saturated isobutyl alcohol

Multiple gel caster (Bio-Rad, Amersham Pharmacia Biotech)

100-ml disposable syringe and flat-tipped needle

Extra plates and spacers

14 × 14-cm acrylic blocks or polycarbonate sheets

250- and 500-ml side-arm flasks (used in gel preparation)

Long razor blade *or* plastic wedge (Wonder Wedge, Amersham Pharmacia Biotech)

Resealable plastic bags

Pour the separating gel

1. Assemble the multiple gel caster according to the manufacturer's instructions.

With the Amersham Pharmacia Biotech unit make sure to insert the large triangular space filler plug in the base of the caster. The plug is removed when casting gradient gels (see Support Protocol 2).

2. Assemble glass sandwiches and stack them in the casting chamber. Stack up to ten 1.5-mm gels and fill in extra space with acrylic blocks or polycarbonate sheets to hold the sandwiches tightly in place. Make sure the spacers are straight along the top, right, and left edges of the glass plates and that all edges of the stack are flush.

The presence of loosely fitting sandwiches in the caster will lead to unevenly cast gels, creating distortions during electrophoresis. Polycarbonate inhibits gel polymerization.

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Table 6.1.8 Recipes for Multiple Single-Concentration Polyacrylamide Gels^a**SEPARATING GEL**

Stock solution ^b	Final acrylamide concentration in separating gel (%) ^c										
	5	6	7	8	9	10	11	12	13	14	15
30% acrylamide/0.8% bisacrylamide	52	62	72	83	93	103	114	124	134	145	155
4× Tris·Cl/SDS, pH 8.8	78	78	78	78	78	78	78	78	78	78	78
H ₂ O	181	171	160	150	140	129	119	109	98	88	78
10% (w/v) ammonium persulfate ^d	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
TEMED	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21

Preparation of separating gel

In a 500-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× Tris·Cl/SDS, pH 8.8 (Table 6.1.1), and H₂O. Degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix; use immediately.

STACKING GEL

In a 250-ml side-arm flask, mix 13.0 ml 30% acrylamide/0.8% bisacrylamide solution, 25 ml 4× Tris·Cl/SDS, pH 6.8 (Table 6.1.1), and 61 ml H₂O. Degas under vacuum ~5 min. Add 0.25 ml 10% ammonium persulfate and 50 μl TEMED. Swirl gently to mix. Use immediately. Failure to form a firm gel usually indicates a problem with the persulfate, TEMED, or both.

^aThe recipes produce about 300 ml of separating gel and 100 ml of stacking gel, which are adequate for ten gels of dimensions 1.5 mm × 14 cm × 14 cm. Volumes were measured using 1.5-mm spacers. For thinner spacers or fewer gels, calculate volumes using the equation in the annotation to step 4. The recipes are based on the SDS (denaturing) discontinuous buffer system of Laemmli (1970).

^bAll reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

^cUnits of numbers in table body are milliliters. The desired percentage of acrylamide in separating gel depends on the molecular size of the protein being separated. See Basic Protocol 1, annotation to step 3.

^dBest to prepare fresh.

Therefore, if polycarbonate sheets are placed in the caster before and after the set of glass sandwiches, the entire set will slide out as one block after polymerization. Placing polycarbonate sheets between each gel sandwich makes them easier to separate from one another after polymerization.

- Place the front sealing plate on the casting chamber, making sure the stack fits snugly. Secure the plate with four spring clamps and tighten the bottom thumb screws.

- Prepare the separating (resolving) gel solution (Table 6.1.8).

A 12-cm separating gel with a 4-cm stacking gel is recommended.

If fewer than ten gels are prepared (Table 6.1.8), use the following formula to estimate the amount of separating gel volume needed:

$$\text{Volume} = \text{gel number} \times \text{height (cm)} \times \text{width (cm)} \times \text{thickness (cm)} + 4 \times \text{gel number} + 10 \text{ ml.}$$

- Using a 100-ml disposable syringe with flat-tipped needle, inject the resolving gel solution down the side of one spacer into the multiple caster. A channel in the silicone plug distributes the solution throughout the whole caster. Avoid introducing bubbles by giving the caster a quick tap on the benchtop once the caster is filled.

6. Overlay the center of each gel with 100 μ l H₂O-saturated isobutyl alcohol and let polymerize for 1 to 2 hr.
7. Drain off the overlay and rinse the surface with 1 \times Tris-Cl/SDS, pH 8.8. If the gels will not be used immediately, skip to step 12.

Pour the stacking gel

8. Prepare the stacking gel solution either singly (see Basic Protocol 1, step 8) or for all the gels at once (Table 6.1.8).

The stacking gel solution should be prepared just before pouring the gel.

9. Fill each sandwich in the caster with stacking gel solution.
10. Insert a comb into each sandwich and let the gel polymerize for 2 hr.

Insert the combs at a 45° angle to avoid trapping air underneath the comb teeth. Air bubbles will inhibit polymerization and cause dents in the wells and a distorted pattern of protein bands.

11. Remove the combs and rinse wells with 1 \times SDS electrophoresis buffer.

Remove the gels from the caster

12. Remove the gels from the caster and separate by carefully inserting a long razor blade or knife between each gel sandwich.

A plastic wedge (Amersham Pharmacia Biotech's Wonder Wedge) also works well.

13. Clean the outside of each gel plate with running water to remove the residual polymerized and unpolymerized acrylamide.
14. Overlay gels to be stored with 1 \times Tris-Cl/SDS, pH 8.8, place in a resealable plastic bag, and store at 4°C until needed (up to 1 week).

SEPARATION OF PROTEINS ON GRADIENT GELS

Gels that consist of a gradient of increasing polyacrylamide concentration resolve a much wider size range of proteins than standard uniform-concentration gels (see Critical Parameters and Troubleshooting). The protein bands, particularly in the low-molecular-weight range, are also much sharper. Unlike single-concentration gels, gradient gels separate proteins in a way that can be represented easily to give a linear plot from 10 to 200 kDa. This facilitates molecular weight estimations.

The quantities given below provide separating gel solution sufficient for two 0.75-mm gels (~7 ml of each concentration) or one 1.5-mm gel (~14 ml of each concentration). Volumes can be adjusted to accommodate gel sandwiches of different dimensions.

Additional Materials (also see Basic Protocol 1)

Light and heavy acrylamide gel solutions (Table 6.1.9 and Table 6.1.10)

Bromphenol blue (optional; for checking practice gradient)

TEMED

Gradient maker (30 to 50 ml, Amersham Pharmacia Biotech SG30 or SG50; or 30 to 100 ml, Bio-Rad 385)

Tygon tubing with micropipet tip

Peristaltic pump (optional; e.g., Markson A-13002, A-34040, or A-34105 minipump)

Whatman 3MM filter paper

ALTERNATE PROTOCOL 5

**Electrophoresis
and
Immunoblotting**

6.1.17

Set up the gradient maker and prepare the gel solutions

1. Assemble the magnetic stirrer and gradient maker on a ring stand as shown in Figure 6.1.2. Connect the outlet valve of the gradient maker to Tygon tubing attached to a micropipet tip that is placed over the vertical gel sandwich. If desired, place a peristaltic pump in line between the gradient maker and the gel sandwich.

A peristaltic pump will simplify casting by providing a smooth flow rate.

2. Place a small stir-bar into the mixing chamber of the gradient maker (i.e., the chamber connected to the outlet).
3. Using the recipes in Table 6.1.9 and Table 6.1.10, prepare light and heavy acrylamide gel solutions. Do not add ammonium persulfate until just before use (step 7).
4. With the outlet port and interconnecting valve between the two chambers closed, pipet 7 ml of light (low-concentration) acrylamide gel solution into the reservoir chamber for one 0.75-mm-thick gradient gel.

Recommended gradient ranges are 5% to 20% for most applications (to separate proteins of 10 to several hundred kilodaltons).

A practice run with heavy and light solutions is recommended. Bromphenol blue should be added to the heavy solution to demonstrate linearity of the practice gradient.

5. Open the interconnecting valve briefly to allow a small amount (~200 μ l) of light solution to flow through the valve and into the mixing chamber.

The presence of air bubbles in the interconnecting valve may obstruct the flow between chambers during casting.

Deaeration is not recommended for either the light or heavy solution. Omitting the deaeration will allow polymerization to proceed more slowly, letting the gradient establish itself in the gel sandwich before polymerization takes place.

6. Add 7 ml of heavy (high-concentration) acrylamide gel solution to the mixing chamber.

Keep the heavy solution on ice until use. Once the ammonium persulfate is added to the heavy solution, it will polymerize without TEMED, albeit more slowly; keeping the solution on ice prevents this. The gel solution will come to room temperature during casting. The higher the percentage of acrylamide, the more severe the problem of premature polymerization is.

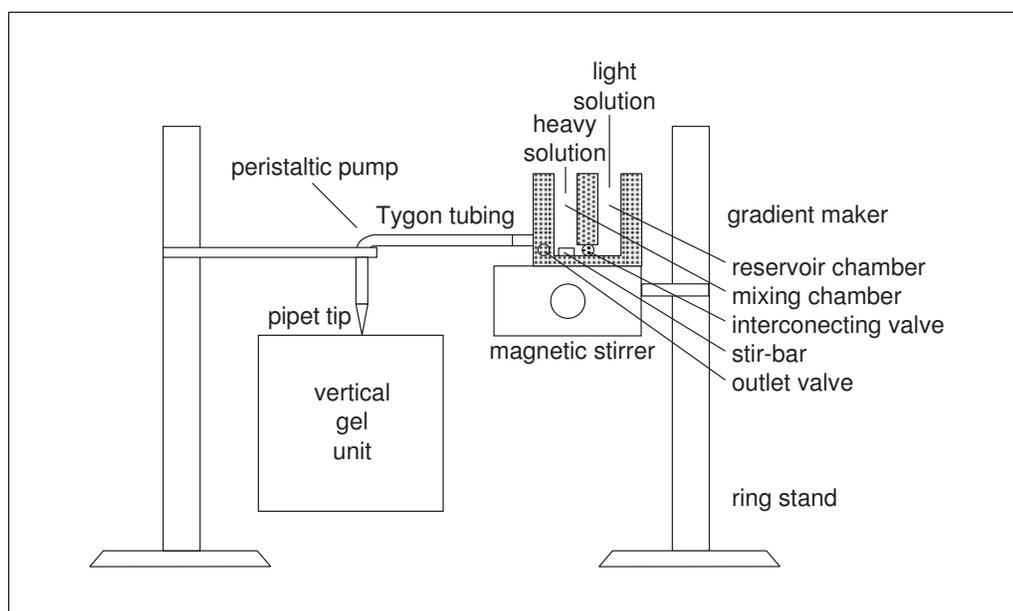


Figure 6.1.2 Gradient gel setup. A peristaltic pump, though not required, will provide better control.

Table 6.1.9 Light Acrylamide Gel Solutions for Gradient Gels^a

Stock solution	Acrylamide concentration of light gel solution (%) ^b									
	5	6	7	8	9	10	11	12	13	14
30% acrylamide/ 0.8% bisacrylamide ^c	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0
4× Tris-Cl/SDS, pH 8.8 ^c	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H ₂ O	8.75	8.25	7.75	7.25	6.75	6.25	5.75	5.25	4.75	4.25
10% (w/v) ammonium persulfate ^d	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05

^aTo survey proteins ≥10 kDa, 5%-20% gradient gels are recommended. To expand the range between 10 and 200 kD, a 10%-20% gel is recommended.

^bNumbers in body of table are milliliters of stock solution. Deaeration is not required. Keep solution at room temperature prior to adding TEMED no longer than 1 hr.

^cSee Table 6.1.1 for preparation.

^dBest to prepare fresh.

Table 6.1.10 Heavy Acrylamide Gel Solutions for Gradient Gels^a

Stock solution	Acrylamide concentration of heavy gel solution (%) ^b											
	10	11	12	13	14	15	16	17	18	19	20	
30% acrylamide/ 0.8% bisacrylamide ^c	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	
4× Tris-Cl/SDS, pH 8.8 ^c	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	
H ₂ O	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5	0	
Sucrose (g)	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	
10% (w/v) ammonium persulfate ^{b,d}	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	

^aDeaeration is not recommended for gradient gels.

^bNumbers in body of table are milliliters of stock solution (except sucrose). Do not add the ammonium persulfate until just before use. The heavy acrylamide will polymerize, albeit more slowly, without the addition of TEMED. Keep the heavy solution on ice after adding ammonium persulfate.

^cSee Table 6.1.1 for preparation.

^dBest to prepare fresh.

7. Add the specified amount of 10% ammonium persulfate and ~2.3 μl TEMED per 7 ml acrylamide solution to each chamber. Mix the solutions in each chamber with a disposable pipet.

Form the gradient and cast the gel

8. Open the interconnecting valve completely.

Some of the heavy solution will flow back into the reservoir chamber containing light solution as the two chambers equilibrate. This will not affect the formation of the gradient.

9. Turn on the magnetic stirrer and adjust the rate to produce a slight vortex in the mixing chamber.
10. Open the outlet of the gradient maker slowly. Adjust the outlet valve to a flow rate of 2 ml/min. If using a peristaltic pump, calibrate the flow rate with a graduated cylinder prior to casting the gel.

Some adjustment of the flow rate may be necessary during casting. If the light solution is not flowing into the mixing chamber, a bubble may be caught in the interconnecting valve.

Quickly close the outlet and cover the top of the reservoir chamber with a gloved thumb. Push down with the thumb to increase the pressure in the chamber and force the air bubble out of the center valve.

11. Fill the gel sandwich from the top. Place the pipet tip against one side of the sandwich so the solution flows down one plate only. The heavy solution will flow into the sandwich first, followed by progressively lighter solution.
12. Watch as the last of the light solution drains into the outlet tube and adjust the flow rate to ensure that the last few milliliters of solution do not flow quickly into the gel sandwich and disturb the gradient.
13. Overlay the gradient gel with H₂O-saturated isobutyl alcohol. Allow the gel to polymerize ~1 hr.

In this gel system, the gel will polymerize from the bottom (i.e., heavy solution) up. Because polymerization is an exothermic reaction, heat can be felt evolving from the bottom of the gel sandwich during polymerization. A sharp optical discontinuity at the gel-overlay interface indicates that polymerization has occurred. In general, 1 hr is adequate for polymerization.

14. Remove the H₂O-saturated isobutyl alcohol and rinse with 1× Tris·Cl/SDS, pH 8.8. Cast the stacking gel (see Basic Protocol 1, steps 8 to 11).

The gel can be covered with 1× Tris·Cl/SDS, pH 8.8, sealed in a plastic bag, and stored for up to 1 week.

Load and run the gel

15. Prepare the protein sample and protein molecular-weight-standards mixture. Load and run the gel (see Basic Protocol 1, steps 13 to 26).

The gel can be stained with Coomassie blue or silver (APPENDIX 3).

16. After staining, dry the gels onto Whatman 3MM or equivalent filter paper.

Gradient gels >0.75 mm thick require special handling during drying to prevent cracking. The simplest approach to drying gradient gels is to use thin gels; ≤0.75-mm gradient gels with ≤20% acrylamide solutions will dry without cracking as long as the vacuum pump is working properly and the cold trap is dry at the onset of drying. For gradient gels >0.75 mm thick, add 3% (w/v) glycerol to the final destaining solution to help prevent cracking. Another method is to dehydrate and shrink the gel in 30% methanol for up to 3 hr prior to drying. Then place the gel in distilled water for 5 min before drying.

SUPPORT PROTOCOL 2

CASTING MULTIPLE GRADIENT GELS

Casting gradient gels in a multiple gel caster has several advantages. In addition to the time savings, batch casting gives gels that are essentially identical. This is particularly important for gradient gels, where slight variations in casting technique can cause variations in protein mobility. The gels may be stored for up to 1 week after casting to ensure internal consistency from run to run during the week. Furthermore, gels with several ranges of concentrations (e.g., 5% to 20% and 10% to 20% acrylamide) can be cast and stored, giving much more flexibility to optimize separations.

Additional Materials (also see Alternate Protocol 5)

Plug solution (see recipe)

Light and heavy acrylamide gel solutions for multiple gradient gels (Table 6.1.11 and Table 6.1.12)

TEMED

H₂O-saturated isobutyl alcohol

Multiple gel caster (Bio-Rad, Amersham Pharmacia Biotech)
Peristaltic pump (25 ml/min)
500- or 1000-ml gradient maker (Bio-Rad, Amersham Pharmacia Biotech)
Tygon tubing

Set up system and pour separating gel

1. Assemble the multiple caster as in casting multiple single-concentration gels (see Support Protocol 1, steps 1 to 3), making sure to remove the triangular space filler plugs in the bottom of the caster.

The plug is used only when casting single-concentration gels.

2. Set up the peristaltic pump (Fig. 6.1.3). Using a graduated cylinder and water, adjust the flow rate so that the volume of the gradient solution plus volume of plug solution is poured in ~15 to 18 min (~25 ml/min).
3. Set up the gradient maker. Close all valves and place a stir-bar in the mixing chamber, which is the one with the outlet port. Attach one end of a piece of Tygon tubing to the outlet of the gradient maker. Run the other end of the tubing through the peristaltic pump and attach it to the red inlet port at the bottom of the caster.

Choose a gradient maker that holds no more than four times the total volume of the gradient solution to be poured (i.e., a 1000- or 500-ml gradient maker).

4. Prepare solutions for the gradient maker (Table 6.1.11 and Table 6.1.12).
5. Add the TEMED to both heavy and light solutions (54 μ l/165 ml) and immediately pour the light (low-concentration) solution into the mixing chamber (the one with the port). Open the mixing valve slightly to allow the tunnel to fill and to avoid air bubbles. Close the valve again and pour the heavy (high-concentration) acrylamide solution into the reservoir chamber.
6. Start the magnetic stirrer and open the outlet valve; then start the pump and open the mixing valve.

In units for casting multiple gels, acrylamide solution flows in from the bottom. To use a multiple casting unit, the light solution is placed in the mixing chamber and the heavy solution in the reservoir. This is the reverse of casting a single gel (see Alternate Protocol 5).

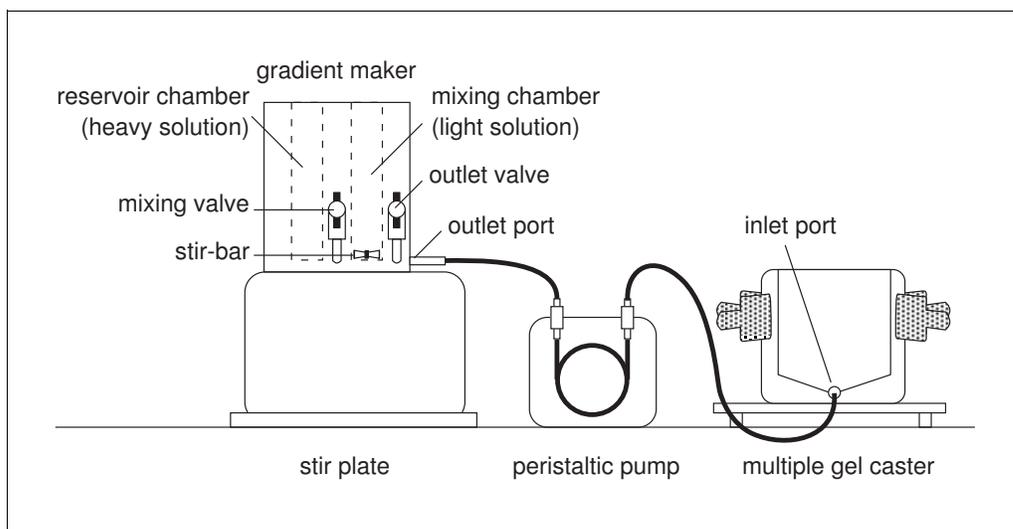


Figure 6.1.3 Setup for casting multiple gradient gels. Casting multiple gradient gels requires a peristaltic pump and a multiple gel caster. Gel solution is introduced through the bottom of the multiple caster.

Table 6.1.11 Light Acrylamide Gel Solutions for Multiple Gradient Gels^{a,b}

Stock solution	Acrylamide concentration of light separating gel solution (%) ^c									
	5	6	7	8	9	10	11	12	13	14
30% acrylamide/ 0.8% bisacrylamide ^d	28	33	39	44	50	55	61	66	72	77
4× Tris·Cl/SDS, pH 8.8 ^d	41	41	41	41	41	41	41	41	41	41
H ₂ O	96	91	85	80	74	69	63	58	52	47
10% (w/v) ammonium persulfate ^e	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55

^aTo survey proteins ≥10 kDa, 5%-20% gradient gels are recommended. To expand the range between 10 and 200 kDa, a 10%-20% gel is recommended.

^bRecipes produce ten 1.5-mm-thick gradient gels with 10 ml extra solution to account for losses in tubing.

^cNumbers in body of table are milliliters of stock solution. Deaeration is not required. Keep solution at room temperature prior to adding TEMED no longer than 1 hr.

^dSee Table 6.1.1 for preparation.

^eBest to prepare fresh.

Table 6.1.12 Heavy Acrylamide Gel Solutions for Multiple Gradient Gels^{a,b}

Stock solution	Acrylamide concentration of heavy gel solution (%) ^c										
	10	11	12	13	14	15	16	17	18	19	20
30% acrylamide/ 0.8% bisacrylamide ^d	55	61	66	72	77	83	88	94	99	105	110
4× Tris·Cl/SDS, pH 8.8 ^d	41	41	41	41	41	41	41	41	41	41	41
H ₂ O	55	50	44	39	33	28	22	17	11	5.5	0
Sucrose (g)	25	25	25	25	25	25	25	25	25	25	25
10% (w/v) ammonium persulfate ^e	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55

^aDeaeration is not recommended for gradient gels.

^bRecipes produce 10 ml extra solution to account for losses in tubing.

^cNumbers in body of table are milliliters of stock solution (except sucrose). Do not add the ammonium persulfate until just before use. The heavy acrylamide will polymerize, albeit more slowly, without the addition of TEMED. Keep the heavy solution on ice after adding ammonium persulfate.

^dSee Table 6.1.1 for preparation.

^eBest to prepare fresh.

Thus, light solution enters the multiple caster first, followed by progressively heavier solution. Finally, the acrylamide solution is stabilized in the multiple caster with a heavy plug solution and allowed to polymerize (see step 8 and manufacturer's instructions).

- When almost all the acrylamide solution is gone from the gradient maker, stop the pump and close the mixing valve. Tilt the gradient maker toward the outlet side and remove the last milliliters of the mix. Do not allow air bubbles to enter the tubing.
- Add the plug solution to the mixing chamber and start the pump. Make sure that no bubbles are introduced. Continue pumping until the bottom of the caster is filled with plug solution to just below the glass plates; then turn off the pump. Clamp the tubing close to the red port of the casting chamber.
- Quickly overlay each separate gel sandwich with 100 μl H₂O-saturated isobutyl alcohol. Use the same amount on each sandwich.

Failure to use the same amount of overlay solution will cause the gel sandwiches to polymerize at different heights.

10. Drain off the overlay and rinse the surface of the gels with 1× Tris·Cl/SDS, pH 8.8.

Pour stacking gel and remove gels from caster

11. Prepare and cast the stacking gel as in casting multiple single-concentration gels (see Support Protocol 1, steps 8 to 11).
12. Remove gels from the caster and clean the gel sandwiches (see Support Protocol 1, steps 12 and 13). Store gels, if necessary, according to the instructions for multiple single-concentration gels (see Support Protocol 1, step 14).

ELECTROPHORESIS IN SINGLE-CONCENTRATION MINIGELS

Separation of proteins in a small-gel format is becoming increasingly popular for applications that range from isolating material for peptide sequencing to performing routine protein separations. The unique combination of speed and high resolution is the foremost advantage of small gels. Additionally, small gels are easily adapted to single-concentration, gradient, and two-dimensional SDS-PAGE procedures. The minigel procedures described are adaptations of larger gel systems.

This protocol describes the use of a multiple gel caster. The caster is simple to use, and up to five gels can be prepared at one time with this procedure. Single gels can be prepared using adaptations in the manufacturer's instructions. A multiple gel caster is the only practical way to produce small linear polyacrylamide gradient gels (see Support Protocol 3).

Materials

Minigel vertical gel unit (Amersham Pharmacia Biotech Mighty Small SE 250/280 *or* Bio-Rad Mini-Protean II) with glass plates, clamps, and buffer chambers

0.75-mm spacers

Multiple gel caster (Amersham Pharmacia Biotech SE-275/295 *or* Bio-Rad Mini-Protean II multicasting chamber)

Acrylic plate (Amersham Pharmacia Biotech SE-217 *or* Bio-Rad 165-1957) *or* polycarbonate separation sheet (Amersham Pharmacia Biotech SE-213 *or* Bio-Rad 165-1958)

10- and 50-ml syringes

Combs (Teflon, Amersham Pharmacia Biotech SE-211A series *or* Bio-Rad Mini-Protean II)

Long razor blade

Micropipet

Additional reagents and equipment for standard denaturing SDS-PAGE (see Basic Protocol 1)

Pour the separating gel

1. Assemble each gel sandwich by stacking, in order, the notched (Amersham Pharmacia Biotech) *or* small rectangular (Bio-Rad) plate, 0.75-mm spacers, and the larger rectangular plate. Be sure to align the spacers properly, with the ends flush with the top and bottom edge of the two plates, when positioning the sandwiches in the multiple gel caster (Fig. 6.1.4).

The protocol described is basically for the Amersham Pharmacia Biotech system. For other systems, make adjustments according to the manufacturer's instructions. Alternatively, precast minigels can be purchased from a number of suppliers (see Table 6.1.3).

The multiple casters from Amersham Pharmacia Biotech have a notch in the base designed for casting gradient gels. A silicone rubber insert fills up this space when casting

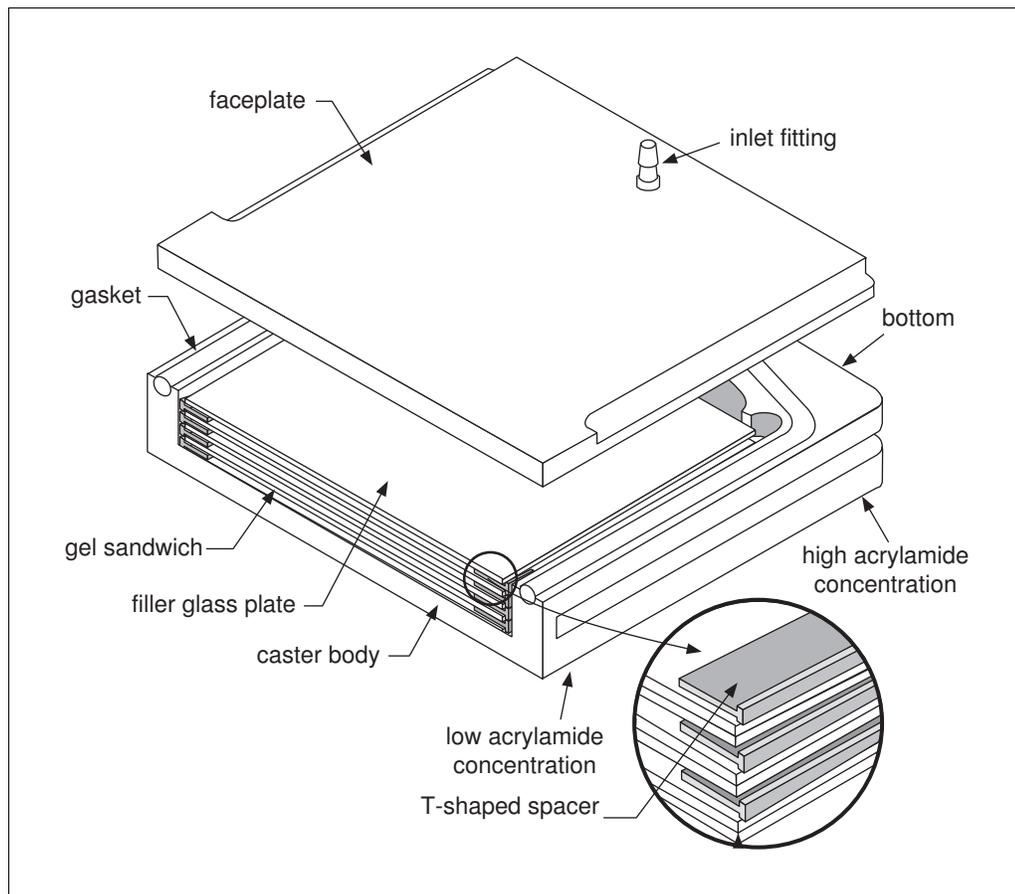


Figure 6.1.4 Minigel sandwiches positioned in the multiple gel caster. Extra glass or acrylic plates or polycarbonate sheets are used to fill any free space in the caster and to ensure that the gel sandwiches are held firmly in place.

single-concentration gels. The Amersham Pharmacia Biotech spacers are T-shaped to prevent slipping. The flanged edge of the spacer must be positioned against the outside edge of the glass plate. Placing a sheet of wax paper between the gel sandwiches will help separate the sandwiches after polymerization.

2. Fit the gel sandwiches tightly in the multiple gel caster. Use an acrylic plate or polycarbonate separation sheet to eliminate any slack in the chamber.

Loosely fitting sandwiches in the caster will lead to unevenly cast gels, creating distortions during electrophoresis.

3. Place the front faceplate on the caster, clamp it in place against the silicone gasket, and verify alignment of the glass plates and spacers.
4. Prepare the separating gel solution as directed in Table 6.1.1. For five 0.75-mm-thick gels, prepare ~30 ml solution (i.e., double the volumes listed).

To compute the total gel volume needed, multiply the area of the gel (e.g., 7.3 × 8.3 cm) by the thickness of the gel (e.g., 0.75 mm) and then by the number of gels in the caster. If needed, add ~4 to 5 ml of extra gel solution to account for the space around the outside of the gel sandwiches.

Do not add TEMED and ammonium persulfate until just before use.

5. Fill a 50-ml syringe with the separating gel solution and slowly inject it into the caster until the gels are 6 cm high, allowing 1.5 cm for the stacking gel.

- Overlay each gel with 100 μ l H₂O-saturated isobutyl alcohol. Allow the gels to polymerize for ~1 hr.

Pour the stacking gel

- Remove the isobutyl alcohol and rinse with 1 \times Tris·Cl/SDS, pH 8.8.

Stacking gels can be cast one at a time with the gel mounted on the electrophoresis unit, or all at once in the multiple caster.

- Practice placing a comb in the gel sandwiches before preparing the stacking gel solution. Press the comb against the rectangular or taller plate so that all teeth of the comb are aligned with the opening in the gel sandwich, then insert into the sandwich. Remove combs after practicing.
- Prepare the stacking gel solution (2 ml per gel) as directed in Table 6.1.1. Fill a 10-ml syringe with stacking gel solution and inject the solution into each gel sandwich.
- Insert combs, taking care not to trap bubbles. Allow gels to polymerize 1 hr.
- Remove the front faceplate. Carefully pull the gels out of the caster, using a long razor blade to separate the sandwiches.

If the gels are left to polymerize for prolonged periods, they will be difficult to remove from the caster.

The gels can be stored tightly wrapped in plastic wrap with the combs left in place inside a sealable bag to prevent drying for ~1 week. Without the stacking gel, the separating gel can be stored for 2 to 3 weeks. Keep gels moist with 1 \times Tris·Cl/SDS, pH 8.8, at 4°C. Do not store gels in the multiple caster.

Prepare the sample, load the gel, and conduct electrophoresis

- Remove the combs and rinse the sample wells with 1 \times SDS electrophoresis buffer. Place a line indicating the bottom of each well on the front glass plate with a marker.
- Fill the upper and lower buffer chambers with 1 \times SDS electrophoresis buffer. The upper chamber should be filled to 1 to 2 cm over the notched plate.
- Prepare the protein sample and protein-standards mixture (see Basic Protocol 1, step 12).
- Load the sample using a micropipet. Insert the pipet tip through the upper buffer and into the well. The mark on the glass plate will act as a guide. Dispense the sample into the well.

For a complex mixture, 20 to 25 μ g protein in 10 μ l SDS sample buffer will give a strongly stained Coomassie blue pattern. Much smaller amounts (1 to 5 μ g) are required for highly purified proteins, and a 10- to 100-fold smaller amount of protein in the same volume (e.g., 10 μ l) is required for silver staining.

- Electrophorese samples at 10 to 15 mA per 0.75-mm gel until the dye front reaches the bottom of the gel (~1 to 1.5 hr).
- Disassemble the gel (see Basic Protocol 1, steps 23 to 26). Proceed with detection of proteins.

PREPARING MULTIPLE GRADIENT MINIGELS

Polyacrylamide gradients not only enhance the resolution of larger-format gels but also greatly improve protein separation in the small format. Casting gradient minigels one at a time is not generally feasible because of the small volumes used, but multiple gel casters make it easy to cast several small gradient gels at one time. The gels are cast from the bottom in multiple casters, with the light acrylamide solution entering first. This is the opposite of casting one gel at a time, in which the heavy solution enters from the top of the gel sandwich and flows down to the bottom.

Additional Materials (also see *Basic Protocol 2*)

Plug solution (see recipe)

Additional reagents and equipment for preparing gradient gels (see *Alternate Protocol 5*)

Set up the system and prepare the gel solutions

1. Assemble minigel sandwiches in the multiple gel caster as described for single-concentration minigels (see *Basic Protocol 2*, steps 1 to 3).
2. Set up the 30-ml gradient maker, magnetic stirrer, peristaltic pump (optional), and Tygon tubing as in Figure 6.1.3. Connect the outlet of the 30-ml gradient maker to the inlet at the base of the front faceplate of the caster.

The monomer solution will be introduced through the inlet at the bottom of the front faceplate of the caster first, followed by progressively heavier solution.

3. Prepare light (Table 6.1.9) and heavy (Table 6.1.10) acrylamide gel solutions. Use ~12 ml of each solution for five 0.75-mm-thick minigels.

Adjust volumes if a different thickness or number of gels is needed. Do not add ammonium persulfate until just before use. Deaeration is not recommended for gradient gels.

4. With the outlet and interconnecting valve closed, add the heavy solution to the reservoir chamber. Briefly open the interconnecting valve to let a small amount of heavy solution through to the mixing chamber, clearing the valve of air.
5. Fill the mixing chamber with light solution. Add 4 μ l TEMED per 12 ml acrylamide solution to each chamber and mix with a disposable pipet.

Form the gradient and cast the gels

6. Turn on the magnetic stirrer. Open the interconnecting valve and allow the chambers to equilibrate. Then slowly open the outlet port to allow the solution to flow from the gradient maker to the multiple caster by gravity (a peristaltic pump may be used for better control). Adjust the flow rate to 3 to 4 ml/min.

Faster flow rates are possible and will also produce good gradients. However, a fast flow increases the potential for introduction of bubbles into the caster.

7. Close the outlet port as the last of the gradient solution leaves the mixing chamber, just before air enters the outlet tube. Fill the two chambers with plug solution and slowly open the outlet once again.
8. Allow the plug solution to push the acrylamide in the caster up into the plates. Close the outlet when the plug solution reaches the bottom of the plates.

A discontinuity between the bottom of the gels and the plug solution will be obvious.

9. Quickly add 100 μ l H₂O-saturated isobutyl alcohol to each gel sandwich. Let the gels polymerize undisturbed for ~1 hr.
10. Prepare and pour the stacking gel (see *Basic Protocol 2*, steps 9 and 10).

Disassemble the system

11. Disconnect the gradient maker, place the caster in a sink, and remove the front faceplate. The plug solution will drain out from the bottom of the caster.
12. Remove the gels (see Basic Protocol 2, step 11).

Gradient minigels can be stored as described for single-concentration minigels (see Basic Protocol 2, step 11 annotation). For instructions on preparing, loading, and running the gels, see Basic Protocol 2, steps 12 to 17.

REAGENTS AND SOLUTIONS

Use Milli-Q water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Anode buffer

121.1 g Tris base (0.2 M final)
500 ml H₂O
Adjust to pH 8.9 with concentrated HCl
Dilute to 5 liters with H₂O
Store at 4°C up to 1 month

Final concentration is 0.2 M Tris-Cl, pH 8.9.

Cathode buffer

12.11 g Tris base (0.1 M final)
17.92 g tricine (0.1 M final)
1 g SDS (0.1% final)
Dilute to 1 liter with H₂O
Do not adjust pH
Store at 4°C up to 1 month

Coomassie blue G-250 staining solution

200 ml acetic acid (20% final)
1800 ml H₂O
0.5 g Coomassie blue G-250 (0.025% final)
Mix 1 hr and filter (Whatman no. 1 paper)
Store at room temperature indefinitely

Phosphate/SDS electrophoresis buffer

Dilute 500 ml of 4× phosphate/SDS, pH 7.2 (Table 6.1.7) with H₂O to 2 liters. Store at 4°C up to 1 month.

Final concentrations are 0.1 M sodium phosphate (pH 7.2)/0.1% (w/v) SDS.

Phosphate/SDS sample buffer, 2× (for continuous systems)

0.5 ml 4× phosphate/SDS, pH 7.2 (Table 6.1.7; 20 mM sodium phosphate final)
0.2 g SDS (2% final)
0.1 mg bromphenol blue (0.001% final)
0.31 g DTT (0.2 M final)
2.0 ml glycerol (2% final)
Add H₂O to 10 ml and mix

Plug solution

0.125 M Tris-Cl, pH 8.8 (APPENDIX 2A)
50% (w/v) sucrose
0.001% (w/v) bromphenol blue
Store at 4°C up to 1 month

Recrystallized SDS (optional)

High-purity SDS is available from several suppliers, but for some sensitive applications (e.g., protein sequencing) recrystallization is useful. Commercially available electrophoresis-grade SDS is usually of sufficient purity for most applications.

Add 100 g SDS to 450 ml ethanol and heat to 55°C. While stirring, gradually add 50 to 75 ml hot H₂O until all SDS dissolves. Add 10 g activated charcoal (Norit 1, Sigma) to solution. After 10 min, filter solution through Whatman no. 5 paper on a Buchner funnel to remove charcoal. Chill filtrate 24 hr at 4°C and 24 hr at –20°C. Collect crystalline SDS on a coarse-frit (porosity A) sintered-glass funnel and wash with 800 ml –20°C ethanol (reagent grade). Repeat crystallization without adding activated charcoal. Dry recrystallized SDS under vacuum overnight at room temperature. Store in a desiccator over phosphorous pentoxide (P₂O₅) in a dark bottle.

If proteins will be electroeluted or electroblotted for protein sequence analysis, it may be desirable to crystallize the SDS twice from ethanol/H₂O (Hunkapiller et al., 1983).

SDS electrophoresis buffer, 5×

15.1 g Tris base (0.125 M final)

72.0 g glycine (0.96 M final)

5.0 g SDS (0.5% final)

H₂O to 1000 ml

Dilute to 1× or 2× for working solution, as appropriate

Do not adjust the pH of the stock solution, as the solution is pH 8.3 when diluted. Store at 0° to 4°C until use (up to 1 month).

***SDS sample buffer, 2×* (for discontinuous systems)**

25 ml 4× Tris-Cl/SDS, pH 6.8 (Table 6.1.1)

20 ml glycerol (20% final)

4 g SDS (4% final)

2 ml 2-ME or 3.1 g DTT (0.2% 2-ME or 0.2 M DTT final)

1 mg bromphenol blue (0.001% final)

Add H₂O to 100 ml and mix

Store in 1-ml aliquots at –70°C

To avoid reducing proteins to subunits (if desired), omit 2-ME or DTT (reducing agent) and add 10 mM iodoacetamide to prevent disulfide interchange.

***SDS sample buffer, 6×* (for discontinuous systems)**

7 ml 4× Tris-Cl/SDS, pH 6.8 (Table 6.1.1)

3.0 ml glycerol (30% final)

1 g SDS (10% final)

0.93 g DTT (0.6 M final)

1.2 mg bromphenol blue (0.012% final)

Add H₂O to 10 ml (if needed)

Store in 0.5-ml aliquots at –70°C

Tricine sample buffer, 2×

2 ml 4× Tris-Cl/SDS, pH 6.8 (Table 6.1.1; 0.1 M)

2.4 ml (3.0 g) glycerol (24% final)

0.8 g SDS (8% final)

0.31 g DTT (0.2 M final)

2 mg Coomassie blue G-250 (0.02% final)

Add H₂O to 10 ml and mix

COMMENTARY

Background Information

Polyacrylamide gels form after polymerization of monomeric acrylamide into polymeric polyacrylamide chains and cross-linking of the chains by *N,N'*-methylenebisacrylamide. The polymerization reaction is initiated by the addition of ammonium persulfate, and the reaction is accelerated by TEMED, which catalyzes the formation of free radicals from ammonium persulfate. Because oxygen inhibits the polymerization process, deaerating the gel solution before the polymerization catalysts are added will speed up polymerization; deaeration is not recommended for the gradient gel protocols because slower polymerization facilitates casting of gradient gels.

Precast gels for commonly used vertical minigel and standard-sized SDS-PAGE apparatuses are available from several manufacturers (Table 6.1.3). Flatbed (horizontal) isoelectric focusing (IEF) and SDS-PAGE gels are not listed. Amersham Pharmacia Biotech supplies a range of horizontal gels for a variety of applications and should be consulted for further information. When using precast gels, pay strict attention to shelf life. In general, manufacturers overrate the shelf life, and the sooner the gels are used, the better. When reasonably fresh, precast gels provide excellent resolution that is as good or better than a typical gel cast in the laboratory.

The most widely used method for discontinuous gel electrophoresis is the system described by Laemmli (1970). This is the denaturing (SDS) discontinuous method used in Basic Protocol 1. A discontinuous buffer system uses buffers of different pH and composition to generate a discontinuous pH and voltage gradient in the gel. Because the discontinuous gel system concentrates the proteins in each sample into narrow bands, the applied sample may be more dilute than that used for continuous electrophoresis.

In the discontinuous system the sample first passes through a stacking gel, which has large pores. The stacking gel buffer contains chloride ions (called the leading ions) whose electrophoretic mobility is greater than the mobility of the proteins in the sample. The electrophoresis buffer contains glycine ions (called the trailing ions) whose electrophoretic mobility is less than the mobility of the proteins in the sample. The net result is that the faster migrating ions leave a zone of lower conductivity between themselves and the migrating protein.

The higher voltage gradient in this zone allows the proteins to move faster and to “stack” in the zone between the leading and trailing ions. After leaving the stacking gel, the protein enters the separating gel. The separating gel has a smaller pore size, a higher salt concentration, and higher pH compared to the stacking gel. In the separating gel, the glycine ions migrate past the proteins, and the proteins are separated according to either molecular size in a denaturing gel (containing SDS) or molecular shape, size, and charge in a nondenaturing gel.

Proteins are denatured by heating in the presence of a low-molecular-weight thiol (2-ME or DTT) and SDS. Most proteins bind SDS in a constant-weight ratio, leading to identical charge densities for the denatured proteins. Thus, the SDS-protein complexes migrate in the polyacrylamide gel according to size, not charge. Most proteins are resolved on polyacrylamide gels containing from 5% to 15% acrylamide and 0.2% to 0.5% bisacrylamide (see Table 6.1.1). The relationship between the relative mobility and log molecular weight is linear over these ranges (Fig. 6.1.5). With the use of plots like those shown in Figure 6.1.5, the molecular weight of an unknown protein (or its subunits) may be determined by comparison with known protein standards (Table 6.1.2). In general, all of the procedures in this unit are suitable for radiolabeled and biotinylated proteins without modification.

Basic Protocol 1 relies on denaturing proteins in the presence of SDS and 2-ME or DTT. Under these conditions, the subunits of proteins are dissociated and their biological activities are lost. A true estimate of a protein's molecular size can be made by comparing the relative mobility of the unknown protein to proteins in a calibration mixture (Fig. 6.1.5). Gradient gels (Alternate Protocol 5) simplify molecular-weight determinations by producing a linear relationship between log molecular weight of the protein and log % T over a much wider size range than single-concentration gels. Although percent acrylamide monomer is a more common measure of gel concentration, % T, the percentage of total monomer (acrylamide plus bisacrylamide) in the solution or gel, is used for molecular weight calculations in gradient gels. The % T of a stained protein is estimated assuming the acrylamide gradient is linear. For example, proteins in the gel shown in Figure 6.1.6 were separated in a 5.1% to 20.5% T acrylamide gradient. The % T of the point

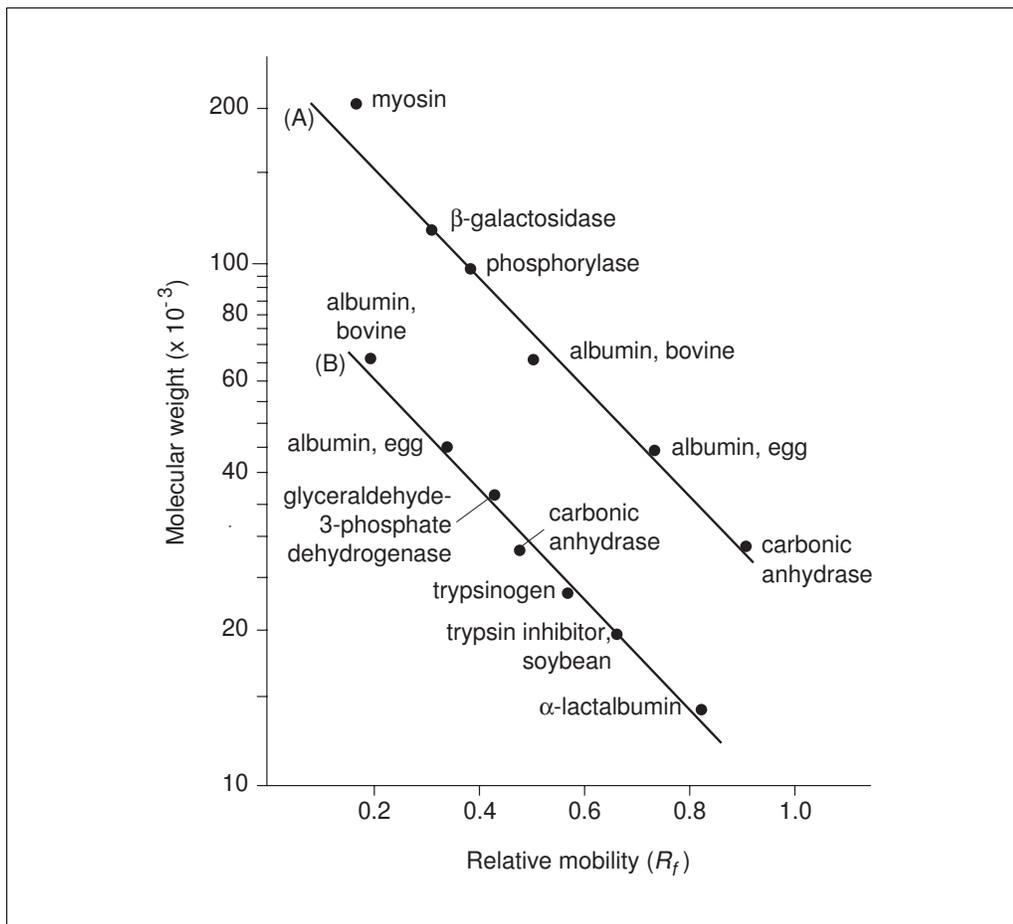


Figure 6.1.5 Typical calibration curves obtained with standard proteins separated by nongradient denaturing (SDS) discontinuous gel electrophoresis based on the method of Laemmli (1970). **(A)** Gel with 7% polyacrylamide. **(B)** Gel with 11% polyacrylamide. (Redrawn with permission from Sigma.)

halfway through the resolving gel is 12.5% T. Simply plotting log molecular mass versus distance moved into the gel (or R_f) also produces a relatively linear standard curve over a fairly wide size range.

If two proteins have identical molecular sizes, they more than likely will not be resolved with one-dimensional SDS-PAGE, and two-dimensional SDS-PAGE should be considered. Unusual protein compositions can cause anomalous mobilities during electrophoresis (see Critical Parameters and Troubleshooting), but similar-sized proteins of widely different amino acid composition or structure may still be resolved from one another using one-dimensional SDS-PAGE. Purified protein complexes or multimeric proteins consisting of subunits of different molecular size will be resolved into constituent polypeptides. Comparison of the protein bands obtained under nonreducing and reducing conditions provides information about the molecular size of disulfide cross-linked com-

ponent subunits. The individual polypeptides can be isolated by electroelution or electroblotting, and the amino acid sequences can be determined.

Both the tricine (Schagger and von Jagow, 1987) and the modified Laemmli (Okajima et al., 1993) peptide separation procedures presented here (Alternate Protocols 1 and 2) are simple to set up and provide resolution down to 5 kDa. To separate peptides below 5 kDa, the tricine procedure must be modified by preparing a 16.5% T, 2.7% C resolving gel that uses a 10% T spacer gel between the stacking and resolving gel (Schagger and von Jagow, 1987). % C is the percentage of cross-linker (bisacrylamide) in the total monomer (acrylamide plus bisacrylamide).

Continuous electrophoresis, where the same buffer is used throughout the tank and gel, is popular because of its versatility and simplicity. The phosphate system described in Alternate Protocol 3 is based on that of Weber et al.

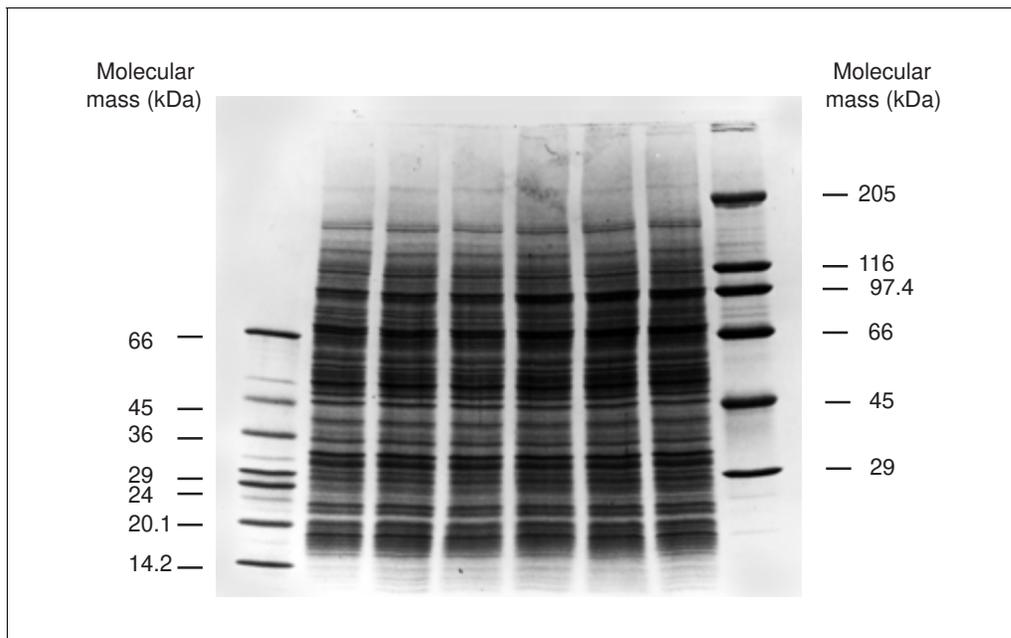


Figure 6.1.6 Separation of membrane proteins by 5.1% to 20.5% T polyacrylamide gradient SDS-PAGE. Approximately 30 μ l of 1 \times SDS sample buffer containing 30 μ g of Alaskan pea (*Pisum sativum*) membrane proteins was loaded in wells of a 14 \times 14-cm, 0.75-mm-thick gel. Standard proteins were included in the outside lanes. The gel was run at 4 mA for ~15 hr.

(1972). Although unable to produce the high-resolution separations of the discontinuous SDS-PAGE procedures, continuous SDS-PAGE uses fewer solutions with one basic buffer and no stacking gel. Artifacts are also less likely to occur in continuous systems. Pepsin, for example, migrates anomalously on Laemmli-based discontinuous SDS-PAGE but has the expected mobility after electrophoresis in the phosphate-based continuous system described here. This is also true of cross-linked proteins.

Multiple gel casting (Support Protocols 1 to 3) is appropriate when gel-to-gel consistency is paramount or when the number of gels processed exceeds five a week. The variety of multiple gel casters, gradient makers, and inexpensive pumps available from major suppliers simplifies the process of casting gels in the laboratory. Alternatively, precast gradient gels are available for most major brands of gel apparatuses (Table 6.1.3).

Minigels (Basic Protocol 2) are generally considered to be in the 8 \times 10-cm size range, although there is considerable variation in exact size. Every technique that is used on larger systems can be translated with little difficulty into the minigel format. This includes standard and gradient SDS-PAGE and separations for immunoblotting and peptide sequencing. Two-

dimensional SDS-PAGE electrophoresis also adapts well, but here the limitation of separation area becomes apparent; for high-resolution separations, large-format gels are required. Gradient minigels (Support Protocol 3) are popular due to the combination of separation range and resolution (Matsudaira and Burgess, 1978). They are particularly useful for separation of proteins prior to peptide sequencing.

Mylar support (Gel Bond) provides a practical way of casting, running, and, staining extremely thin gels. When gels <0.75 mm thick are used, reagents have much better access both into and out of the gel, reducing staining time in both Coomassie blue and silver staining. Double and broadened images caused by differential migration of the protein across the thickness of the gel are minimized, improving resolution.

Critical Parameters and Troubleshooting

If an electrophoretically separated protein will be electroeluted or electroblotted for sequence analysis, the highest-purity reagents available should be used. If necessary, SDS can be purified by recrystallization following the procedure given in Reagents and Solutions.

If the gels polymerize too fast, the amount of ammonium persulfate should be reduced by

one-third to one-half. If the gels polymerize too slowly or fail to polymerize all the way to the top, use fresh ammonium persulfate or increase the amount of ammonium persulfate by one-third to one-half. The overlay should be added slowly down the spacer edge to prevent the overlay solution from crashing down and disturbing the gel interface.

After a separating gel is poured, it may be stored with an overlay of the same buffer used in the gel. Immediately prior to use, the stacking gel should be poured; otherwise, there will be a gradual diffusion-driven mixing of buffers between the two gels, which will cause a loss of resolution.

The protein of interest should be present in 0.2- to 1- μ g amounts in a complex mixture of proteins if the gel will be stained by Coomassie blue. Typically, 30 to 50 μ g of a complex protein mixture in a total volume of <20 μ l is loaded on a 0.75-mm-thick slab gel (16 cm, 10 wells).

When casting multiple gradient gels, eliminate all bubbles in the outlet tubing of the gradient maker. If air bubbles get into the outlet tube, they may flow into the caster and then up through the gradient being poured, causing an area of distortion in the polymerized gel. Air bubbles are not so great a problem when casting single gradient gels from the top. As the gels are cast, the stirrer must be slowed so that the vortex in the mixing chamber does not allow air to enter the outlet.

Uneven heating of the gel causes differential migration of proteins, with the outer lanes moving more slowly than the center lanes (called smiling). Increased heat transfer eliminates smiling and can be achieved by filling the lower buffer chamber with buffer all the way to the level of the sample wells, by maintaining a constant temperature between 10° to 20°C, and by stirring the lower buffer with a magnetic stirrer. Alternatively, decrease the heat load by running at a lower current.

If the tracking dye band is diffuse, prepare fresh buffer and acrylamide monomer stocks. If the protein bands are diffuse, increase the current by 25% to 50% to complete the run more quickly and minimize band diffusion, use a higher percentage of acrylamide, or try a gradient gel. Lengthy separations using gradient gels generally produce good results (Fig. 6.1.6). Check for possible proteolytic degradation that may cause loss of high-molecular-weight bands and create a smeared banding pattern.

If there is vertical streaking of protein bands, decrease the amount of sample loaded on the

gel, further purify the protein of interest to reduce the amount of contaminating protein applied to the gel, or reduce the current by 25%. Another cause of vertical streaking of protein bands is precipitation, which can sometimes be eliminated by centrifuging the sample or by reducing the percentage of acrylamide in the gel.

Proteins can migrate faster or slower than their actual molecular weight would indicate. Abnormal migration is usually associated with a high proportion of basic or charged amino acids (Takano et al., 1988). Other problems can occur during isolation and preparation of the protein sample for electrophoresis. Proteolysis of proteins during cell fractionation by endogenous proteases can cause subtle band splitting and smearing in the resulting electrophoretogram (electrophoresis pattern). Many endogenous proteases are very active in SDS sample buffers and will rapidly degrade the sample; thus, first heating the samples to 70° to 100°C for 3 min is recommended.

In some cases, heating to 100°C in sample buffer will cause selective aggregation of proteins, creating a smeared layer of Coomassie blue-stained material at the top of the gel (Gallagher and Leonard, 1987). To avoid heating artifacts and also prevent proteolysis, the use of specific protease inhibitors during protein isolation and/or lower heating temperatures (70° to 80°C) have been effective (Dhugga et al., 1988).

Although continuous gels suffer from poor band sharpness, they are less prone to artifacts caused by aggregation and protein cross-linking. If streaking or aggregation appear to be a problem with the Laemmli system, then the same sample should be subjected to continuous SDS-PAGE to see if the problem is intrinsic to the Laemmli gel or the sample.

If the protein bands spread laterally from gel lanes, the time between applying the sample and running the gel should be reduced in order to decrease the diffusion of sample out of the wells. Alternatively, the acrylamide percentage should be increased in the stacking gels from 4% to 4.5% or 5% acrylamide, or the operating current should be increased by 25% to decrease diffusion in the stacking gel. Use caution when adding 1 \times SDS electrophoresis buffer to the upper buffer chamber. Samples can get swept into adjacent wells and onto the top of the well arm.

If the protein bands are uneven, the stacking gel may not have been adequately polymerized. This can be corrected by deaerating the stacking

gel solution thoroughly or by increasing the ammonium persulfate and TEMED concentrations by one-third to one-half. Another cause of distorted bands is salt in the protein sample, which can be removed by dialysis, gel filtration, or precipitation. Skewed protein bands can be caused by an uneven interface between the stacking and separating gels, which can be corrected by starting over and being careful not to disturb the separating gel while overlaying with isobutyl alcohol.

If a run takes too long, the buffers may be too concentrated or the operating current too low. If the run is too short, the buffers may be too dilute or the operating current too high.

If double bands are observed, the protein may be partially oxidized or partially degraded. Oxidation can be minimized by increasing the 2-ME concentration in the sample buffer or by preparing a fresh protein sample. If fewer bands than expected are observed and there is a heavy protein band at the dye front, increase the acrylamide percentage in the gel.

Anticipated Results

Polyacrylamide gel electrophoresis done under denaturing and reducing conditions should resolve any two proteins, except two of identical size. Resolution of proteins in the presence of SDS is a function of gel concentration and the size of the proteins being separated. Under nondenaturing conditions, the biological activity of a protein will be maintained.

Comparison of reducing and nonreducing denaturing gels can also provide valuable information about the number of disulfide cross-linked subunits in a protein complex. If the subunits are held together by disulfide linkages, the protein will separate in denaturing gels as a complex or as smaller-sized subunits under nonreducing or reducing conditions, respectively. However, proteins separated on nonreducing denaturing gels appear more diffuse and exhibit less overall resolution than those separated on reducing gels.

Gradient gels provide superior protein-band sharpness and resolve a larger size range of proteins, making them ideal for most types of experiments in spite of being more difficult to prepare. Molecular-weight calculations are simplified because of the extended linear relationship between size and protein position in the gel. Increased band sharpness of both high- and low-molecular-weight proteins on the same gel greatly simplifies survey experiments, such as gene expression studies where the characteristics of the responsive protein are not

known. Furthermore, the increased resolution dramatically improves autoradiographic analysis. Preparation of gradient gels is straightforward, although practice with gradient solutions containing dye is recommended. The gradient gels can be stored for several days at 0° to 4°C before casting the stacking gel.

Time Considerations

Preparation of separating and stacking gels requires 2 to 3 hr. Gradient gels generally take 5 min to cast singly. Casting multiple single-concentration gels requires an additional 10 min for assembly. Casting multiple gradient gels takes 15 to 20 min plus assembly time. It takes 4 to 5 hr to run a 14 × 14-cm, 0.75-mm gel at 15 mA (70 to 150 V), and 3 to 4 hr to run a 0.75-mm gel at 20 mA (80 to 200 V). Overnight separations of ~12 hr require 4 mA per 0.75-mm gel. It takes 4 to 5 hr to run a 1.5-mm gel at 30 mA. Electrophoresis is normally performed at 15° to 20°C, with the temperature held constant using a circulating water bath. For air-cooled electrophoresis units, lower currents and thus longer run times are recommended.

It takes ~1 hr to run a 0.75-mm minigel at 20 mA (100 to 120 V). Separation times are not significantly different for gradient minigels.

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An excellent book describing gel electrophoresis of proteins.

Contributed by Sean R. Gallagher
Motorola Corporation
Tempe, Arizona

Immunoblotting and Immunodetection

UNIT 6.2

Immunoblotting (often referred to as western blotting) is used to identify specific antigens recognized by polyclonal or monoclonal antibodies. Protein samples are solubilized, usually with sodium dodecyl sulfate (SDS) and reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol (2-ME). Following solubilization, the material is separated by SDS-PAGE (UNIT 6.1). The antigens are then electrophoretically transferred in a tank (see Basic Protocol 1) or a semidry transfer apparatus (see Alternate Protocol 1) to a nitrocellulose, PVDF, or nylon membrane, a process that can be monitored by reversible staining (see Support Protocol 1) or by Ponceau S staining (see Support Protocol 2). Previously stained gels may also be blotted (see Alternate Protocol 2).

The transferred proteins are bound to the surface of the membrane, providing access for reaction with immunodetection reagents. All remaining binding sites are blocked by immersing the membrane in a solution containing either a protein or detergent blocking agent. After probing with the primary antibody, the membrane is washed and the antibody-antigen complexes are identified with horseradish peroxidase (HRPO) or alkaline phosphatase enzymes coupled to the secondary anti-IgG antibody (e.g., goat anti-rabbit IgG). The enzymes are attached directly (see Basic Protocol 2) or via an avidin-biotin bridge (see Alternate Protocol 3) to the secondary antibody. Chromogenic or luminescent substrates (see Basic Protocol 3 and Alternate Protocol 4) are then used to visualize the activity. Finally, membranes may be stripped and reprobed (see Support Protocol 3).

NOTE: When handling gels and membranes wear powder-free gloves.

PROTEIN BLOTTING WITH TANK TRANSFER SYSTEMS

In this procedure, blotting is performed in a tank of buffer with the gel in a vertical orientation, completely submerged between two large electrode panels. In some systems up to four gels can be transferred at one time. For difficult-to-transfer proteins (>100 kDa or hydrophobic; e.g., myosin), tank blotting is preferable to semidry systems (see Basic Protocol 2) because prolonged transfers are possible without buffer depletion. However, transfers >1 hr at high power require cooling using a heat exchanger and a circulating water bath that can maintain a constant transfer temperature of 10° to 20°C.

Materials

- Samples for analysis
- Protein molecular weight standards (UNIT 6.1), prestained (Sigma or Bio-Rad) or biotinylated (Vector Labs or Sigma)
- Transfer buffer (see recipe)
- Powder-free gloves
- Scotch-Brite pads (3M) or equivalent sponge
- Whatman 3MM filter paper or equivalent
- Transfer membrane: 0.45- μ m nitrocellulose (Millipore or Schleicher & Schuell), PVDF (Millipore Immobilon P), neutral nylon (Pall Biotodyne A), or positively charged nylon (Pall Biotodyne B; Bio-Rad Zetabind) membrane
- Electroblotting apparatus (EC Apparatus, Bio-Rad, or Amersham Pharmacia Biotech)
- Indelible pen (e.g., Paper-Mate ballpoint) or soft lead pencil
- Additional reagents and equipment for gel electrophoresis (UNIT 6.1) and staining proteins in gels and on membranes (see Support Protocol 1)

NOTE: Deionized, distilled water should be used throughout this protocol.

**BASIC
PROTOCOL 1**

**Electrophoresis
and
Immunoblotting**

Electrophorese the protein sample

1. Prepare antigenic samples and separate proteins using small or standard-sized one- or two-dimensional gels (UNIT 6.1). Include prestained or biotinylated protein molecular weight standards in one or more gel lanes.

The protein markers will transfer to the membrane and conveniently indicate membrane orientation and sizes of proteins after immunostaining.

A variety of gel sizes and percentages of acrylamide can be used (UNIT 6.1). Most routinely used are either 14 cm × 14 cm × 0.75-mm gels or 8 cm × 10 cm × 0.75-mm minigels. Acrylamide concentrations vary from 5% to 20%, but are usually in the 10% to 15% range.

Assemble the immunoblot sandwich

2. When electrophoresis is complete, disassemble gel sandwich and remove stacking gel. Equilibrate gel 30 min at room temperature in transfer buffer.

Oil from hands blocks the transfer.

Match the appropriate transfer buffer to the membrane (see Reagents and Solutions).

Gel equilibration is required to prevent a change in the size of the gel during transfer. Any shift in gel dimension will result in a blurred transfer pattern.

3. Assemble transfer sandwich in a tray large enough to hold the plastic transfer cassette. Fill with transfer buffer so that cassette is covered.

The transfer cassette should be assembled under buffer to minimize trapping of air bubbles. Use Figure 6.2.1 as a guide to assembly.

4. On bottom half of plastic transfer cassette, place Scotch-Brite pad or sponge, followed by a sheet of filter paper cut to same size as gel and prewet with transfer buffer.

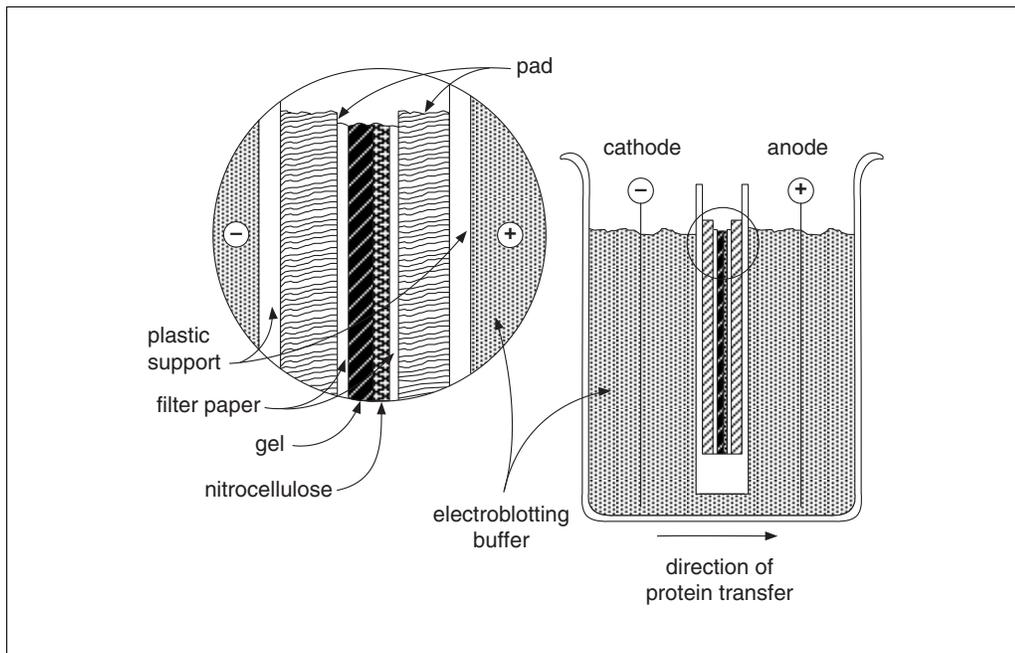


Figure 6.2.1 Immunoblotting with a tank blotting unit. The polyacrylamide gel containing the protein is laid on a sheet of filter paper. The uncovered side of the gel is overlaid with a sheet of membrane precut to the size of the gel plus 1 to 2 mm on each edge, then this membrane is overlaid with another sheet of filter paper. The filter paper containing the gel and membrane is sandwiched between Scotch-Brite pads. This sandwich is placed in a plastic support, and the entire assembly is placed in a tank containing transfer buffer. For transfer of negatively charged protein, the membrane is positioned on the anode side of the gel. For transfer of positively charged protein, the membrane is placed on the cathode side of the gel. Charged proteins are transferred electrophoretically from the gel onto the membrane. Transfer is achieved by applying a voltage of 100 V for 1 to 2 hr (with cooling) or 14 V overnight.

5. Place gel on top of filter paper. The side of the gel touching the paper arbitrarily becomes the cathode side of the gel (i.e., ultimately toward the negative electrode when positioned in the tank). Remove any air bubbles between gel and filter paper by gently rolling a test tube or glass rod over surface of gel.

Any bubbles between the filter paper, gel, and membrane will block current flow and prevent protein transfer. This problem is indicated on the membrane by sharply defined white areas devoid of transferred protein.

Proteins are usually negatively charged in transfer buffer and move toward the positive anode. However, some proteins may be positively charged. An additional membrane placed on the cathode side of the gel will bind these proteins.

6. Prepare transfer membrane. Cut membrane to same size as gel plus 1 to 2 mm on each edge. Place into distilled water slowly, with one edge at a 45° angle. Equilibrate 10 to 15 min in transfer buffer.

The water will wick up into the membrane, wetting the entire surface. If it is inserted too quickly into the water, air gets trapped and will appear as white blotches in the membrane. Protein will not transfer onto these areas.

This wetting procedure works for nitrocellulose and nylon membranes only. PVDF membranes are hydrophobic and will not wet simply from being placed into distilled water or transfer buffer. For these membranes, first immerse 1 to 2 sec in 100% methanol, then equilibrate 10 to 15 min with transfer buffer. Do not let membrane dry out at any time. If this occurs, wet once again with methanol and transfer buffer as described above.

7. Moisten surface of gel with transfer buffer. Place prewetted membrane directly on top side of gel (i.e., anode side) and remove all air bubbles as in step 5.

Poor contact between the gel and membrane will cause a swirled pattern of transferred proteins on the membrane. Some proteins will transfer as soon as the gel is placed on the membrane; repositioning the gel or membrane can result in a smeared or double image on the developed blot.

The use of 0.2- μ m membranes may improve retention of smaller-molecular-weight proteins.

8. Wet another piece of Whatman 3MM filter paper, place on anode side of membrane, and remove all air bubbles. Place another Scotch-Brite pad or sponge on top of this filter paper.

9. Complete assembly by locking top half of the transfer cassette into place (Fig. 6.2.1).

It is important to orient the sandwich so that the membrane faces the anode (positively charged) side of the tank.

Transfer proteins from gel to membrane

10. Fill tank with transfer buffer and place transfer cassette containing sandwich into electroblotting apparatus in correct orientation. Connect leads of power supply to corresponding anode and cathode sides of electroblotting apparatus.

Transfer buffer should cover the electrode panels but should not touch the base of the banana plug.

11. Electrophoretically transfer proteins from gel to membrane for 30 min to 1 hr at 100 V with cooling or overnight at 14 V (constant voltage), in a cold room.

Transfer time is dependent on the thickness and the percent acrylamide of the gel, as well as the size of the protein being transferred. In general, proteins are transferred within 1 to 6 hr, but high-molecular-weight molecules may take longer. Overnight transfer at low voltage is reliable and convenient. Cooling (at 10° to 20°C) is required for transfers >1 hr at high power. Heat exchanger cooling cores using a circulating water bath are placed into the transfer unit for cooling.

12. Turn off the power supply and disassemble the apparatus. Remove membrane from blotting apparatus and note orientation by cutting a corner or marking with a soft lead pencil or Paper-Mate ballpoint pen.

Many ballpoint inks come off, but Paper-Mate stays on the membrane.

Membranes can be dried and stored in resealable plastic bags at 4°C for 1 year or longer at this point. Prior to further processing, dried PVDF membranes must be placed into a small amount of 100% methanol to wet the membrane, then in distilled water to remove the methanol.

13. Stain gel for total protein with Coomassie blue to verify transfer efficiency. If desired, stain membrane reversibly to visualize transferred proteins (see Support Protocol 1), or irreversibly with Coomassie blue, India ink, naphthol blue, or colloidal gold.

These staining procedures are incompatible with nylon membranes.

If membrane shows significant staining on the backside, either the gel was heavily overloaded or the membrane has poor protein-binding capacity (see Troubleshooting). In either case, protein-binding sites on the side facing the gel are saturated, allowing protein to migrate to the other side of the membrane. Nitrocellulose in particular will show diminished binding capacity with age or poor storage conditions (e.g., high temperature and humidity). In addition, some proteins simply do not bind well to a particular matrix. By using several membrane sheets in place of one, the protein can be detected as it passes through each consecutive sheet. This will give an indication of how efficiently the membrane binds to a particular protein.

14. Proceed with immunoprobng and visual detection of proteins (see Basic Protocols 2 and 3 and Alternate Protocols 3 and 4).

ALTERNATE PROTOCOL 1

PROTEIN BLOTTING WITH SEMIDRY SYSTEMS

Even and efficient transfer of most proteins is also possible with semidry blotting, a convenient alternative to tank transfer systems. Instead of being placed vertically into a tank filled with transfer buffer, the gel is held horizontally between buffer-saturated blotting paper that is in contact with the electrodes (Fig. 6.2.2), greatly reducing the amount of buffer required. The electrodes are close together, giving high field strengths and rapid transfer with a standard electrophoresis power supply. Prolonged transfers (>1 hr) are not recommended; tank blotting (see Basic Protocol 1) should be used for proteins that require long blotting times for efficient transfer.

Additional Materials (also see Basic Protocol 1)

Six sheets of Whatman 3MM filter paper or equivalent, cut to size of gel and saturated with transfer buffer

Semidry transfer unit (Amersham Pharmacia Biotech, Bio-Rad, or Sartorius)

1. Prepare samples and separate proteins using small or standard-sized one- or two-dimensional gels (UNIT 6.1).

Because transfer efficiency depends on many factors (e.g., gel concentration and thickness, protein size, shape, and net charge) results may vary. Below is a guideline for 0.75-mm-thick SDS-PAGE gels transferred by semidry blotting.

Percent acrylamide (resolving gel)	Size range transferred (~100% efficiency)
5–7	29–150 kDa
8–10	14–66 kDa
13–15	<36 kDa
18–20	<20 kDa

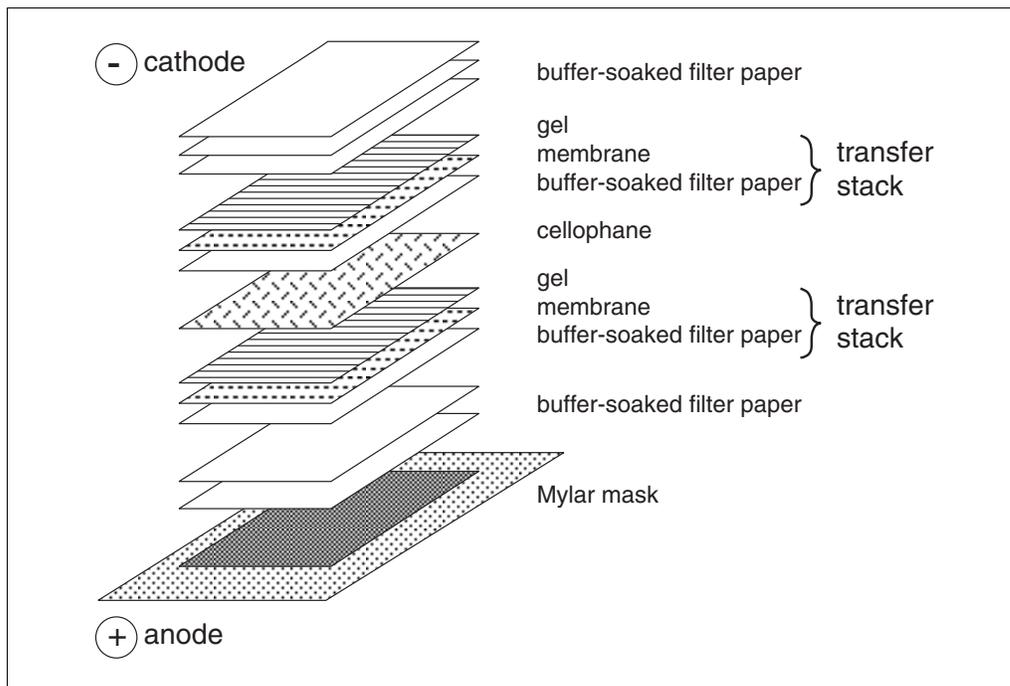


Figure 6.2.2 Immunoblotting with a semidry transfer unit. Generally, the lower electrode is the anode, and one gel is transferred at a time. A Mylar mask (optional in some units) is put in place on the anode. This is followed by three sheets of transfer buffer-soaked filter paper, the membrane, the gel, and finally, three more sheets of buffer-soaked filter paper. To transfer multiple gels, construct transfer stacks as illustrated, and separate each with a sheet of porous cellophane. For transfer of negatively charged protein, the membrane is positioned on the anode side of the gel. For transfer of positively charged protein, the membrane is placed on the cathode side of the gel. Transfer is achieved by applying a maximum current of 0.8 mA/cm^2 of gel area. For a typical minigel ($8 \times 10 \text{ cm}$) and standard-sized gel ($14 \times 14 \text{ cm}$), this means 60 and 200 mA, respectively.

2. Prepare transfer membrane (see Basic Protocol 1, step 6).
3. Disassemble gel sandwich. Remove and discard stacking gel.

Equilibration of the separating gel with transfer buffer is not normally required for semidry blotting, but it may improve transfer in some cases.

4. Place three sheets of filter paper saturated with transfer buffer on the anode (Fig. 6.2.2).

Most transfer units are designed so that negatively charged proteins move downward toward either a platinum or graphite positive electrode (anode).

CAPS transfer buffer, pH 10.5 (see recipe for transfer buffer) can be used in place of the Tris/glycine/methanol transfer buffer of Basic Protocol 1. CAPS buffer should be used if the protein is to be sequenced right on the membrane (Moos, 1992), as glycine will interfere with this procedure.

The filter paper should be cut to the exact size of the gel. This forces the current to flow only through the gel and not through overlapping filter paper. Some manufacturers (e.g., Amersham Pharmacia Biotech) recommend placing a Mylar mask on the lower platinum anode. With an opening that is slightly less than the size of the gel, the mask forces the current to flow through the gel and not the surrounding electrode area during transfer.

5. Place equilibrated transfer membrane on top of filter paper stack. Remove all bubbles between membrane and filter paper by rolling a test tube over surface of membrane.

Any bubbles in the filter paper stack or between the filter paper, membrane, and gel will block current flow and prevent protein transfer. This problem is indicated on the membrane by sharply defined white areas devoid of transferred protein.

6. Place gel on top of membrane. Gently roll a test tube over surface of gel to ensure intimate contact between gel and membrane and to remove any interfering bubbles.

Poor contact between the gel and membrane will cause a swirled pattern of transferred proteins on the membrane. Some proteins will transfer as soon as the gel is placed on the membrane; repositioning the gel or membrane can result in a smeared or double image on the developed blot.

7. Complete the transfer stack by putting the three remaining sheets of filter paper on top of gel. Roll out bubbles as described above.

Multiple gels can be transferred using semidry blotting. Simply put a sheet of porous cellophane (Amersham Pharmacia Biotech) or dialysis membrane (Bio-Rad or Sartorius) equilibrated with transfer buffer between each transfer stack (Fig. 6.2.2). Transfer efficiency is dependent on the position of the transfer stack in the blotting unit and for critical applications transferring one gel at a time is recommended. The gel next to the anode tends to be more efficiently transferred when blotting more than one gel at a time.

Transfer proteins from gel to membrane

8. Place top electrode onto transfer stack.

Most units have safety-interlock features and can only be assembled one way. Consult manufacturer's instructions for details.

Once assembled, do not move the top electrode. This can shift the transfer stack and move the gel relative to the membrane. Some transfer will occur as soon as the gel contacts the membrane, and any shifting of the transfer stack after assembly will distort the transfer pattern.

9. Carefully connect high-voltage leads to the power supply (see UNIT 6.1 for safety precautions). Apply constant current to initiate protein transfer. Transfers of 1 hr are generally sufficient.

In general, do not exceed 0.8 mA/cm² of gel area. For a typical minigel (8 × 10 cm) and standard-sized gel (14 × 14 cm) this means ~60 and 200 mA, respectively.

Monitor the temperature of the transfer unit directly above the gel by touch. The unit should not exceed 45°C. If the outside of the unit is warm, too much current is being applied. Note that units with graphite electrodes are more prone to heating, because graphite has much more resistance to current flow than platinum or steel electrodes.

10. After transfer, turn off power supply and disassemble unit. Remove membrane from transfer stack, marking orientation as in step 12 of Basic Protocol 1. Proceed with staining and immunoprobng (see Basic Protocol 1, steps 13 and 14).

ALTERNATE PROTOCOL 2

BLOTTING OF STAINED GELS

Gels stained with Coomassie blue R250 can be effectively immunoblotted by the following procedure, based on Perides et al. (1986) and Dionisi et al. (1995). Briefly, the stained gel is soaked in a series of solutions designed to increase the solubility of the proteins after staining and fixation. After transfer, the membranes are treated with 45% or 100% methanol to decrease the Coomassie blue bound to the membrane prior to processing for chromogenic development. For chemiluminescent development, removal of the Coomassie blue is generally not needed.

Materials

Destained gel containing proteins of interest
25 mM Tris base/192 mM glycine/1% SDS
25 mM Tris base/192 mM glycine/0.1% SDS

1. Soak destained gel containing proteins of interest in distilled water for 15 min.
2. Equilibrate gel with 25 mM Tris base/192 mM glycine/1% SDS for 1 hr with gentle agitation.
3. Transfer gel to 25 mM Tris base/192 mM glycine/0.1% SDS and equilibrate 30 min with gentle agitation.

To increase transfer efficiency of larger proteins, the gel should be transferred to the above solution with 6 M urea for an additional 30 min.

4. Proceed with transfer (see Basic Protocol 1, steps 2 to 12).

For the most efficient transfer and binding to the membrane, the transfer buffer should contain SDS.

5. After transfer, soak membranes for 10 to 30 min in 45% methanol (nitrocellulose) or 100% methanol (nylon or PVDF) to remove the bound Coomassie blue.

This step is not needed if using chemiluminescent reactions or radiolabeled protein A for immunodevelopment. Destaining of the nitrocellulose membrane is enhanced by adding a small ball of laboratory tissue to the methanol to absorb the Coomassie blue.

6. Proceed with immunoprob ing and visual detection of proteins (see Basic Protocols 2 and 3 and Alternate Protocols 3 and 4).

REVERSIBLE STAINING OF TRANSFERRED PROTEINS

To verify transfer efficiency, nitrocellulose and PVDF membranes can be reversibly stained. This method will not work on nylon membranes.

Additional Materials (also see Basic Protocol 1)

Ponceau S solution (see recipe)

Additional reagents and equipment for photographing membranes

1. Following protein transfer to nitrocellulose or PVDF (see Basic Protocol 1 or Alternate Protocol 1), place membrane in Ponceau S solution 5 min at room temperature.
2. Destain 2 min in water. Photograph membrane if required and mark the molecular-weight-standard band locations with indelible ink.
3. Completely destain membrane by soaking an additional 10 min in water.

QUANTITATION OF PROTEIN WITH PONCEAU S

In addition to qualitatively visualizing proteins on membranes after blotting, Ponceau S provides a convenient method for quantifying the amount of protein in a given lane. By eluting the dye off the strip and reading in a spectrophotometer (A_{525}), an internal control value of protein on a lane is obtained. This value is used to correct for any differences in protein loading from lane to lane. Comparison of the Ponceau S value to the chemiluminescent or chromogenic immunodetection value determined by densitometry provides a straightforward correction for lane-to-lane variation. This method works best for complex mixtures where the immunodetected protein represents a small proportion of the total protein (Klein et al., 1995).

Additional Materials (also see Basic Protocol 1)

Spectrophotometer and 2-ml cuvette

SUPPORT PROTOCOL 1

SUPPORT PROTOCOL 2

Electrophoresis and Immunoblotting

6.2.7

1. Following protein transfer to nitrocellulose, PVDF, or nylon (see Basic Protocol 1 or Alternate Protocol 1), stain membrane, photograph, and destain (see Support Protocol 1).

Membranes should be destained until the background becomes white.

2. Mark lanes with a soft pencil and cut lanes into strips.
3. Place each strip into 7 ml of distilled water for 7 min and remove the resulting solution. If any particulates are visible, centrifuge 30 min at 2000 rpm to remove them.
4. Read A_{525} in a 2-ml cuvette.

Any variation in gel-to-gel sample loading and blotting efficiency will be reflected in a change in A_{525} of the sample lanes when compared to the control. The change in A_{525} can be calibrated to a known amount of protein loaded on the control lane. This will be a relative value, however, since the transfer out of the gel and binding to the membrane is rarely 100%.

IMMUNOPROBING WITH DIRECTLY CONJUGATED SECONDARY ANTIBODY

Immobilized proteins are probed with specific antibodies to identify and quantitate any antigens present. The membrane is immersed in blocking buffer to fill all protein-binding sites with a nonreactive protein or detergent. Next, it is placed in a solution containing the antibody directed against the antigen (primary antibody). The blot is washed and exposed to an enzyme-antibody conjugate directed against the primary antibody (secondary antibody; e.g., goat anti-rabbit IgG). Antigens are identified by chromogenic or luminescent visualization (see Basic Protocol 3 and Alternate Protocol 4) of the antigen/primary antibody/secondary antibody/enzyme complex bound to the membrane. Tween 20 is a common alternative to protein-blocking agents when using nitrocellulose or PVDF filters.

Materials

Membrane with transferred proteins (see Basic Protocol 1 or Alternate Protocol 1)

Blocking buffer appropriate for membrane and detection protocol (see recipe)

Primary antibody specific for protein of interest

TTBS (nitrocellulose or PVDF) or TBS (nylon; see *APPENDIX 2A* for recipes)

Secondary antibody conjugate: horseradish peroxidase (HRPO)—or alkaline phosphatase (AP)—anti-Ig conjugate (Cappel, Vector Labs, Kirkegaard & Perry, or Sigma; dilute as indicated by manufacturer and store frozen in 25- μ l aliquots until use)

Heat-sealable plastic bag

Powder-free gloves

Plastic box

1. Place membrane in heat-sealable plastic bag with 5 ml blocking buffer and seal bag. Incubate 30 min to 1 hr at room temperature with agitation on an orbital shaker or rocking platform.

Usually 5 ml buffer is sufficient for two to three membranes (14 \times 14-cm size). If membrane is to be stripped and reprobed (see Support Protocol 3), blocking buffer must contain casein (for AP systems) or nonfat dry milk.

Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

2. Dilute primary antibody in blocking buffer.

Primary antibody dilution is determined empirically but is typically 1/100 to 1/1000 for a polyclonal antibody (Fig. 6.2.3), 1/10 to 1/100 for hybridoma supernatants, and \geq 1/1000

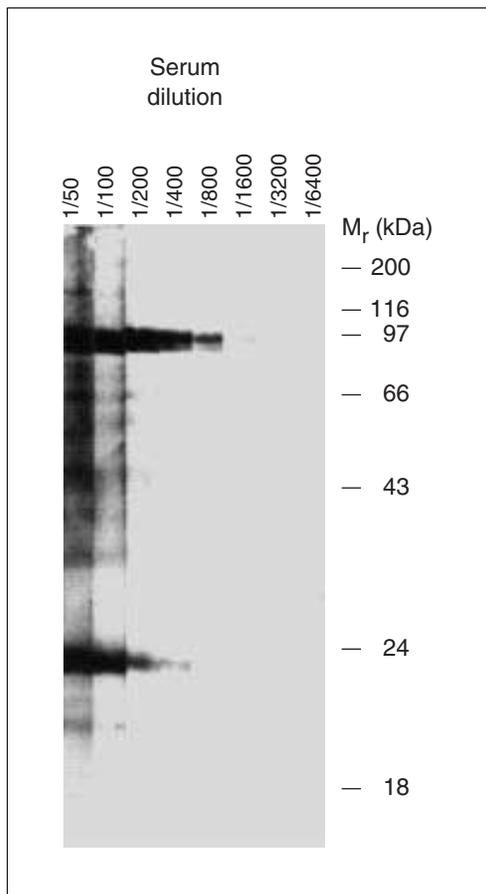


Figure 6.2.3 Serial dilution of primary antibody directed against the 97-kDa catalytic subunit of the plant plasma membrane ATPase. Blot was developed with HRPO-coupled avidin-biotin reagents according to the second alternate protocol and visualized with 4-chloro-1-naphthol (4CN). Note how background improves with dilution.

for murine ascites fluid containing monoclonal antibodies. Ten- to one-hundred-fold higher dilutions can be used with alkaline phosphatase- or luminescence-based detection systems. Both primary and secondary antibody solutions can be used at least twice, but long-term storage (i.e., >2 days at 4°C) is not recommended.

3. Open bag and pour out blocking buffer. Replace with diluted primary antibody and incubate 30 min to 1 hr at room temperature with constant agitation.

Usually 5 ml diluted primary antibody solution is sufficient for two to three membranes (14 × 14-cm size). Incubation time may vary depending on conjugate used.

When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove membrane from plastic bag with gloved hand. Place in plastic box and wash four times by agitating with 200 ml TTBS (nitrocellulose or PVDF) or TBS (nylon), 10 to 15 min each time.

5. Dilute secondary antibody HRPO- or AP-anti-Ig conjugate in blocking buffer.

Commercially available enzyme-conjugated secondary antibody is usually diluted 1/200 to 1/2000 prior to use (Harlow and Lane, 1988).

6. Place membrane in new heat-sealable plastic bag, add diluted HRPO- or AP-anti-Ig conjugate, and incubate 30 min to 1 hr at room temperature with constant agitation.

When using plastic incubation trays, see step 3 annotation for proper antibody solution volumes.

7. Remove membrane from bag and wash as in step 4. Develop according to appropriate visualization protocol (see Basic Protocol 3 or Alternate Protocol 4).

**IMMUNOPROBING WITH AVIDIN-BIOTIN COUPLING TO
SECONDARY ANTIBODY**

The following procedure is based on the Vectastain ABC kit from Vector Labs (see *SUPPLIERS APPENDIX*). It uses an avidin-biotin complex to attach horseradish peroxidase (HRPO) or alkaline phosphatase (AP) to the biotinylated secondary antibody. Avidin-biotin systems are capable of extremely high sensitivity due to the multiple reporter enzymes bound to each secondary antibody. In addition, the detergent Tween 20 is a popular alternative to protein-blocking agents when using nitrocellulose or PVDF filters.

Additional Materials (also see *Basic Protocol 2*)

Blocking buffer appropriate for membrane and detection protocol (see recipe)

TTBS (nitrocellulose or PVDF) or TBS (neutral or positively charged nylon; see *APPENDIX 2A* for recipes)

Vectastain ABC (HRPO) or ABC-AP (AP) kit (Vector Labs) containing the following: reagent A (avidin), reagent B (biotinylated HRPO or AP), and biotinylated secondary antibody (request membrane immunodetection protocols when ordering)

1. Equilibrate membrane in appropriate blocking buffer in heat-sealed plastic bag with constant agitation using an orbital shaker or rocking platform. For nitrocellulose and PVDF, incubate 30 to 60 min at room temperature. For nylon, incubate ≥ 2 hr at 37°C.

TTBS is well suited for avidin-biotin systems. For nylon, protein-binding agents are recommended. Because nonfat dry milk contains residual biotin, which will interfere with the immunoassay, it must be used in the blocking step only. If membrane is to be stripped and reprobed (see Support Protocol 3), blocking buffer must contain casein (for AP systems) or nonfat dry milk.

Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

2. Prepare primary antibody solution in TTBS (nitrocellulose or PVDF) or TBS (nylon).

Dilutions of sera containing primary antibody generally range from 1/100 to 1/100,000. This depends in large part on the sensitivity of the detection system. With high-sensitivity avidin-biotin systems, dilutions from 1/1000 to 1/100,000 are common. Higher dilutions can be used with AP- or luminescence-based detection systems. To determine the appropriate concentration of the primary antibody, a dilution series is easily performed with membrane strips. Separate antigens on a preparative gel (i.e., a single large sample well) and immunoblot the entire gel. Cut 2- to 4-mm strips by hand or with a membrane cutter (Schleicher and Schuell; Inotech) and incubate individual strips in a set of serial dilutions of primary antibody. The correct dilution should give low background and high specificity (Fig. 6.2.3).

3. Open bag, remove blocking buffer, and add enough primary antibody solution to cover membrane. Incubate 30 min at room temperature with gentle rocking.

Usually 5 ml diluted primary antibody solution is sufficient for two to three membranes (14 × 14-cm size). Incubation time may vary depending on conjugate used.

When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove membrane from bag and place in plastic box. Wash membrane three times over a 15-min span in TTBS (nitrocellulose or PVDF) or TBS (nylon). Add enough TTBS or TBS to fully cover the membrane (e.g., 5 to 10 ml/strip or 25 to 50 ml/whole membrane).

5. Prepare biotinylated secondary antibody solution by diluting two drops biotinylated antibody with 50 to 100 ml TTBS (nitrocellulose or PVDF) or TBS (nylon).

This dilution gives both high sensitivity and enough volume to easily cover a large 14 × 14-cm membrane.

6. Transfer membrane to fresh plastic bag containing secondary antibody solution. Incubate 30 min at room temperature with slow rocking, then wash as in step 4.

When using plastic incubation trays, see step 3 annotations for proper antibody solution volumes.

7. While membrane is being incubated with secondary antibody, prepare avidin-biotin-HRPO or -AP complex. Mix two drops Vectastain reagent A and two drops reagent B into 10 ml TTBS (nitrocellulose or PVDF) or TBS (nylon). Incubate 30 min at room temperature, then further dilute to 50 ml with TTBS or TBS.

Diluting the A and B reagents to 50 ml expands the amount of membrane that can be probed without greatly affecting sensitivity. Sodium azide is a peroxidase inhibitor and should not be used as a preservative. Casein, nonfat dry milk, serum, and some grades of BSA may interfere with the formation of the avidin-biotin complex and should not be used in the presence of avidin or biotin reagents (Gillespie and Hudspeth, 1991; Vector Labs).

8. Transfer membrane to avidin-biotin-enzyme solution. Incubate 30 min at room temperature with slow rocking, then wash over a 30-min span as in step 4.
9. Develop membrane according to the appropriate visualization protocol (see Basic Protocol 3 or Alternate Protocol 4).

VISUALIZATION WITH CHROMOGENIC SUBSTRATES

Bound antigens are typically visualized with chromogenic substrates. The substrates 4CN, DAB/NiCl₂, and TMB are commonly used with horseradish peroxidase (HRPO)-based immunodetection procedures, while BCIP/NBT is recommended for alkaline phosphatase (AP)-based procedures (see Table 6.2.1). After incubation with primary and secondary antibodies, the membrane is placed in the appropriate substrate solution. Protein bands usually appear within a few minutes.

Materials

Membrane with transferred proteins and probed with antibody-enzyme complex
(see Basic Protocol 2 or Alternate Protocol 3)

TBS (APPENDIX 2A)

Chromogenic visualization solution (Table 6.2.1)

Additional reagents and equipment for gel photography

1. If final membrane wash (see Basic Protocol 2, step 7, or see Alternate Protocol 3, step 8) was performed in TTBS, wash membrane 15 min at room temperature in 50 ml TBS.

The Tween 20 in TTBS interferes with 4CN development (Bjerrum et al., 1988).

2. Place membrane into chromogenic visualization solution. Bands should appear in 10 to 30 min.
3. Terminate reaction by washing membrane in distilled water. Air dry and photograph for a permanent record.

BASIC PROTOCOL 3

Table 6.2.1 Chromogenic and Luminescent Visualization Systems^a

System	Reagent ^b	Reaction/Detection	Comments ^c
Chromogenic			
HRPO-based	4CN	Oxidized products form purple precipitate	Not very sensitive (Tween 20 inhibits reaction); fades rapidly upon exposure to light
	DAB/NiCl ₂ ^d	Forms dark brown precipitate	More sensitive than 4CN but potentially carcinogenic; resulting membrane easily scanned
	TMB ^e	Forms dark purple stain	More stable, less toxic than DAB/NiCl ₂ ; may be somewhat more sensitive ^e ; can be used with all membrane types; kits available from Kirkegaard & Perry, TSI, Moss, and Vector Labs
AP-based	BCIP/NBT	BCIP hydrolysis produces indigo precipitate after oxidation with NBT; reduced NBT precipitates; dark blue-gray stain results	More sensitive and reliable than other AP-precipitating substrates; note that phosphate inhibits AP activity
Luminescent			
HRPO-based	Luminol/H ₂ O ₂ / <i>p</i> -iodophenol	Oxidized luminol substrate gives off blue light; <i>p</i> -iodophenol increases light output	Very convenient, sensitive system; reaction detected within a few seconds to 1 hr
AP-based	Substituted 1,2-dioxetane-phosphates (e.g., AMPPD, CSPD, Lumigen-PPD, Lumi-Phos 530 ^f)	Dephosphorylated substrate gives off light	Protocol described gives reasonable sensitivity on all membrane types; consult instructions of reagent manufacturer for maximum sensitivity and minimum background (see Troubleshooting)

^aAbbreviations: AMPPD or Lumigen-PPD, disodium 3-(4-methoxy-spiro{1,2-dioxetane-3,2'-tricyclo[3.3.1.1.1^{3,7}] decan}-4-yl)phenyl phosphate; AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; 4CN, 4-chloro-1-naphthol; CSPD, AMPPD with substituted chlorine moiety on adamantane ring; DAB, 3,3'-diaminobenzidine; HRPO, horseradish peroxidase; NBT, nitroblue tetrazolium; TMB, 3,3',5,5'-tetramethylbenzidine.

^bRecipes and suppliers are listed in Reagents and Solutions except for TMP, for which use of a kit is recommended.

^cSee Commentary for further details.

^dDAB/NiCl₂ can be used without the nickel enhancement, but it is much less sensitive.

^eMcKimm-Breschkin (1990) reported that if nitrocellulose filters are first treated with 1% dextran sulfate for 10 min in 10 mM citrate-EDTA (pH 5.0), TMB precipitates onto the membrane with a sensitivity much greater than 4CN or DAB, and equal to or better than that of BCIP/NBT.

^fLumi-Phos 530 contains dioxetane phosphate, MgCl₂, CTAB (cetyltrimethylammonium bromide), and fluorescent enhancer in a pH 9.6 buffer.

ALTERNATE PROTOCOL 4

VISUALIZATION WITH LUMINESCENT SUBSTRATES

Antigens can also be visualized with luminescent substrates. Detection with light offers both speed and enhanced sensitivity over chromogenic and radioisotopic procedures. After the final wash, the blot is immersed in a substrate solution containing luminol for horseradish peroxidase (HRPO) systems or dioxetane phosphate for alkaline phosphatase (AP) systems, sealed in thin plastic wrap, and placed firmly against film. Exposures range from a few seconds to several hours, although typically strong signals appear within a few seconds or minutes.

Immunoblotting and Immunodetection

Additional Materials (also see Basic Protocol 3)

- Luminescent substrate buffer: 50 mM Tris-Cl, pH 7.5 (HRPO; APPENDIX 2A) or dioxetane phosphate substrate buffer (alkaline phosphatase; see recipe)
- Nitro-Block solution (AP reactions only): 5% (v/v) Nitro-Block (Tropix) in dioxetane phosphate substrate buffer, prepared just before use
- Luminescent visualization solution (Table 6.2.1)
- Clear plastic wrap
- Additional reagents and equipment for autoradiography (UNIT 6.3)

NOTE: See Troubleshooting section for suggestions concerning optimization of this protocol, particularly when employing AP-based systems.

1. Equilibrate membrane in two 15-min washes with 50 ml substrate buffer.
For blots of whole gels, use 50 ml substrate buffer; for strips, use 5 to 10 ml/strip.
2. For AP reactions using nitrocellulose or PVDF membranes: Incubate 5 min in Nitro-Block solution, followed by 5 min in substrate buffer (volumes as in step 1).
Nitro-Block enhances light output from the dioxetane substrate in reactions using AMPPD, CSPD, or Lumigen-PPD concentrate. It is required for nitrocellulose and recommended for PVDF membranes. It is not needed for Lumi-Phos 530, AP reactions on nylon membranes, or HRPO-based reactions on any type of membrane. Lumi-Phos 530 is not recommended for nitrocellulose membranes.
3. Transfer membrane to visualization solution. Soak 30 sec (HRPO reactions) to 5 min (AP reactions; volumes as in step 1).
Alternatively, lay out a square of plastic wrap and pipet 1 to 2 ml visualization solution into the middle. Place membrane on the plastic so that the visualization solution spreads out evenly from edge to edge. Fold wrap back onto membrane, seal, and proceed to step 5.
4. Remove membrane, drain, and place face down on a sheet of clear plastic wrap. Fold wrap back onto membrane to form a liquid-tight enclosure.
To ensure an optimal image, only one layer of plastic should be between the membrane and film. Sealable bags are an effective alternative. Moisture must not come in contact with the X-ray film.
5. In a darkroom, place membrane face down onto film.
Do this quickly and do not reposition; a double image will be formed if the membrane is moved while in contact with the film. A blurred image is usually caused by poor contact between membrane and film; use a film cassette that ensures a tight fit.
6. Expose film for a few seconds to several hours.
Typically, immunoblots produce very strong signals within a few seconds or minutes. However, weak signals may require several hours to an overnight exposure. If no image is detected, expose film 30 min to 1 hr, and if needed, overnight (see Troubleshooting).
7. If desired, wash membrane in two 15-min washes of 50 ml TBS and process for chromogenic development (see Basic Protocol 3).
Chemiluminescent and chromogenic immunoblotting can be easily combined on a single blot to provide a permanent visual marker of a known protein. First probe membrane with the chemiluminescent reactions to record on film. If stripping and reprobing is needed, then process by wetting and NaOH treatment (see Support Protocol 3). For the last reaction, use chromogenic development to produce a permanent visual record of the blot. Alternatively, once the film record of the chemiluminescent blot is recorded, the blot can be rinsed briefly with distilled water and placed in the appropriate chromogenic solution for chromogenic development of the blot. This results in a permanent reference stain on the blot for comparison to the more easily scanned and quantitated film record.

STRIPPING AND REUSING MEMBRANES

This stripping procedure works with blotted membranes from one- and two-dimensional gels as well as with proteins blotted from previously stained gels (Suck and Krupinska, 1996). Reprobing PVDF membranes that have been developed with chemiluminescent reagents is simple and straightforward. All residual antibodies are removed from the membrane by first rewetting it in water and then briefly treating it with NaOH. Although repeated reprobing can lead to loss of signal, up to five reprobings generally are feasible. The blot should have been previously blocked with 5% nonfat dry milk prior to treatment.

Materials

0.2 M NaOH

1. Wash blot 5 min in distilled water.

In order to effectively reprobe the membranes, casein (for AP systems) or nonfat dry milk must be used as the blocking agent. Chromogenic development leaves a permanent stain on the membrane that is difficult to remove, and should not be used when reprobing. The stain can interfere with subsequent analysis if reactive bands from sequential immunostainings are close together.

2. Transfer to 0.2 M NaOH and wash 5 min.
3. Wash blot 5 min in distilled water.
4. Proceed with immunoprobng procedure (see Basic Protocol 2 and Alternate Protocol 3).

Casein or nonfat dry milk is recommended as blocking agent when reprobing membranes.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX. For selection of appropriate chromogenic or luminescent solutions, and for definition of abbreviations, see Table 6.2.1.

Alkaline phosphate substrate buffer

100 mM Tris-Cl, pH 9.5
100 mM NaCl
5 mM MgCl₂

BCIP/NBT visualization solution

Mix 33 μ l NBT stock (100 mg NBT in 2 ml at 70% DMF, stored <1 year at 4°C) and 5 ml alkaline phosphate substrate buffer (see recipe). Add 17 μ l BCIP stock (100 mg BCIP in 2 ml of 100% DMF, stored <1 year at 4°C) and mix. Stable 1 hr at room temperature.

Recipe is from Harlow and Lane (1988). Alternatively, BCIP/NBT substrates may be purchased from Sigma, Kirkegaard & Perry, Moss, and Vector Labs.

Blocking buffer

Colorimetric detection:

For nitrocellulose and PVDF: 0.1% (v/v) Tween 20 in TBS (TTBS; APPENDIX 2A).

For neutral and positively charged nylon: Tris-buffered saline (TBS; APPENDIX 2A) containing 10% (w/v) nonfat dry milk. Prepare just before use.

TTBS can be stored \leq 1 week at 4°C.

Luminescence detection:

For nitrocellulose, PVDF, and neutral nylon (e.g., Pall Biodyne A): 0.2% casein (e.g., Hammarsten grade or I-Block; Tropix) in TTBS (APPENDIX 2A). Prepare just before use.

continued

For positively charged nylon: 6% (w/v) casein/1% (v/v) polyvinyl pyrrolidone (PVP) in TTBS (APPENDIX 2A). With constant mixing, add casein and PVP to warm (65°C) TTBS. Stir for 5 min. Cool before use. Prepare just before use.

4CN visualization solution

Mix 20 ml ice-cold methanol with 60 mg 4CN. Separately mix 60 µl of 30% H₂O₂ with 100 ml TBS (APPENDIX 2A) at room temperature. Rapidly mix the two solutions and use immediately.

DAB/NiCl₂ visualization solution

5 ml 100 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
100 µl DAB stock (40 mg/ml in H₂O, stored in 100-µl aliquots at -20°C)
25 µl NiCl₂ stock (80 mg/ml in H₂O, stored in 100-µl aliquots at -20°C)
15 µl 3% H₂O₂
Mix just before use

CAUTION: Handle DAB carefully, wearing gloves and mask; it is a carcinogen.

Suppliers of peroxidase substrates are Sigma, Kirkegaard & Perry, Moss, and Vector Labs.

Dioxetane phosphate substrate buffer

1 mM MgCl₂
0.1 M diethanolamine
0.02% sodium azide (optional)
Adjust to pH 10 with HCl and use fresh

Traditionally, the AMPPD substrate buffer has been a solution containing 1 mM MgCl₂ and 50 mM sodium carbonate/bicarbonate, pH 9.6 (Gillespie and Hudspeth, 1991). The use of diethanolamine results in better light output (Tropix Western Light instructions).

Alternatively, 100 mM Tris·Cl (pH 9.5)/100 mM NaCl/5 mM MgCl₂ can be used (Sandhu et al., 1991).

Dioxetane phosphate visualization solution

Prepare 0.1 mg/ml AMPPD or CSPD (Tropix) or Lumigen-PPD (Lumigen; see Table 6.2.1) substrate in dioxetane phosphate substrate buffer (see recipe). Prepare just before use. Lumi-Phos 530 (Boehringer Mannheim or Lumigen) is a ready-to-use solution and can be applied directly to the membrane.

This concentration of AMPPD substrate (240 µM) is the minimum recommended by Tropix Western Light. Ten-fold lower concentrations can be used but require longer exposures.

Luminol visualization solution

0.5 ml 10× luminol stock [40 mg luminol (Sigma) in 10 ml DMSO]
0.5 ml 10× *p*-iodophenol stock [optional; 10 mg (Aldrich) in 10 ml DMSO]
2.5 ml 100 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
25 µl 3% H₂O₂
H₂O to 5 ml
Prepare just before use

Recipe is from Schneppenheim et al. (1991). Premixed luminol substrate mix (Mast Immunosystems; Amersham ECL; Du Pont NEN Renaissance; Kirkegaard & Perry LumiGLO) may also be used. *p*-iodophenol is an optional enhancing agent that increases light output. Luminol and *p*-iodophenol stocks can be stored for ≤6 months at -20°C.

Ponceau S solution

Dissolve 0.5 g Ponceau S in 1 ml glacial acetic acid. Bring to 100 ml with water. Prepare just before use.

Transfer buffer

Add 18.2 g Tris base and 86.5 g glycine to 4 liters of water. Add 1200 ml methanol and bring to 6 liters with water. The pH of the solution is ~8.3 to 8.4. For use with PVDF filters, decrease methanol concentration to 15%; for nylon filters, omit methanol altogether.

CAPS transfer buffer can also be used. Add 2.21 g cyclohexylaminopropane sulfonic acid (CAPS; free acid), 0.5 g DTT, 150 ml methanol, and water to 1 liter. Adjust to pH 10.5 with NaOH and chill to 4°C. For proteins >60 kDa, reduce methanol content to 1% (Moos, 1992).

COMMENTARY

Background Information

Immunoprecipitation has been widely used to visualize the antigens recognized by various antibodies, both polyclonal and monoclonal (UNIT 7.2). However, there are several problems inherent with this method, including the requirement for radiolabeling of antigen, co-precipitation of tightly associated macromolecules, occasional difficulty in obtaining precipitating antibodies, and insolubility of various antigens (Talbot et al., 1984).

To circumvent these problems, electroblotting (Towbin et al., 1979)—subsequently popularized as western blotting or immunoblotting (Burnette, 1981)—was conceived. Immunoblotting is a rapid and sensitive assay for the detection and characterization of proteins that works by exploiting the specificity inherent in antigen-antibody recognition. It involves the solubilization and electrophoretic separation of proteins, glycoproteins, or lipopolysaccharides by SDS-PAGE (UNIT 6.1) or urea-PAGE, followed by quantitative transfer and irreversible binding to nitrocellulose, PVDF, or nylon. This technique has been useful in identifying specific antigens recognized by polyclonal or monoclonal antibodies and is highly sensitive (1 ng of antigen can be detected).

Electroblotting of previously stained gels is a convenient way to visualize and document the gel prior to immunoblotting. Transfer efficiencies at all molecular weights will be lower with fixed and stained gels. This is particularly true of proteins >50 kDa (Perides et al., 1986). The additional incubation in 6 M urea will significantly increase transfer efficiency of all proteins and is required for proteins >50 kDa.

Ponceau S staining provides an easy method for calibrating and quantitating the amount of material on a nitrocellulose or PVDF blot. An alternative to this method is to use an internal protein control with a separate antibody probe, but these tend to be expensive and time-consuming to use. Other applications for Ponceau S calibration include monitoring transfer effi-

ciency under varied conditions for optimization of tank and semidry blotting.

Immunoblotted proteins can be detected by chromogenic or luminescent assays (see Table 6.2.1 for a description of the reagents available for each system, their reactions, and a comparison of their advantages and disadvantages). Luminescent detection methods offer several advantages over traditional chromogenic procedures. In general, luminescent substrates increase the sensitivity of both HRPO and phosphatase systems without the need for radioisotopes. Substrates for the latter have only recently been applied to protein blotting (see Gillespie and Hudspeth, 1991; Sandhu et al., 1991; Bronstein et al., 1992). Luminescent detection can be completed in as little as a few seconds; exposures rarely go more than 1 hr. Depending on the system, the luminescence can last for 3 days, permitting multiple exposures of the same blot. Furthermore, the signal is detected by film, and varying the exposure can result in more or less sensitivity. Luminescent blots can be easily erased and reprobbed because the reaction products are soluble and do not deposit on the membrane (see below). Compared to chromogenic development, the luminescent image recorded on film is easier to photograph and to quantitate by densitometry.

Alkaline phosphatase-based luminescent protocols that achieve maximum sensitivity with minimum background can be complex, and the manufacturer's instructions should be consulted (see Reagents and Solutions). The procedure described in Alternate Protocol 4 gives reasonable sensitivity on nitrocellulose, PVDF, and nylon membranes with a minimum of steps.

Critical Parameters

First and foremost, the antibody being used should recognize denatured antigen. Nonspecific binding of antibodies can occur, so control antigens and antibodies should always be run in parallel. Time of transfer and primary anti-

body and conjugate dilutions should always be optimized.

A variety of agents are currently used to block binding sites on the membrane after blotting (Harlow and Lane, 1988). These include Tween 20, PVP, nonfat dry milk, casein, BSA, and serum. A 0.1% (v/v) solution of Tween 20 in TBS (TTBS), a convenient alternative to protein-based blocking agents, is recommended for chromogenic development of nitrocellulose and PVDF membranes (Blake et al., 1984). In contrast to dry milk/TBS blocking solution (BLOTTO), TTBS is stable and has a long shelf life at 4°C. Furthermore, TTBS generally produces a clean background and permits subsequent staining with India ink. However, even with the application of such standard blocking procedures as 5% to 10% milk protein or 0.05% to 0.1% Tween 20, background can still be a significant problem. If this happens, using a blocking protein (e.g., goat, horse, or rabbit normal serum) from the same species as the primary antibody can reduce the background, presumably by reducing cross-reactivity between the primary antibodies and the blocking agent. Combinations of blocking agents can also be effective. Thus, 0.1% human serum albumin (HSA) and 0.05% Tween 20 in TBS is recommended when probing Immobilon-P membranes with human serum (Craig et al., 1993). However, this can also lead to overall loss of antigen signal, requiring a tenfold increase in the primary antibody (serum) concentration to achieve an adequate background free antigen signal.

When using chemiluminescent detection for immunoblotting, high background frequently occurs, particularly for strong signals (Pampori et al., 1995). Several methods are available for reducing the background from chemiluminescent reactions. These include changing the type and concentration of blocking agents (see above), optimizing antibody concentrations, letting the reaction proceed for several minutes before exposing to film, or simply limiting the exposure time of the film on the blot. These procedures are not always successful, however, and can lead to inconsistent results. An alternative approach is to reduce the concentration of reagents ten-fold. This effectively removes the background and has a number of advantages which include lower cost, increased signal-to-noise ratio, and reduced detection of cross-reacting species.

Two types of nylon membrane are used for western transfer—neutral (e.g., Pall Biodyne A) and positively charged (e.g., Pall Biodyne

B). Although the positively charged membranes have very good protein-binding characteristics, they tend to give a higher background. These membranes remain positively charged from pH 3 to pH 10. Neutral nylon membranes are also charged, having a mix of amino and carboxyl groups that give an isoelectric point of 6.5. Because of their high binding capacity, positively charged membranes are popular for protein applications using luminescence.

Nylon membranes require more stringent blocking steps. Here 10% nonfat dry milk in TBS is recommended for chromogenic development. During luminescence development, however, background is a more significant problem. Compared to dry milk, purified casein has minimal endogenous alkaline phosphatase activity (AP activity leads to high background) and is therefore recommended as a blocking agent for nitrocellulose, PVDF, and nylon membranes. Positively charged nylon requires much more stringent blocking with 6% (w/v) casein and 1% (v/v) polyvinylpyrrolidone (PVP). Because nonfat dry milk and casein may contain biotin that will interfere with avidin-biotin reactions, subsequent steps are done without protein-blocking agents when using these systems. If background is a problem, highly purified casein (0.2% to 6%) added to the antibody incubation buffers may help.

If reprobing is desired, blots can be air dried and stored at 4°C for 3 months after chemiluminescence detection. After drying, store in a sealed freezer bag until use. Repeated probing will lead to a gradual loss of signal and increased background. However, this will depend in part on the properties of the sample.

If the primary procedure is problematic due to loss of sensitivity or an increase in the background, then two possible alternative procedures for stripping membranes are recommended. The first uses 2-mercaptoethanol and SDS (Kaufmann et al., 1987; Tesfaigzi et al., 1994). Briefly, the membranes are incubated in 2% SDS/100 mM Tris-Cl, pH 7.4/100 mM 2-mercaptoethanol for 30 min at 70°C, effectively removing primary and secondary antibodies. As with the primary procedure recommended above, the repeated probing should be done with caution due to the potential loss of detection signal, and 5% nonfat dry milk is required as a blocking agent. The milk blocking agent facilitates antibody removal from the blot (Kaufmann et al., 1987). The second uses guanidine-HCl. For nylon and PVDF membranes (do *not* use with nitrocellulose), incubate the immunoblot in 7 M guanidine-HCl for

10 min at room temperature. (The short wash time is critical, as guanidine-HCl is a very strong denaturant, so do not leave the filter in this solution >15 min.) Pour off excess guanidine-HCl and then rinse the membrane several times in 1× TTBS. Reblock the membrane and proceed with the standard immunoblotting procedure. Membranes stripped using this procedure can generally be reused three or four times.

Troubleshooting

There are several problems associated with immunoblotting. The antigen is solubilized and electrophoresed in the presence of denaturing agents (e.g., SDS or urea), and some antibodies may not recognize the denatured form of the antigen transferred to the membrane. The results observed may be entirely dependent on the denaturation and transfer system used. For example, zwitterionic detergents have been shown to restore the antigenicity of outer membrane proteins in immunoblotting (Mandrell and Zollinger, 1984). Gel electrophoresis under nondenaturing conditions can also be performed.

Other potential problems include high background, nonspecific or weak cross-reactivity of antibodies, poor protein transfer or membrane binding efficiency, and insufficient sensitivity. For an extensive survey and discussion of immunoblotting problems and artifacts, see Bjerum et al. (1988).

If no transfer of protein has occurred, check the power supply and electroblot apparatus to make sure that the proper electrical connections were made and that power was delivered during transfer. In addition, check that the correct orientation of filter and gel relative to the anode and cathode electrodes was used.

If the transfer efficiency using the tank system appears to be low, increase the transfer time or power. Cooling (using the unit's built-in cooling cores) is generally required for transfers >1 hr. At no time should the buffer temperature go above 45°C. Prolonged transfers (>1 hr) are not possible in semidry transfer units due to rapid buffer depletion.

Alternatively, the transfer buffer can be modified to increase efficiency. Adding SDS at a concentration of 0.1% to the transfer buffer improves the transfer of all proteins out of the gel, particularly those above 60 to 90 kD in size. Lowering the concentration of methanol will also improve the recovery of proteins from the gel. These procedures are tradeoffs. Methanol improves the binding of proteins to PVDF and nitrocellulose, but at the same time hinders

transfer. With SDS present, transfer efficiency is improved, but the SDS can interfere with protein binding to the membrane. Nylon and PVDF membranes are particularly sensitive to SDS interference. If needed, 0.01% to 0.02% SDS may be used in PVDF membrane transfer buffers (Millipore, 1990). SDS and methanol should not be used in the transfer buffer for nylon (Peluso and Rosenberg, 1987).

Gel cross-linking and thickness also have a profound effect on the transfer efficiency. In general, 0.5- to 0.75-mm-thick gels will transfer much more efficiently than thicker gels (e.g., 1.5 mm thick). Gels with a higher acrylamide percentage will also transfer less efficiently. Proteins can be particularly difficult to transfer from gradient gels, and a combination of longer transfer times, thin gels, and the addition of SDS to the transfer buffer may be needed.

If the protein bands are diffuse, check the transfer cassette. The gel must be held firmly against the membrane during transfer. If the transfer sandwich is loose in the cassette, add another thin sponge or more blotter paper to both sides.

Occasionally, a grid pattern will be apparent on the membrane after tank transfer. This is caused by having either the gel or the membrane too close to the sides of the cassette. Correct this by adding more layers of filter paper to diffuse the current flowing through the gel and membrane. Use a thinner sponge and more filter paper if necessary.

If air bubbles are trapped between the filter and the gel, they will appear as clear white areas on the filter after blotting and staining. Take extra care to make sure that all bubbles are removed.

Insufficient blocking or nonspecific binding of the primary or secondary antibody will cause a high background stain. A control using pre-immune serum or only the secondary antibody will determine if these problems are due to the primary antibody. Try switching to another primary blocking agent; protein blocking agents may weakly cross-react. Lowering the concentration of primary antibody should decrease background and improve specificity (Fig. 6.2.3).

Due to the nature of light and the method of detection, certain precautions are warranted when using luminescent visualization (e.g., Harper and Murphy, 1991). Very strong signals can overshadow nearby weaker signals on the membrane. Because light will pipe through the membrane and the surrounding plastic wrap, overexposure will produce a broad diffuse im-

age on the film. The signal can also saturate the film, exposing the film to a point whereby increased exposure will not cause a linear increase in the density of the image on the film.

With the alkaline phosphatase substrate AMPPD, nitrocellulose, PVDF, and nylon membranes require 2, 4, and 8 to 12 hr, respectively, to reach maximum light emission. In addition, PVDF is reported to give a stronger signal than nitrocellulose (Tropix Western Light instructions). Positively charged nylon requires special blocking procedures to minimize background (Gillespie and Hudspeth, 1991). These procedures include using a blocking and primary antibody solution containing 6% casein, 1% polyvinylpyrrolidone-40 (PVP-40), 3 mM NaN₃, 10 mM EDTA, and PBS, pH 6.8. Prior to use, the casein must be heated to 65°C to reduce alkaline phosphatase activity in the casein itself. In addition, maximum sensitivity has been observed when free biotin or biotinylated proteins are removed by pretreating the casein with avidin-agarose (Sigma).

Anticipated Results

Immunoblotting should result in the detection of one or more bands. Although antibodies directed against a single protein should produce a single band, degradation of the sample (e.g., via endogenous proteolytic activity) may cause visualization of multiple bands of slightly different size. Multimers will also form spontaneously, causing higher-molecular-weight bands on the blot. If simultaneously testing multiple antibodies directed against a complex protein mixture (e.g., using patient sera against SDS-PAGE-separated viral proteins in AIDS western blot test), multiple bands will be visualized. Typically, picogram to nanogram sensitivities are common in protein blotting and immunodetection procedures.

Time Considerations

The entire immunoblotting procedure can be completed in 1 to 2 days, depending on transfer time and type of gel. Gel electrophoresis requires 4 to 6 hr on a regular gel and 1 hr on a minigel. Transfer time can be 1 hr (high-power transfer) to overnight. Blocking, conjugate incubation, and washing each take 30 min to 1 hr. Finally, substrate incubation requires 10 to 30 min (chromogen) and a few seconds to several hours (luminescence).

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Details peroxidase-based luminescent detection methods.

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Detection and Quantitation of Radiolabeled Proteins in Gels and Blots

This unit presents procedures for visualizing and quantitating radiolabeled proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; UNIT 6.1) or affixed to filter membranes. Autoradiography (see Basic Protocol) is the most common method by which this is accomplished, and X-ray film is the traditional recording medium. The use of autoradiography with gels requires that the gel be dried prior to being placed in contact with the film (see Support Protocol 1). The decay of radioactive materials within the dried gel or filter leaves an image on the film that reflects its distribution in the sample. Film images can be quantified by densitometry (see Support Protocol 4) to obtain a relative measure of the amount of radioactivity in the sample.

The use of X-ray films for autoradiography, however, suffers from two drawbacks: lack of sensitivity and a limited linear range over which the image density reflects the amount of radioactivity. Lack of sensitivity can be overcome by fluorography (see Alternate Protocol 1) or by the use of intensifying screens (see Support Protocol 2), both of which enhance the radioactive signal. Ensuring that the exposure is within a linear range requires careful controls; film is often preflashed (see Support Protocol 3) to increase the linear measurement range for weakly radioactive samples, and it is important to ensure that the film not be saturated to attain strong radioactive signals. Sensitivity and linear ranges of measurement can be greatly extended by using a phosphor imaging system (see Alternate Protocol 2). Phosphor imaging also makes it much faster and easier to quantify radioactive samples.

To enhance radioactive signals, solid-state scintillation is frequently employed to convert the energy released by radioactive molecules to visible light. This is accomplished in several different ways. In fluorography (see Alternate Protocol 1) organic scintillants are incorporated into the sample to increase the proportion of emitted energy detected from low-energy β particles (e.g., from ^3H , ^{14}C , and ^{35}S). Another method uses high-density, fluorescent “intensifying screens” (see Support Protocol 2), which are placed next to the sample and used to capture the excess energy of γ rays (e.g., those produced by ^{125}I) and high-energy β particles (e.g., from ^{32}P).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by your local radiation safety officer (also see APPENDIX 1D).

AUTORADIOGRAPHY

Autoradiography uses X-ray film to visualize and quantitate radioactive molecules that have been electrophoresed through agarose or polyacrylamide gels (UNIT 6.1), hybridized to filters (e.g., immunoblots; UNIT 6.2), or chromatographed through paper or thin-layer plates. A photon of light or the β particles and γ rays released from radioactive molecules “activate” silver bromide crystals on the film emulsion. This renders them capable of being reduced through the developing process to form silver metal (a “grain”). The silver grains on the film form the image.

The choice of film is critical for autoradiography. Double-coated films (e.g., Kodak X-Omat AR and Fuji RX) contain two emulsion layers on either side of a polyester support and are most commonly used for autoradiography (Laskey and Mills, 1977). Double-coated films are ideal for detecting the high-energy β particles emitted by ^{32}P and ^{125}I ,

BASIC PROTOCOL

Electrophoresis and Immunoblotting

6.3.1

since they can penetrate the polyester support and expose both emulsion layers. These films are normally used with calcium tungstate (CaWO_4) intensifying screens at reduced temperature (-70°C); they are highly sensitive to the blue light emitted by these screens. The green-light-sensitive BioMax MS film (Kodak) is a double-coated film spectrally matched to the blue- and green-light-emitting BioMax MS intensifying screen. The BioMax MS film/BioMax MS intensifying screen system normally gives greatest sensitivity to ^{32}P (four times greater than that of X-Omat AR film with CaWO_4 screens).

Single-coated films, containing one emulsion layer (e.g., Kodak BioMax MR), are optimized for direct-exposure techniques with medium-energy radioisotopes (e.g., ^{14}C , ^{35}S , and ^{33}P , but not ^3H). The majority of the β particles emitted by these isotopes cannot pass through the polyester support of double-coated films, and therefore the emulsion layer on the other side of the film is useless. Even though direct exposure with single-coated films gives better clarity for medium-energy isotopes, single-coated films often require longer exposure times. Fluorography, therefore, is often used to enhance sensitivity. The blue-light-sensitive double-coated X-Omat AR film is generally used for fluorography with 2,5-diphenyloxazole (PPO; which emits at 388 nm), sodium salicylate (which emits at 420 nm), and commercial fluorographic solutions and sprays (e.g., from Amersham and DuPont NEN; which emit light in the blue end of the spectrum).

Materials

Fixed and dried gel (see Support Protocol 1) or filter (e.g., from immunoblotting;
UNIT 6.2)

Developer: Kodak developer and replenisher, prepared according to the manufacturer's instructions, 18° to 20°C

Fixer: Kodak fixer and replenisher, prepared according to the manufacturer's instructions, 18° to 20°C

Metal film cassette or paper film cassette with particle-board supports and metal binder clips

Plastic wrap (e.g., Saran Wrap)

X-ray film

Trays to hold film processing solutions

Clips for hanging film

1. In a darkroom illuminated with a safelight, place the sample (e.g., dried gel or filter) in the film cassette. Cover the sample with plastic wrap to prevent it from sticking to the film and contaminating the cassette with radioactivity.

The safelight should be a bulb of <15 W that is equipped with a Kodak GBX-2 red filter (or equivalent).

Fluorescent glow-in-the-dark ink (available at craft stores) is a convenient way to mark samples exposed to film. The ink can be spread on adhesive labels, which in turn are placed on the plastic wrap around the edge of the sample. If exposed to light prior to autoradiography, the ink will fluoresce and expose the film, making it possible to orient the film image on the dried gel after developing.

2. Place a sheet of X-ray film on top of the sample, then close and secure the film cassette (see Fig. 6.3.1).

If preflashed film is used for direct exposure (see Support Protocol 3), the exposed side should face the sample. Preflashed film should be used if sample is weakly radioactive or if quantitation of the radioactivity is desired. For single-coated film, the emulsion layer should face the sample.

If a paper cassette is used, particle-board supports the same size as the cassette should be placed on either side and secured with the metal binder clips. This will ensure that the sample and film are held in contact and do not shift during exposure.

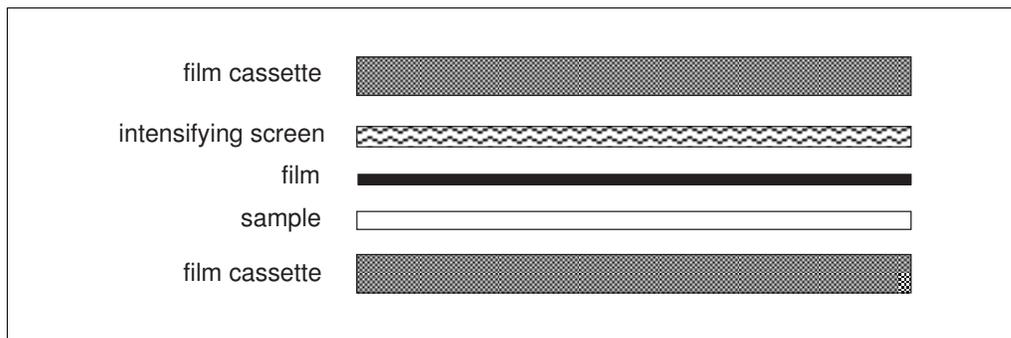


Figure 6.3.1 Autoradiography setup: intensifying screen, film, and sample in film cassette.

- Expose the film for the desired length of time and at the appropriate temperature.

Time of exposure will depend on the strength of the radioactivity in the sample and, in most cases, will have to be determined empirically by making multiple exposures for different lengths of time. To help estimate exposure time, a Geiger counter can often be used to detect the relative amount of radioactivity in the sample. With experience, this can help alleviate the trial and error often associated with obtaining the optimum exposure. Time of exposure and use of internal controls are particularly important if quantitative comparisons between experiments are desired.

- After exposure, return cassette to the darkroom and remove film for developing.

If the film was exposed at -70°C , allow the cassette to come to room temperature before developing. This will avoid static discharge, which can cause black dots or stripes on the autoradiogram.

Automated film developers are also available and can be used to develop the film.

- Immerse the film for 5 min in 18° to 20°C developer, then wash 1 min in running water at room temperature.

Shorter periods of time in developer will yield a lighter image. The amount of time in developer, therefore, can be used to roughly control intensity of the image.

- Immerse the film for 5 min in 18° to 20°C fixer, then wash for 15 min in running water.

- Hang the film to dry.

The orientation of the film with respect to the gel can be determined by the images of the fluorescent markers.

FIXING AND DRYING GELS FOR AUTORADIOGRAPHY

SDS-PAGE gels containing radiolabeled proteins should be fixed and dried before exposure to film. This will prevent the gel from sticking to the film, improve the sharpness of the image, and increase sensitivity slightly. However, if the specific activity of the sample is high or the detection method is sensitive (e.g., where a phosphor imager is used; see Alternate Protocol 2), then fixing and drying the gel may not be necessary. Gel dryers are available from a number of manufacturers (e.g., Bio-Rad), most of which use heat and a vacuum to accelerate the drying process.

SUPPORT PROTOCOL 1

Electrophoresis and Immunoblotting

6.3.3

Materials

Gel from SDS-PAGE (UNIT 6.1)

Fixing solution: 10% (v/v) glacial acetic acid/20% (v/v) methanol in H₂O

Alternative fixing solution (for gels with $\geq 15\%$ acrylamide or thicker than 1.5 mm): 3% (v/v) glycerol/10% (v/v) glacial acetic acid/20% (v/v) methanol in H₂O

Glass dish

Rotary shaker

Filter paper (Whatman 3MM) in sheets at least 1 to 2 cm larger than gel

Plastic wrap (e.g., Saran Wrap)

Gel dryer with vacuum pump

1. After electrophoresis, remove the gel and the supporting glass plates from the electrophoresis apparatus and place in a glass dish. Carefully remove the upper glass plate by gently prying apart the corners with a metal spatula. Make a notch in the upper right hand corner of the gel for orientation.

Since the gel contains radiolabeled proteins, be sure to follow the necessary guidelines for handling radioactivity. Everything that comes in contact with the gel is potentially radioactive.

- 2a. *For gels with $<15\%$ acrylamide and <1.5 mm thick:* Place the glass dish in a fume hood and pour enough fixing solution into the dish to cover the gel. Place the dish on a rotary shaker and gently rotate until 5 min after all of the blue color from the bromphenol blue in the sample buffer (if used) has disappeared (~30 min total).

The bromphenol blue typically used in SDS-PAGE sample buffer will turn yellow as the acidic fixing solution diffuses into the gel.

During fixing, the gel will typically slide off the lower glass plate, which can be removed.

- 2b. *For gels with $\geq 15\%$ acrylamide or >1.5 mm thick:* Fix gel as in step 2a, but soak 1 hr in alternative fixing solution.

The glycerol in the alternative fixing solution should help prevent cracking during drying.

3. Pour off the fixing solution and rinse the gel for a few minutes with deionized water.

CAUTION: Remember that solutions that come in contact with the gel are potentially radioactive.

4. Carefully pour off the water and position the gel in the center of the glass dish. Be sure that any excess water is drained. Place a sheet of Whatman 3MM filter paper, at least 1 to 2 cm larger than the gel, over the gel.

The gel will stick to the filter paper, which will allow it to be lifted and turned over with the gel side facing up.

5. Cover gel with plastic wrap. Smooth the wrap with a piece of tissue paper to remove any air bubbles or wrinkles.
6. Place a piece of filter paper on the gel support of the gel dryer to prevent contamination of the dryer by radioactivity.
7. Place the filter paper/gel/plastic wrap sandwich on the filter paper in the gel dryer with the plastic sheet facing up.
8. Position the rubber sealing gasket of the gel dryer over the gel. Set the appropriate heat setting on the gel dryer (normally 80°C; 60°C if the gel contains a fluor). Apply the vacuum and allow the gel to dry (typically 2 hr for a gel of 1 mm thickness).

Removing the gel before it is completely dry can lead to cracking; it is therefore not a good idea to rush the drying process. A rough indication of whether the gel is dry can be obtained by feeling the gel under the sealing gasket. If the gel is dry, it should be warm over the entire surface.

9. Remove gel from dryer and proceed with autoradiography (see Basic Protocol).

USE OF INTENSIFYING SCREENS

Intensifying screens are used to enhance the film image generated by radioactive molecules (Laskey and Mills, 1977; Laskey, 1980). They are used strictly in conjunction with strong β -emitting isotopes such as ^{32}P or γ -emitting isotopes such as ^{125}I . Emissions from these forms of radiation will frequently pass completely through a film, but they can be absorbed by an intensifying screen which fluoresces and exposes the film with multiple photons of light. While an intensifying screen will substantially enhance the film image as compared with direct exposure (Table 6.3.1), some loss of image resolution will occur due to light scatter. Intensifying screens are distributed by most laboratory supply companies (e.g., Fisher, Sigma, and Kodak).

As shown in Figure 6.3.1, the film should be placed between the sample and the intensifying screen. Preflashed film (see Support Protocol 3) should be used if the sample is weakly radioactive or if quantitation of the radioactivity is desired. The preflashed side of the film should be placed adjacent to the intensifying screen. For very weakly radioactive samples, a second screen can be placed on the other side of the radioactive sample (i.e., screen, then sample, then film, then screen), but this causes further loss in resolution due to light scatter. Also, the sample and sample support must be sufficiently transparent to allow light from the second screen to reach the film. The film should be exposed at -70°C to stabilize the silver bromide crystals activated by the radioactivity or the light emitted from the screen.

Table 6.3.1 Different Methods for Isotope Detection and Their Sensitivities^a

Isotope	Method ^b	Sensitivity ^c	Enhancement over direct autoradiography ^d
^{125}I	S	100	16
^{32}P	S	50	10.5
^{14}C	F	400	15
^{35}S	F	400	15
^3H	F	8000	>100

^aExposures conducted at -70°C using preexposed film.

^bS, intensifying screen; F, fluorography using PPO.

^cDefined as dpm/cm^2 required for detectable image ($A_{540} = 0.02$) in 24 hr.

^dDirect autoradiography for comparison was performed on Kodirex film (Laskey, 1980).

SUPPORT PROTOCOL 2

PREFLASHING (PREEXPOSING) FILM

Silver bromide crystals that are activated by light, β particles, or γ rays are highly unstable and quickly revert back to their stable form. The absorption of several photons increases their stability but does not ensure development; approximately five photons of light are required to obtain a 50% probability that any single silver bromide crystal will be developed during film processing. This inefficiency means that film images produced by very low levels of exposure will be disproportionately faint. However, two measures can be taken to maximize efficiency and linearity of exposure at the low levels commonly encountered in ordinary use. First, the film should be preexposed to a hypersensitizing flash of light, which provides several photons per silver bromide crystal and stably activates them without providing enough exposure to cause them to become developed. This allows a linear relationship to be drawn between the film image and the amount of radioactivity in the sample. Second, film exposure should be conducted at low temperatures (-70°C) to slow the reversal of activated silver bromide crystals to their stable form (Laskey and Mills, 1975).

Film can be hypersensitized by exposure to a flash of light (<1 msec) provided by a photographic flash unit or a stroboscope before being placed onto the radioactive sample for exposure of the autoradiogram (Laskey and Mills, 1975, 1977). As the optimal light intensity required for preexposure varies with the type of film and the flash unit being used, the ideal exposure is best determined empirically as described below.

Materials

Stroboscope or flash unit (e.g., Auto 22 Electronic Flash from Vivitar or Sensitize Pre-Flash from Amersham Pharmacia Biotech)
Neutral-density filter (Kodak)
Orange filter (Wratten 22; Kodak)
X-ray film
Spectrophotometer

1. Cover the stroboscope or flash unit with the neutral-density and orange filter.

This serves to decrease the intensity of emitted light, particularly the blue wavelengths to which X-ray films are most sensitive. Filters are not required for the Amersham flash unit.

2. Place the film perpendicular to the light source at a distance of ≥ 50 cm to ensure uniform illumination.
3. Expose a series of test films for different flash lengths, then develop them (see Basic Protocol).

An uneven fog level on film can be remedied by placing a porous paper diffuser, such as Whatman no. 1 filter paper, between the film and the light source.

4. Cut the films into pieces that fit into a cuvette holder of a spectrophotometer and measure the absorbance at 540 nm.

Choose an exposure time that causes the absorbance of the preexposed film to increase by 0.15 with respect to film that was not preexposed.

FLUOROGRAPHY

Organic scintillants can be included in radioactive samples to obtain autoradiograms of weak β -emitting isotopes such as ^3H , ^{14}C , and ^{35}S . The scintillant fluoresces upon absorption of β particles from these isotopes, facilitating film exposure. Fluorographs of radioactive molecules in polyacrylamide gels have traditionally used the scintillant PPO (2,5-diphenyloxazole; Laskey and Mills, 1975). PPO, however, has largely been replaced with commercial scintillation formulations that reduce the amount of preparation time and are considerably safer to use. These scintillants (e.g., Enhance from NEN Life Science) come with complete instructions for their use. In addition, spray applicators are also available that can be used on filters or thin-layer plates. The expected levels of image enhancement obtained through fluorography are listed in Table 6.3.1. Sodium salicylate can also be used for fluorography as described below (Chamberlain, 1979). It yields levels of image enhancement comparable to organic scintillants, although it sometimes causes a more diffuse film image. The conditions should work for most standard sizes and thicknesses of gels.

CAUTION: Gloves should be worn at all times; sodium salicylate can elicit allergic reactions and is readily absorbed through the skin.

Materials

Polyacrylamide gel

1 M sodium salicylate, pH 5 to 7, freshly prepared

Additional reagents and equipment for fixing and drying gels (see Support Protocol 1)

1. If gel is acid-fixed, soak for 1 to 5 hr in ~20 vol water to prevent precipitation of salicylic acid from the sodium salicylate.
2. Soak gel 30 min in 10 vol of 1 M sodium salicylate, pH 5 to 7.
To prevent cracking of gels with >15% acrylamide or thicker than 1.5 mm, 2% (v/v) glycerol can be added to the 1 M sodium salicylate.
3. Dry the gel (see Support Protocol 1) and proceed with autoradiography (see Basic Protocol).

DENSITOMETRY

Film images obtained by autoradiographic methods can be quantified by densitometry. Densitometers work by comparing the intensity of light transmitted through a sample with the intensity of the incident light. The amount of light transmitted will be proportional to the amount of radioactivity in the gel, provided that the film has been properly preexposed (see Support Protocol 3). The linear range of correctly preexposed film is 0.1 to 1.0 absorbance units. However, if the preexposure is excessive—i.e., an increase of >0.2 absorbance units (A_{540}) treated film/untreated film—smaller amounts of radioactivity will produce disproportionately dense images. Autoradiograms that exceed an absorbance of 1.4 absorbance units (A_{540}) have saturated all available silver bromide crystals and also cannot be evaluated quantitatively.

Densitometers are available from several manufacturers (e.g., Molecular Dynamics, Bio-Rad, and UVP). Most models come with software that facilitates calculations and allows the user to define the region of the film to be measured. Procedures for the use of these machines vary and instructions are provided by the manufacturer. Densitometers are also available that measure light reflected from a sample. Reflectance densitometers are useful in instances where the sample medium is completely opaque—e.g., filters that have been probed using nonradioactive colorimetric detection assays.

PHOSPHOR IMAGING

Phosphor imaging screens can be used as an alternative to film for recording and quantifying autoradiographic images (Johnston et al., 1990). They can detect radioisotopes such as ^{32}P , ^{125}I , ^{14}C , ^{35}S , and ^3H . There are several advantages of phosphor imaging over film: (1) linear dynamic ranges are 5 orders of magnitude, compared to ~ 1.5 orders of magnitude for film (Fig. 6.3.2); (2) exposure times are 10 to 250 times faster than with film; (3) quantification is much easier and faster, and most imagers come with software to directly analyze data; (4) fluorography and gel drying are often unnecessary because of the sensitivity of phosphor imaging; and (5) phosphor screens can be reused indefinitely if handled carefully.

Phosphor imaging screens are composed of crystals of BaFBr:Eu^{+2} . When the screen is exposed to ionizing radiation such as α , β , or γ radiation, or wavelengths of light shorter than 380 nm, the electrons from Eu^{+2} are excited and then trapped in an "F-center" of the BaFBr^- complex; this results in the oxidation of Eu^{+2} to Eu^{+3} , which forms the latent image on the screen. After exposure, the latent image is released by scanning the screen with a laser (633 nm). During scanning, Eu^{3+} reverts back to Eu^{+2} , releasing a photon at 390 nm. The luminescence can then be collected and measured in relation to the position of the scanning laser beam. The result is a representation of the latent image on the storage phosphor imaging plates. The image can then be viewed on a video monitor and analyzed with the aid of appropriate software.

Some companies (e.g., Bio-Rad) offer different screens for use with different isotopes. They vary principally in the protective coating on the screen, which is optimized for low- or high-energy β particles or γ rays. No coating is typically used for weak β emitters such as tritium. More recently, screens have also been developed that measure chemiluminescence. Such screens are particularly valuable for use with many nonradioactive labeling protocols.

The protocol below is for the PhosphorImager system from Molecular Dynamics; other phosphor imaging systems are available from Bio-Rad, Imaging Research, and National Diagnostics.

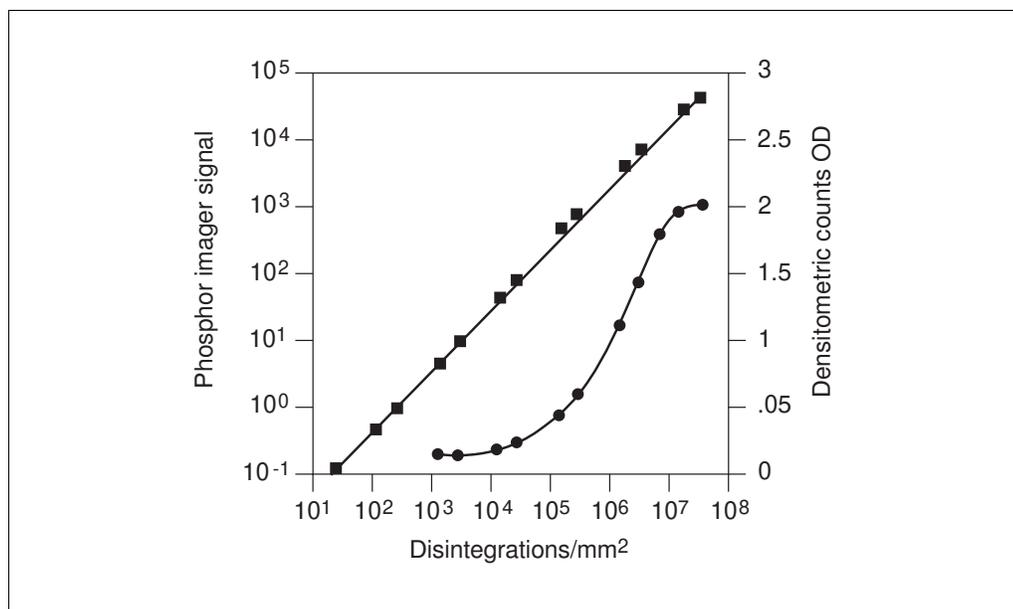


Figure 6.3.2 ^{32}P dilution series quantified on Model GS-525 phosphor imager (squares), compared to film (circles). Image courtesy of Bio-Rad, Hercules, Calif.

Materials

Gel or filter (e.g., from immunoblotting; *UNIT 6.2*)
PhosphorImager system (Molecular Dynamics) including:
ImageEraser light box
Exposure cassette with phosphor screen
Scanning software

1. Erase any latent image on the phosphor screen left by a previous user, or caused by background radiation, by exposure to visible light.

The PhosphorImager system comes with an extra-bright light box (ImageEraser) for this purpose. Standard laboratory light boxes may also be used.

2. Cover gel or filter with plastic wrap to protect the exposure cassette. Place wrapped gel or filter in the PhosphorImager cassette and close to begin exposure.

The gel does not have to be dried for this procedure. The phosphor screen is affixed to the lid of the cassette. Exposure times are typically one-tenth of the time required for film exposure.

3. After exposure, slide the screen face down into the PhosphorImager system.
4. Select the scanning area using the software supplied with the PhosphorImager and start scanning.

The blue light emitted during scanning is collected to produce the latent image.

5. Analyze and quantitate the image using the software provided.
6. Erase the phosphor screen by exposing it to visible light as in step 1.

COMMENTARY

Background Information

The ability to detect radiolabeled proteins is critical to many studies in cell biology. A variety of labeling methods are described throughout this manual, many of which are used to follow protein purification, protein processing, or the movement of proteins within the cell. More often than not, detection of radiolabeled proteins is coupled with the resolving power of SDS polyacrylamide gel electrophoresis (SDS-PAGE; *UNIT 6.1*). Radiolabeled proteins separated on gels can be used directly to obtain an autoradiographic image. Alternatively, proteins separated by SDS-PAGE are frequently transferred to membranes (*UNIT 6.2*) and detected using radiolabeled probes such as antibodies and ^{125}I -labeled protein A. The autoradiographic image, whether generated on film or a phosphor screen, reflects the distribution of the radioactive proteins on the two-dimensional surface of the gel or filter. Molecular sizes of radiolabeled proteins, therefore, can be determined by correlating their positions with molecular markers. Also, the density of the band images can be used to determine the relative

quantities of the radiolabeled proteins in the sample.

Critical Parameters

The sensitivity of the detection device and the strength of the radioactive signal are the two most important parameters for autoradiography. Sensitivity can be enhanced by treating samples with fluors or by using intensifying screens (Table 6.3.2). Because phosphor imaging is 10 to 250 times more sensitive than film (Johnston et al., 1990), this technology makes it possible to monitor radioactive samples that would previously have gone undetected with film.

A second important parameter is the range over which the measurement device is linear. Film requires preflashing in order for the intensity of the image to be linear with respect to the amount of radioactivity, particularly for weakly radioactive samples (Laskey and Mills, 1975, 1977). Phosphor imaging offers a much wider linear range of measurement (5 orders of magnitude compared to 1.5 for film; Johnston et al., 1990). This makes it possible to accurately

Table 6.3.2 Film Choice and Exposure Temperature for Autoradiography

Isotope	Enhancement method	Film	Exposure temperature
³ H	Fluorography	Double-coated	-70°C
³⁵ S, ¹⁴ C, ³² P	None	Single-coated	Room temperature
³⁵ S, ¹⁴ C, ³² P	Fluorography	Double-coated	-70°C
³² P, ¹²⁵ I	CaWO ₄ intensifying screens	Double-coated	-70°C

quantitate very weak or very strong radioactive samples.

Troubleshooting

Cracking is one of the most common problems encountered when drying gels. This often occurs if the gel is removed from the dryer before it has adequately dried or if drying temperatures are too high. To overcome this problem, drying times should be extended and the performance of the vacuum pump and heater unit should be checked. For many gels, particularly for those with high percentages of polyacrylamide or >1.5 mm thick, cracking can be reduced by using an alternative fixing solution containing glycerol (3% glycerol/10% glacial acetic acid/20% methanol; see Support Protocol 1).

Among the biggest problems encountered in autoradiography are images that are either too weak or too intense. Such problems can be solved by varying the exposure time. Estimating initial exposure time is difficult, since the amount of radioactivity in the sample is often unknown. A Geiger counter can offer some guidance with certain isotopes. For highly exposed film, the length of time in developer can be reduced to produce a lighter image. It is particularly important to remember that if accurate quantification of the film image is desired, film must be preflashed so that there is a linear relationship between the amount of radioactivity in the sample and the image intensity.

Artifacts, such as black spots and stripes, can be avoided during developing by making sure that no moisture comes in contact with the film and that films exposed at -70°C are brought to room temperature before developing. Also, it must be noted that β particles from weak isotopes such as ³H cannot penetrate plastic wrap, and plastic wraps can attenuate signals from ³⁵S and ¹⁴C up to two-fold.

Anticipated Results

The protocols described here should yield a film image of a gel that can be quantified, stored, and photographed for publication.

Time Considerations

Fixing a gel will require ~45 min. Drying will take an additional 2 hr for a gel 1 mm in thickness. Incorporation of a fluor will add ~45 min to the processing time.

For gels >1.5 mm thick or with >15% acrylamide, an additional 30 min will be required for fixing and ~30 additional minutes will be required for drying.

The length of exposure for films in autoradiography can range from a few minutes to a few weeks, depending on the strength of the radioactivity in the sample. Most exposures last from several hours to a few days. Exposure time can be reduced more than 10-fold with a phosphor imager.

Literature Cited

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Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis combines two different electrophoretic separating techniques in perpendicular directions to provide a much greater separation of complex protein mixtures than either of the individual procedures. The most common two-dimensional technique uses isoelectric focusing (IEF) (see Basic Protocols 1 and 2) followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in a perpendicular direction (see Basic Protocol 3). This combination of isoelectric point (pI) and size separation is the most powerful tool for protein separations currently available. After staining, proteins appear on the final two-dimensional gel as round or elliptical spots instead of the rectangular bands observed on one-dimensional gels. Although the total separating power of large-format two-dimensional gels is estimated to be >5000 spots per gel, in practice a single two-dimensional separation of a complex mixture such as a whole-cell or tissue extract may produce 1000 to 2000 well-resolved spots when a sensitive detection method is used.

Until recently, the most common IEF procedures were based on the use of soluble ampholytes, relatively small organic molecules with various isoelectric points and buffering capacities. The pH gradient for IEF gels is produced when the soluble ampholytes migrate in the gel matrix until they reach their isoelectric point. Because stable pH gradients outside the pH 4.0 to 8.0 range are difficult to create with soluble ampholytes, alternative protocols using nonequilibrium conditions are required to resolve proteins with pI values below 4.0 (see Alternate Protocol 1 for acidic proteins) or above 8.0 (see Alternate Protocol 2 for basic proteins). One of the more important limitations of soluble ampholytes is the difficulty in obtaining highly reproducible pH profiles, especially when very narrow pH ranges are needed.

An increasingly attractive alternative to soluble ampholytes is the use of immobilized pH gradient (IPG) gels (see Basic Protocol 2). In this system, the buffering side chains are covalently incorporated into the acrylamide matrix, and any pH range and curve shape can be generated by pouring a gradient gel using two solutions that differ in ampholyte composition rather than acrylamide concentration. As with tube gels, the initial electrophoresis is followed by a second separation using SDS-PAGE in a perpendicular direction (see Basic Protocol 4). The use of IPG gels has recently increased, for at least three major reasons: many of the technical problems associated with their use have been solved or substantially minimized, reproducible premade IPG gels are now commercially available, and lately strong interest has arisen in using two-dimensional gels for proteome analysis studies (analyzing and comparing the complete protein profiles of cell lines, tissue samples, or single-celled organisms).

Another common two-dimensional electrophoresis format is a nonreducing/reducing electrophoretic separation (see Alternate Protocol 3), which provides useful information about intersubunit disulfides or protein-protein complexes that have been cross-linked using a bifunctional chemical cross-linker containing a disulfide bond within the linker region.

This unit also includes support protocols describing pI standards and pH profile measurements (see Support Protocol 1), electrophoresis of immobilized pH gradient gels (see Support Protocol 2), casting Immobiline gels (see Support Protocol 3), preparation of tissue culture cells and solid tissues for isoelectric focusing (see Support Protocols 4 and 5), preparation of molecular weight standards for two-dimensional gels (see Support Protocol 6), and two-dimensional protein databases (see Support Protocol 7).

NOTE: High-purity water (e.g., Milli-Q water or equivalent) is essential for all solutions. For cautions relating to electricity and electrophoresis, see Safety Considerations in the introduction to *UNIT 6.1*.

**BASIC
PROTOCOL 1**

**HIGH-RESOLUTION EQUILIBRIUM ISOELECTRIC FOCUSING
IN TUBE GELS**

This protocol describes the preparation of broad-range first-dimension gels using soluble ampholytes that resolve proteins with pI values between approximately 4.0 and 8.0, and is based on the original procedure described by O'Farrell (1975). The procedure presented here refers specifically to 3-mm IEF tube gels (first-dimension) combined with 1.5-mm-thick 16 × 16-cm (size of separating gel) second-dimension gels (see Basic Protocol 3) and may be easily adapted to a variety of different gel sizes (see Table 6.4.1). A 3-mm IEF gel has a total protein capacity of ~500 µg for complex protein mixtures such as whole-cell extracts. The maximum capacity of any single protein spot is ~0.5 to 5 µg, depending on the solubility of the protein near its isoelectric point and the separation distance from any near neighbors.

In this protocol, gels are cast and prefocused before the sample is loaded. The proteins are then separated according to isoelectric point, and the gels are extruded from the tubes and stored. Measuring pH profiles in IEF gels is a convenient and accurate method for determining pI (see Support Protocol 1). To provide optimal reproducibility, multiple gels should be cast and run simultaneously. This is especially important for comparative studies involving complex mixtures of proteins.

The IEF gels may be cast either by pouring the gel solution into the gel tubes (steps 3a to 7a) or by using hydrostatic pressure (steps 3b to 7b). Pouring the gel solution into the gel tubes is convenient for 3-mm-diameter IEF gels and requires only a minimal excess of reagents. Because the gels are cast using a long needle and syringe, for narrower gels, where the needle does not fit inside the gel tube, casting using hydrostatic pressure is more appropriate. This method requires a larger excess of reagents and special casting cylinders. Many types of ampholytes are readily available from different suppliers to form the desired pH profiles. As ampholytes may vary significantly in their performance, careful selection of the appropriate ampholytes is usually necessary (see Commentary).

Materials

- Chromic acid, in acid-resistant container
- Urea (ultrapure)
- 30% acrylamide/0.8% bisacrylamide (see recipe)
- 20% (w/v) Triton X-100 (see recipe)
- Ampholytes (e.g., pH 3-10/2D; ESA)
- TEMED (*N,N,N',N'*-tetramethylethylenediamine)
- 2.5% (w/v) ammonium persulfate (see recipe; prepare immediately before use)
- 8 M urea (see recipe; prepare immediately before use)
- 0.1 M orthophosphoric acid (H₃PO₄; see recipe)
- 0.1 M NaOH (make fresh daily)
- Lysis buffer (see recipe)
- Protein samples to be analyzed
- Equilibration buffer (see recipe)
- 2-Mercaptoethanol
- Isoelectric focusing apparatus (e.g., Protean II xi 2D from Bio-Rad or equivalent)
with glass tubes, casting stand, buffer chambers, rubber grommets, and plugs
- 37°C water bath

110°C oven
10-ml syringe equipped with filter capsule (0.22 or 0.45 μm , e.g., Costar $\mu\text{Star LB}$)
10-ml syringe equipped with blunt needle [e.g., 20-G \times 6 in. (15 cm) or 18-G \times 6 in. (15 cm)]
Large glass cylinder sealed at bottom with Parafilm (optional, for hydrostatic pressure casting method only)
2000-V power supply
60-ml syringe
Metal or plastic scoop
Dry ice pellets

Wash tubes and prepare the gel mixture

1. Remove the glass tubes from a chromic acid-filled container. Extensively wash the tubes with water, using high-purity water for the last wash. Dry the tubes at least 1 hr in an oven at 110°C and store them at room temperature, covered with aluminum foil.

To prevent gels from sticking to the glass tubes, gel tubes have to be very clean. Satisfactory results are obtained by storing the tubes in chromic acid between uses and washing them shortly before use. Because drying the tubes requires at least 1 hr, cleaning steps should be performed the day before gels will be cast.

CAUTION: *Chromic acid is highly corrosive; follow supplier's precautions carefully.*

2. Prepare the gel solution by mixing:

16.9 g urea
4.0 ml of 30% acrylamide/0.8% bisacrylamide
3.0 ml of 20% (w/v) Triton X-100
7.5 ml water
3.0 ml ampholytes.

Briefly warm the mixture in a 37°C water bath to solubilize urea if needed.

To minimize decomposition of urea, never warm any solutions containing urea above 37°C, use ultrapure urea, and prepare solutions immediately before use.

Choice of ampholyte composition is one of the key factors determining the quality of isoelectric focusing separations. Substantial differences in performance, resolution, and shape of the pH gradient formed may be observed with different combinations of ampholytes and with ampholytes from different suppliers. ESA's ampholytes (pH 3-10/2D) are suited for most applications and give reproducible results.

Although purity of all reagents is important, the purity of urea and choice of ampholytes are among the most critical factors for the quality and performance of isoelectric focusing. Most commercially available reagents marketed specifically for two-dimensional gel electrophoresis should be suitable, although individual lots of reagents from any supplier may provide variability and/or unacceptable results.

Cast gels by pouring

- 3a. Wrap one end of each glass tube with Parafilm and mount the tube in a casting stand. Mark all the tubes to indicate the desired gel height.

For reproducible results, all gels should be the same height.

- 4a. Filter the gel solution using a 10-ml syringe equipped with a syringe-tip filter capsule. Briefly degas the gel solution (~5 min) either by sonication or under vacuum. Then add 42.5 μl TEMED and 187.5 μl of 2.5% (w/v) ammonium persulfate solution to the filtered gel mixture and swirl gently to mix.

- 5a. Using a 10-ml syringe with a blunt needle, fill each glass tube with gel solution to the desired height. Make sure there are no air bubbles trapped in the gel.

A needle is the best choice for casting gels if tubes of 3-mm inner diameter are used. For narrower tubes, the use of hydrostatic pressure is more appropriate (see steps 3b to 7b, below). For long gels the needle can be extended by inserting a piece of capillary polyethylene tubing over the needle tip. The amount of gel solution described in step 2 is sufficient for sixteen 3-mm tube gels that are 16 cm long.

- 6a. Immediately overlay each gel with ~50 μ l of 8 M urea.

A pipettor with a capillary pipet tip is a convenient tool for overlaying with urea. Avoid mixing the overlay and gel solutions. Polymerization starts to occur ~15 min after the addition of TEMED and ammonium persulfate. It is essential that the gels be poured and overlaid before significant polymerization has occurred.

- 7a. Let the gels polymerize at least 3 hr prior to use.

Urea decomposes at a substantial rate at room temperature; therefore, the gels should be used the same day they are cast.

Cast gels using hydrostatic pressure

- 3b. Place a rubber band around the gel tubes so they form a tight bundle. Place the bundle inside a larger glass cylinder that is sealed at the bottom with several layers of Parafilm. All tubes must be precisely vertical.

The dimensions of the larger cylinder depend on the dimensions and number of gel tubes. Excessive space will require more gel solution to cast the gels.

- 4b. Filter the gel solution using a 10-ml syringe and filter capsule. Degas the gel solution briefly (~5 min) either with sonication or under vacuum. Add 42.5 μ l TEMED and 187.5 μ l of 2.5% ammonium persulfate solution and swirl.

- 5b. Pipet the gel solution into the bottom of the glass cylinder. Gently run water down the outside of the tube bundle using a wash bottle. Keep adding water until the gel mix reaches the desired height.

Hydrostatic pressure will force the gel solution into the tubes. Sufficient gel solution must be used to obtain the desired gel height while avoiding forcing any water into the tubes. The volume of gel solution required can be estimated as follows: number of gels \times 3.14 \times (tube internal radius in cm)² \times height in cm + ~10 ml to keep a safe level of gel mix at the bottom of the casting cylinder. As water is less dense than the gel solution, the water level will be slightly higher than the level of gel solution inside the tubes.

- 6b. Overlay the gels with 8 M urea.

Urea decomposes at a substantial rate at room temperature; therefore, the gels should be used the same day they are cast.

- 7b. Let the gels polymerize at least 3 hr prior to use.

Mount the gels in the electrophoresis unit

8. Prepare the lower electrode solution by degassing the proper amount of 0.1 M H₃PO₄ under vacuum with stirring for at least 5 min. Fill the bottom electrophoresis chamber.

The amount of phosphoric acid depends on the length of the gel tubes and the type of electrophoresis unit. The solution should cover the entire gel for good heat dissipation. Approximately 3 liters are required for Protean II xi 2D electrophoresis units.

9. Remove the gel tubes from the casting stand, remove the Parafilm from the tube bottoms, and inspect gels for irregularities or trapped air bubbles. Discard imperfect gels. If using gels cast with hydrostatic pressure, remove the bundle of tubes en bloc, cut off excess acrylamide with a razor blade, and then rinse away remaining acrylamide particles from the outside of each tube.

10. Place a rubber grommet on the top of the tube. Approximately 5 mm of the tube should be visible above the upper edge of the grommet.
11. Mount the tube with the grommet in the upper reservoir and plug any unused holes.

After the tube is seated, its lower end must be submerged in the lower electrode solution. Be sure to remove any air bubbles trapped at the bottom of the tube by shaking or tapping the tube gently. Alternatively, with some units bubbles can be dislodged by raising and lowering the tubes or by using a long curved needle and syringe.

Prefocus the gels

12. Prepare the 0.1 M NaOH upper electrode solution by degassing under vacuum with stirring for at least 5 min.

The amount of upper electrode solution necessary depends on the type of electrophoresis chamber. If a Bio-Rad Protean II xi 2D apparatus is used, 1 liter of 0.1 M NaOH is sufficient for both prefocusing and the separation.

13. Remove the 8 M urea overlay from the top of the gels using a Pasteur pipet and place ~50 μ l lysis buffer on the top of each gel.
14. Overlay lysis buffer with the degassed 0.1 M NaOH to fill the gel tubes. Avoid mixing of NaOH with the lysis buffer.
15. Pour the degassed 0.1 M NaOH into the upper chamber, making sure that all the gel tubes are covered with the electrode solution. Check carefully for leaks and air bubbles, then place lid on apparatus.
16. Connect the electrodes to a power supply by the red (+) lead to the lower chamber and the black (-) lead to the upper chamber.

The voltages and currents used during electrophoresis are dangerous and potentially lethal. Safety considerations are given in the Electricity and Electrophoresis section of UNIT 6.1.

17. Prefocus for 30 min using 500 V constant voltage.

Load the samples

18. Turn off power supply (see Safety Considerations in UNIT 6.1), disconnect leads, and remove lid. Using a 60-ml syringe, remove the electrode solution (0.1 M NaOH) from the upper chamber.
19. Remove the electrode solution and the overlay solution from each tube. Be careful not to damage the gel surface.
20. Place ~50 μ l lysis buffer on the top of each gel. Wait at least 2 min.
21. Remove the lysis buffer from the tubes.

Rinsing the gels with lysis buffer removes any residual NaOH and protects the samples against exposure to high pH.

22. Load protein samples to be analyzed and carefully overlay each sample with ~50 μ l lysis buffer diluted with water 8:2 (v/v). Avoid mixing the buffer with the sample.

The overlay solution protects samples from direct contact with the strong base used as an upper electrode solution. Dilution of the lysis buffer with water is necessary to decrease the density so the overlay does not mix with the sample.

A 3-mm-i.d. \times 16-cm-long IEF gel has a total protein capacity of ~500 μ g for whole-cell extracts and other complex protein mixtures. The maximum capacity for any single protein spot is ~0.5 to 5 μ g, depending on its solubility near its isoelectric point and the separation distance from any near neighbors. Preparation of relatively pure protein samples for

isoelectric focusing is generally straightforward. The sample usually may be prepared in one of the following ways: dialyze into any compatible low-ionic-strength buffer; lyophilize in a volatile or compatible low-ionic-strength buffer and dissolve in lysis buffer; or precipitate the protein using trichloroacetic acid (TCA) and redissolve in lysis buffer. For preparing extracts from cultured cells and from tissue samples, see Support Protocol 4 and Support Protocol 5, respectively.

The minimum sample concentration of protein or radioactivity has to be sufficient for the desired detection method. For complex protein mixtures such as tissue or cell extracts, a 500- μg total load is recommended for Coomassie blue staining or electroblotting (UNIT 6.2) for subsequent structural analysis, a 50- μg total protein load should be sufficient for silver staining or immunoblotting, and no less than 100,000 counts/gel is recommended for proteins labeled with ^3H , ^{14}C , or ^{35}S for autoradiography purposes. Sample volumes should be $<150\ \mu\text{l}$ for 3-mm gels and $<40\ \mu\text{l}$ for 1.5-mm gels. This implies at least a 5 $\mu\text{g}/\mu\text{l}$ protein concentration in the sample for gels to be stained with Coomassie blue.

23. Carefully fill all tubes with 0.1 M NaOH. Avoid mixing the NaOH solution with the overlay solution and the sample.
24. Fill the upper reservoir with 0.1 M NaOH. Be sure that all gel tubes are covered with the solution.

Run the gels

25. Connect the electrodes to a power supply with red (+) to the lower chamber and black (-) to the upper chamber.
26. Focus for a total of 12,000 Vhr.

Unlike other electrophoretic techniques, in IEF the volt-hour is the most common unit describing the “time” of isoelectric focusing. The initial voltage is usually set according to the desired number of volt-hours in a way that is convenient for the operator (i.e., so that the separation will run overnight), but it should not be $<400\ \text{V}$. The upper voltage limit is restricted by heat released in the gels during isoelectric focusing. At constant voltage the current will be the highest during the first hour of separation. The initial current will be strongly influenced by the ionic strength of the samples loaded onto the gels. An initial voltage of $<800\ \text{V}$ is recommended for 3-mm gels loaded with samples containing less than 100 mM salts/buffers; the voltage could be increased to 1200 V after $\sim 1\ \text{hr}$, if cooling is used. The current is a derivative of voltage and is never preset for isoelectric focusing purposes. Some power supplies allow preprogramming the desired number of volt-hours and continuously adjust voltage and current during the isoelectric focusing procedure (constant power). The total number of volt-hours is a major factor that affects separation in the first dimension. Optimal focusing time will vary for different ampholyte combinations, but 12,000 Vhr is a reasonable value for most systems. To achieve a total of 12,000 Vhr set the power supply to 667 V for 18 hr. These conditions are convenient for an overnight separation and do not require use of a cooling unit. Higher voltages can be used but may cause overheating of gels unless a highly efficient cooling system is employed. The maximum practical voltage decreases with increased gel tube inner diameter. Focusing for too long may cause cathodic drift and result in a shifted pH profile in the gel, whereas focusing for a short time will decrease resolution.

Extrude and store gels

27. Turn off power supply and carefully disconnect leads. Detach the lid and remove the NaOH solution from the upper reservoir of the electrophoresis chamber using a 60-ml disposable plastic syringe.
28. Remove one gel tube at a time from the chamber.
29. Using a 10-ml syringe equipped with a blunt needle, slowly and carefully inject water between the gel and glass tube. Start from the bottom of the tube, then repeat the procedure from the top. The gel should slide out of the glass tube.

It is convenient to let the gel slide from the glass tube onto a metal or plastic scoop, which facilitates transfer of the gel into a storage vial. It is relatively easy to break the gel during extrusion, and practicing on several unused gels is recommended. To extrude smaller-diameter gels, use water pressure generated by a syringe connected to the gel tube with Tygon tubing. If clean, unscratched glass tubes are used, extrusion should be easy.

30. Using the scoop, slide the gel into a 4.5-ml cryovial containing 3 ml equilibration buffer and 50 μ l 2-mercaptoethanol. Close the vial, incubate exactly 5 min at room temperature, then freeze by placing the tube horizontally on top of dry ice pellets. Do not move or agitate the tube while the sample is freezing.

The IEF gels may be run on a second-dimension gel immediately (see Basic Protocol 3), or can be stored at -80°C for many weeks. Even when the second-dimension is to be run immediately, extruded gels should be frozen after a carefully controlled incubation time at room temperature, such as the 5 min cited above for 3-mm-i.d. gels, to minimize diffusion of proteins out of the IEF gel. This short incubation before freezing will allow glycerol to diffuse into the gel. Too short an incubation or agitation during freezing can result in gel breakage. The total incubation time in equilibration buffer (sum of the time prior to freezing and after thawing) is critical and should be carefully controlled. Insufficient incubation time in equilibration buffer will not allow sufficient time for SDS to diffuse into the gel and saturate sites on the proteins. Excessive incubation times can result in appreciable protein losses due to diffusion out of the highly porous IEF gel.

CONDUCTING pH PROFILE MEASUREMENTS

Standards with different isoelectric points can help in evaluating the performance of a specific system and determining the effective pH range in the isoelectric focusing gel. Many pI standards are commercially available from different suppliers. It is most useful to separate a mixture of standard proteins that is prepared from several individual proteins or purchased as a preformulated kit. This mixture should be run in parallel with experimental samples on a separate reference gel. It is generally not recommended to run pI standards together in the same gel with samples because of possible interference with migration and identification of proteins of interest. Instead of analyzing standard proteins, a more precise evaluation of the pH profile can be made by directly measuring the pH throughout the gel using either a surface pH electrode or the following procedure.

1. Prepare and focus one or two gels (see Basic Protocol 1, steps 1 to 26) without any sample in parallel with experimental samples.
2. Prepare 20 to 40 glass test tubes each containing 1 ml high-purity, degassed water for each gel that will be used to measure the pH gradient (measurements on duplicate gels are recommended).

The number of tubes required per gel equals twice the gel length (in cm).

3. After electric focusing is completed, extrude the blank gels (see Basic Protocol 1, steps 27 to 29). Briefly rinse the gels with water.

After extrusion, gel surfaces may be contaminated with electrode solutions. Rinsing with water is essential for obtaining reliable pH profiles.

4. Place the gel on a glass plate with a plastic ruler below the plate. Cut the gel into 0.5-cm pieces using a sharp razor blade.
5. Place each gel piece in a test tube containing 1 ml water.

Do not mix the order of samples because each gel piece represents a single pH profile data point.

6. Place all test tubes on a shaker and shake gently for 1 hr at room temperature.

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Electrophoresis and Immunoblotting

6.4.7

7. Read the pH of each solution and plot the pH profile as a function of the distance from the top of the gel.

ALTERNATE PROTOCOL 1

NONEQUILIBRIUM ISOELECTRIC FOCUSING OF VERY ACIDIC PROTEINS

Basic Protocol 1 is sufficient for separating proteins with isoelectric points greater than ~3.5 to 4.0. For very acidic proteins, however, a nonequilibrium system is needed. The major features of this method are utilization of a shorter focusing time (without reaching equilibrium), a modified ampholyte mixture, and different electrode solutions.

Additional Materials (also see Basic Protocol 1)

- 10% (w/v) ammonium persulfate (prepare immediately before use)
- Concentrated sulfuric acid (used in lower chamber electrode solution)
- Ampholytes, pH 2-11 (used in upper chamber electrode solution)

To analyze very acidic proteins, follow Basic Protocol 1 with these exceptions in the indicated steps:

2. When preparing the gel solution, use the following mixture of ampholytes: 2.4 ml ampholytes pH 2.5-4 and 0.6 ml ampholytes pH 2-11.
4. Following the procedure for casting gels by pouring, add 100 μ l of 10% ammonium persulfate solution, swirl, add 42.5 μ l TEMED, and swirl again.

Gel mixtures containing entirely or predominantly very acidic or very basic ampholytes are generally difficult to polymerize. Use of an increased ammonium persulfate concentration and adherence to the proper order of adding the reagents should ensure polymerization.

8. Prepare the bottom chamber electrode solution by adding 4.5 ml concentrated sulfuric acid to 3 liters water. Degas at least 5 min.

Omit steps 12 to 19 (do not prefocus the gels).

20. Remove the 8 M urea (polymerization overlay solution) and place ~50 μ l lysis buffer on top of each gel. Wait at least 2 min, then remove the lysis buffer.
23. Carefully fill all tubes with the upper chamber electrode (anode) solution prepared by mixing pH 2-11 ampholytes with water in a 1:40 ratio.
24. Fill the upper buffer chamber (anode) with the solution described in step 23.

Iminodiacetic acid (10 mM) may be a more economical alternative anode solution.

26. Focus for a total of 4000 Vhr.

ALTERNATE PROTOCOL 2

NONEQUILIBRIUM ISOELECTRIC FOCUSING OF BASIC PROTEINS

In general, most equilibrium IEF gel systems using soluble ampholytes produce pH gradients that do not exceed pH 8.0 on the basic end, yet many proteins have higher pI values. For this reason samples containing very basic proteins are usually focused using a nonequilibrium system. In an equilibrium system, proteins are loaded on the basic end of the gel and migrate toward the acidic end until they reach a pH equal to their pI. In nonequilibrium systems, the sample is loaded on the acidic end of the gel, and focusing is terminated after a relatively short time (fewer volt-hours).

To run nonequilibrium IEF gels, follow the procedure previously described (see Basic Protocol 1) with these alterations in the indicated steps:

8. Use 0.1 M NaOH as the lower electrode solution.

Electrode solutions and electrodes are reversed in this procedure relative to equilibrium isoelectric focusing.

Omit steps 12 to 19 (do not refocus the gels).

20. Remove the 8 M urea (polymerization overlay solution) and place 50 μ l lysis buffer on top of each gel. Wait at least 2 min, then remove the lysis buffer.
23. After loading the samples and overlaying with lysis buffer diluted with water 8:2 (v/v) as in Basic Protocol 1, use 0.1 M H_3PO_4 instead of NaOH to fill all gel tubes.
24. Use 0.1 M H_3PO_4 as the upper electrode solution.
25. Reverse the connection of electrodes—i.e., connect the red (+) lead to the upper chamber and the black (–) lead to the lower chamber.
26. Focus for a total of 3000 to 5000 Vhr.

The optimal number of volt-hours depends on the nature of the sample and the ampholytes used. The values recommended above may need to be adjusted empirically.

ISOELECTRIC FOCUSING USING IMMOBILIZED pH GRADIENT GEL STRIPS

In immobilized pH gradient (IPG) gels, the ampholytes are covalently linked to the acrylamide matrix, which facilitates production of highly reproducible gradients as well as very narrow pH gradients for optimal resolution of minor charge differences. A variety of precast gels and all the necessary equipment are commercially available from either Amersham Pharmacia Biotech or Bio-Rad. Equipment and chemicals are also available for the user to cast gels in the laboratory (see Support Protocol 3), although precast gels are likely to suffice for the majority of applications. Narrow strips of precast IEF gels (Immobiline DryStrips or Ready Strip IPG strips) may be used to achieve a first-dimension separation for two-dimensional gel electrophoresis, and broader precast slab gels (Immobiline DryPlates) can be used to compare multiple samples after IEF separation only (see Support Protocol 2 and Table 6.4.3). In this protocol, precast Immobiline DryStrips from Amersham Pharmacia Biotech are rehydrated overnight using the reswelling cassette (one to twelve sample strips may be handled at a time); samples are applied using sample cup holders and gel strips are isoelectric focused overnight. This procedure has been adapted from instruction booklets provided by Amersham Pharmacia Biotech with Immobiline Dry Strip Kits and with the Immobiline DryStrip reswelling tray. Recently, both Amersham Pharmacia Biotech and Bio-Rad have developed newer IEF systems, the IPGphor Isoelectric Focusing System and the Protean IEF Cell, respectively. These systems simplify IPG strip handling and the overall isoelectric focusing procedure. The IPGphor system also integrates IPG strip reswelling and electrophoresis steps in a single strip holder. These systems include very high-voltage power supplies integrated with efficient cooling units to permit more rapid isoelectric focusing. In general, premade commercial IPG strips from any supplier can be used with any isoelectric focusing device that can physically accommodate the strip. The appropriate user manuals should be consulted for specific details.

Since there is a greater selection of pH ranges for premade Immobiline DryPlates than DryStrips, it is sometimes convenient to cut DryPlates into strips prior to rehydrating the gel to obtain narrower pH ranges where needed. See Basic Protocol 4 for details concerning preparing and running the second-dimension gel.

Wear gloves throughout the procedure and handle the Immobiline DryStrips with forceps where feasible to prevent extraneous protein contamination of the gels and gel solutions.

BASIC PROTOCOL 2

Electrophoresis and Immunoblotting

6.4.9

Table 6.4.1 Rehydration Solutions for Immobiline DryStrips^a

Component	Final conc.	DryStrip type		
		3-10L	3-10NL	4-7L
Ultrapure urea ^b	7 M	2.1 g	2.1 g	2.1 g
Thiourea ^b	2 M	0.76 g	0.76 g	0.76 g
CHAPS ^c	2%	0.1 g	0.1 g	0.1 g
Pharmalyte pH 3-10	1:50 ^d	100 µl		
Pharmalyte pH 4-6.5			50 µl	100 µl
Pharmalyte pH 8-10.5			25 µl	
Ampholine pH 6-8			25 µl	
DTT	0.3%	75 mg	75 mg	75 mg
Bromphenol blue	Trace	A few grains	A few grains	A few grains
Milli-Q water		To 5 ml	To 5 ml	To 5 ml

^aRehydration solutions should be prepared fresh immediately before use or stored as frozen aliquots and should be filtered using a 0.2-µm filter. Minimize total time the solution is at room temperature prior to use to minimize decomposition of urea. If the reswelling tray is used, ~250 or 400 µl rehydration solution is required per 11- or 18-cm DryStrip, respectively.

^bA "total urea" concentration of 9 M is typically used. Thiourea is more effective than urea for minimizing protein precipitation during isoelectric focusing, but its solubility is lower. The combination of 7 M urea/2 M thiourea usually results in superior sample solubilization and isoelectric focusing as compared with 9 M urea alone.

^cThe optimal detergent and detergent concentration should be empirically determined. Other common alternatives are Triton X-100 and octyl-glucoside. The detergent used must be nonionic or zwitterionic to avoid high current and consequent overheating during isoelectrofocusing.

^dAlternatively, soluble ampholytes are available as preformulated "IPC buffer" concentrates for each type of IPG strip.

Thoroughly clean all equipment with a mild laboratory detergent solution, rinse well with Milli-Q water, and allow to dry before using. Solutions containing 10 M urea may be heated *briefly* to 30° to 40°C to aid in solubilization.

Materials

- Urea (ultrapure)
- CHAPS or Triton X-100
- Pharmalyte 3-10, 4-6.5, and/or 8-10.5 soluble ampholytes (see Table 6.4.1; Amersham Pharmacia Biotech)
- Ampholine pH 6-8 (Amersham Pharmacia Biotech)
- DTT (dithiothreitol)
- Bromphenol blue
- Precast Immobiline DryStrips (Amersham Pharmacia Biotech)
- DryStrip cover fluid (Amersham Pharmacia Biotech)
- Immobiline DryStrip kit (Amersham Pharmacia Biotech) including:
 - Cathode electrode
 - Anode electrode
 - Sample cup bar
 - Tray
 - Sample cups
 - Immobiline strip aligner
 - IEF electrode strips
 - Sample application pieces
 - Instruction manual

continued

Protein sample to be analyzed
Lysis buffer (see recipe)
Immobiline DryStrip reswelling tray (Amersham Pharmacia Biotech)
Forceps
Filter paper
Glass plate
Flatbed electrophoresis unit (Amersham Pharmacia Biotech Multiphor II or equivalent)
Recirculating cooling water bath
Power supply (minimum capacity of 3000 to 3500 V)
Petri dishes
Additional reagents and equipment for protein detection by staining (*APPENDIX 3*) and/or for electroblotting (*UNIT 6.2*, optional)

Rehydrate the Immobiline DryStrip(s)

1. Prepare an appropriate rehydration solution for the type of DryStrips to be used as described in Table 6.4.1 (~400 μ l rehydration solution per 18-cm DryStrip).

The rehydration solution should contain 7 M urea/2 M thiourea, and 2% CHAPS or another appropriate detergent (zwitterionic or nonionic) such as Triton X-100, NP-40, or n-octylglucoside should be included in the rehydration solution to aid in sample solubility. The optimal detergent and detergent concentration may vary with type of sample and should be determined empirically.

One possible method of loading large sample volumes onto IPG gels is to add the sample directly to the rehydration solution. Sample loading during rehydration is preferred when using the IPGphor system with its single strip holder for both rehydration and isoelectric focusing.

Solutions containing urea should be filtered using a 0.2- μ m filter before use.

2. Slide the protective lid off the reswelling tray and level the tray by adjusting the leveling feet until the leveling bubble is centered.
3. For an 18-cm gel, pipet 350 to 400 μ l of rehydration solution into a slot of the reswelling tray. Move the pipet along the length of the well while adding the solution to spread it evenly throughout the length of the slot. Avoid excessive air bubble formation while pipetting this solution.

If the IPGphor system is used, pipet the rehydration solution containing the protein sample into each cleaned ceramic holder.

4. Remove the protective cover from the Immobiline DryStrips and gently place them, gel side down, into the prepared slot.

To facilitate their removal after rehydration, the strips should be oriented with their pointed ends at the sloped end of the slots in the rehydration tray. Be careful not to trap any air bubbles under the gel strips.

5. Overlay each strip with 2 to 3 ml of DryStrip cover fluid to prevent evaporation and urea crystallization. Slide the protective lid into place and allow gels to rehydrate overnight (~16 hours) at room temperature.

Shorter rehydration times can be used, although a minimum of 6 to 8 hr is usually needed to completely and reproducibly rehydrate the gels. Do not substantially exceed 16 hr as extensive incubation, especially rehydrating gels over a weekend, increases potential problems due to evaporation and subsequent urea crystallization. In addition, long incubation times increase the extent of urea decomposition, which will increase the risk of amino group modification on proteins by the cyanate produced from urea decomposition.

If the IPGphor system is used, a low voltage (30 to 40 V) can be applied during rehydration, which improves isoelectric focusing and protein yields of some samples. With this device,

protocols can be preprogrammed so as to allow isoelectric focusing to immediately follow rehydration. Hence, both rehydration with low voltage and isoelectric focusing can be completed overnight, since ~8 hr is sufficient for rehydration, and up to 8000 V can be used in later stages of isoelectric focusing to shorten the total focusing time.

6. After the overnight rehydration, slide the lid off the reswelling tray. Place a forceps tip into the slight depression under each strip and remove the strip. Gently blot any excess oil or moisture from the plastic backing of the rehydrated strips with filter paper. A damp piece of filter paper may also be used to blot the surface of the gel. Some of the paper may adhere to the gel and should be gently peeled away. The gel is now ready to be placed in the strip aligner on the cooling plate of the electrophoresis unit.

Do not allow the gel to dehydrate prior to placing it on the cooling plate in step 11. (Steps 7 to 10 should be completed prior to removing the strips from the reswelling tray.)

Run the first dimension

7. Level the Multiphor II electrophoresis unit, then connect it to a circulating cooling water bath. Allow it to cool to 15°C for 1 to 2 hr to ensure even cooling. Do not cool below 15°C to prevent precipitation of urea in the gels.
8. Pipet ~5 ml DryStrip cover fluid onto the surface of the Multiphor II cooling plate. Position the Immobiline DryStrip tray on the cooling plate oriented with the red (+, anodic) electrode at the top, near the cooling tubes.

Avoid large air bubbles between the cooling plate and the tray (small bubbles should not cause a problem).

9. Connect the red and black electrode leads on the tray to their respective positions on the Multiphor II unit. Pour 10 ml of DryStrip cover fluid into the tray. Place the Immobiline DryStrip aligner on top of the oil, groove side up.

Avoid getting oil on top of the strip aligner. The possible presence of small air bubbles under the strip aligner is not important.

10. Cut two electrode strips to a length of 11 cm (regardless of the number of DryStrips used). Place the electrode strips onto a clean glass plate and soak each one with 0.5 ml Milli-Q water. Blot with a Kimwipe or tissue paper to remove excess water.

The electrode strips should be evenly soaked and just damp after blotting. Excessive water could cause sample streaking.

11. Transfer the strips from step 6 to adjacent grooves in the aligner tray. Position the rounded (acidic) end of each strip near the top of the tray at the red electrode (anode) near the cooling tubes, and the square end at the bottom of the tray near the black electrode (cathode). Be sure that the edges of all gel strips at the anode end are lined up evenly.

12. Place the blotted electrode strips from step 9 on top of the gel surface of the DryStrips near the anode and cathode ends of the gel. Position the red (anode) and black (cathode) electrodes on top of the electrode strips at their respective ends.

After the electrodes have been pressed down on top of the electrode strips, check that the gel strips have not shifted position.

13. Push the sample cups onto the sample cup bar. Place the sample cup bar near the anode end of the gel so that the small spacer arm just touches the electrode and the sample cups are nearest to the electrode, but do not allow the cups to touch the gel.

The sample cups should face the nearest electrode. The acidic end of the gel can usually be used for sample application; however, the optimal loading position may need to be determined empirically for different types of samples. At high protein concentrations and/or at non-optimal pH, samples may precipitate in the gel at the loading position.

14. Position one sample cup above each gel strip and push down to ensure good contact between the bottom of the sample cup and the gel strip. Make sure the gel strips have not shifted position.
15. Pour 70 to 80 ml of DryStrip cover fluid into the tray (it will cover the gels). If oil leaks into the sample cups, adjust the cups to stop leakage. When there is no leakage into the sample cups, add enough cover fluid to the tray to completely cover the sample cups (~150 ml).
16. Pipet protein samples (in lysis buffer) into the sample cups by underlayering. The sample should sink to the bottom of the cup. Check for leakage of the sample out of the sample cup.

Samples should either be lyophilized and then solubilized in lysis buffer, or diluted 9 parts lysis buffer to 1 part sample. The maximum volume each sample cup holds is 100 μ l. The complexity of the sample, the sample solubility at the loading concentration and pH used, the thickness of the second-dimension gel, and the detection method to be employed should be considered when deciding how much protein to load. As a starting reference, typical loading ranges for 1.0- to 1.5-mm-thick 18-cm \times 18-cm gels would be ~5 to 20 ng per major spot for silver staining and ~1 to 5 μ g per major spot for Coomassie blue staining. When very complex samples are used such as whole cell extracts, total protein loads are likely to be ~20 to 100 μ g for silver staining and ~200 to 1000 μ g for Coomassie blue staining. The salt concentration in samples should be kept <50 mM and, if the sample contains SDS, the final SDS concentration should be <0.25%.

17. Place the lid on the Multiphor II unit and connect the leads to a power supply. Focus the gels with constant voltage for 2 to 3 hr at 500 V followed by 12 to 16 hr at 3500 V for a total of 40 to 60 kWhr. Refer to the user manual for exact recommended voltage conditions for each type of Immobiline DryStrip.

The optimal number of Vhr will depend upon the pH range of the Immobiline Strip used, the type of sample, and the sample load and volume; therefore, the optimal Vhr should be empirically determined for different applications.

18. When isoelectric focusing is complete, disconnect the power supply and remove the cover from the Multiphor II unit. Remove the electrodes, electrode strips, and sample cup bar from the tray.

If gels are to be run in the second dimension immediately after isofocusing, steps 1 to 3 of Basic Protocol 4 should be completed prior to terminating isofocusing.

19. Using forceps, remove the DryStrips from the tray. If the gels are to be run in the second dimension immediately, place in a petri dish with the support film along the wall of the dish and proceed directly to equilibration of the gel (see Basic Protocol 4, step 4). Alternatively, gels may be stored sealed in a plastic bag at -80°C until ready to run the second-dimension gel.

Gels may be stored at least 2 to 3 months at -80°C . Do not place in the equilibration buffers required for the second dimension prior to storage.

ELECTROPHORESIS ON IMMOBILIZED pH GRADIENT GELS

In this protocol, after precast IEF gels (Immobiline DryPlates) from Amersham Pharmacia Biotech are rehydrated, samples are loaded and subjected to isoelectric focusing. Gels are typically run at 2500 to 3500 V and require focusing times of 2 to 7 hr. Protein samples may be detected by conventional methods such as Coomassie blue or silver staining. Isoelectric points can be determined with the use of pI calibration proteins; alternatively, because the gradient is linear, one can measure the migration distance across the gel and estimate the pI at each location. As noted in Basic Protocol 2, Immobiline DryPlates can be cut into 3-mm-wide strips to use as the first dimension of two-dimensional gels, since

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DryPlates are available in narrower pH ranges than DryStrips. DryPlates can also be used as described in this protocol to simultaneously separate multiple samples in a single dimension. Applications of this method include initial screening of samples to determine the optimal pH gradient prior to running more time-consuming two-dimensional gels, prescreening fractions from a chromatographic purification step prior to running two-dimensional gels, and evaluation of charge heterogeneity of purified proteins.

Additional Materials (also see *Basic Protocol 2*)

Precast DryPlate gel (Amersham Pharmacia Biotech)
Repel-Silane (Amersham Pharmacia Biotech)
Paraffin oil
Protein samples to be analyzed
Reswelling Cassette kit (Amersham Pharmacia Biotech) including:
125 × 260 × 3-mm glass plate with 0.5-mm U frame
125 × 260 × 3-mm glass plate
Silicone tubing
Pinchcock
Clamps
20-ml syringe
Roller (Amersham Pharmacia Biotech)
Whatman no. 1 filter paper
Flatbed electrophoresis unit (Amersham Pharmacia Biotech Multiphor II)
10° or 15°C cooling water bath
Electrode strips
Sample applicator strip or sample application pieces
Power supply (minimum capacity 3000 to 3500 V)
Additional reagents and equipment for protein detection by staining (*APPENDIX 3*)
and for electroblotting (*UNIT 6.2*; optional)

Rehydrate the gel

1. Remove precast gel from packaging. If the entire gel is not needed, cut off the required number of lanes and reseal the unused gel. Mark the polarity of the gel section to be used by cutting a small triangle off the anode corner. Handle the gel by the support film only.

It is critical that the lanes are cut from the gel in the proper orientation to preserve the pH gradient (see Fig. 6.4.1), and polarity must be indicated for proper orientation of electrodes later in the procedure.

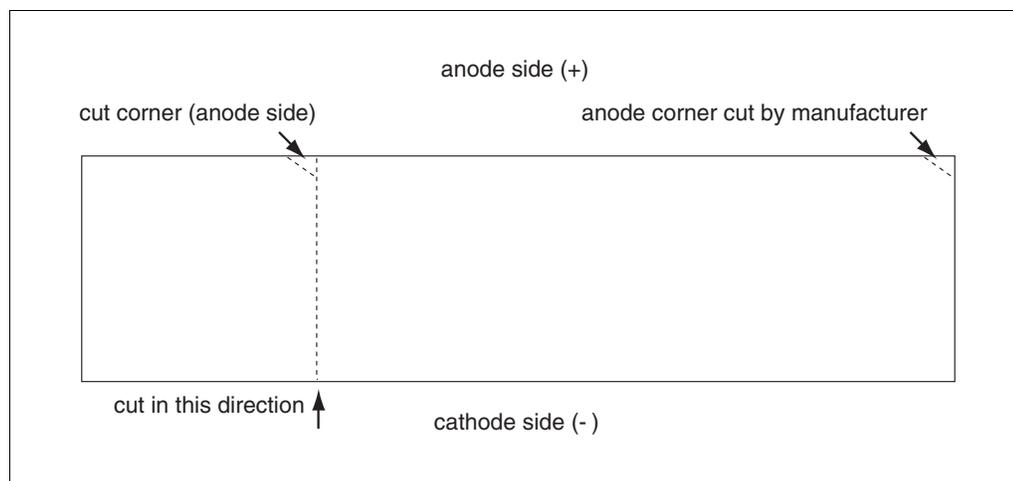


Figure 6.4.1 Marking orientation of a precast IPG gel when only a portion of the gel is used.

2. Use the Reswelling Cassette to rehydrate the gel. Connect silicone tubing through hole in the bottom corner of the U-frame plate, seal with silicone glue, and connect the pinchcock to the other end of the tubing. Place a glass plate on a clean flat surface and wet with a few drops of water. Place the gel on the plate, gel side up. Gently roll with a clean rubber roller to remove any air bubbles.

3. Cover the plate and gel with the plate fitted with the U frame.

The U-frame plate should be coated with a thin layer of Repel-Silane to prevent the gel from sticking to the plate.

4. Place clamps around the edges of the plates, making sure the seal is tight.
5. Slowly fill the cassette with the desired rehydration solution using a 20-ml syringe connected to the silicone tubing and let stand for the recommended amount of time. Precool electrophoresis unit 1 to 2 hr prior to electrophoresis (see step 9).

Reswelling with water for 2 to 3 hr is normally sufficient. If using additives such as urea, Triton, glycerol, or reducing agents, allow the gel to rehydrate overnight. Additives can be used to improve solubility of proteins near their isoelectric point. Reducing reagents such as DTT are used to reduce disulfide bonds.

6. When gel has been allowed to rehydrate completely, remove the clamps and gently pry the plates apart.
7. Moisten a piece of filter paper with water and place on top of the gel, then layer with a piece of dry filter paper.
8. Gently blot the gel by rolling over the dry filter paper with the rubber roller to remove excess water. The gel is now ready to be placed on the cooling plate.

Do not let the gel dehydrate prior to placing it on the cooling plate in step 11.

Run the gel

9. Connect the flatbed electrophoresis unit to a recirculating cooling water bath. Allow to cool to 10°C for 1 to 2 hr to ensure even cooling. If the gel has been rehydrated in the presence of urea, do not cool below 15°C so that urea does not precipitate.
10. Pipet 2 to 3 ml paraffin oil onto the surface of the cooling plate.
11. Position the gel on the cooling plate, being careful not to trap air bubbles between the gel and the plate. Orient the gel so that the polarity of the gel matches the polarity of the cooling plate.
12. Soak two electrode strips with ~3 ml water, then blot to remove excess water.
13. Lay a blotted electrode strip along each long edge of the gel. Cut off the ends of the electrode strip so that it does not extend beyond the edge of the gel.
14. Load protein samples to be analyzed onto the gel. Use an applicator strip for sample volumes between 5 and 20 μl (make sure contact between the strip and the gel is uniform). Use sample application pieces for sample volumes >20 μl . Remove the application pieces halfway through focusing. For sample volumes of 2 to 10 μl , samples may be spotted directly on the gel without using applicator strips. See manufacturer's instructions for further details.

An important experimental consideration is the position in the pH gradient where the sample is applied. The acidic end of the gel can usually be used for sample application; however, the optimal loading position may need to be determined empirically for different types of samples. At high protein concentrations and/or at nonoptimal pHs, samples may precipitate in the gel at the loading position.

Samples should contain <50 mM salt or buffer components; greater concentrations will cause local overheating of the gel. If possible, salt-free samples should be solubilized or dialyzed in the rehydration buffer.

15. Align the electrodes with the electrode strips, put the safety lid in place, and connect the apparatus to the power supply. Conduct electrophoresis at 3000 V.

Broad-range IPG gels, such as Immobiline DryPlate, pH 3-10, should be run at ~3000 V for 2 to 4 hr. Narrower-range gels are also run at 3000 V but may require a focusing time of 4 to 7 hr.

16. After removing gels from the electrophoresis apparatus, detect proteins using any conventional staining technique such as Coomassie blue or silver staining.
17. Preserve the gels by sealing in a plastic bag or by drying for a permanent record. Alternatively, electrotransfer the proteins on the gel to a membrane.

To dry a gel, presoak it first in a preservation solution. For silver-stained gels, use a solution of 5% to 10% (w/v) glycerol/30% (v/v) ethanol; for Coomassie blue-stained gels, use a solution of 5% to 10% (w/v) glycerol/16% (v/v) ethanol/8% (w/v) acetic acid. After soaking the gel, place it on a glass plate gel side up, cover with a cellophane sheet soaked in preservation solution, and allow to dry at room temperature.

For electrotransfer (UNIT 6.2), use film remover to remove the plastic support film from the gel. Electrotransfer of proteins to a polyvinylidene difluoride (PVDF) membrane using a Multiphor II NovaBlot transfer kit (Amersham Pharmacia Biotech) is recommended. Transferring IPG gels requires special procedures; see the transfer kit manual for instructions.

SUPPORT PROTOCOL 3

CASTING AN IMMOBILINE GEL

An alternative to precast IPG gels is the use of Amersham Pharmacia Biotech Immobilines to cast immobilized pH gradient gels with customized pH gradients and ranges, including very narrow pH ranges, to improve separation of proteins with small charge differences. This protocol describes the general procedure of casting custom-made Immobiline gels. The Reswelling Cassette used in Basic Protocol 2 for rehydrating gels is employed for casting the gels, which are polyacrylamide gels poured with a gradient of Immobilines following instructions provided by Amersham Pharmacia Biotech application note 324.

Additional Materials (also see *Support Protocol 2*)

GelBond PAG film (Amersham Pharmacia Biotech)
Immobiline solutions (Amersham Pharmacia Biotech)
2.5% (v/v) glycerol

Gradient maker
Orbital shaker

Additional materials and equipment for rehydrating immobilized pH gradient gels
(see Basic Protocol 2)

Cast the gel

1. Coat the plate with the U frame with Repel-Silane to prevent the gel from sticking to the glass plate.
2. Place a glass plate on a clean, flat surface and wet with several drops of water. Cover with a sheet of GelBond PAG film, hydrophilic side up. Use the roller to remove any air bubbles trapped between the film and the glass plate.
3. Place the plate with the U frame on top of the GelBond PAG film. Clamp the plates together on three sides.

4. Mix the Immobililine solutions following the instructions provided by Amersham Pharmacia Biotech application note 324 to prepare the desired pH range. Cast the pH gradient gel using a gradient maker.

Once the catalysts have been added to the gel solution, it is important to work quickly to ensure that the gradient is poured before polymerization occurs. See UNIT 6.1 for instructions for using gradient makers.

5. Do not disturb the gel during the first 10 min to allow the gradient to stabilize. Allow the gel to polymerize 1 hr in a 50°C oven.

Dry and store the gel

6. Allow the gel to cool to room temperature, then disassemble the cassette. Cut off a small corner to label the anode end of the gel.
7. Wash the gel 2 to 3 hr with 200 to 300 ml water. Use an orbital shaker and change the water two or three times.

At this stage the gel may be used immediately (if no additives are needed) or dried as described in steps 8 to 10.

8. Wash the gel 30 min to 1 hr with 200 to 300 ml of 2.5% (v/v) glycerol.
9. Place the gel on a glass plate (gel side up) in a dust-free environment and allow to dry at room temperature overnight.
10. Store the dried gel in a sealed plastic bag at –20°C for up to 2 months.

The gels may be rehydrated when needed (see Support Protocol 2).

PREPARING TISSUE CULTURE CELL EXTRACTS FOR ISOELECTRIC FOCUSING

SUPPORT PROTOCOL 4

Preparation of samples containing relatively pure proteins for isoelectric focusing is generally straightforward (see Basic Protocol 1, step 22). In contrast, complex samples such as whole-cell extracts, tissue extracts, or subcellular fractions are more difficult to prepare for successful isoelectric focusing. Solubility limitations both prior to isoelectrofocusing and during focusing restrict analysis of these complex samples to protocols that include nonionic detergents and urea (see Basic Protocol 1). In addition, the presence of DNA and RNA in crude cell extracts further complicates isoelectric focusing. The protocol presented below is suitable for preparing samples from cell cultures and is based on quantities compatible with silver staining or Coomassie blue staining. If smaller cell numbers and high-sensitivity detection methods such as autoradiography are used, volumes and quantities should be adjusted as needed.

In this protocol the cells are harvested and washed in phosphate-buffered saline (PBS) with proteolysis inhibitors, then lysed in Tris/SDS buffer using sonication, after which the total protein concentration is determined in the lysate. The lysate is further treated with a mixture of DNase and RNase, and additional SDS and reducing agent are added. At this stage, the samples can be stored at –80°C for an extended time or, after addition of urea and lysis buffer, may be loaded directly onto prefocused IEF gels. Filtration of the final sample prior to loading onto the IEF gel is essential for quality of isoelectric focusing.

Electrophoresis and Immunoblotting

6.4.17

Materials

Cell culture flasks containing cells of interest
PBS with proteolysis inhibitors (PBS/I buffer; see recipe)
Dry ice/ethanol (optional, for freezing samples)
Tris/SDS buffer (see recipe)
BCA protein assay kit (Pierce)
DNase and RNase solution (see recipe)
20% (w/v) SDS (*APPENDIX 2E*)
2-Mercaptoethanol
Urea (ultrapure)
Lysis buffer (see recipe)

50-ml centrifuge tube
Centrifuge with rotor (e.g., Beckman JS-4.2), 4°C
1- to 2-ml cryovials
Microcentrifuge, 4°C
Sonicator with microtip
0.2- μ m microcentrifuge filter units (e.g., Millipore Ultrafree-MC filter units)

Harvest and wash the cells

1. Place cell culture flasks containing cells of interest on ice.
2. Rapidly wash cells three times with 2 to 6 ml PBS/I buffer. Keep the flasks on ice.
The required volume of PBS/I buffer depends on the flask size. For example, use 2 ml for a 25-cm² tissue culture flask and 6 ml for a 75-cm² tissue culture flask.
3. Add 2 to 6 ml PBS/I buffer to the flask, scrape the cells using a cell scraper, and transfer the suspension to a 50-ml centrifuge tube. Repeat this step with another 2 to 6 ml buffer to ensure complete transfer of the cells.
Cells grown in suspension are washed in an analogous manner using repetitive centrifugation.
4. Collect the cells by centrifuging 15 min at 2600 \times g (3000 rpm in Beckman JS-4.2 rotor), 4°C.
5. Discard the supernatant and resuspend the cells in a small volume of PBS/I buffer.
6. Transfer the cell suspension to a labeled cryovial.
The weight of the empty cryovial can be determined prior to use if the wet weight of the cell pellet is desired as a reference value rather than cell number, radioactivity, or another criterion.
7. Microcentrifuge the cells 15 min at maximum speed, 4°C.
8. Remove and discard the supernatant using a pipettor or Pasteur pipet. Weigh the vial containing the cell pellet. Record the wet weight of the cell pellet (in mg).
9. Freeze the cell pellet in a dry ice/ethanol mixture (optional).
Frozen cells can be stored at -80°C for at least several months.

Prepare the cell pellets for isoelectric focusing

10. Retrieve cell pellets from -80°C storage if samples were frozen.
11. Add 400 μ l Tris/SDS buffer per 50 to 100 mg cell pellet wet weight. Keep the cells on ice at all times.
The total amount of protein in the pellet is roughly 5% of the wet pellet weight.

12. Sonicate the sample three times for 3 sec using a sonicator with a microtip at medium power. Keep the samples on ice during sonication.

Use pulse sonication or wait at least 5 min between sonications. Minimizing heat generation is essential because substantial proteolysis can occur if the sample warms appreciably. If additional sonication is necessary (i.e., if the sample is not homogeneous), let the sample cool down on ice before the next series of sonications.

13. Run a BCA protein assay to determine the protein concentration if sample will be loaded on that basis. For maximum accuracy use the same amount of Tris/SDS buffer in standards as in experimental samples.

14. Prepare labeled cryovials to store aliquots of the sample, if desired. Precool vials on ice prior to making aliquots.

The amount of protein per aliquot depends on the anticipated future uses of the sample, as repeated freezing and thawing should be avoided. About 500 µg/gel whole-cell extract is a maximum load for preparative purposes using 3-mm gels (i.e., isolation of proteins for sequencing or other structural work). Approximately 50 µg/gel is an appropriate load for silver staining. The final protein concentration after completion of the protocol (steps 15 to 20) will equal the concentration found by protein assay divided by 1.1, owing to the addition of reagents after the protein assay step.

15. Add 20 µl DNase and RNase solution per 400 µl Tris/SDS buffer used for sonication (step 11). Incubate 10 min on ice.

16. Add 20 µl of 20% SDS solution and 5 µl of 2-mercaptoethanol per 400 µl Tris/SDS buffer used in step 11. Incubate 5 min at 37°C.

17. Quickly divide samples into previously prepared cryovials and immediately freeze aliquots using a dry ice/ethanol bath. Store at –80°C. Samples stored at –80°C are stable ≥1 year.

Work quickly to minimize potential proteolysis.

This step may be omitted if the samples are to be loaded on IEF gels immediately. Generally, the total amount of sample greatly exceeds the amount required for an IEF gel, and freezing aliquots is beneficial. To avoid potential reproducibility problems, all samples should be processed identically.

Prepare the samples for isoelectric focusing

18. If cell extracts were frozen, thaw samples and immediately add dry urea to 9 M final concentration.

The amount of urea (in mg) equals 0.83 times the sample volume (in µl). For example, use 83 mg urea per 100 µl sample. The final volume of the sample (with urea added) equals 1.6 times the initial volume (160 µl in the same example).

19. Add an equal volume of lysis buffer (160 µl in the above example) and warm briefly if necessary to dissolve urea.

20. Filter samples using a 0.2-µm microcentrifuge filter unit by microcentrifuging at maximum speed, room temperature, until the entire sample has passed through the filter. Load the desired volume onto the IEF gel.

If a 500-µg total protein load per 3-mm gel is desired (a practical maximum load for most whole-cell extracts), the protein concentration determined during the protein assay has to be ≥5 µg/µl. If the sample is less concentrated, the sample volume required will be too large for a 3-mm IEF gel. Alternatively, sample loads can be based on cell numbers, radioactivity, or any other appropriate reference (see Basic Protocol 1, step 22).

PREPARING PROTEINS IN TISSUE SAMPLES

Tissue samples are usually solubilized in lysis buffer using homogenization. After centrifugation, the protein sample can be loaded onto the first-dimension gel. In general, much higher sample-to-sample variability is expected when tissue samples are analyzed.

Materials

Tissue samples
Lysis buffer (see recipe)
Dounce homogenizer or equivalent
Ultracentrifuge and rotor (e.g., Beckman Ti70), 2°C

1. Place tissue sample in a Dounce homogenizer, add ~2 ml lysis buffer per 100 mg tissue, and homogenize the sample on ice (e.g., 3 to 5 strokes).
2. Let the mixture stand a few minutes, then transfer to an appropriately sized ultracentrifuge tube depending on total sample volume.
3. Centrifuge 2 hr at $100,000 \times g$ (e.g., 33,000 rpm in a Beckman Ti70 rotor for $100,000 \times g$), or 1 hr at $200,000 \times g$, 2°C.
4. Divide the supernatant into aliquots and freeze at -80°C or immediately load an appropriate volume onto the IEF gel.

SECOND-DIMENSION ELECTROPHORESIS OF IEF TUBE GELS

Second-dimension gels are identical to those described in *UNIT 6.1* except for sample loading, which requires a broad, flat well. A broad well can be cast using an appropriate two-dimensional comb if the second-dimension gel thickness is slightly larger than that of the first-dimension gel. Alternatively, when the second-dimension gel is being cast, water can be layered over the entire surface of the gel to produce a flat surface that will accommodate the first-dimension gel.

Narrow analytical isoelectric focusing gels (≤ 1.5 mm) that fit between the glass plates of the second-dimension gel do not generally require a stacking gel, although a stacking gel may improve resolution under some circumstances. Stacking gels are essential when first-dimension gels > 1.5 mm are loaded on reduced-thickness second-dimension gels, for example, when 3-mm first-dimension gels are loaded on 1.5-mm second-dimension gels. To ensure the best reproducibility, casting multiple second-dimension gels in a multigel casting stand is strongly recommended. This is especially important when gradient gels are used for the second-dimension and/or critical comparisons of multiple samples are planned.

This protocol describes all the specific steps required for successfully casting and running the second-dimension gel. The use of beveled plates and an agarose overlay is especially important when 3-mm IEF gels are loaded onto 1.5-mm second-dimension gels.

Materials

2% (w/v) agarose (see recipe)
Equilibration buffer (see recipe)
Isoelectric focusing gels containing protein samples to be analyzed (see Basic Protocol 1)
Piece of agarose containing molecular weight standards (see Support Protocol 6)
Beveled glass plates
Boiling water bath
Metal or plastic scoop
Additional reagents and equipment for linear and gradient Laemmli gels (*UNIT 6.1*)

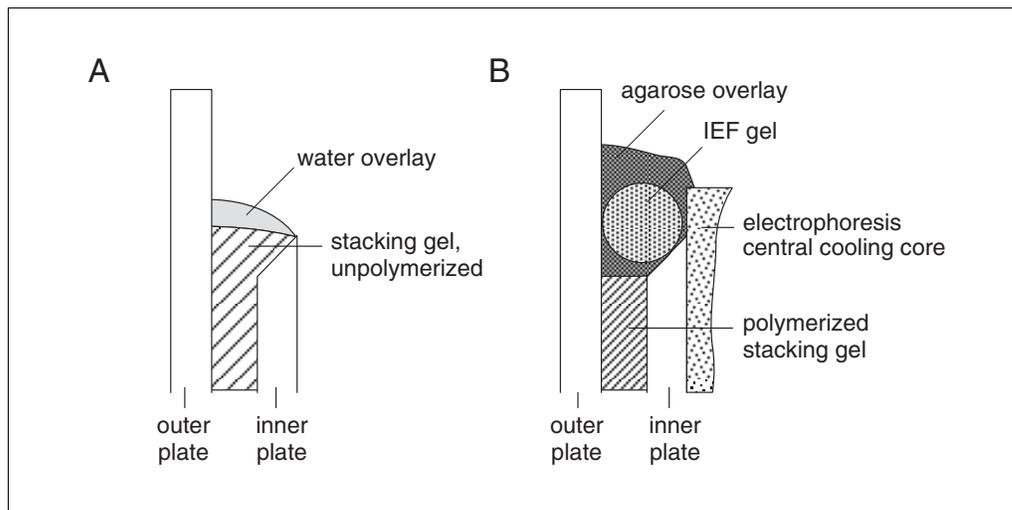


Figure 6.4.2 Casting the second-dimension gel and loading the IEF gel. **(A)** The stacking gel solution should reach to the upper edge of the beveled plate, and then the gel solution has to be overlaid with a minimum volume of water. The water will stay on the surface because of surface tension. **(B)** After polymerization, the gel is mounted on the central cooling core of the electrophoresis unit, and the equilibrated IEF gel is placed on top of the polymerized stacking gel. Excess buffer is removed, and the IEF gel is overlaid with hot agarose/equilibration buffer mixture. After the agarose solidifies, the upper electrophoresis chamber is filled with buffer.

Cast the second-dimension gels

1. Assemble the glass-plate sandwich of an electrophoresis apparatus, using a beveled plate for the shorter side of the gel sandwich.

A beveled plate provides more space for a thicker IEF gel and will accommodate a first-dimension gel that is at least 1 to 2 mm larger than the thickness of the second-dimension gel.

2. If the thickness of the first-dimension gel exceeds that of the second-dimension gel, pour a separating gel of the desired acrylamide concentration and immediately overlay with water to produce a smooth surface. The separating gel height should be a minimum of 2 cm below the top of the beveled plate to accommodate the stacking gel.
3. After the separating gel has polymerized (a sharp interface between the polymerized gel and the water overlay will reappear), remove the overlay, rinse the gel surface with water, and pour the stacking gel. The stacking gel solution should reach to the top of the bevel. Immediately overlay the stacking gel solution with a minimum amount of water, which will adhere owing to the surface tension (see Fig. 6.4.2A).

A water overlay of the stacking gel provides a smooth surface and better contact between the IEF gel and second-dimension gel. A small volume of water has to be used to avoid lowering the upper edge of the stacking gel below the edge of the beveled plate. The stacking gel height must be between 1.5 and 2 cm. The solution is filled to the top of the bevel so that after the slight shrinkage that occurs during polymerization the top of the polymerized gel will be near the bottom of the bevel (see Fig. 6.4.2B).

Load the isoelectric focusing gels onto the second-dimension gels

4. Assemble second-dimension gels in an electrophoresis chamber. Do not pour electrophoresis buffer into the upper chamber.
5. Melt 2% (w/v) agarose in a boiling water bath and add an equal volume of equilibration buffer for use in step 11. Keep agarose/equilibration buffer in the boiling water bath until step 11 is completed.

- Retrieve isoelectric focusing gels containing protein samples to be analyzed from storage. Incubate cryotubes containing frozen IEF gels in a 37°C water bath for 15 min for a 3-mm tube gel. A 5- to 7-min incubation is sufficient for 1.5-mm or thinner IEF gels. Do not agitate during thawing, as vigorous agitation of a partially thawed gel can break the gel.

During this thawing/equilibration step, SDS in the equilibration solution in which the gels were frozen diffuses into the gel matrix and binds to proteins in the IEF gel. The length of incubation in the equilibration buffer is critical because insufficient saturation of proteins with SDS will contribute to vertical streaks on staining. On the other hand, extended incubation in equilibration buffer will result in excessive loss of proteins owing to diffusion of protein out of the gel, which is especially critical for thin IEF gels. For this reason, it is recommended that after extrusion from the IEF tube IEF gels be initially incubated for 5 min to allow adequate diffusion of glycerol into the gel to minimize gel breakage, followed by freezing on dry ice (see Basic Protocol 1, step 30). This is desirable even if the second-dimension gel will be run directly after isoelectric focusing, as it is the most feasible way of precisely controlling the equilibration time while the remaining gels in the IEF run are extruded.

- Pour the gel and equilibration solution out of the cryovial onto a metal or plastic scoop. Carefully remove excess equilibration buffer with a pipet.
- Place a few milliliters of electrophoresis buffer on the top of the second-dimension gel.
- Slowly slide the IEF gel off the scoop and onto the top of the second-dimension gel. Remove all air bubbles trapped between the gels. Remove excess electrophoresis buffer from the top of the second-dimension gel.

The basic end of the gel may be placed on either the left or right side of the second-dimension gel. However, once a convention is established, all gels should be oriented the same way. The acidic end of the IEF gel can be recognized in two ways: the bromphenol blue will usually be yellow, and a bulge (increased gel diameter) will be present.

- Place a piece of agarose containing molecular weight standards (see Support Protocol 6) beside the basic side of the IEF gel (optional).

Note that when molecular weight standards are used, the isoelectric focusing gel has to be shorter than the width of the second-dimension gel.

- Carefully overlay the IEF gel (and the gel piece with standard proteins) with the hot agarose/equilibration buffer mixture (~2 ml/gel) prepared in step 5. Let the agarose solidify.

The agarose prevents the IEF gel from shifting position and ensures good contact between the IEF and second-dimension gels.

- Carefully pour electrophoresis buffer into the upper reservoir, taking care to avoid disturbing the agarose-covered IEF gel.
- Connect electrodes and run the gels.

See UNIT 6.1 for electrophoresis conditions.

BASIC PROTOCOL 4

SECOND-DIMENSION ELECTROPHORESIS OF IPG GELS

In this protocol, vertical gel electrophoresis is used as the second dimension for IPG gels in an analogous manner to the protocol described for the second dimension of IEF tube gels (see Basic Protocol 3). One difference is the use of second-dimension gel spacers or gel apparatus that will accommodate an 18-cm-long Immobiline DryStrip; Bio-Rad offers a conversion kit to increase the gel width from 16 cm to 18 cm, and Amersham Pharmacia Biotech offers the Iso-Dalt gel system. The use of beveled plates is not necessary as the 0.5-mm strips are narrower than the second-dimension gel (1.0 or 1.5 mm thick). Another change involves a two-step equilibration of the strips prior to electrophoresis.

Additional Materials (also see Basic Protocol 3)

DryStrip equilibration solutions 1 and 2 (see recipes; prepare fresh in step 4)
Immobiline IPG DryStrip with focused protein (see Basic Protocol 2)
Platform shaker

Cast the second-dimension gel

1. Assemble the glass-plate sandwich of an electrophoresis apparatus, using gel plates wide enough to accommodate an 18-cm-long DryStrip gel.

Beveled plates are not necessary. If the spacers are not wide enough to accommodate an 18 cm gel, the ends of the gel strip may be trimmed away from the IPG gel so that it will fit on top of the second dimension; however, some very basic or acidic proteins may be lost.

2. Pour a separating gel of the desired acrylamide concentration and immediately overlay with water to produce a smooth surface.

The separating gel should be a minimum of ~2.5 cm below the top of the inner plate to accommodate a 2-cm stacking gel.

3. After the separating gel has polymerized, remove the water overlay, rinse the gel surface with water to remove any unpolymerized acrylamide, and pour the stacking gel to a height of 0.5 cm from the top of the plate. Overlay with water to produce a smooth surface.

A water overlay provides a smooth surface for better contact between the Immobiline DryStrip and the second dimension gel. The stacking gel height should be ~2 cm.

Load the Immobiline IPG DryStrip gel onto the second-dimension gel

4. Prepare Immobiline DryStrip equilibration solutions 1 and 2 (see recipe).
5. Assemble the second-dimension gels in a electrophoresis chamber. Do not pour electrophoresis buffer into the upper chamber.
6. Melt 2% (w/v) agarose in a boiling water bath. Mix a solution of 1 part 2% agarose to 2 parts equilibration solution 2.

Keep agarose/equilibration buffer mixture in boiling water bath until step 11 is completed.

The agarose prevents the IPG DryStrip from shifting position and ensures good contact between the IEF and second-dimension gels.

7. Using forceps, remove the IPG gels from the electrophoresis tray after isoelectric focusing is complete or from the -80°C freezer (see Basic Protocol 2, step 19) and place each strip in a separate petri dish with the support film side of the strip facing the petri dish wall. Add 15 ml of DryStrip equilibration buffer 1. Cover and place on a platform shaker for 10 min.

Strips may be run in the second dimension immediately after isofocusing or after storage at -80°C . If the strips have been stored at -80°C , remove them from the freezer; then place in petri dish as stated and continue with the equilibration procedure.

8. Discard equilibration buffer 1 and add 15 ml of equilibration buffer 2. Cover and place on a platform shaker for 10 min.
9. Dampen a piece of filter paper and place on a glass plate. Remove the DryStrips from equilibration buffer 2. Place each strip on its edge on the filter paper to remove any excess buffer.

Strips should not be left in this position for >10 min, or spot sharpness may be affected.

10. Add a small amount of SDS electrode buffer along the glass plate above the second-dimension gel. Place the DryStrip gel in the well with the gel facing out and the basic side to the left. Push the DryStrip down so that it is firmly in contact with the stacking gel of the second-dimension gel. Remove excess running buffer.
11. Overlay the IPG gel strip with the agarose/equilibration buffer (from step 6) and allow agarose to solidify.
12. Carefully pour electrophoresis buffer into the upper reservoir, taking care to avoid disturbing the agarose-embedded IPG DryStrip.
13. Connect electrodes and run the gels.

See UNIT 6.1 for electrophoresis conditions.

**SUPPORT
PROTOCOL 6**

**PREPARING MOLECULAR WEIGHT STANDARDS FOR
TWO-DIMENSIONAL GELS**

Molecular weight markers are usually necessary for the identification of proteins or as references to describe experimental proteins on two-dimensional gels. In many cases, molecular weight markers are required only at the beginning of a project. Once the system is established, common proteins in the sample (e.g., actin or tubulin) provide sufficient references for molecular weight identification on subsequent gels. To minimize any differences in migration of the molecular weight standards and isoelectric focused proteins, the standard proteins should be loaded on the second-dimension gel in the same manner as the IEF gel. This protocol describes the preparation of standards in solidified agarose. The agarose pieces may be stored at -80°C for at least a year and provide a convenient source of standards for the second-dimension gel. The procedure described is recommended for 3-mm IEF gels. Narrow standards in solidified agarose (made in tubes ≤ 1.5 mm in diameter) can be prepared by the same method, but extrusion of the thinner agarose gel without breaking is more difficult. The protocol supplies molecular weight markers containing ~ 2.5 μg of each standard suitable for Coomassie blue staining or 0.25 μg of each standard for silver staining.

Materials

Molecular weight standards (Table 6.1.2)

1 \times SDS sample buffer (UNIT 6.1)

2% (w/v) agarose (see recipe)

Boiling water bath

Glass tubes (3-mm inner diameter)

Plastic or metal tray

1. Prepare 3 ml molecular weight standards in 1 \times SDS sample buffer using 250 μg of each standard.

The stated amount is appropriate for Coomassie blue staining of gels. If silver staining is planned, use 25 μg of each standard.

2. Mix the standards with 2 ml of 2% (w/v) agarose melted in a boiling water bath.
3. Prepare clean glass tubes by wrapping one end with Parafilm. Pour the hot mixture into the tubes and let the agarose solidify.
4. Carefully extrude the agarose from the tubes.
5. Cut agarose rods into 5-mm pieces using a razor blade.
6. Freeze all pieces separately on a plastic or metal tray using dry ice.
7. Collect frozen pieces in a plastic bottle and store at -80°C . The standards may be stored ≥ 1 year.

DIAGONAL GEL ELECTROPHORESIS (NONREDUCING/ REDUCING GELS)

ALTERNATE
PROTOCOL 3

Protein subunit compositions and cross-linked protein complexes can be analyzed by two-dimensional gel electrophoresis using separation under nonreducing conditions in the first dimension followed by reduction of disulfide bonds and separation under reducing conditions in the second dimension. Most proteins will migrate equal distances in both dimensions, forming a diagonal pattern. Proteins containing interchain disulfide bonds will be dissociated into individual subunits and can be resolved in the second-dimension gel.

The approach is similar to that described for two-dimensional gel electrophoresis (see Basic Protocol 3) except, in this protocol, the first-dimension gels are nonreducing (i.e., 2-mercaptoethanol or dithiothreitol is omitted from sample buffer) SDS-denaturing gels instead of isoelectric focusing gels. Use of 1.2-mm tube gels for the first-dimension separation and 1.5-mm slab gels for the second-dimension run is recommended.

Additional Materials (also see Basic Protocol 3)

- Separating and stacking gel solutions (see Table 6.1.1)
- 1× SDS sample buffer *without* reducing agents (UNIT 6.1)
- Reducing buffer (see recipe)
- 1.5% (w/v) agarose in reducing buffer (see recipe; optional, for securing first-dimension gel on second-dimension gel)
- Two-dimensional comb (optional)
- Additional reagents and equipment for casting tube gels (see Basic Protocol 1), SDS-PAGE (UNIT 6.1), and protein staining (APPENDIX 3)

Pour and run the first-dimension gel

1. Clean and dry 1.2-mm glass gel tubes for the first-dimension gel (see Basic Protocol 1, step 1).
2. Prepare a separating gel solution with the desired percentage acrylamide (Table 6.1.1); omit the stacking gel for the first dimension.

Stacking gels can usually be avoided in the first dimension by keeping sample volumes small (i.e., $\leq 10 \mu\text{l}$).

Less than 200 μl of gel solution is required to cast a single 1.2-mm tube gel 12 cm in length. Adjust the amounts from Table 6.1.1 accordingly.

3. Cast the first-dimension polyacrylamide gels in 1.2-mm tubes using a syringe with a long needle (see Basic Protocol 1, step 5a). Overlay with water and allow the gels to polymerize.
4. Prepare samples in 1× SDS sample buffer without any reducing reagents (i.e., no 2-mercaptoethanol or DTT). Load the samples and electrophorese until the tracking dye is ~1 cm from the bottom of the tube.

Reduce sample and run the second-dimension gel

5. Extrude the gel from the tube (see Basic Protocol 1, steps 27 to 29).
6. Place the extruded gel in a test tube containing 5 ml reducing buffer. Equilibrate 15 min at 37°C with gentle agitation.
7. Cast the second-dimension separating and stacking gels (see Basic Protocol 3, steps 1 to 3), making sure that the top of the stacking gel is at least 5 mm below the top of

Electrophoresis
and
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6.4.25

the short glass plate. Layer water across the entire stacking gel or use a two-dimensional comb.

Most two-dimensional gel combs have a separate small well for a standard or reference sample.

The use of beveled plates (see Basic Protocol 3, steps 1 to 3) is not essential but is still preferred because it will facilitate loading of the first-dimension gel. In this procedure, the first-dimension gel will fit between the glass plates if 1.2-mm tubes are used for the first dimension and 1.5-mm gels are used for the second dimension.

8. Load the first-dimension gel onto the second-dimension gel. Remove any air bubbles trapped between the gels.

If the first-dimension gel does not remain securely in place, it can be embedded using 1.5% (w/v) agarose in reducing buffer.

9. Carefully pour electrophoresis buffer into the upper electrophoresis chamber and electrophorese using voltages and times appropriate for the gel type selected.

Parameters for electrophoresis are given in UNIT 6.1.

SUPPORT PROTOCOL 7

USING TWO-DIMENSIONAL PROTEIN DATABASES

Computerized image acquisition and manipulation constitute the only practical method for systematic qualitative and quantitative evaluation of complex protein patterns from different samples that are to be compared by high-resolution two-dimensional gel analysis. Examples of experimental applications include comparisons of tumor cells or tissues with appropriate normal controls and comparisons of a single cell line under different experimental conditions.

There are currently a number of commercially available image acquisition/computer systems specifically designed for comparing two-dimensional gels and storing associated information in a database. The systems include both hardware and the necessary software for comparing different gels and producing databases containing the two-dimensional protein patterns, with options for annotating specific spots and producing quantitative comparisons among large numbers of different samples. With most systems, images can be acquired from either stained gels or autoradiographs. The equipment used to obtain two-dimensional gel images includes laser scanners, video cameras, and phosphoimagers. After image acquisition, software running on a microcomputer or workstation is used to refine the image, detect spots, and match spots between different gels.

It is essential that very high-quality, reproducible gels be used for computerized comparisons. The greatest dynamic range in protein abundance for a single two-dimensional gel can be obtained using autoradiography or phosphoimaging (UNIT 6.3). With these methods, up to several thousand spots can be compared and tracked. A representative reference gel or a composite image can be stored and used as a reference for future experiments.

Information related to each spot on the two-dimensional pattern, including the quantity of protein in the indicated spot on different gels used in the comparison, can be archived and updated. Other known information related to a specific spot can also be added to the investigator-built database, including the pI, molecular weight, amino acid composition, sequence, and/or identity of the protein and any other important attributes correlated with the indicated spot. A number of research groups, including those of Garrels and Celis (Garrels, 1989; Garrels and Franza, 1989; Celis et al., 1991), have extensively characterized hundreds of spots from specific cell lines and have used multiple methods to characterize proteins of interest. The most definitive methods for establishing the identi-

ties for proteins of interest detected by computer-assisted comparisons are protein sequence analysis and, more recently, mass spectrometry of tryptic fragments. Both methods are compatible with the quantities of protein that can be recovered from two-dimensional gels.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

30% acrylamide/0.8% bisacrylamide

30 g acrylamide
0.8 g bisacrylamide
H₂O to 100 ml

Filter solution through 0.2- to 0.45- μ m filter (e.g., Micro Filtration Systems, cellulose nitrate, 0.2 μ m). Store at 4°C (stable at least 3 months).

CAUTION: Acrylamide is a neurotoxin. Wear gloves and a dust mask when handling solid acrylamide. Wear gloves when working with acrylamide solution. Never pipet acrylamide solutions by mouth.

Agarose in reducing buffer, 1.5% (w/v)

Mix 0.15 g agarose and 10 ml reducing buffer (see recipe). Heat in boiling water bath until dissolved. Prepare immediately before use.

Agarose, 2% (w/v)

Mix 2 g agarose and 100 ml water. Stir on a hot plate until dissolved. Keeping the solution near 100°C, divide by placing 5-ml aliquots in 25-ml glass screw-cap tubes. Let the aliquots solidify. Store at 4°C (stable at least 3 months).

Ammonium persulfate, 2.5% (w/v)

0.25 g ammonium persulfate
H₂O to 10 ml
Prepare immediately before use

DNase and RNase solution

Mix 2.50 ml of 1 M Tris·Cl, pH 7.0 (*APPENDIX 2A*), 250 μ l of 1 M MgCl₂, (*APPENDIX 2A*), and 2.2 ml water. Add to 5 mg DNase (Worthington) and dissolve DNase. Add 2.5 mg RNase (in solution; Worthington). Mix well, divide into 50- or 100- μ l aliquots, and store at -80°C (stable at least 1 year).

The volume of RNase solution needed is variable and depends on the protein concentration, which is reported on the vial label (in mg/ml). The volume of RNase added should be <200 μ l; if a larger volume is used, the amount of H₂O should be decreased proportionally.

Final concentrations are 0.5 M Tris·Cl, pH 7.0, 0.1 M MgCl₂, 0.1% (w/v) DNase, and 0.05% RNase.

DryStrip equilibration solutions

20 ml 1 M Tris·Cl, pH 6.8 (*APPENDIX 2A*)
72 g ultrapure urea
60 ml glycerol
2 g sodium dodecyl sulfate (SDS)
67 ml Milli-Q-purified water

For solution 1: Add 50 mg DTT per 10 ml of equilibration buffer

For solution 2: Add 0.45 g iodoacetamide and a few grains bromphenol blue per 10 ml of equilibration buffer

Make fresh immediately before use

Final concentrations are 50 mM Tris·Cl, pH 6.8; 6 M urea; 30% glycerol; and 1% SDS in a final volume of 200 ml.

EDTA, 2% (w/v)

2 g Na₂EDTA

H₂O to 100 ml

Adjust to pH 7.0 with NaOH

Store at room temperature (stable several months)

Titrate while dissolving. EDTA is difficult to dissolve without addition of NaOH even when the disodium salt is used.

Equilibration buffer

3 g SDS

7.4 ml 2% (w/v) EDTA, pH 7.0 (see recipe)

10 ml glycerol

2 ml 1.0 M Tris-Cl, pH 8.65 (*APPENDIX 2A*)

0.3 ml bromphenol blue (saturated solution in H₂O)

H₂O to 100 ml

Store at room temperature (stable for several weeks)

Final concentrations are 3% (w/v) SDS, 0.4 mM EDTA, 10% (v/v) glycerol, and 20 mM Tris-Cl, pH 8.65.

Leupeptin, 2 mg/ml

20 mg leupeptin

10 ml water

Divide into convenient volumes

Store at -20°C (stable at least 1 year)

Lysis buffer

2.59 g urea (ultrapure)

1.6 ml H₂O

0.25 ml 2-mercaptoethanol

0.3 ml ampholytes

1.0 ml 20% (w/v) Triton X-100 solution (see recipe)

Prepare immediately before use

Use same ampholytes as for the IEF gel formulation. To dissolve urea, warm the mixture in a 30°C water bath if necessary.

Orthophosphoric acid (H₃PO₄), 0.1 M

13.7 ml 85% phosphoric acid

Water to 2 liters

Make fresh daily

Must be degassed prior to use.

PBS, 10×

152 g NaCl

24 g monobasic sodium phosphate, anhydrous

1600 ml H₂O

Adjust pH to ~6.7 with NaOH

Add H₂O to 2 liters

Store at room temperature (stable at least 1 month)

The 1× solution should be pH 7.3 to 7.5. Final concentrations are 130 mM NaCl and 10 mM sodium phosphate.

PBS with proteolysis inhibitors (PBS/I buffer)

20 ml 10× PBS (see recipe)

20 ml 2% (w/v) EDTA, pH 7.0 (see recipe)

200 μ l 0.15 M phenylmethylsulfonyl fluoride (PMSF) in 2-propanol
100 μ l 2 mg/ml leupeptin (see recipe)
200 μ l 1 mg/ml pepstatin (see recipe)
Adjust to pH 7.2 with HCl
Add H₂O to 200 ml
Prepare immediately before use

Diisopropyl fluorophosphate (DFP) is a better serine protease inhibitor than PMSF at lower temperatures (0° to 4°C); however, although both compounds are toxic, exceptional caution must be exercised with DFP owing to its volatility. If DFP is used, work in a chemical fume hood and carefully follow the supplier's precautions. DFP and PMSF have half-lives on the order of hours in aqueous neutral solutions, and the degradation rate increases rapidly as the pH is increased above neutral. Make aqueous solutions immediately before use. Use of 1 M NaOH is convenient to inactivate residual DFP or PMSF.

Final concentrations are 10 mM sodium phosphate, 130 mM NaCl, 0.2% EDTA, 0.15 mM PMSF, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin.

Pepstatin, 1 mg/ml

10 mg pepstatin
10 ml anhydrous ethanol
Divide into convenient volumes
Store at -20°C (stable at least 1 year)

Thaw aliquots and mix well immediately before use.

Reducing buffer

0.5 g dithiothreitol (DTT)
0.1 g SDS
1.51 g Tris base
Adjust to pH 6.8 with HCl
Add H₂O to 100 ml
Prepare fresh every time

Final concentrations are 0.5% (w/v) DTT, 0.1% (w/v) SDS, and 125 mM Tris·Cl, pH 6.8.

Tris/SDS buffer

0.3 g SDS
0.6 g Tris base
Adjust to pH 8.0 with HCl
Add H₂O to 100 ml
Divide into 5-ml aliquots
Store at -80°C (stable at least 1 year)

Final concentrations are 0.3% (w/v) SDS and 50 mM Tris·Cl, pH 8.0.

Triton X-100 solution, 20% (w/v)

3 g Triton X-100
12 ml H₂O
Warm in 37°C water bath to dissolve Triton X-100
Store at 4°C (stable ~2 weeks)

Urea, 8 M

0.75 g ultrapure urea
1.0 ml H₂O
Prepare immediately before use

Avoid heating above room temperature.

COMMENTARY

Background Information

Two-dimensional gel electrophoresis, using isoelectric focusing followed by SDS-PAGE, is the single most powerful analytical method currently available for separating complex protein mixtures such as whole-cell or tissue extracts. It is therefore a valuable method for following disease-related changes or for detecting changes in protein expression under diverse experimental conditions. To achieve maximum reproducibility between samples to be compared, multiple gels should be cast and run simultaneously (Anderson and Anderson, 1978a,b).

Despite the exceptionally high resolving power of the method, the total number of proteins that can be resolved in a single two-dimensional gel is only ~1000 to 2000, whereas the total number of proteins in a single mammalian cell type is likely to be at least 10 to 20 times higher. Therefore, the old guideline that a single spot on a two-dimensional gel is a single protein needs to be revised as analytical detection methods improve. Conversely, a single protein (single gene product) can produce multiple, usually adjacent spots in the isoelectric focusing dimension owing to variable degrees of chemical or posttranslational modification. Common examples of variable posttranslational modifications that can usually be detected on two-dimensional gels include phosphorylation, glycosylation, and acetylation. Examples of chemical modifications that cause charge heterogeneity include deamidation of side-chain amines (usually asparagines), oxidation of sensitive side chains, and modification of lysines. Potential modification of lysines by urea is particularly important because urea rapidly decomposes to form cyanate, which readily reacts with amino groups, especially above pH 7. Despite potential side reactions, urea is the most useful IEF additive for maintaining solubility of proteins near their isoelectric points.

Two-dimensional PAGE has become a valuable preparative tool for protein isolation in addition to its historical role as an analytical method. The sensitivity of many protein analysis methods has improved to the point where one or several spots from two-dimensional gels are sufficient for protein identification using mass spectrometry methods.

Commercially available equipment for running two-dimensional gels can be divided into four groups based on size: microgels (Amersham Pharmacia Biotech Phast system), minigels (e.g., Bio-Rad or Amersham Pharmacia Biotech), standard or full-sized gels (e.g., Bio-Rad or Amer-

sham Pharmacia Biotech), and large or “giant” gels (ESA Investigator 2D gel system or Amersham Pharmacia Biotech Iso-Dalt gel system). In general, the larger the gel, the better the final resolution, but as gel size increases so do costs, difficulty of gel handling, and time requirements.

Standard-size gels provide adequate resolution for most applications and are relatively easy to handle. A 3-mm soluble ampholyte tube IEF gel or a 0.5 mm × 3 mm × 18-cm Immobiline gel has a total protein capacity of ~500 µg for complex protein mixtures such as whole-cell extracts. The maximum capacity for any single protein spot is ~0.5 to 5 µg, depending on the solubility of the protein near its isoelectric point and the separation distance from any near neighbors. A variety of alternative gel sizes, their limits, and their advantages are summarized in Table 6.4.2. The lower protein limit for any of the systems is determined strictly by the available detection methods.

Proteins can be detected in two-dimensional gels by the same wide range of techniques used for one-dimensional gels. Autoradiography (UNIT 6.3), silver staining, and electroblotting to PVDF membranes (UNIT 6.2) followed by colloidal gold or colloidal silver staining or immunodetection (UNIT 6.2) are among the most sensitive techniques available. If a larger amount of protein is available, Coomassie blue staining of the gel or amido black staining of a PVDF membrane after electrotransfer (UNIT 6.2) would be the detection methods of choice.

The major technical limitation in two-dimensional gel electrophoresis is gel-to-gel variation. Even when extreme care is exercised to produce highly reproducible first- and second-dimension gels, some gel-related variability among gels cast at the same time is likely to persist. Another source of variability includes differences in extraction or recovery of proteins during sample solubilization and handling. Maximizing resolution and reproducibility is especially important if computerized comparisons of two-dimensional gels of complex protein mixtures such as cell or tissue extracts are being attempted.

Isoelectric focusing using soluble ampholytes

Soluble ampholytes are mixtures of low-molecular-weight organic compounds with differing side-chain pK_a values that provide buffering capacity. In an IEF gel, the ampholytes migrate to their isoelectric point, where they provide buffer-

Table 6.4.2 Size Options for Two-Dimensional Gel Electrophoresis

Gel type	First-dimension gel		Second-dimension gel ^a		Purpose	Comments ^b
	Diameter <i>D</i> (mm)	Length <i>L</i> (cm)	Thickness <i>T</i> (mm)	Height <i>H</i> (cm)		
Microgels/minigels ^c	<1.5	<10	< <i>D</i>	<10	Analytical	1-4
	<1.5	<10	> <i>D</i>	<10	Analytical	3-6
	>1.5	<10	< <i>D</i> ^d	<10	Analytical/preparative	1,2,7,8
Full-size gels ^e	<1.5	12-18	< <i>D</i>	12-18	Analytical	1-4
	<1.5	12-18	> <i>D</i>	12-18	Analytical	3-6
	>1.5	12-18	< <i>D</i> ^d	12-18	Analytical/preparative	1,2,7,8
Giant gels ^f	<1.5	>20	> <i>D</i>	>20	Analytical	4-6,9
	>1.5	>20	< <i>D</i> ^d	>20	Analytical/preparative	1-3,7

^aThe second-dimension gel width has to be at least equal to the IEF tube gel height.

^bKey to comments: (1) tube gel cannot be placed directly on top of second-dimension gel, and use of agarose is recommended; (2) use of stacking gel is recommended; (3) extrusion and handling are relatively difficult; (4) total protein load is limited to usually $\leq 50 \mu\text{g}$ for whole-cell or tissue extracts; (5) tube gel can be placed directly on top of second-dimension gel, and use of agarose is not necessary; (6) use of a stacking gel is not necessary; (7) total protein load capacity is relatively large; (8) extrusion and handling are relatively simple; (9) extrusion and handling are very difficult.

^cMinigel systems provide rapid separations with moderate resolution. Microgels (Phastgels) are precast gels that are slightly smaller than most minigels.

^dUse of second-dimension gels thicker than 1.5 mm is generally not recommended owing to difficulty with either efficient staining or efficient electroblotting.

^eFull-size gels provide resolution satisfactory for most applications.

^fGiant gels provide very good resolution. Specialized equipment is required, such as Investigator 2D (ESA), Iso-Dalt (Hoeffer Pharmacia), or homemade giant-size gel systems.

ing capacity and hence produce stable pH gradients. In theory, any desired pH gradient could be produced by blending ampholytes with appropriate pK_a values. In practice, it is relatively easy to produce pH gradients from \sim pH 3.5 or 4.0 to pH 8.0, but stable soluble gradients outside this range are usually not technically feasible. Within these pH limits, some manipulation of the gradient shape and pH range is possible by blending different amounts of specific pH range ampholytes. For example, 0.50 ml of pH 5-7 ampholytes plus 0.25 ml of pH 4-8 ampholytes can be used instead of 0.75 ml of pH 4-8 ampholytes alone to increase the separation distance of proteins in the pH 5.0 to 7.0 range.

Basic Protocol 1 is based on use of 3-mm first-dimension isoelectric focusing gels and 1.5-mm second-dimension gels using the Bio-Rad two-dimensional gel apparatus (Protean II xi 2D). The method can be easily adapted to equipment from other suppliers or to different-sized gels by adjusting the quantities of reagents used. The protocol uses 8 M urea and Triton X-100 as solubilizing agents. Solubilization of the protein sample applied to the gel as well as maintenance of solubility during electric focusing are the most

critical factors influencing the quality of separation in the first dimension. The most common modification to Triton X-100-based procedures is addition of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) to the gel and solubilizing buffer mixtures. Addition of SDS to complex samples such as tissue or cell extracts can also enhance reproducible solubilization of the largest possible subset of proteins. Although SDS is charged, in the presence of Triton X-100 it is separated from proteins during focusing and migrates to the acidic end of the gel. Regardless of the method used to maintain solubility, some proteins (especially those >100 kDa) tend to precipitate at pH values approaching their isoelectric point and thus produce horizontal smears on the final second-dimension gel.

Basic Protocol 1 requires several modifications for successful separation of very acidic (Alternate Protocol 1) or very basic (Alternate Protocol 2) proteins. In both cases, prefocusing of the gels must be avoided, and the isoelectrofocusing time has to be reduced. A short separation time does not allow the system to reach equilibrium and is used to establish the desired

Table 6.4.3 Commercially Available Precast IPG Gels and Immobiline Chemicals

Name	Use	Available pH range
Immobiline DryPlate ^a	Running one-dimensional immobilized pH gradient gels	pH 4.0-7.0, pH 4.2-4.9, pH 4.5-5.4, pH 5.0-6.0, pH 5.6-6.6
Immobiline DryStrip ^a	Running first dimension in two-dimensional gels	
110 mm		pH 4-7L, pH 3-10L
180 mm		pH 4-7L, pH 3-10L ^c , pH 3-10NL ^d
IPG Ready Strips ^b	Running first dimension in two-dimensional gels	
7 cm		pH 3-10, pH 4-7, pH 3-6, pH 5-8, pH 7-10
11 cm		pH 3-10, pH 4-7, pH 3-6, pH 5-8, pH 7-10
Immobiline II ^a	Creating custom gradient immobilized pH gradient gels	pK 3.6, pK 4.6, pK 6.2, pK 7.0, pK 8.5, pK 9.3

^aFrom Amersham Pharmacia Biotech.

^bFrom Bio-Rad.

^cA linear gradient with maximum resolution above pH 7.0.

^dA nonlinear gradient with best resolution at pH 5.0-7.0.

pH gradient. Separation of very acidic proteins requires modifications of gel and electrode solutions. Separation of very basic proteins has to be performed with the positions of electrodes and electrode solutions reversed in the electrophoresis chamber.

The total protein load per gel depends on the complexity of the sample, the solubility of proteins in the sample, and the diameter of the first-dimension gel. Approximately 4 times as much total sample can be applied to a 3-mm gel compared with a 1.5-mm gel. Another advantage of a larger-diameter isoelectric focusing gel is that extrusion and gel handling are easier owing to improved strength of the gel. If care is exercised in loading the first-dimension gel onto the 1.5-mm second-dimension gel, the final resolution will be similar to that obtained using smaller-diameter IEF gels. The separating or resolving power of a system is dependent on the quality of ampholytes used, the slope of the pH gradient, and the lengths of both first- and second-dimension gels.

Immobilized pH gradient gels

In immobilized pH gradient (IPG) gels (Basic Protocol 2), the pH gradient is an integral part of the polyacrylamide matrix (Strahler and Hanash, 1991). Because the pH gradient is covalently associated with the polyacrylamide gel matrix, precise, reproducible, and very high-resolution separations can be achieved. A variety of precast gels and all the necessary equipment are commercially available from either Amersham Pharmacia

Biotech or Bio-Rad. Reproducible two-dimensional gels can be obtained by running a sample on a narrow strip of immobilized pH gradient gel and then running it in a vertical SDS second-dimension gel. Isoelectrofocusing is performed in a horizontal electrophoresis unit in which multiple gel strips may be run simultaneously. Equipment and reagents are also available for the user to cast custom Immobiline gels in the laboratory.

Some of the major advantages of using precast IPG gels are their ease of use and high reproducibility, the time savings realized, and the fact that precision narrow-range gradients can be used to resolve small charge differences. Because the pH gradient is covalently coupled to the polyacrylamide gel matrix, the pH gradient remains stable and linear during prolonged electrophoresis, thus ensuring reproducibility. This is in contrast to conventional IEF gels, where gradient drift occurs during prolonged electrophoresis. Additionally, the precast gels can be rehydrated in water or in solutions with one or more additives such as urea, CHAPS or Triton X-100, carrier ampholytes, glycerol, and reducing agents, which may help to increase protein solubility. Precast gel strips are available with pH ranges such as pH 3.0 to 10.0 or pH 4.0 to 7.0 as well as narrower ranges (as DryPlates). In addition, a variety of Immobilines permits the user to cast IPG gels with customized pH gradients of any gradient range and shape between pH 3.0 and pH 10.0.

Table 6.4.3 lists types of commercially available gels and Immobilines. With the Immobiline

system, the apparent pI of a given protein may be slightly different from that determined by other methods. Therefore, it is recommended that a broad-range gradient be tried initially, followed by a narrower-range gradient, if needed.

Diagonal gel electrophoresis

Diagonal gel electrophoresis is a form of two-dimensional analysis useful for investigating the subunit composition of multisubunit proteins containing interchain disulfide bonds (Goverman and Lewis, 1991). Proteins are electrophoresed in the first-dimension in a tube gel (or a slab gel) under nonreducing conditions. The proteins are then reduced in situ, and the first gel (or a strip thereof) is layered onto a second gel and electrophoresed. In the second gel, the proteins migrate at right angles to the original, first-dimension migration. Most cellular proteins are not disulfide-linked and will fall on the “diagonal” in this system; that is, they migrate approximately equal distances in both directions during electrophoresis and lie approximately on the diagonal line connecting opposite corners of the gel. On reduction, component subunits of proteins connected by interchain disulfide bonds will resolve below the diagonal because the individual subunits migrate faster than the disulfide-linked complex during the second electrophoresis. Some proteins with internal disulfide bonds, but no interchain disulfides, may migrate slightly above the diagonal because internal disulfides can produce a more compact molecular shape (causing faster migration in the first dimension).

Investigator 2D gel system

The Investigator 2D gel system was introduced in 1990 by Millipore as the first commercial “large” or “giant” format two-dimensional gel system designed for analytical purposes, although analogous homemade units had been reported earlier (Garrels, 1979; Young et al., 1983). This product line, including precast first- and second-dimension gels, can now be purchased from ESA. The Investigator 2D gel system uses larger gel sizes than the gels described in this unit as well as a number of novel approaches and reagents designed to enhance resolution and gel-to-gel reproducibility. The major features of this system include the following: an increased length of the first-dimension gel (20 cm), an increased length and width of the second-dimension gel (20 × 22 cm), a thread reinforcement of the isoelectric focusing gel, temperature control during electrophoresis of the second-dimension gel, and use of a special high-tensile-strength acrylamide to fa-

cilitate handling of the large, thin second-dimension gel. Narrow gel tubes (1.2 mm) for the first-dimension are standard, but 3-mm-inner-diameter tubes are available to accommodate larger protein loads. The 3-mm IEF gels do not have the thread reinforcement. One modification related to the above protocol includes addition of 0.3% (w/v) CHAPS in the gel solution, which is intended to improve the solubility of proteins and their migration and separation in the gel. The system is supplied with a manual that adequately describes the method.

Critical Parameters and Troubleshooting

Although no individual steps in two-dimensional gel analysis are exceptionally difficult, the large number of steps involved increase the likelihood and possible severity of errors or problems. Several steps are especially critical and may require optimization. The first is sample preparation. Proteins applied to IEF gels have to be completely solubilized. Residual precipitate or even soluble aggregates are likely to cause artifacts on the end of the tube gel or position on the IPG strip where the sample is loaded. Precipitated or aggregated proteins may also interact with soluble proteins, causing components with normally good solubility to coprecipitate or migrate anomalously.

Two general rules of thumb apply to sample solubility: (1) the more complex the sample or the more crude the extract, the more likely problems will be encountered with sample solubility, and (2) the higher the protein load applied to the gel, the more likely solubility problems will arise. Care must also be taken to avoid proteolysis during sample preparation, especially when complex, impure samples are used. If samples are frozen either before or after solubilization, they should be stored at -80°C and should not be subjected to repeated freeze-thawing. Addition of SDS to the solubilization solution increases the solubilization of some proteins; however, SDS should only be used when the IEF gels contain urea and Triton X-100, as the solubilizing agents are required for effective separation of the strongly anionic SDS molecules from the proteins during isoelectric focusing. Heating of samples in urea-containing solutions must be avoided because urea readily decomposes to cyanate, which reacts with amino groups and causes charge heterogeneity. High concentrations of salts and buffers in the sample should be avoided. Ionic compounds increase the conductivity of the sample and can result in localized overheating, especially for Immobiline gels.

Samples analyzed on immobilized pH gradient gels should not contain precipitates. The concentration of salts and buffer ions in the sample should be kept to a minimum (<50 mM) to avoid local overheating of the gel during electrophoresis. If the sample forms aggregates or precipitates at the point of application, apply the sample to a different location (different pH) on the gel, or load the sample during the rehydration step.

Early decisions that must be made include the size of the gels required and whether a soluble ampholyte system or an Immobiline gel will be used. The major consideration affecting appropriate gel size is the degree of resolution needed. In general, the smallest gel format should be selected that will provide the needed degree of resolution, because smaller gels are easier, less expensive, and faster to run. Therefore, quick screening of samples or analysis of relatively simple samples can easily be accomplished with microgels or minigels. In contrast, if detailed qualitative or quantitative comparisons of cell or tissue extracts are planned, standard-size or large gels are indicated. Similarly, 3-mm or larger first-dimension tube gels followed by 1.5-mm second-dimension gels in the standard or large format are indicated if the two-dimensional gels will be used for preparative isolation of a protein for applications such as raising antibodies or conducting structural analysis. Immobilized pH gradient gels should very seriously be considered as an alternative to soluble ampholyte gels for most separations owing to their stable and reproducible pH profiles. The IPG gels are particularly appropriate when a narrow pH range is required.

Immobilized pH gradient gels are typically run at 2500 to 3500 V and typically require a focusing time of 16 to 18 hr. Use of high voltage (8,000 to 10,000 V) integrated power supplies/cooling units can shorten focusing times to <4 hr. Optimal focusing conditions may be experimentally determined by applying the sample to different positions on the gel and estimating the time for the migration patterns to coincide. Some proteins may require longer run times to reach their pI. Because there is no gradient drift, the potential problems with longer run times are limited to sample modifications or drying out of the gel. These problems usually can be minimized by including a reducing agent in the rehydration solution and coating the top surface of the gel with paraffin oil.

The quality of the first-dimensional separation is strongly dependent on the purity of the reagents used, especially the urea and ampholytes. One fairly commonly encountered frustration, when soluble ampholyte gels are used, is that different

batches of ampholytes from the same supplier will sometimes produce markedly different two-dimensional gel patterns. Therefore, it is advisable to purchase an adequate supply of a single lot of ampholytes to meet anticipated needs for an entire study where such an approach is feasible. However, whereas ampholytes usually have a reasonably long shelf life at 4°C (usually up to a year), shelf life as well as total ampholyte requirements often cannot be predicted with much certainty.

When any doubt arises about the purity or quality of ampholytes or any other reagent, the reagent should be replaced immediately. Constant monitoring of the system performance, especially when changing lots of ampholytes, urea, or acrylamide, can help minimize potential reagent-associated problems. In most cases, the best standard for a given two-dimensional gel system is an experimental sample or control that is available in sufficient quantity so that many replicate aliquots can be frozen and stored for an extended time at -80°C; alternatively, a sample that can be reproducibly prepared over a long time frame would make an acceptable standard. Such an experimental standard or reference is more likely to detect subtle, but experimentally important, changes in the two-dimensional gel system than commercially available IEF or SDS gel standard mixes.

Another critical factor is equilibration of the first-dimension gel in the second-dimension equilibration buffer. During this step, urea diffuses out of the IEF gel while SDS and reducing reagent diffuse into the gel. If the gel is inadequately saturated with SDS, vertical streaking will result. However, if the gel is incubated in the equilibration buffer for an extended time, a substantial amount of the protein can rapidly diffuse out of the large-pore IEF gel. Losses arising from diffusion can be critical for any experiment because different proteins will diffuse at different rates, but rigorous control of the incubation step is especially important if quantitative comparisons among gels are planned. The simplest method of controlling the incubation time is to freeze the extruded IEF gel after a carefully controlled 5-min incubation in the equilibration buffer; any additional equilibration incubation time required can then be incorporated and carefully controlled when the sample is thawed for loading onto the second-dimension gel.

Another crucial step is loading of the equilibrated IEF gel onto the top of the second-dimension gel. Any irregularity or obstruction between the two gels, including particles of dirt or air bubbles, will affect the flow of current and disrupt

the resolution of proteins in the final gel. Similarly, poor contact or any movement of the IEF gel during electrophoresis of the proteins out of the IEF gel into the second-dimension gel will lead to artifacts. Therefore, it is advisable to embed the IEF gel in a buffered agarose matrix to ensure good electrical contact between the gels and to prevent gel movement after electrophoresis is initiated.

Finally, the choice of second-dimension gel composition and separation conditions can influence the quality of results. A proper percentage of acrylamide should be selected to optimize resolution within the desired molecular mass range. If gradient gels are needed, use of a multiple gel casting stand is the best way to ensure reproducibility among samples within a single experiment. Further details on optimization of two-dimensional gel systems are presented by Hochstrasser et al. (1988).

When no technical, sample-related, or reagent-related problems are encountered, the final stained two-dimensional gels should contain numerous rounded or slightly elliptical spots. Typically, more than 1000 spots can be detected on a standard 16 × 14-cm gel when using a sensitive staining protocol such as silver staining or autoradiography and a whole-cell extract as a sample. Some horizontal streaks for most high-molecular-mass proteins (proteins exceeding ~100 kDa) are common owing to the decreased solubility of larger proteins near their isoelectric points, even in the presence of urea and nonionic detergent. However, excessive horizontal smearing of proteins smaller than 100 kDa indicates poor isoelectric focusing, which could be related to one or more of the following factors: sample improperly solubilized or contaminated with interfering substances such as large nucleic acid molecules, poor purity of reagents (check the urea first), poor-quality ampholytes, or insufficient isoelectric focusing (total volt-hours too low). It is important to note that in general the solubility of any protein is the lowest near its isoelectric point, but there are vast differences among proteins in terms of both the minimum concentration where precipitation becomes a problem and the degree to which precipitation can be prevented by adding different solubilization agents. The best conditions for maintaining solubility during isoelectric focusing for a given sample type must be determined empirically, although the most universal conditions are those described in the protocols in this unit. In contrast, IEF systems that do not use any detergents or denaturants are limited to that fairly small percentage of proteins which maintain good solubility near their isoelectric point.

If high-molecular-weight proteins are expected but are not present in the final two-dimensional gel, check the sample preparation protocol as well as the sample storage conditions. The most likely problem is proteolysis during sample preparation. Multiple freeze-thawing cycles could contribute to this problem. Vertical smears on the two-dimensional gel suggest (1) insufficient equilibration of the IEF gel (not enough SDS bound to the proteins), (2) poor contact between the IEF and second-dimension gels, or (3) problems related to the stacking gel (too short or wrong buffer). Use of a stacking gel is especially important when large-diameter IEF gels are loaded onto smaller second-dimension gels.

Omission of Triton X-100 or other nonionic or zwitterionic detergent from the final sample loaded on the gel can yield poor results, especially for samples containing SDS, because the amount of detergent in the IEF gels alone may be insufficient to remove bound SDS from proteins. The presence of Triton X-100 in the sample is especially important when SDS sample buffer is used to solubilize protein samples (i.e., after immunoprecipitation). The amount of Triton X-100 in the lysis buffer is normally sufficient for effective dissociation of SDS from proteins. If poor results are encountered with SDS-containing samples, try decreasing the final SDS concentration and/or increasing the final Triton X-100 concentration in the sample.

If no proteins are detected on the gel, check whether (1) the total protein load is appropriate for the detection method used, (2) the orientation of electrical connections is wrong or electrical connection during isoelectric focusing is poor (all gels from that run will be blank), (3) an air bubble obstructs current in a single IEF tube, or (4) the electrical connection is incorrect or is poor during the second-dimension gel separation. Careful monitoring of current and voltage at the beginning, during, and at the end of electrophoretic separations is strongly recommended. Recording the initial and final current and voltage will also facilitate troubleshooting. Additional guidelines for troubleshooting and evaluating artifacts in two-dimensional gel electrophoresis are described by Dunbar (1987).

Anticipated Results

A two-dimensional electrophoretic separation of proteins should produce a pattern of round or elliptical spots separated from one another. The pI range of the separated proteins as well as the observed molecular weight range depend on the first-dimension isoelectric focusing protocol and the percentage of acrylamide used for the second-

dimension gel. A complex protein mixture such as a whole-cell extract should produce more than 1000 silver-stained spots distributed over most of the gel area. Fewer spots will be detected with less sensitive detection techniques such as Coomassie blue staining. On the other hand, separation of radiolabeled proteins and use of multiple exposures permit detection of many low-abundance proteins.

Time Considerations

Time requirements are very dependent on gel size and whether an external cooling unit is used to permit faster separations. Isoelectric focusing using the standard-size gel format described in Basic Protocol 1 with 3-mm tubes is most conveniently done in an overnight run of ~16 to 18 hr. This separation time can be decreased to ~5 to 6 hr using higher voltages and an external cooling device. Isoelectric focusing of 18-cm-long IPG gels requires ~16 to 18 hr. Extruding a set of sixteen IEF tube gels and freezing them in equilibration buffer takes 1 to 2 hr, including setup time. Preparing and running SDS gels is described in UNIT 6.1. It takes ~30 min to thaw, equilibrate, and load two second-dimension gels.

Overall, if standard-size gels are used without external cooling, it will take ~3 working days before the results of two-dimensional electrophoresis are obtained. A single person can conveniently run about 16 two-dimensional gels in one week, depending on the amount of electrophoresis equipment available. The rate-limiting step in most laboratories is running the second-dimension gels because 16 soluble ampholyte IEF gels or 12 IPG gels can be focused in one run, but loading, running, and detecting results from 12 to 16 second-dimension gels requires substantial operator time and electrophoresis equipment.

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Key Reference

Hochstrasser et al., 1988. See above.

Discusses methods for improving and troubleshooting two-dimensional separation.

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One-Dimensional Electrophoresis Using Nondenaturing Conditions

UNIT 6.5

Nondenaturing or “native” electrophoresis—i.e., electrophoresis in the absence of denaturants such as detergents and urea—is an often-overlooked technique for determining the native size, subunit structure, and optimal separation of a protein. Because mobility depends on the size, shape, and intrinsic charge of the protein, nondenaturing electrophoresis provides a set of separation parameters distinctly different from mainly size-dependent denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; *UNITS 6.1*) and charge-dependent isoelectric focusing (IEF; *UNIT 6.4*). Two protocols are presented below. Continuous PAGE (see Basic Protocol) is highly flexible, permitting cationic and anionic electrophoresis over a full range of pH. The discontinuous procedure (see Alternate Protocol) is limited to proteins negatively charged at neutral pH but provides high resolution for accurate size calibration.

CONTINUOUS ELECTROPHORESIS IN NONDENATURING POLYACRYLAMIDE GELS

**BASIC
PROTOCOL**

Separation of proteins by nondenaturing electrophoresis requires the same type of equipment used for denaturing slab gels (*UNIT 6.1*) and is adaptable to a range of gel sizes (e.g., from 7.3×8.3 -cm minigels to 14×16 -cm full-size gels) and matrix types (e.g., single-concentration and gradient gels). This protocol outlines straightforward procedures for making acrylamide solutions, casting separating gels (stacking gels are omitted), loading samples, and conducting electrophoresis. Continuous systems, although flexible, do not give the high-resolution separation found in discontinuous systems (see Alternate Protocol).

Separation in a continuous system (i.e., in which the same buffer is used for preparing acrylamide solutions and filling electrophoresis chambers) is governed by pH, and this protocol describes four types of buffers useful over discrete ranges from pH 3.7 to pH 10.6. Use of unadjusted acetic acid gel buffer can extend the range to pH 2.0. The choice of pH and thus the buffer system depends on the protein being studied (i.e., its isoelectric point) and often must be determined empirically. In general, the system should be between pH 5.0 and 8.0 for optimal results. Extremes of pH can lead to precipitation or denaturation of the protein. Acrylamide concentrations are empirically determined, but the higher the percent acrylamide, the sharper the protein bands.

It is important to include native protein standards in the electrophoresis runs. Several manufacturers supply standards for isoelectric focusing that are also suitable for native electrophoresis. The standards have a range of isoelectric points and will carry a net positive, negative, or zero charge depending on the pH of the gel system. Alternatively, Sigma supplies a standard kit that is useful for calculating molecular weights under neutral pH, nondenaturing conditions. The samples and standard proteins, should be used at concentrations of ~ 1 to $2 \mu\text{g}/\mu\text{l}$.

Materials

- 4 \times acetic acid gel buffer (200 mM acetic acid, pH 3.7 to 5.6; see recipe)
- 4 \times phosphate gel buffer (400 mM sodium phosphate, pH 5.8 to 8.0; see recipe)
- 4 \times Tris gel buffer (200 mM Tris·Cl, pH 7.1 to 8.9; see recipe)
- 4 \times glycine gel buffer (200 mM glycine, pH 8.6 to 10.6; see recipe)
- 300 mM sodium sulfite (0.38 g in 10 ml H₂O; used in acetic acid gel preparation)
- Protein samples to be analyzed

**Electrophoresis
and
Immunoblotting**

6.5.1

Contributed by Sean R. Gallagher

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Supplement 5

Native protein standards

Electrophoresis buffer: appropriate 4× gel buffer diluted to 1× with H₂O

75-ml side-arm flask (used in gel preparation)

Additional reagents and equipment for gel electrophoresis (UNIT 6.1) and staining proteins in gels (APPENDIX 3)

Prepare the gel

1. Assemble the glass-plate sandwich of the gel electrophoresis unit and secure it to the casting stand.

Either single-concentration or gradient gels can be used in the minigel or standard-size format. Gradient gels will enhance the band sharpness of the separated proteins.

2. Prepare acrylamide solutions according to the recipes in Table 6.5.1, Table 6.5.2, Table 6.5.3, or Table 6.5.4, adding the ammonium persulfate and TEMED just before use.

Deaeration of the solution before the polymerization catalysts are added will speed polymerization by removing inhibitory oxygen, but this is not generally required. The pH used depends on many factors. The most important are the pI values of both the protein of interest and any contaminants, as well as protein mobility and protein solubility. Determining which pH and thus which buffer system to use is largely empirical. However, extremes of pH (<4.0 and >9.0) can lead to denaturation and should be avoided. Prior knowledge of the pI of a protein (UNITS 6.4) allows determination of the net charge under

Table 6.5.1 Recipes for Acetic Acid Nondenaturing Polyacrylamide Gels^a: pH Range 3.7 to 5.6^b

Stock solution ^c	Final acrylamide concentration in gel (%) ^d						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
300 mM sodium sulfite ^e	0.4	0.4	0.4	0.4	0.4	0.4	0.4
4× acetic acid gel buffer	10	10	10	10	10	10	10
H ₂ O	22.58	19.28	15.98	12.48	9.28	5.96	2.68
10% (w/v) ammonium persulfate ^{e,f}	0.3	0.3	0.3	0.3	0.3	0.3	0.3
TEMED ^f	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 300 mM sodium sulfite, 4× acetic acid gel buffer (see Reagents and Solutions), and H₂O. If desired to speed polymerization, degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

^aThe recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.

^bThe pH range can be extended to ~2.0 (the pH of acetic acid) by using unadjusted acetic acid in place of 4× acetic acid gel buffer, although there is little buffering capacity at this pH.

^cAll reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

^dUnits of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.

^eMust be freshly made. Sodium sulfite is needed for efficient polymerization at acid pH.

^fAdded just before polymerization.

Table 6.5.2 Recipes for Phosphate Nondenaturing Polyacrylamide Gels^a: pH Range 5.8 to 8.0

Stock solution ^b	Final acrylamide concentration in gel (%) ^c						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
4× phosphate gel buffer	10	10	10	10	10	10	10
H ₂ O	23.08	19.78	16.48	12.98	9.78	6.46	3.18
10% (w/v) ammonium persulfate ^{d,e}	0.2	0.2	0.2	0.2	0.2	0.2	0.2
TEMED ^e	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× phosphate gel buffer (see Reagents and Solutions), and H₂O. If desired, degas under vacuum ~5 min to speed polymerization. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

^aThe recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.

^bAll reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

^cUnits of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.

^dMust be freshly made.

^eAdded just before polymerization.

the separation conditions (i.e., if gel pH < protein pI, the protein will have a net positive charge; if gel pH > protein pI, the protein will be negatively charged).

3. Pour gel to 2 cm from the top of the gel mold and insert the comb. Avoid trapping air bubbles under the comb teeth.

Air bubbles will cause small semicircular depressions in the well and lead to distortions in the protein banding pattern.

4. Allow gel solution to polymerize 1 to 2 hr.

Polymerization is indicated by a sharp optical discontinuity around the wells.

Prepare samples and load the wells

5. Solubilize the protein sample to be analyzed using 5% (w/v) sucrose in water or dilute (1 to 5 mM) gel buffer if possible. Also prepare native protein standards.

The concentration of protein will vary depending on the sample complexity and detection method. For Coomassie blue staining of highly enriched samples such as the standards, use 1 to 2 mg/ml (1 to 2 μg/μl). For more complex mixtures, use 5 to 10 mg/ml (5 to 10 μg/μl). Load 10- to 100-fold less for silver staining. In general, samples should be loaded in a minimum volume, preferably 10 to 20 μl for 0.75- and 1.5-mm-thick gels, respectively. With thin gels, this means using a more concentrated protein sample.

6. Remove comb carefully and rinse wells with electrophoresis buffer (appropriate 4× gel buffer diluted to 1×).

Rinsing with electrophoresis buffer is needed to remove residual unpolymerized acrylamide monomer, which will continue to polymerize after comb removal, creating uneven wells that may interfere with sample loading.

Table 6.5.3 Recipes for Tris Nondenaturing Polyacrylamide Gels^a: pH Range 7.1 to 8.9

Stock solution ^b	Final acrylamide concentration in gel (%) ^c						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
4× Tris gel buffer	10	10	10	10	10	10	10
H ₂ O	23.08	19.78	16.48	12.98	9.78	6.46	3.18
10% (w/v) ammonium persulfate ^{d,e}	0.2	0.2	0.2	0.2	0.2	0.2	0.2
TEMED ^e	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× Tris gel buffer (see Reagents and Solutions), and H₂O. If desired, degas under vacuum ~5 min to speed polymerization. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

^aThe recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.

^bAll reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

^cUnits of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.

^dMust be freshly made.

^eAdded just before polymerization.

7. Fill wells with electrophoresis buffer. If desired, prerun gel.

The gel can be prerun at this point to remove any charged material such as ammonium persulfate from the gel prior to loading the sample. Assemble the electrophoresis unit and fill the buffer chambers with electrophoresis buffer. Run the gel at 300 V until the current no longer drops. This should take ~30 min. Disassemble the unit, discard the buffer, and proceed to the next step.

8. Carefully load up to 10 µl (0.75-mm gels) or 20 µl (1.5-mm gels) sample per lane as a thin layer at the bottom of the wells. Load control wells with native protein standards. Add an equal volume of electrophoresis buffer to any empty wells to prevent spreading of adjoining lanes.

Mobility (R_f) markers require special consideration in nondenaturing gel systems. For cationic systems, cytochrome c (pI ~9 to 10, 5 to 10 µg/lane) works well as an R_f marker. Bromphenol blue (10 µg/ml) is a suitable marker for anionic systems. The marker should be included in the solubilization buffer with the sample.

Perform the separation

9. Assemble the gel unit, fill the upper and lower buffer chambers with electrophoresis buffer, and connect the unit to the power supply. Set current to 30 mA for a 1.5-mm-thick gel (15 mA for a 0.75-mm-thick gel).

If the protein is negatively charged under the separation conditions, then the standard SDS-PAGE electrode polarity should be used (proteins will migrate to the anode or positive electrode; see UNIT 6.1). If the protein is positively charged, then the electrodes should be reversed at the power supply (i.e., red high-voltage cable to the black output and black high-voltage lead to the red output) so the positively charged protein migrates to the negative cathode.

Table 6.5.4 Recipes for Glycine Nondenaturing Polyacrylamide Gels^a: pH Range 8.6 to 10.6

Stock solution ^b	Final acrylamide concentration in gel (%) ^c						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
4× glycine gel buffer	10	10	10	10	10	10	10
H ₂ O	23.08	19.78	16.48	12.98	9.78	6.46	3.18
10% (w/v) ammonium persulfate ^{d,e}	0.2	0.2	0.2	0.2	0.2	0.2	0.2
TEMED ^e	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× glycine gel buffer (see Reagents and Solutions), and H₂O. If desired, degas under vacuum ~5 min to speed polymerization. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

^aThe recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.

^bAll reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

^cUnits of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.

^dMust be freshly made.

^eAdded just before polymerization.

10. Continue electrophoresis until the R_f marker reaches the bottom of the gel.

For minigels, electrophoresis will take 1 to 2 hr. Standard gels require 4 to 6 hr runs.

11. Turn off power supply, disassemble the unit, and remove gel from sandwich.

12. Stain the gel according to APPENDIX 3.

NATIVE DISCONTINUOUS ELECTROPHORESIS AND GENERATION OF MOLECULAR WEIGHT STANDARD CURVES (FERGUSON PLOTS)

ALTERNATE PROTOCOL

One straightforward approach to discontinuous native electrophoresis is to leave out the SDS and reducing agent (DTT) from the standard Laemmli SDS-PAGE protocol (UNIT 6.1). The gels are prepared as described in UNIT 6.5 except that the sample buffer contains no SDS or DTT (samples are not heated), and the gel and electrophoresis solutions are prepared without SDS. This protocol illustrates the separation of standard proteins at four different concentrations of acrylamide and how the results are used to construct a molecular weight standard curve (Ferguson plot) without the need for SDS. By plotting relative mobility against %T (percentage weight per volume of acrylamide plus bisacrylamide in the gel), the presence of isoforms and multimeric proteins can also be detected.

Materials

4× Tris·Cl, pH 8.8 (1.5 M Tris·Cl; APPENDIX 2A)

4× Tris·Cl, pH 6.8 (0.5 M Tris·Cl)

Protein sample of interest

2× Tris/glycerol sample buffer (see recipe)

Electrophoresis and Immunoblotting

6.5.5

Native protein standards (e.g., Sigma nondenatured protein molecular weight kit)
Tris/glycine electrophoresis buffer (see recipe)

1. Assemble the glass-plate sandwich of the gel electrophoresis unit and place it in the casting stand.
2. Prepare and cast the gels, using 4× Tris·Cl, pH 8.8, for the separating gel and 4× Tris·Cl, pH 6.8, for the stacking gel instead of the SDS-containing counterparts (Table 6.1.1). Prepare a minimum of four separate gels at different acrylamide concentrations.

A typical range of concentrations is from 5% to 12.5% (e.g., 5%, 7.5%, 10%, 12.5% acrylamide). As with SDS-PAGE, typical gel thickness ranges from 0.75 to 1.5 mm. The 0.75-mm-thick gels are recommended because they offer a combination of fast staining and high resolution.

3. Mix protein sample of interest 1:1 with 2× Tris/glycerol sample buffer to attain a 1 to 2 µg/µl final concentration. Also prepare native protein standards. Remove comb, rinse wells, and load 10 to 20 µl per well for Coomassie brilliant blue staining, and 1 to 2 µl for silver staining.

Some proteins must be dissolved in 50 mM NaCl or water to become fully solubilized prior to mixing with the sample buffer (Sigma, 1986).

4. Assemble gel electrophoresis unit, using Tris/glycine electrophoresis buffer to fill both lower and upper buffer chambers. Connect power supply and conduct electrophoresis.

Conditions for separation are the same as for discontinuous SDS-PAGE (i.e., 30 mA for 1.5-mm-thick gels, 15 mA for 0.75-mm-thick gels). For standard-size gels the separation takes 4 to 5 hr; for minigels, 1 to 2 hr is required. Alternatively, standard gels can be run at 4 to 6 mA/gel overnight.

5. After the bromphenol blue R_f marker has reached the bottom of the gel, fix and stain the proteins in the gels according to APPENDIX 3. Estimate relative mobilities of the proteins.

An example of a stained gel is shown in Figure 6.5.1. A minimum of four gel concentrations is recommended. In Figure 6.5.1, Sigma native molecular weight standards were separated on 5%, 7.5%, 10%, and 12.5% acrylamide gels (5.1%, 7.7%, 10.3%, and 12.8% T, respectively).

6. Plot $\log R_f$ against gel concentration (% T) (Fig. 6.5.2). Determine the slope of K_r using linear regression.
7. Plot $-\log K_r$ of the curves from step 6 against \log molecular weight of the standards (Fig. 6.5.3). Determine the slope using linear regression.
8. Estimate the size of the standards and unknowns from the generated curve (Ferguson plot).

Use the curve generated by linear regression to estimate the predicted size of the standard for comparison to the actual size stated by the supplier. This indicates the accuracy of the curve. The $-\log K_r$ value (y) of the unknown is then used to predict the molecular weight (x).

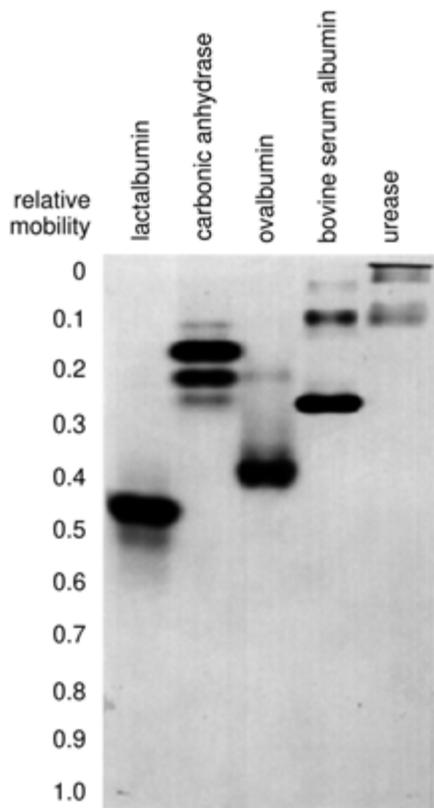


Figure 6.5.1 Separation of native protein standards under nondenaturing conditions by discontinuous polyacrylamide gel electrophoresis at 12.8% T. Approximately 20 μg protein was loaded per lane on a 1.5-mm-thick, 16-cm-long gel. The gel and samples were prepared according to the Alternate Protocol and were electrophoresed 16 hr at 6 mA. Proteins were stained with Coomassie blue.

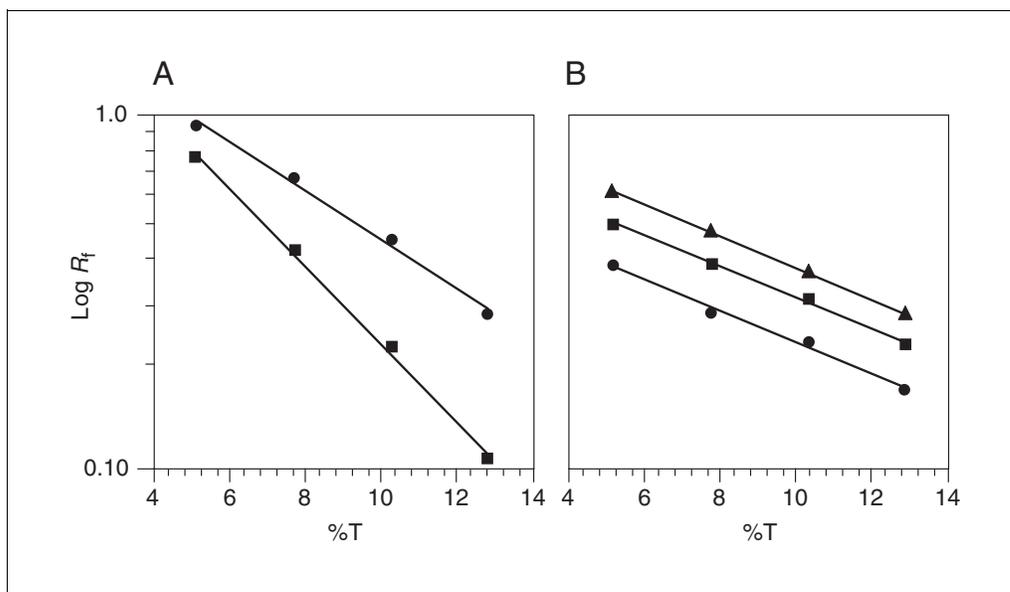


Figure 6.5.2 Effect of %T on the relative mobility of several native proteins. The relative mobility (R_f) of the standard proteins shown in Figure 6.5.1 was determined at four different gel concentrations and plotted as $\log R_f$ against %T. See text for details. (A) BSA monomer (squares) and dimer (circles); (B) carbonic anhydrase isoforms.

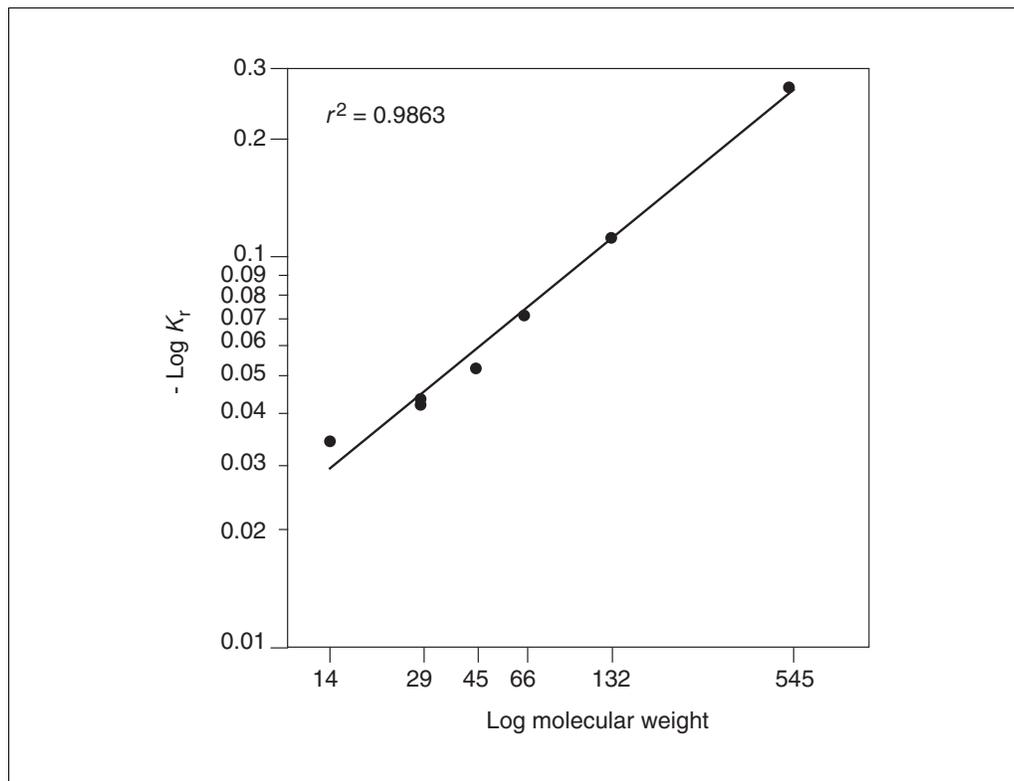


Figure 6.5.3 Native molecular weight standard curve. The $-\log$ slope of the line (K_r) from Figure 6.5.2 is plotted against log molecular weight of the standards.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Acetic acid gel buffer, 4 \times (200 mM acetic acid, pH 3.7 to 5.6)

11.49 ml glacial acetic acid
 Add to 500 ml H₂O
 Adjust to pH 3.7 to 5.6 with 1 M NaOH
 Add H₂O to 1000 ml
 Store up to 1 month at 4°C

Glycine gel buffer, 4 \times (200 mM glycine, pH 8.6 to 10.6)

15.01 g glycine
 Add to 500 ml H₂O
 Adjust to pH 8.6 to 10.6 with 1 M NaOH
 Add H₂O to 1000 ml
 Store up to 1 month at 4°C

Phosphate gel buffer, 4 \times (400 mM sodium phosphate, pH 5.8 to 8.0)

55.2 g NaH₂PO₄·H₂O
 Add to 500 ml H₂O
 Adjust to pH 5.8 to 8.0 with 1 M NaOH
 Add H₂O to 1000 ml
 Store up to 1 month at 4°C

Tris gel buffer, 4× (200 mM Tris·Cl, pH 7.1 to 8.9)

24.23 g Tris base
Add to 500 ml H₂O
Adjust to pH 7.1 to 8.9 with 1 M HCl
Add H₂O to 1000 ml
Store up to 1 month at 4°C

Tris/glycerol sample buffer, 2×

25 ml 0.5 M Tris·Cl, pH 6.8 (APPENDIX 2A)
20 ml glycerol
1 mg bromphenol blue
Add H₂O to 100 ml and mix
Store in 1-ml aliquots up to 6 months at -70°C

Tris/glycine electrophoresis buffer

15.1 g Tris base
72.0 g glycine
H₂O to 5000 ml
Store up to 1 month at 4°C

COMMENTARY

Background Information

Under nondenaturing conditions, in which protein activity, native charge, and conformation are sustained, electrophoretic separation depends on many factors, including size, shape, and charge. Characteristics such as intrinsic molecular weight (i.e., in the absence of denaturation), the number of isoforms, and the presence of multimeric proteins can be determined with nondenaturing electrophoresis (often called native electrophoresis).

The most important application of nondenaturing electrophoresis is in the determination of native protein size (see Alternate Protocol). Ferguson plots (reviewed by Andrews, 1986) were first described for starch gels (Ferguson, 1964) and then for polyacrylamide gels (Hedrick and Smith, 1968). Ferguson plots are prepared by separating proteins under nondenaturing conditions at several different gel concentrations. As the acrylamide concentration (%T) is increased, the relative mobility (R_f) of the protein decreases. This is plotted as log relative mobility (on the y axis) versus %T (on the x axis) to produce a straight line. The slope of this line is referred to as the retardation coefficient (K_r) and measures how effectively a protein is slowed by the increase in %T. Large proteins will be retarded much more significantly than small proteins with increasing gel concentration, with the size of the protein being proportional to the slope of the curve. Once the K_r plots for several size standards are generated (Fig. 6.5.2), the K_r values are plotted against

the molecular weight of the standard proteins using a log-log graph (Fig. 6.5.3). The retardation coefficient also depends on a large number of other variables including temperature, pH, buffer type, ionic strength, and %C (percent bisacrylamide cross-linker). All these factors should be kept constant for a given experiment.

In addition to estimated size, other types of information are available from the Ferguson plots (Rodbard and Chrambach, 1971; Andrews, 1986). For example, if two components differ in size but have the same charge per unit size (e.g., for a multimeric protein with identical subunits), curves similar to those illustrated by BSA monomer and dimer (Fig. 6.5.2A) will result. Note that when the curve is extrapolated back to 0% T, it is evident that the monomer and the dimer have similar free solution mobilities. Furthermore, as the acrylamide concentration is increased, the separation between the two also increases. However, if two proteins have similar sizes but different amounts of charge, the curves will be parallel on the log plot. This is illustrated by the carbonic anhydrase isoforms (Fig. 6.5.2B). In this example, optimal separation of the isoforms occurs at the lower concentrations of acrylamide as this is a log plot.

Further applications of nondenaturing electrophoresis include preparative purification. The pH of the gel determines the net charge on the protein. Below its isoelectric point (pI) a protein will have a net positive charge, whereas above its pI it will have a net negative charge.

In general, most proteins will be positively charged at pH 2.0 to 4.0; above pH 8.0, most proteins will be negatively charged. As these general guidelines imply, the majority of proteins have isoelectric points between pH 4.0 and 8.0. There are, however, many exceptions. A protein with a highly acidic isoelectric point (e.g., pepsin, with a pI of 2.2) will remain negatively charged at a pH down to its pI. Although a full range of pH options are given, extremes of pH (<4.0 and >9.0) should be avoided, if possible, to minimize denaturation or inactivation. By picking an appropriate electrophoresis pH, it is possible to ensure that the protein of interest will be either positively or negatively charged so that it can be selectively run into the gel, excluding a large proportion of contaminants that have the opposite or no charge. Furthermore, the pH conditions determine the mobility and can be adjusted to ensure a difference in mobility between the protein of interest and contaminants.

Continuous gel systems (see Basic Protocol) offer the most flexibility in terms of separation design. The pH can be tailored so that a given protein has a net positive, neutral, or negative charge. Depending on the polarity of the gel, the protein can then be excluded from or electrophoresed into the gel. Discontinuous gels have a fixed pH and gel polarity. For the nondenaturing Laemmli gel presented in the Alternate Protocol, the proteins of interest should have an isoelectric point of ≤ 7.0 in order to be negatively charged so that they move into the gel. Other more basic and more acidic discontinuous gel systems can be found in Hames (1990) and Schägger (1994).

Critical Parameters

The success of a gel separation under nondenaturing conditions depends on many factors, and two of the most important are protein solubility and isoelectric point. The protein must be soluble at the pH and the ionic strength of the gel, and it must be charged at that pH in order to move into the gel. If the protein experiences a pH below its isoelectric point, then it will have a net positive charge and will move to the negative electrode. Note that this is the reverse of typical SDS-PAGE. If the protein experiences a pH above its isoelectric point, it will have a net negative charge and will migrate to the positive electrode.

Solubility is a complex issue. Membrane-associated and other hydrophobic proteins are difficult to separate by nondenaturing electrophoresis (Schägger, 1994). Nonionic deter-

gents at concentrations up to 1% and solubilizing reagents such as urea (4 to 8 M) can be used, but these reagents, especially urea, are likely to alter the protein's conformation and most likely the isoelectric point by exposing previously hidden charged groups. If detergent or urea must be included for solubilization, the minimum required to solubilize the protein should be used. Schägger (1994) lists several nonionic detergents suitable for solubilization. Among the more popular are octylglucoside and CHAPS. In general, detergents should be used near the critical micelle concentration (CMC; 0.001% to 1%, depending on the detergent).

The gel concentration has a dramatic effect on resolution and should be optimized in order to achieve the best separation and band sharpness. In general, increasing the %T will improve band sharpness.

Troubleshooting

Gel polymerization at acid pH can be problematic, and sodium sulfite is needed for efficient polymerization (Andrews, 1986). Both the ammonium persulfate and the sodium sulfite must be freshly made, and the highest quality reagents available should be used. Furthermore, the gel solutions should be at room temperature for effective polymerization.

If the protein does not enter the gel and no stained material is present at the well surface, try reversing the polarity of the electrode. If material concentrates at the top of the gel, try lowering the acrylamide concentration. Stained material at the top of the gel may also indicate poor solubilization, and increasing the ionic strength of the solubilization buffer or adding a small amount of urea and/or nonionic detergent may be required.

Anticipated Results

Proteins will resolve depending on their solubility and native charge at the chosen pH. Ideally, a distinct band representing the protein of interest will be visible. If the band is diffuse, then increasing the gel concentration or using a gradient gel will improve resolution. If the band is not visible, then the protein may be at its isoelectric point or may have moved out of the gel because it had the wrong charge. Continuous gel systems, although more versatile, will give lower resolution than discontinuous gels. Detergents or other solubilizing agents such as urea may be needed to fully solubilize and resolve the protein. Once the conditions that resolve the protein are determined, Ferguson plots will give indications of multiple

subunit structure, native size, and potential isoform relationships.

Time Considerations

Separations will be complete when the tracking dye or protein reaches the bottom of the gel. For minigels, this generally takes 1 to 2 hr, using 15 or 30 mA for 0.75- or 1.5-mm-thick gels, respectively. Standard-format gels require 4 to 5 hr at 15 or 30 mA for 0.75- or 1.5-mm-thick gels, respectively. Standard-format gels can also run overnight at 4 to 6 or 8 to 12 mA for 0.75- or 1.5-mm-thick gels, respectively.

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Key Reference

Andrews, 1986. See above.

Covers a variety of electrophoretic techniques, including nondenaturing electrophoresis and Ferguson plots.

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Staining Proteins in Gels

Polyacrylamide gel electrophoresis (PAGE) is a powerful method for the resolution of protein mixtures according to the proteins' charge-to-mass ratio (nondenaturing-PAGE; UNIT 6.5), mass (SDS-PAGE; UNIT 6.1), isoelectric point (isoelectric focusing) or combinations of these properties (two-dimensional PAGE; UNIT 6.4). A number of protocols have been developed to visualize the separated protein bands following electrophoresis. In this unit, four different methods, which are widely used in cell biology laboratories, are described. These are: staining with the dye Coomassie blue (Basic Protocol 1 and Alternate Protocol 1), silver staining (Basic Protocol 2), fluorescent staining with SYPRO Ruby (Basic Protocol 3), and negative staining with zinc (Basic Protocol 4).

Staining with Coomassie blue (Basic Protocol 1) is a relatively simple and very popular method, albeit less sensitive than other procedures. A variation of this protocol, suitable for staining proteins in isoelectric focusing gels, is described under Alternate Protocol 1. Staining with silver (Basic Protocol 2) is probably the most sensitive method, although it is time-consuming and incompatible with some downstream applications (see Commentary). The remaining two methods (Basic Protocols 3 and 4) are more sensitive than Coomassie blue staining and recommended for downstream applications where protein fixation needs to be avoided (e.g., analysis of biological activity). Some of the protocols can be combined. For instance, some researchers prefer to stain gels first with Coomassie blue (Basic Protocol 1) or zinc (Basic Protocol 4) and, if higher sensitivity is necessary, subsequently with silver (Basic Protocol 2). Further details on the differences between the methods, as well as guidelines for their selection according to specific applications, are discussed in the Commentary.

In the following protocols, the volumes of solutions are indicated in "gel volumes." When staining slab mini-gels, 10 gel vol correspond to ~50 to 80 ml.

STAINING PROTEIN GELS WITH COOMASSIE BLUE

Coomassie brilliant blue R-250, a triphenylmethane anionic dye, binds avidly to almost all proteins in either native or denatured states and is widely used for detection of proteins in polyacrylamide gels. In this protocol, proteins separated by either nondenaturing- or SDS-PAGE (one- or two-dimensional) are fixed and stained by soaking the gel in a Coomassie blue staining solution; the unbound dye is subsequently removed by washing with destaining solution to yield blue protein bands on a clear background.

Materials

- Polyacrylamide gel containing protein(s) of interest (see UNIT 6.1)
- Coomassie blue staining solution (see recipe)
- Destaining solution: 30% (v/v) methanol/10% (v/v) acetic acid in distilled water (store up to 4 months at room temperature)
- Storage solution: 7% (v/v) acetic acid/5% (v/v) methanol in distilled water (store up to 4 months at room temperature)
- Plastic container with lid (pipet tip containers are appropriate for staining mini-gels)
- Platform shaker (optional)

CAUTION: Glacial acetic acid and methanol are volatile and toxic. The destaining and storage solutions should be prepared in a chemical fume hood, and gloves should be worn throughout the staining procedure.

BASIC PROTOCOL 1

Electrophoresis and Immunoblotting

6.6.1

NOTE: All steps should be performed at room temperature.

1. Remove polyacrylamide gel from electrophoresis assembly and place it in a plastic container with lid containing a large excess (~10 gel vol) of Coomassie blue staining solution.
2. Incubate with gentle agitation for ≥ 20 min for a gel ≤ 1 mm thick or ≥ 1 hr for a gel > 1 mm thick.

The use of a platform shaker is recommended. Most proteins are fixed due to the presence of methanol in the staining solution; gels can therefore be left in staining solution for many hours (i.e., overnight) without any adverse effect, unless the protein(s) of interest is very small (< 5 kDa) and can be lost by diffusion due to incomplete fixation.

3. Remove the Coomassie blue staining solution and rinse the gel briefly with distilled water.

The Coomassie blue staining solution can be reused several times.

4. Add ~10 gel vol of destaining solution and incubate with gentle agitation until the solution becomes as dark as the gel matrix.
5. Discard the destaining solution.
6. Repeat steps 4 and 5 several times until a clear background is obtained.

Both the total amount of destaining solution and the destaining time can be reduced by placing a piece of adsorbent material with affinity for the Coomassie dye (e.g., Whatman 3MM filter paper) inside the container having the gel in destaining solution.

NOTE: Avoid excessive incubation of the gel with destaining solution as it can result in decreased sensitivity due to dissociation of protein-dye complexes.

7. Add ~10 gel vol storage solution and incubate for 10 to 15 min with gentle agitation.

At this point, the gel can be stored at 4°C for several months in a plastic container or sealed plastic bag containing storage solution. Alternatively, it can be soaked in 2% (v/v) glycerol for 15 to 30 min, placed onto Whatman 3MM filter paper, and subsequently dried on a vacuum system.

ALTERNATE PROTOCOL 1

STAINING PROTEIN GELS WITH COOMASSIE BLUE AFTER ISOELECTRIC FOCUSING

In polyacrylamide gel isoelectric focusing, proteins are resolved based on their isoelectric points on a pH gradient generated by a mixture of ampholytes. Many commercially available ampholytes bind Coomassie blue and, therefore, interfere with protein detection. To overcome this problem, the following procedure involves treatment with trichloroacetic acid (TCA) to fix proteins while removing ampholytes and uses CuSO_4 in the staining solution to help reduce the background (Righetti and Drysdale, 1974).

Additional Materials (see also *Basic Protocol 1*)

Polyacrylamide isoelectric focusing gel with protein(s) of interest (e.g., *UNITS 6.4 & 15.4*)

20% (w/v) TCA solution in water

IEF Coomassie blue stock solution (see recipe)

10% (w/v) CuSO_4 in distilled water (store for up to 4 months at room temperature)

CAUTION: TCA is extremely caustic. Protect eyes and wear gloves when preparing and handling TCA solutions.

1. Make fresh staining solution by mixing 90 ml of destaining solution, 10 ml of IEF Coomassie blue stock solution and 1 ml of 10% (w/v) CuSO₄ in distilled water.
2. Remove polyacrylamide gel from isoelectric focusing assembly and place it in a plastic container with lid containing 5 to 10 gel vol of 20% (w/v) TCA solution in water.
3. Incubate for 30 to 60 min with gentle agitation.

The use of a platform shaker is recommended. In this step, proteins are selectively fixed while ampholytes are removed from the gel.
4. Remove the TCA solution and add ~10 gel vol destaining solution.

Do not reuse the TCA solution; dispose of it following applicable safety regulations for chemical waste. In this step, the TCA is removed from the gel.
5. Incubate 15 min with gentle agitation.
6. Discard the destaining solution and add freshly prepared staining solution (from step 1).
7. Incubate with gentle agitation for ≥20 min for a gel ≤1 mm thick or ≥1 hr for a gel >1 mm thick.
8. Remove the staining solution and rinse the gel briefly with distilled water.

Do not reuse the staining solution.
9. Destain the gel (see Basic Protocol 1, steps 4 to 7).

STAINING PROTEIN GELS WITH SILVER

Silver staining is based on the selective reduction of silver ions at sites of the gel that contain proteins and other macromolecules. The following protocol is adapted from that of Blum et al. (1987), and involves pretreatment of a fixed polyacrylamide gel with thiosulfate (step 6) followed by impregnation with silver nitrate (step 9) and color development with formaldehyde at a high pH (steps 12 and 13). Pretreatment with thiosulfate significantly increases the sensitivity and improves the contrast of protein staining. In addition, the presence of low concentrations of thiosulfate during color development reduces nonspecific staining of the gel surface. The procedure is applicable for both nondenaturing- (UNIT 6.5) and SDS-PAGE (one- or two-dimensional; UNIT 6.1 and UNIT 6.4) and yields dark (brown to black) protein bands on a clear background.

Materials

- Polyacrylamide gel containing protein(s) of interest (see UNITS 6.1, 6.4 & 6.5), either unfixed or fixed and stained with Coomassie blue (see Basic Protocol 1 and Alternate Protocol 1)
- Deionized water (HPLC grade or Milli-Q)
- 50% (v/v) ethanol in deionized water
- Fixative solution (see recipe)
- Thiosulfate solution (see recipe)
- Silver nitrate solution (see recipe)
- Developer solution (see recipe)
- 50% (v/v) methanol/12% (v/v) acetic acid in deionized water
- 50% (v/v) methanol in water

continued

**BASIC
PROTOCOL 2**

**Electrophoresis
and
Immunoblotting**

6.6.3

Plastic container with lid (pipet tip containers are appropriate for staining mini-gels)
Clean plastic containers (at least six; lids of pipet tip containers are appropriate for staining mini-gels)
Aluminum foil
Platform shaker (optional)

CAUTION: Glacial acetic acid and methanol are volatile and toxic; solutions containing these solvents should be prepared in a chemical fume hood and handled with care.

NOTE: All solutions should be prepared in deionized water (Milli-Q or HPLC grade). At all times wear gloves that have been rinsed extensively with distilled water. Handle the gel by using clean forceps having blunt tips or by touching the corners with clean, powder-free gloves. All steps should be performed at room temperature.

Fix the gel

1. Remove polyacrylamide gel from electrophoresis assembly and place it in a plastic container with lid containing a large excess (~10 gel vol) of fixative solution.

Steps 1 and 2 are for unfixed gels only. If the polyacrylamide gel was previously fixed and/or stained with Coomassie blue (Basic Protocol 1 or Alternative Protocol 1), proceed directly to step 3.

2. Incubate for ≥ 30 min with gentle agitation.

The use of a platform shaker is recommended.

Pretreat the gel

3. Transfer the gel to a clean plastic container having ~10 gel vol of 50% ethanol in deionized water. Incubate 20 min with gentle agitation.

4. Replace the solution with another ~10 gel vol of 50% (v/v) ethanol in deionized water. Incubate 20 min with gentle agitation. Repeat this step once (total of three pretreatment steps).

The plastic container used in steps 3 and 4 can be reused in step 16. Discard the ethanol solution and rinse the container briefly with deionized water.

5. Transfer the gel to a clean plastic container having ~10 gel vol deionized water and incubate 5 to 10 min with gentle agitation.

6. Discard the water and add ~10 gel vol thiosulfate solution. Incubate for exactly 1 min with gentle agitation.

Incubations longer than 1 min will result in increased background staining.

7. Transfer the gel to a clean plastic container having ~10 gel vol deionized water and incubate exactly 20 sec.

8. Change the water in the container and incubate exactly 20 sec. Perform this step twice (for a total of three).

The plastic container used in steps 7 and 8 can be reused in steps 10 and 11 but should first be rinsed thoroughly with deionized water.

Stain the gel

9. Transfer the gel to a clean plastic container containing 5 to 10 gel vol silver nitrate solution. Cover the container with aluminum foil and incubate 20 min with gentle agitation.

Light exposure causes the reduction of silver ions and may therefore result in increased background staining.

10. Transfer the gel to a clean plastic container having ~10 gel vol deionized water and incubate exactly 20 sec.
11. Change the water in the container and incubate again exactly 20 sec.

The plastic container used in steps 10 and 11 can be reused in steps 14 and 15 but should first be rinsed with deionized water.

Develop the gel stain

12. Transfer the gel to a clean plastic container having ~10 gel vol developer solution.
13. Incubate with gentle agitation until the protein band(s) of interest becomes visible (or until the gel matrix begins to get too dark).

This step lasts a few minutes (≤ 10 min) and requires continuous visual inspection as the protein bands may develop rapidly, and long incubations may result in high background staining. Since some developing will still occur during steps 14 and 15, it is advisable to proceed to the next step as soon as the staining is judged to be optimal.

14. Transfer the gel to a clean plastic container having ~10 gel vol deionized water. Incubate 30 sec.
15. Change the water in the container and incubate an additional 30 sec.
16. Transfer the gel to a clean plastic container having ~10 gel vol 50% methanol/12% acetic acid in deionized water. Incubate 10 min with gentle agitation.
17. Remove the solution from the container and add ~10 gel vol of 50% methanol.

The gel can be stored in this solution for several months at 4°C. Alternatively, the gel can be rehydrated by soaking in water for 5 to 10 min, soaked in 2% (v/v) glycerol for another 15 to 30 min, placed onto Whatman 3MM filter paper, and then dried on a vacuum system.

FLUORESCENCE DETECTION OF PROTEINS IN GELS

In this method, proteins separated on polyacrylamide gels [either nondenaturing- (UNIT 6.5) or SDS-PAGE, one- or two-dimensional (UNIT 6.1 or UNIT 6.4)] are incubated with SYPRO Ruby, a commercially available fluorescent compound that interacts specifically with proteins. Following incubation, protein bands can be readily visualized using standard 300-nm transillumination.

Materials

Polyacrylamide gel containing protein(s) of interest (see UNIT 6.1, UNIT 6.4, or UNIT 6.5)
SYPRO Ruby protein gel stain (Molecular Probes)
Distilled water

Plastic container with lid (pipet tip containers are appropriate for staining mini-gels)

Platform shaker (optional)

300-nm UV transilluminator

Photographic camera or CCD camera (optional)

CAUTION: The potential toxicity of the SYPRO Ruby dye, which comprises an organic component and ruthenium, has not been fully evaluated. Gloves should be worn throughout the staining procedure. For disposal, the stain solution should be poured through activated charcoal, and the dye adsorbed to activated charcoal destroyed in a chemical incinerator following local environmental regulations.

NOTE: Protect SYPRO Ruby protein gel stain from light. If the plastic container with lid used for staining is transparent, cover it completely with aluminum foil.

BASIC PROTOCOL 3

Electrophoresis and Immunoblotting

6.6.5

NOTE: All steps should be performed at room temperature.

1. Remove polyacrylamide gel from electrophoresis assembly and place it in a plastic container with lid containing a large excess (~10 gel vol) of SYPRO Ruby protein gel stain.

2. Incubate 3 hr with gentle agitation.

The use of a platform shaker is recommended.

3. Remove the SYPRO Ruby protein gel stain and rinse the gel briefly with distilled water.

Do not reuse the stain solution. Follow local recommendations for the disposal of potentially toxic organic compounds.

4. Add ~10 gel vol distilled water and incubate 10 min with gentle agitation.

5. Change the distilled water in the container and incubate another 10 min with gentle agitation.

6. Visualize protein bands by fluorescence on a 300-nm UV transillumination unit.

Images can be acquired using a photographic camera with appropriate film (e.g., Polaroid 667 black-and-white print film) or a CCD camera.

CAUTION: *Use UV-rated eye protection to avoid direct exposure of the eyes to the UV light.*

At this point, the gel is ready for downstream applications (e.g., electroelution and immunoblotting).

BASIC PROTOCOL 4

REVERSIBLE PROTEIN STAINING WITH ZINC

In this method, protein bands separated on SDS-PAGE gels [one- or two-dimensional (*UNIT 6.1* or *UNIT 6.4*)] are visualized as clear spots on an opaque white background, the latter being generated by precipitation of SDS with zinc ions. This negative staining procedure is sensitive, fast, and completely reversible upon removal of zinc with a chelating agent.

Materials

Polyacrylamide gel containing protein(s) of interest (see *UNIT 6.1*)

GelCode E-Zinc Reversible Stain Kit (Pierce), containing:

E-Zinc Stain

E-Zinc Developer

E-Zinc Eraser

Distilled water

Plastic container with lid (pipet tip containers are appropriate for staining mini-gels)

Platform shaker (optional)

NOTE: All steps should be performed at room temperature.

Stain the gel

1. Remove polyacrylamide gel from electrophoresis assembly and place it in a plastic container with lid containing ~10 gel vol E-Zinc Stain solution.

2. Incubate 10 min with gentle agitation.

The use of a platform shaker is recommended.

3. Remove the E-Zinc Stain solution from the container and replace it with ~10 gel vol E-Zinc Developer solution.

Do not reuse the stain solution.

4. Incubate 1 to 2 min with gentle agitation. Proceed to the next step as soon as the staining is judged to be optimal.
5. Remove the E-Zinc Developer solution from the container and replace it with ~10 gel vol distilled water.

Do not reuse the developer solution.

Wash the gel

6. Incubate 1 min with gentle agitation.
7. Change the water in the container.
8. Visualize the protein bands by placing the polyacrylamide gel on a dark (black or blue) surface.

The stained gel can be kept in water for several weeks at 4°C.

Solubilize the SDS/zinc precipitate (optional)

9. Incubate the gel with ~10 gel vol E-Zinc Eraser with gentle agitation until the gel matrix is completely clear (~5 to 10 min).

These two steps involve solubilization of the SDS/zinc precipitate to completely destain the gel, thus allowing further analysis of the protein band(s) of interest.

10. Rinse the gel with distilled water.

At this point, the gel is ready for downstream applications (e.g., electroelution, immunoblotting, etc.).

REAGENTS AND SOLUTIONS

Use HPLC-grade or Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Coomassie blue staining solution

For 100 ml: Dissolve 0.1 g Coomassie brilliant blue R-250 in a mixture of 30 ml methanol and 20 ml water. Subsequently, add 40 ml of water and 10 ml of glacial acetic acid. Store for up to 4 months at room temperature.

CAUTION: Glacial acetic acid and methanol are volatile and toxic and should be handled in a chemical fume hood.

This solution is 0.1% (w/v) Coomassie brilliant blue R-250 in methanol/acetic acid/water (3:1:6 v/v/v).

Developer solution

Dissolve 6 g Na₂CO₃ in 98 ml water. Add 50 µl of 37% formaldehyde and 2 ml of thiosulfate solution (see recipe). Prepare fresh before use.

CAUTION: Formaldehyde is toxic and carcinogenic. Wear gloves and handle the concentrated (37%) solution in a chemical hood.

Fixative solution

Mix 50 ml of methanol with 12 ml glacial acetic acid and add water to 100 ml. Add 50 µl of 37% formaldehyde. Prepare fresh before use.

CAUTION: Glacial acetic acid and methanol are volatile and toxic. Formaldehyde is toxic and carcinogenic. Wear gloves and prepare the solution in a chemical fume hood.

IEF Coomassie blue stock solution

Dissolve 0.2% (w/v) Coomassie brilliant blue R-250 and 60% (v/v) methanol in water. Store for up to 4 months at room temperature.

Silver nitrate solution

Dissolve 0.2 g AgNO₃ in 100 ml water. Add 75 µl of 37% formaldehyde. Protect the solution from light. Prepare fresh before use.

CAUTION: *Formaldehyde is toxic and carcinogenic. Wear gloves and handle the concentrated (37%) solution in a chemical fume hood.*

Discard the solution if it becomes cloudy.

Thiosulfate solution

Dissolve 20 mg Na₂SO₄·5H₂O in 100 ml water. Prepare fresh before use.

COMMENTARY

Background Information

The first protocols developed for the visualization of protein bands after electrophoresis in polyacrylamide or other gel matrices relied on the use of dyes with selective avidity for polypeptides. Among these dyes, which included amido black 10B and fast green FCF, the Coomassie blue dyes gave higher sensitivity (Wilson, 1979) and became routinely used. A large number of staining protocols using Coomassie brilliant blue R-250 as a dye have been published (see Neuhoff et al., 1985, and references therein). The protocol described in this unit (Basic Protocol 1) involves simultaneous protein fixation and staining in a solution containing the dye in methanol/acetic acid/water, followed by removal of the unbound dye by washing with methanol/acetic acid/water. This is the most commonly used protocol for protein staining in gels. Proposed variations to this procedure include using no methanol to retain small polypeptides (Schägger and von Jagow, 1987), decreasing the concentrations of both methanol and acetic acid to limit protein fixation (Rosenfeld et al., 1992), and using ion-pairing agents to reduce background (Choi et al., 1996). Protocols using a colloidal form of Coomassie brilliant blue G-250, which rival in sensitivity those using Coomassie brilliant blue R-250, have also been described (see for example: Neuhoff et al., 1985; DeSilva, 1995).

A dramatic increase in sensitivity was achieved with the introduction of protein staining methods based on the selective reduction of silver ions to form metallic silver images (Switzer et al., 1979). The method relies on the autocatalytic reduction of silver, a key phenomenon of the photographic process, and differences in reduction potential of silver at the

sites occupied by macromolecules relative to that at the rest of the gel matrix. The differences in reduction potentials can be manipulated to obtain either a positive or negative image of protein bands. Positive silver staining protocols comprise the following stages: (1) fixation of proteins and elimination of interfering substances (e.g., amino acids, Tris, SDS), (2) sensitization with an agent to increase sensitivity and/or contrast, (3) impregnation with silver solution (silver nitrate, basic silver ammonia or silver-diamine complex), (4) controlled rinse to remove silver ions not associated with proteins, (5) image development, and (6) image development stopping (reviewed by Rabilloud et al., 1994). The procedure described in Basic Protocol 2 (Blum et al., 1987) involves sensitization with thiosulfate, impregnation with silver nitrate, and image development using a dilute formaldehyde solution at high pH. Small amounts of thiosulfate included in the developer solution help to keep the background low by complexing silver ions that otherwise could form spurious silver deposits in the gel matrix. This procedure is highly sensitive and compatible with virtually all types of PAGE systems. Unlike other silver staining protocols, which yield a mixture of positive and negative bands or different band colors, the images of the protein bands are rather uniform. A drawback of this and other silver staining methods is that they can also stain other macromolecules, namely DNA, RNA and bacterial lipopolysaccharides.

Procedures for the fluorescent staining of proteins in polyacrylamide gels include covalent modification with fluorescamine or fluorescein isothiocyanate, and noncovalent staining with the hydrophobic probes 1-anilino-naphthalene-8-sulfonic acid or Nile red (see

Steinberg et al., 1996, and references therein). A family of fluorescent protein stain reagents, including SYPRO Orange, SYPRO Red, and SYPRO Ruby, has recently been introduced and reported to be highly sensitive and protein specific (Steinberg et al., 1996). These reagents detect protein-SDS complexes, rather than protein functional groups, and are compatible with most downstream applications. Known interferences are colored stains or prosthetic groups, Triton X-100, and the excess of SDS at the migration front. A simple protocol for protein staining with SYPRO Ruby, which is commercially available and said to be the most sensitive dye within the SYPRO family, is described in Basic Protocol 3.

A different group of protein staining methods for SDS-PAGE are based on the reversible formation of SDS precipitates, thus allowing visualization of protein bands as clear areas on an opaque background. Although SDS precipitation can be simply achieved by lowering the temperature of the gel to 0° to 4°C for a few hours, faster and more sensitive methods involve the formation of insoluble complexes with heavy metal salts such as copper (Garfin, 1990) or zinc (Fernández-Patron et al., 1995a). A protocol for rapid, reversible staining with zinc is described in Basic Protocol 4. The protocol uses a kit that is commercially available at a reasonable price. If cost is a major issue, however, the reader is referred to the protocol described by Fernández-Patron et al. (1995a).

Additional staining methods not discussed in this unit include radiolabeling with [³⁵S]thiourea and silver bromide (Wallace and Saluz, 1992), combined Coomassie blue/zinc staining (Fernández-Patron et al., 1995b) and mixed Evans blue/rhodamine-B staining (Na et al., 1994).

Critical Parameters

Comparison of staining procedures

None of the staining methods is optimal for all types of applications. The choice of the most appropriate method for a particular experiment will depend on a number of considerations, including: sensitivity, simplicity, duration, long-term documentation, quantitation, and downstream applications. The following guidelines are restricted to the procedures described in this unit, namely staining with Coomassie blue (Basic Protocol 1 and Alternate Protocol 1), silver (Basic Protocol 2), SYPRO Ruby (Basic Protocol 3) and zinc (Basic Protocol 4).

Sensitivity

Figure 6.6.1 shows a comparative experiment in which serial dilutions of protein standards were run on SDS-PAGE (UNIT 6.1) and subsequently stained following the four Basic Protocols described in this unit. Among the four methods, silver (Fig. 6.6.1B) and Coomassie blue staining (Fig. 6.6.1A) gave the highest and lowest sensitivity, respectively. The detection limit of fluorescent staining with SYPRO Ruby could be greatly enhanced by the integration effect of a CCD or photographic camera. Thus, while by direct observation (on a UV transillumination unit) SYPRO Ruby staining was significantly less sensitive than negative zinc staining, both methods gave comparable sensitivities upon the use of a CCD camera to integrate the signal of SYPRO Ruby-stained bands (Fig. 6.6.1C and D).

Simplicity

While staining with Coomassie blue, SYPRO Ruby, or zinc are relatively simple methods, silver staining involves a number of steps that need to be carefully controlled, most notably sensitization with thiosulfate (Basic Protocol 2, step 6), rinsing with water before and after silver impregnation (Basic Protocol 2, steps 7 to 8 and 10 to 11, respectively) and image development (Basic Protocol 2, step 13). In fact, the duration of the image development step critically influences the sensitivity of silver staining, thus resulting in experiment-to-experiment variations in band color intensity. Staining with SYPRO Ruby requires a UV transilluminator to visualize the protein bands and the careful disposal of this potentially toxic dye (see Basic Protocol 3).

Documentation

Gels stained with Coomassie blue, silver, or zinc can be stored at 4°C for several weeks without significant decrease in sensitivity. For long-term storage, gels stained with Coomassie blue, silver, or SYPRO Ruby can be equilibrated in 2% (w/v) glycerol and dried on a gel dryer, although in the case of SYPRO Ruby-stained gels this can result in decreased sensitivity. For photography or digital image acquisition, the blue bands of Coomassie blue-stained gels have less contrast than the dark brown/black bands of silver-stained gels, and SYPRO Ruby-stained gels require the use of an appropriate photographic film (e.g., Polaroid 667 black and white film) or a sensitive CCD camera. Negatively zinc-stained gels can

be problematic for photography or imaging on a CCD camera.

Quantitative analysis

Scanning densitometric analyses of protein bands from stained polyacrylamide gels have been used to determine the relative abundance of proteins in complex mixtures, the purity of protein samples, and even the stoichiometry of multisubunit protein complexes. These quantitative analyses are performed assuming identical staining properties of the different proteins

in the sample (i.e., that their staining results in the same band intensity per protein mass unit). This assumption is not necessarily correct, especially for Coomassie blue or silver staining where band intensities are known to be influenced by the proteins' amino acid compositions. Another limitation is that measurements should be made only within a linear range. Staining with SYPRO dyes, which interact with the protein-SDS complex rather than protein functional groups, has been reported to give relatively less protein-to-protein variability and

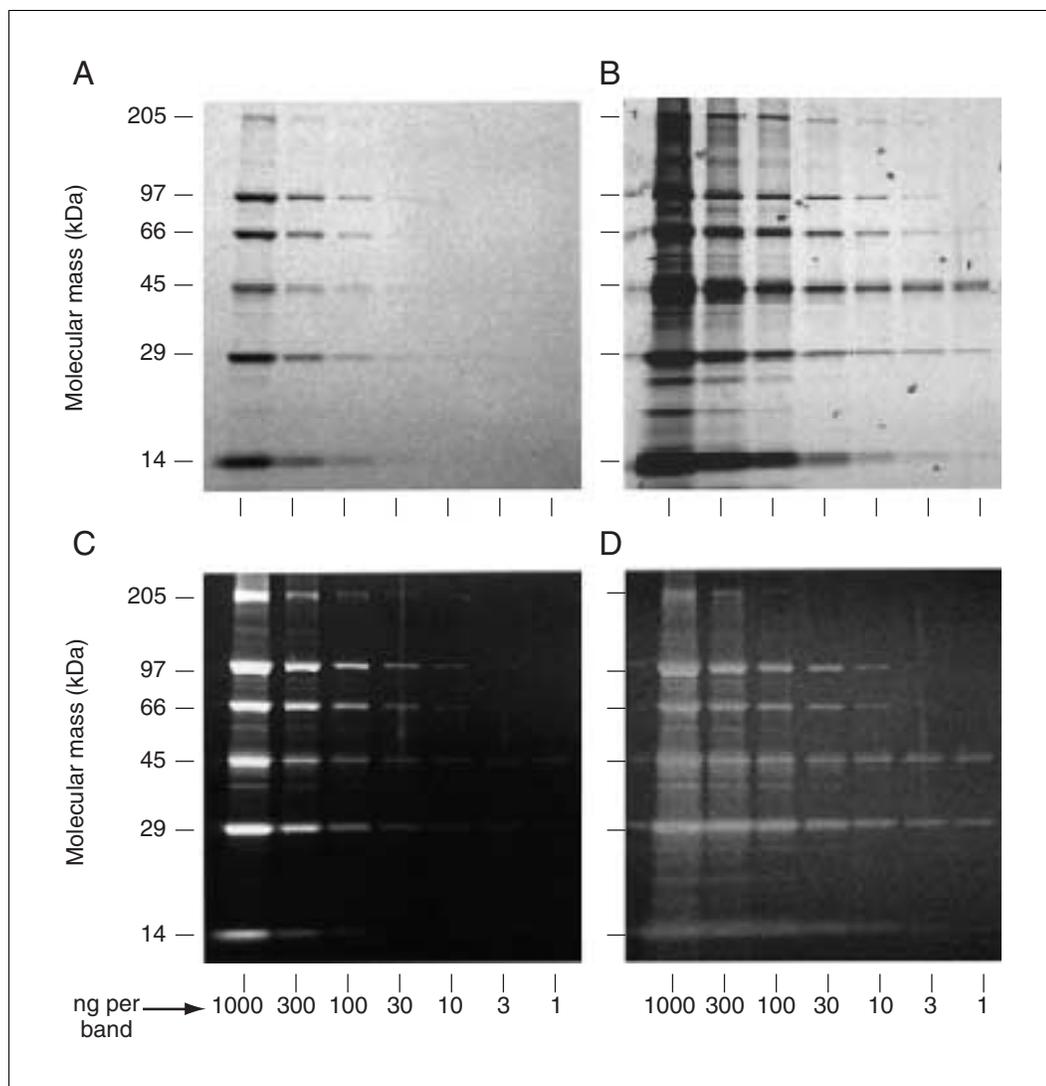


Figure 6.6.1 Comparison of the protein staining methods described in this unit. Serial dilutions of a mixture of rabbit muscle myosin (~205 kDa), rabbit muscle phosphorylase b (~97 kDa), bovine serum albumin (~66 kDa), chicken ovalbumin (~45 kDa), bovine erythrocyte carbonic anhydrase (~29 kDa) and chicken egg lysozyme (~14 kDa) were resolved by SDS-PAGE on a 4% to 20% T gradient gel and stained with: (A) Coomassie blue (Basic Protocol 1), (B) silver (Basic Protocol 2), (C) SYPRO Ruby (Basic Protocol 3), or (D) zinc (Basic Protocol 4). The amounts (in nanograms) per band loaded on each lane are indicated at the bottom of the figure. Images of stained gels were acquired on a Bio-Rad Gel Doc 1000 gel documentation system, using a standard UV (300 nm) transilluminator with (A, B, D) or without (C) a UV/white light conversion screen (Bio-Rad). The image shown in (C) was acquired using an integration time of 0.6 sec in order to optimize sensitivity.

Table 6.6.1 Troubleshooting Guide for Staining with Coomassie Blue (Basic Protocol 1)

Problem	Cause	Solution
<i>No bands are detected</i>		
Background is completely clear	Amount of protein(s) below detection limit	Check protein concentration in original sample. Stain the gel with silver (Basic Protocol 2).
Background is dark blue	Insufficient destaining	Continue to destain the gel until the background is clear (steps 4 to 6).
<i>Protein bands are too faint</i>		
	Staining solution is too old (i.e., methanol has evaporated)	Prepare new Coomassie blue staining solution.
	Insufficient staining time	Re-stain the gel using a longer staining time (step 2)
	Excessive destaining	Re-stain the gel monitoring band and background color during destaining (steps 4 to 6)
<i>High background areas</i>		
High background restricted to lanes with samples	Interfering compounds in the samples	Fix proteins with TCA before staining (see Alternate Protocol 1, steps 2 to 5).
Blue spots at the gel surface	Powder or dirt deposited on the surface during handling of the gel	Use clean, powder-free gloves. Remove powder or dirt by gently touching the surface with a powder-free glove.

Table 6.6.2 Troubleshooting Guide for Staining with Silver (Basic Protocol 2)

Problem	Cause	Solution
<i>Protein bands are absent or too faint</i>		
Background is clear	Amount of protein(s) below detection limit	Check protein concentration in original sample.
	Insufficient image development	Re-stain the gel extending the image development step (step 13) until background begins to get too dark.
	Formaldehyde is too old (white polymers in concentrated solution)	Use formaldehyde from a fresh concentrated solution.
	Silver nitrate solution is not fresh	Prepare fresh silver nitrate solution just before use. Discard the solution if it becomes cloudy.
Background is too dark	Gel has been overdeveloped	Monitor image development (step 13) continuously. Proceed to the next step as soon as the staining is considered satisfactory.
	Poor quality of reagents	Use reagents of the highest purity available. Prepare the solutions using HPLC-grade water.
Band images and background color develop too fast	Concentration of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) in developer and thiosulfate solutions is not optimal	Increase sodium thiosulfate concentration in both solutions (e.g., 2- to 3-fold). Do not reuse the solutions.
Metallic silver is deposited on the gel surface	Powder or dirt deposited on the surface during gel handling	Handle the gels with clean forceps or gloves that have been rinsed with water.
	Metallic silver present in the silver nitrate solution	Protect the solution from light. Discard the solution if it gets cloudy.

Table 6.6.3 Troubleshooting Guide for Fluorescent Staining (Basic Protocol 3)

Problem	Cause	Solution
<i>Protein bands are absent or too faint</i>		
Clear background	Amount of protein(s) below detection limit	Check protein concentration in original sample. Stain the gel with silver (Basic Protocol 2).
	Direct eye visualization is not sensitive enough	Visualize the bands by using a CCD camera or by photography.
	Photographic or imaging conditions are not appropriate	Use sensitive photographic films (Polaroid 667 black-and-white or similar film) or appropriate CCD camera. Increase exposure (integration) time.
	Stain solution too old or inactivated by light	Use fresh stain solution. Protect it from the light. Do not reuse it.
Highly fluorescent background	Protein(s) of interest pre-stained or containing colored prosthetic groups	If possible, remove prosthetic group(s) from proteins and avoid using pre-stained proteins. Otherwise, use an alternative staining method (e.g., Basic Protocol 2 or Basic Protocol 4).
	Too much unbound dye remains in the gel matrix	Rinse the gel further with water (i.e., repeat steps 4 and 5).
	Gel is placed for visualization on an autofluorescent plastic surface (e.g., Saran Wrap)	Place the gel directly onto the surface of the UV transillumination unit.
Heavy stain at the SDS-PAGE migration front	Gel is attached to a polyester surface with affinity for the dye (e.g., backing material of PhastGels)	Remove the gel from the polyester surface before visualization.
	SYPRO dyes bind micellar SDS at the migration front	Allow the SDS front to migrate out of the gel.
Odd marks at the gel surface	Portions of the gel squeezed or have powder deposits due to handling	Handle the gel gently, wearing gloves that have been rinsed with water.

Table 6.6.4 Troubleshooting Guide for Staining with Zinc (Basic Protocol 4)

Problem	Cause	Solution
Protein bands are absent or too faint	Insufficient contrast due to the use of clear surfaces for visualization	Visualize the bands by placing the gel against a dark surface.
	Gel is overdeveloped	Destain the gel (steps 9 and 10) and stain again monitoring the appearance of bands against a dark surface.
Blackground does not turn opaque	Amount of protein(s) below detection limit	Check protein concentration in original sample. Stain the gel with silver (Basic Protocol 2).
	Stain solution or developer are too old	Use fresh stain and developer solutions (do not reuse them).

longer linear ranges than the other staining methods (Steinberg et al., 1996). Nevertheless, in order to check the validity of the measurements it is always advisable to load in the gel, together with the sample(s) to be quantitated, internal controls or a calibration curve with standard protein(s).

Downstream applications

Although PAGE was initially used only for analytical purposes, a large variety of micro-techniques have been developed that use PAGE as a preparative step. Proteins separated by one- or two-dimensional PAGE can be treated in-gel with proteases (Rosenfeld et al., 1992) or cyanogen bromide (Córdoba et al., 1997) to obtain peptide fragments for microsequencing, electroeluted, or transferred to nitrocellulose or poly(vinylidene difluoride) (PVDF) membranes (UNIT 6.2) for further analysis such as immunodetection or Edman degradation. Proteins separated by nondenaturing PAGE (UNIT 6.5), or sometimes even by SDS-PAGE, can be analyzed in-gel for enzymatic or ligand-binding activity. Both the SYPRO Ruby and negative zinc staining methods are compatible with virtually all of these applications. A notable exception is activity analysis of proteins separated by nondenaturing PAGE which are sensitive to SDS, as both staining procedures require the presence of SDS for band visualization. On the other hand, Coomassie blue and silver gel staining involve protein fixation and, therefore, are compatible with fewer downstream applications. It is worth mentioning, however, that Coomassie-stained proteins can be efficiently electrotransferred to PVDF membranes for Edman degradation sequencing (U. Hellman, pers. commun.), although for immunoblot analysis the Coomassie blue dye is a known interference. Coomassie blue staining has been widely used to visualize protein bands destined for in-gel generation of peptides for microsequencing, mainly because these downstream applications have not been sensitive enough to allow analysis of protein bands below the detection limit of the dye. Recent methodological improvements, however, have made it possible to obtain sequence information from as little as 5 ng of protein from polyacrylamide gels stained with silver (Wilm et al., 1996).

Troubleshooting

Tables 6.6.1 to 6.6.4 summarize common problems that may arise during staining of proteins in polyacrylamide gels. Before using any of the methods to analyze a “real” sample,

it is advisable to test the method first on a gel containing known amounts of standard proteins (e.g., see Fig. 6.6.1).

Anticipated Results

The protocols described in this unit should allow detection of submicrogram amounts of protein separated by PAGE as exemplified by Figure 6.6.1.

Time Considerations

By far, negative staining with zinc is the fastest method among the ones described in this unit. Images can be obtained within 20 min after the polyacrylamide gel is removed from the electrophoresis unit. Silver staining requires 30 min for fixation and another ~2.5 hr to complete the procedure. Both the Coomassie blue and SYPRO Ruby procedures require ~3.5 hr for obtaining optimal protein-to-background staining ratio. Suboptimal staining with SYPRO Ruby can be obtained by incubating the gel with stain solution for 60 to 90 min and skipping the subsequent washing steps (Basic Protocol 3, steps 4 and 5). In the case of Coomassie blue staining, binding of the dye to proteins is a fast process that depends mainly on its rate of diffusion into the gel matrix (Hitchman and Ekstrom, 1994). Removal of the unbound Coomassie dye from the gel, which also relies on diffusion, can be accelerated by placing an adsorbent material with affinity for the dye (e.g., Whatman 3MM filter paper) into the destaining solution with the gel or by increasing the temperature of destaining (e.g., to 37°C). In both cases, however, caution should be taken to avoid excessive destaining that could decrease sensitivity.

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Agarose Gel Electrophoresis of Proteins

UNIT 6.7

This unit describes the use of agarose gel as a matrix for the electrophoresis of proteins. Although agarose is widely used as a material for molecular sieving, it is not often used for the electrophoresis of proteins. When it is used for this purpose, it is generally employed for the electrophoresis of very large proteins. In other electrophoresis procedures it is part of a composite polyacrylamide/agarose system. The system described below utilizes agarose alone as the gel matrix. As with acrylamide gels, the proteins thus separated may be transferred to a membrane (immunoblotting) for further analysis, or the proteins may be identified directly in the gel using stains or labeled antibodies. A particular advantage of the method described in the Basic Protocol is the preparation of the gel in a horizontal electrophoresis bed which saves time and eliminates many of the difficulties commonly encountered in the preparation of acrylamide or composite gels in a vertical preparation apparatus.

These protocols describe methods for the separation and identification of von Willebrand factor (vWF), an extremely large plasma protein that is comprised of multimers ranging from 850,000 to 20,000,000 Da. The methods can be applied to other mixtures containing large proteins, multimeric proteins, and other large protein complexes such as fibrinogen and fibrin complexes (Shainoff, 1991). While the use of an SDS buffer system allows separation of proteins on the basis of size, nondenatured proteins can be separated in “native” agarose gels if their charge and configuration allow for satisfactory partitioning. Von Willebrand factor multimers are stable in SDS due to their disulfide linkages, and the protocols below utilize SDS. The agarose gel electrophoresis and blotting with immunodetection procedure (see Basic Protocol) utilizes identification of protein by a specific antibody followed by chemiluminescent detection methods. Major advantages of this method include the technical ease of preparing the gel, increased sensitivity, and a much shorter turn-around time than in-gel antibody analysis (see Alternate Protocol). It also eliminates the use of radioactive isotopes. In addition, the primary and secondary antibodies can be easily removed and the membrane (PVDF) probed again with a different detection antibody. This allows the laboratory the opportunity to detect a second protein or antigenic site on the same protein and also a “second chance” to correct an omission in the procedure, especially for samples in limited supply (UNIT 6.2); however, the immunoblotting step of such large proteins must be done with care in order to assure the adequate transfer of the largest proteins. The Alternate Protocol uses direct identification of the protein in the gel by a specific radiolabeled antibody, eliminating the immunoblotting step, but sacrificing sensitivity.

AGAROSE GEL ELECTROPHORESIS AND BLOTTING WITH IMMUNODETECTION

The following is a method for separating a complex mixture of human plasma proteins by continuous SDS horizontal (submerged) agarose gel electrophoresis. The very large multimers of circulating plasma von Willebrand factor (or more highly purified preparations of this protein) are identified using a specific antibody and visualized using a chemiluminescent reagent. This protocol utilizes a 20 × 25-cm horizontal gel apparatus. Dry agarose is weighed, mixed with electrophoresis buffer, and melted in a hot water bath. The agarose is allowed to partially cool, then is poured into a horizontal casting frame with a Teflon comb in place, and allowed to solidify. The gel is covered with 1 to 2 mm cold electrophoresis buffer and the comb is carefully removed. Prepared samples containing the proteins of interest are diluted with sample buffer and loaded into wells. Electrophoresis is carried out for 3 to 6 hours at 4°C. The gel is placed into a vertical tank transfer

**BASIC
PROTOCOL**

**Electrophoresis
and
Immunoblotting**

6.7.1

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Supplement 15

apparatus (UNIT 6.2), and the proteins are transferred overnight onto an immobilization matrix (PVDF membrane). The membrane is blocked and probed with a polyclonal primary antibody specific for the antigen of interest and, after washing, a conjugated secondary antibody is introduced. After incubation the membrane is washed again, and the protein bands of interest are illuminated with a chemiluminescent detection system.

Materials

SeaKem HGT(P) agarose (Bio Whittaker or equivalent)
1× electrophoresis buffer, 4°C (see recipe)
Protein samples
2× sample buffer (see recipe)
0.25× transfer buffer without methanol (see recipe)
Blocking buffer (UNIT 6.2) containing 5% (w/v) nonfat dry milk, fresh
Antibodies:
 Primary: rabbit anti-vWF (Dako)
 Secondary: donkey horseradish peroxidase–linked anti-rabbit Ig (Amersham Pharmacia)
ECL Western Blotting Analysis system (Amersham Pharmacia)
Aluminum foil
Boiling waterbath (optional)
Horizon 20-25 horizontal electrophoresis apparatus (Life Technologies) or equivalent
Teflon comb (e.g., 1 × 9–mm, 20-well)
Pipet with fine tip or equivalent
0.45- μ m Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore)
Additional reagents and equipment for protein transfer to membranes and immunoblotting (UNIT 6.2)

Cast agarose gels

1. Weigh 1.2 g SeaKem HGT(P) agarose and transfer to a 250-ml flask containing 200 ml of 1× electrophoresis buffer. Add a Teflon-coated magnetic stir bar and tightly cover the flask with aluminum foil.
2. Heat in a boiling water bath, with slow mixing to avoid bubbles, until clear. Alternatively, microwave until boiling

CAUTION: Whenever a solution is heated in a microwave the chance of superheating (“boil up”) is always present. Protective gloves, gown, and eyewear should be worn at all times.

3. Assemble Horizon 20-25 horizontal electrophoresis apparatus or equivalent according to manufacturer’s recommendations.

The wedge shaped casting dams of the Horizon (Life Technologies) horizontal apparatus are easily placed in backwards, resulting in leaking of liquid agarose. Care should be exercised so that the dams form a perpendicular angle with the UVT tray.

4. Cool agarose to 55° to 60°C and pour the molten agarose into the electrophoresis apparatus to a depth of 4 mm.

It is often helpful to prewarm the electrophoresis apparatus with warm water or in an oven to avoid cooling the agarose and causing the agarose to solidify unevenly.

5. Carefully insert the desired (e.g., 1 × 9–mm, 20-well) Teflon comb with care to avoid bubbles.

Removing the comb while the agarose is still in a molten state and then reinserting often eliminates the formation of bubbles under the teeth of the comb.

6. After the agarose has solidified, place the apparatus at 4°C and allow the agarose to age 20 to 30 minutes.

The electrophoresis is to be carried out at 4°C. The apparatus may either be placed in a cold room, the electrophoresis buffer can be circulated through a refrigeration unit, or the apparatus can be packed in wet ice.

7. Overlay the solidified gel with 2 to 3 mm electrophoresis buffer, 4°C.
8. Remove the sample comb by lifting vertically in one smooth motion.

It is helpful to hold the gel down with the gloved fingers of the other hand to keep the gel from being pulled up when the comb is removed.

Prepare and load sample

9. Dilute the protein samples to twice the desired concentration using distilled water. Immediately add the diluted sample to an equal volume of 2× sample buffer.
10. Load a sample volume of 10 to 15 µl into the bottom of each sample well using a pipet with a fine tip or equivalent.

Prior to sample loading examine each sample well to ascertain that air bubbles are not trapped in the well.

Electrophorese gel (also see UNIT 6.1)

11. Run samples into the gel matrix at a constant current of 25 mA for ~30 min or until the sample dye has completely entered the gel.
12. Pause the electrophoresis and decrease the level of the electrophoresis buffer to ~1 mm above the upper surface of the gel. Increase the constant current to 50 mA and run for an additional 3 to 4 hr, or until the marker dye has migrated at least 6 to 7 cm.

Blotting the gel

13. Perform immunoblotting to a 0.45-µm Immobilon-P polyvinylidene fluoride (PVDF) membrane in a tank transfer system as described (UNIT 6.2), with the exception of the following conditions:
 - a. Electrophoretically transfer at a constant current of 100 mA at 4°C overnight.
 - b. Use 0.25× transfer buffer without methanol.
 - c. Use blocking buffer containing 5% (w/v) nonfat dry milk.

Immunodetect protein

14. Perform immunoprobng with directly conjugated secondary antibody as described (UNIT 6.2), except with the following variations which are specific for immunodetection of von Willebrand Factor protein:
 - a. Dilute primary antibody, rabbit anti-vWF, to a concentration of 1:4000.
 - b. Dilute secondary antibody, donkey horseradish peroxidase–linked anti-rabbit Ig, to a concentration of 1:2000.

Visualization

15. Visualize von Willebrand factor protein on the PVDF membrane by meticulously following the recommendations enclosed in the ECL Western Blotting Analysis system.

Visualization with Luminescent Substrates is discussed in UNIT 6.2. To determine the optimal concentration of antibody, run a preliminary gel followed by immunoblotting. Cut the blot into several vertical test strips, each containing 1 to 2 lanes. Use the supplier's recommended concentration of antibody as a starting point and process the test strips with 3- to 10- to 30-fold increased and decreased antibody concentrations in separate containers.

ALTERNATE PROTOCOL

AGAROSE GEL ELECTROPHORESIS WITH IN-GEL ANTIBODY ANALYSIS

This alternative protocol describes a method for separating large plasma proteins using SDS agarose electrophoresis and visualizing the protein of interest directly in the gel with a ^{125}I -radiolabeled rabbit antibody, in this case, to human von Willebrand factor protein, and autoradiography (see also *UNIT 6.3*). The methodology applied to agarose electrophoresis of von Willebrand factor is outlined below. The gel preparation consists of mating two glass plates on a horizontal surface separated by a 0.5-mm spacer. A sheet of GelBond support film is fixed to the glass plate to provide support for the agarose. Agarose at a concentration of 1.35% (w/v) is poured between the glass plate and the spacer plate. After the gel is solidified, the apparatus is disassembled and 1.0×0.1 -cm wells are punched into the gel. The gel is placed into a horizontal electrophoresis chamber, presoaked wicks are attached to the gel, and sample volumes of 8 μl are loaded. The prepared samples are electrophoresed until the sample dye has migrated 8 to 10 cm. The gel is immediately fixed with isopropanol/glacial acetic acid fixing solution. After the gel is washed, it is blocked with ethanolamine/BSA. The gel, after a second series of washes, is incubated with ^{125}I -labeled anti-vWF antibody for 10 to 24 hours at room temperature. After extensive washing and drying the gel is placed in a cassette with film and exposed 1 to 5 days.

Materials

- In-gel sample buffer, fresh (see recipe)
- Borate saline buffer (BSB; see recipe)
- Sample
- 0.5% (w/v) bromphenol blue in H_2O
- Isopropanol
- Agarose gel buffer (see recipe)
- SeaKem HGT(P) agarose (FMC/BioWhittaker Molecular)
- Agarose running buffer (see recipe)
- Fixing buffer (see recipe)
- Blocking buffer, in-gel (see recipe)
- ^{125}I -labeled rabbit anti-human vWF polyclonal antibody (Dako #A0082):
radiolabel using protocol of choice and immunopurify (Hoyer and Shainoff,
1980; also see *UNIT 7.10*)
- 2% (w/v) human IgG (see recipe)
- High-salt wash buffer (see recipe)
- 12×75 -mm polypropylene tube
- $12.5 \times 26.0 \times 0.3$ -cm glass plate (Amersham Pharmacia Biotech)
- 12.5×24.0 -cm spacer plate with adherent 0.5-mm spacers (Amersham Pharmacia
Biotech)
- 12.4×25.8 -cm GelBond film (Amersham Pharmacia Biotech)
- Flexiclamps (Amersham Pharmacia Biotech)
- 20-ml syringe
- 60°C oven
- Aluminum foil
- Gelman Delux electrophoresis chamber (Gelman Sciences) or equivalent

104 × 253-mm paper electrophoresis electrode wicks (Amersham Pharmacia Biotech)
Flattened no. 2 cork borer
Forceps, fine
Horizontal rotary mixer
Forced hot-air dryer (optional)
Kodak X-Omatic film cassette with Lanex screens and film

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by your local radiations safety officer (also see *APPENDIX 1D*).

Prepare sample

1. Prepare 3 ml fresh in-gel sample buffer.
2. Label a 12 × 75-mm polypropylene tube for each sample to be assayed. Add 20 μl borate saline buffer (BSB), 120 μl sample buffer, and 10 μl sample into each tube.
3. Cover each tube, vortex gently, and incubate 2 hr at 37°C.
4. Add 8 μl of 0.5% bromphenol blue to each sample and mix gently.

Prepare agarose gel

5. Prepare a boiling water bath by placing approximately 50 ml distilled water in a 250-ml beaker and incubating on a hot plate with magnetic stirring capabilities.
6. Use isopropanol to clean the 12.5 × 26.0 × 0.3-cm glass plate and 12.5 × 24.0-cm spacer plate with its adherent 0.5-mm spacers. Dry with a lint-free tissue (e.g., Kimwipe).

It is advisable, because of the fragile nature of this gel, to prepare sufficient materials to pour a gel in reserve in the eventuality that one is rendered unusable.

7. Place a few milliliters of distilled water on the glass plate and adhere the hydrophilic side of a 12.4 × 25.8-cm GelBond film. Express any trapped air or excess water with a lint-free tissue.
8. Mate the glass plate with the adherent GelBond film and the spacer plate. Clamp with two flexiclamps.

The gel-forming sandwich should consist of the glass plate (the larger of the two plates) with an adherent piece of GelBond film (hydrophilic side to the glass plate) and the spacer plate with its adherent spacer bars placed on top of the GelBond (Fig. 6.7.1).

9. Place the clamped plates and a 20-ml syringe in a 60°C oven for ~10 minutes to equilibrate.
10. Measure 40 ml agarose gel buffer into a 50-ml Erlenmeyer flask and add a Teflon-coated magnetic stir bar. Pour 0.54 g SeaKem HGT(P) agarose into the flask, tightly cover with aluminum foil, and place the flask into the boiling water bath (step 5). Dissolve the agarose with constant stirring. After the solution becomes clear, continue boiling an additional 10 min.
11. Remove the heated glass plate assembly (gel-forming sandwich) and the 20-ml syringe from the oven. Quickly fill the heated syringe with 20 ml hot agarose, and holding the plate assembly at an ~75° angle, fill the narrow space in the plate assembly using a back and forth motion to prevent bubbles from being trapped in the gel.

Use of a needle with the syringe is optional.

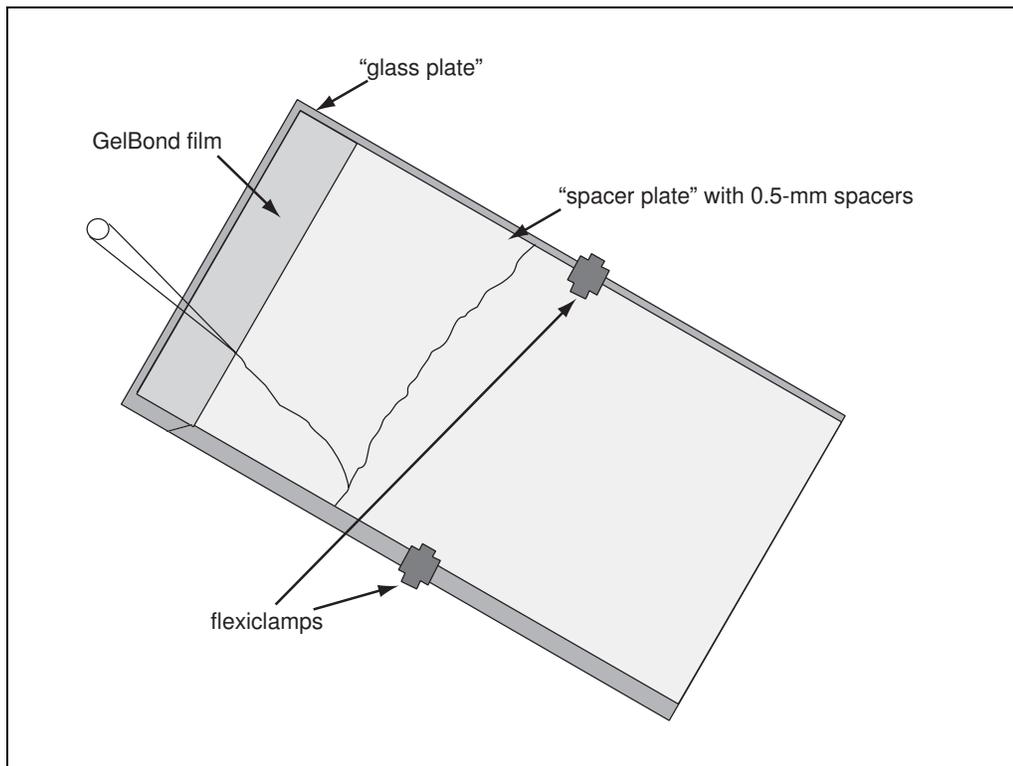


Figure 6.7.1 Diagram of casting gel in the Alternate Protocol. The layers include (back to front): the “glass plate” (the larger of the two plates), GelBond film (hydrophilic side adherent to the glass plate), and the “spacer plate” with attached 0.5-mm spacer bars. The agarose fills the narrow space between the GelBond and the “spacer plate.”

12. After filling, lay the plate assembly flat and allow to cool and solidify ~45 min.

Electrophoresis gel

13. Position the Gelman Delux electrophoresis chamber or equivalent electrophoresis apparatus on a flat surface and fill each electrode chamber with 400 to 450 ml agarose running buffer. Place 104 × 253-mm paper electrophoresis electrode wicks in each chamber to equilibrate in running buffer.
14. Remove the flexiclamps. Insert a thin spatula blade between the spacer plate and the gel attached to the glass plate. Carefully pry upward to separate the spacer plate. Examine the gel against a bright light for bubbles, thin areas, or areas of separation from the GelBond film.

The agarose gel should now be attached to the GelBond support backing and be easily handled.

15. Punch the required number of 0.1 × 10-mm wells using a flattened no. 2 cork borer. Use a fine set of forceps to remove the agarose from the interior of each well.

It is helpful to construct a template to place under the gel to facilitate punching the wells in an evenly spaced straight line. Also, it is necessary to work quickly because these gels are very thin and dry out rapidly.

16. Place a glass plate across the bridge on the electrophoresis chamber and place the gel attached to the GelBond on top of the plate. Affix the presoaked wicks (step 13) on each side of the gel so that there is continuity between the agarose running buffer in the electrode chambers and the gel.

17. Load 8 μ l of sample into each well. Place the cover over the electrophoresis apparatus and connect the power supply.
18. Electrophorese at 25 V for ~30 min at room temperature to allow the samples to enter the gel matrix. Increase the power supply to 50 V and continue electrophoresis until the dye marker has migrated 8 to 10 cm from the wells (~3 to 5 hr).

Fix gel

21. Add 200 ml fixing buffer to a container appropriate to the size of the gel. Carefully remove the paper wicks and place the gel in fixing buffer 1 hr without agitation.

The gel can also fix overnight in fixing buffer for a convenient stopping point.

22. Wash the gel 1 hr with 200 to 300 ml BSB with gentle mixing on a horizontal rotary mixer.

A prerinse ~1 to 2 min before the wash with ~100 ml BSB is recommended

23. Prepare 250 ml fresh in-gel blocking buffer. Block gel 1 hr with gentle mixing.
24. Prerinse gel with ~100 ml BSB. Wash 1 hr with 200 to 300 ml BSB.

Immunodetect protein

25. Prepare a solution of dilute 125 I-labeled rabbit anti-human vWF polyclonal antibody in BSB to a concentration of $\sim 2 \times 10^6$ cpm in a volume sufficient to cover the gel. Add 0.5 ml of 2% human IgG to the antibody solution.

The majority of the gels can be covered with 50 to 70 ml.

In an effort to minimize the volume of radioactive solutions, the authors' laboratory keeps a supply of dedicated plasticware to accommodate a wide variety of gel sizes. Strict laboratory precaution should be exercised in the preparation, handling, and disposal of radioactive materials.

26. Incubate the gel in the antibody solution at least 16 to 24 hr with gentle mixing.
27. Discard the antibody solution, adhering to standard radiation safety waste disposal protocols.
28. Wash the gel 1 hr in 200 ml high-salt wash buffer with gentle mixing using a horizontal rotary mixer.
29. Repeat the wash an additional three to four times.

The washes should be monitored and continued until all excess 125 I is removed.

30. Wash 1 hr in 200 ml distilled water. Repeat once.
31. Dry the gel with a forced hot-air dryer directed on the gel, or air dry the gel.

Autoradiograph the gel

32. Place the gel in a Kodak X-Omatic film cassette with a Lanex screen. Under dark room conditions, place a piece of Kodak X-Omat film on the gel and then incubate the cassette at -70°C . Expose for an appropriate amount of time.

Times vary from 1 to 5 days. It is helpful to secure the gel to the cassette with tape to keep it from changing position.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.

Agarose gel buffer

0.05 M Na₂HPO₄

0.1% (w/v) SDS

Adjust pH to 7.0 with concentrated HCl

Filter and store up to 3 months at room temperature

Agarose running buffer

0.1 M Na₂HPO₄

0.1% (w/v) SDS

Adjust pH to 7.0 with concentrated HCl

Store up to 3 months at room temperature

Blocking buffer, in-gel

15 ml 16.6 M ethanolamine (1.0 M)

250 mg fatty-acid-free Fraction V BSA

Add H₂O to ~200 ml

Adjust pH to 8.0 with concentrated HCl

Add H₂O to 250 ml

Prepare fresh on the day of use

Borate saline buffer (BSB)

15.4 g boric acid (36 mM)

65.06 g NaCl (143 mM)

1.40 g NaOH (0.005 N)

Add 1.56 ml concentrated HCl to adjust pH to 7.83

Adjust volume to 7 liters with H₂O

Store up to 3 months at room temperature

Electrophoresis buffer, 1×

Dilute 10× TAE (see recipe) 1/10 in water. Add 10 ml of 20% (w/v) SDS (**APPENDIX 2A**) per 2 liters. Final concentrations are 40 mM Tris-acetate, 1 mM EDTA, and 0.1% (w/v) SDS. Final pH is 7.8 to 8.3. Store up to 1 week at room temperature.

The solution is also referred to as 1× TAE-SDS. Two liters are required for the application.

Fixing buffer

50 ml isopropanol (25%)

20 ml glacial acetic acid (10%)

130 ml H₂O

Prepare fresh on the day of use

High-salt wash buffer

56.78 g Na₂HPO₄ (0.1 M final)

233.6 g NaCl (1 M final)

Adjust pH to 7.0 with concentrated HCl

Add H₂O to 4 liters

Store up to 3 months at room temperature

Human IgG, 2%

2.0 g human IgG in 100 ml BSB (see recipe)
Store in aliquots up to 1 year at -20°C

In-gel sample buffer*Stock solution*

0.01M Na_2HPO_4
Adjust pH to 7.0 with HCl
Filter and store up to 3 months at room temperature

Working solution

To 3 ml stock solution add:
20.64 mg iodoacetamide
37.5 mg SDS
Prepare fresh

Sample buffer, 2×

20 ml 10× TAE (see recipe)
1 ml 20% (w/v) SDS (APPENDIX 2A)
20 ml glycerol
0.2 g bromphenol blue
Add H_2O to 200 ml
Store up to 1 year at room temperature

TAE, 10×

48.4 g Tris base (400 mM)
20 ml 0.5M EDTA (10 mM; APPENDIX 2A)
Adjust pH to 7.8 with glacial acetic acid
Adjust volume to 1 liter
Store up to 1 year at room temperature

Transfer buffer without methanol, 0.25× and 10×*For a 10× solution*

250 mM Tris·Cl, pH 8.3 (APPENDIX 2A)
1.92 M glycine
1.0% (w/v) SDS
Store up to 1 year at room temperature

This is the stock solution for transfer buffer and is also known as Tris/glycine/SDS (TG/SDS).

For a 0.25× solution

Dilute 10× transfer buffer 1/40 in distilled water. Final concentrations are 6.25 mM Tris·Cl, 48 mM glycine, and 0.025% (w/v) SDS. Final pH is 8.3. Store up to 1 week at room temperature.

Four liters are required for the application.

Table 6.7.1 Troubleshooting Guide for Agarose Electrophoresis and Immunoblotting

Problem	Possible cause	Solution
<i>Agarose electrophoresis</i>		
Run time too long or too short	Buffer concentration too high or too low Voltage too high or too low	Verify buffer preparation Review and increase or decrease voltage or current settings
Band spreads into other lanes	Sample diffusing out of well or into surrounding gel	Minimize time for sample loading and start electrophoresis promptly
Samples leak underneath gel	Bottom of well torn when removing comb	Remove comb slowly
Bands migrate at different rates (visualized as “smiles,” “frowns,” and “sneers”)	Gel cast unevenly Uneven heat distribution	Use level to verify that the apparatus is level Decrease power settings. Cool buffer to 4°C. Circulate buffer.
Bands not in straight lines (visualized as a “wiggle”)	Artifacts in wells	Flush wells with electrophoresis buffer Inspect wells for trapped air bubbles
Bromphenol turns yellow	pH change during electrophoresis	Verify pH of buffer Circulate buffer during run
<i>Immunoblotting</i>		
High background	Insufficient blocking	Increase concentration and/or time of blocking step
	Overdevelopment	Remove membrane from substrate after 1 min
	Protein contamination	Wash or replace fiber pads and clean apparatus
	Incomplete washing	Increase wash time and volume Review recipe, especially Tween 20
	Primary or secondary antibody too concentrated	Review supplier’s recommendations Run test strips to optimize reactions
Weak signal or no reaction	Sample load insufficient	Increase amount of sample
	Low antibody specificity	Increase antibody concentration
	Antigen not transferred	Increase transfer time. Stain membrane for protein transfer.
	Conjugate not active	Mix more thoroughly

COMMENTARY

Background Information

The major use of agarose in protein analysis is its application as a matrix for molecular sieving; however, it is also widely used as a material that can be modified to form an affinity matrix for affinity chromatography. Because of its ability to separate proteins of very large size, it is also utilized for electrophoresis and preparation of very large proteins, ranging from several million to approximately fifty-thousand daltons. Agarose has the advantage of being

nontoxic. In addition, it may be melted to allow recovery and further studies of the separated protein, and an excised band may be directly injected into an animal for immunization. Also, the use of native gels allows separation and recovery of nondenatured proteins for functional studies.

The electrophoresis procedure outlined above illustrates the ability of agarose to separate proteins of molecular weights that exceed

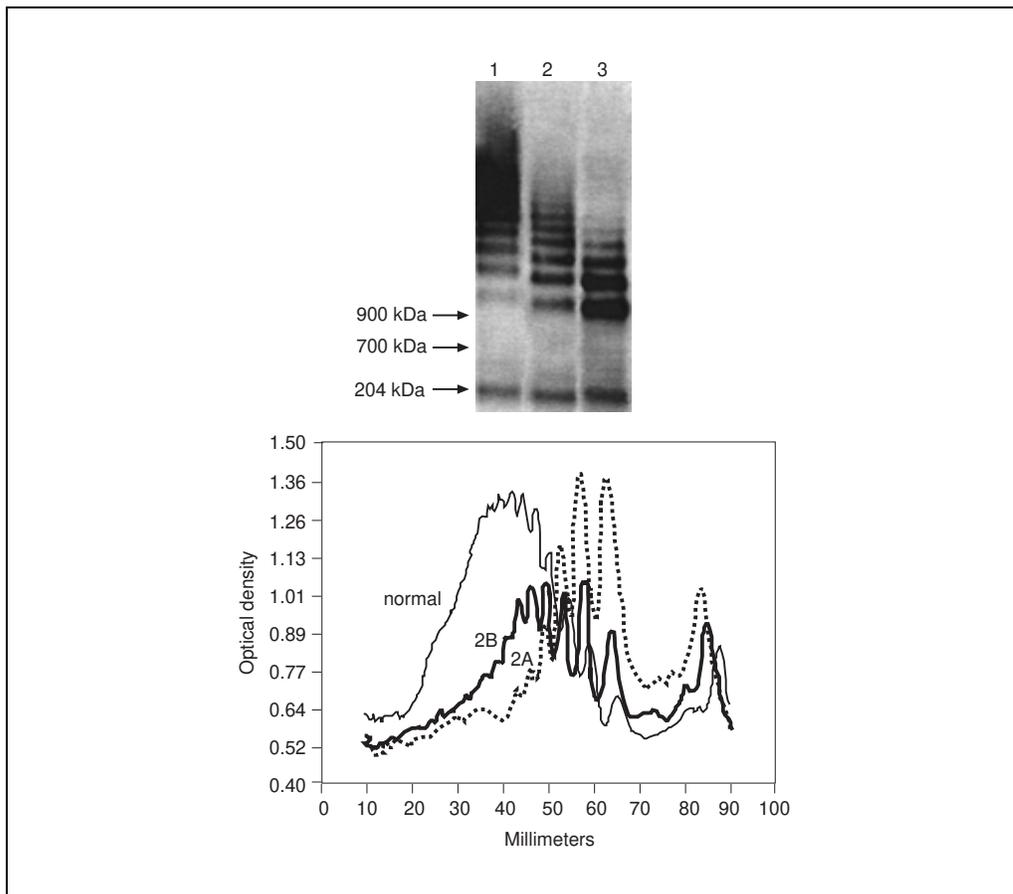


Figure 6.7.2 Luminograph of vWF multimers from normal plasma (lane 1), von Willebrand disease Type 2B plasma (lane 2), and von Willebrand disease Type 2A plasma (lane 3). A densitometric tracing is seen below. Reproduced with permission from Krizek and Rick (2000).

1×10^6 Da. For smaller proteins, higher concentrations of agarose (e.g., 3%) and shorter blotting times may be used. In instances where electroblotting cannot be carried out because of precipitation of the proteins during transfer due to separation from detergent, the proteins can be immobilized in the gel to prevent diffusion before immuno-identification. Immobilization of the proteins also allows for the use of sequential antibodies for identification of protein bands. Additionally, in-gel identification may also be important if there is uneven transfer of proteins due to dissimilar transfer characteristics. Immobilization of the proteins in the gel is accomplished with the use of an agarose gel that is modified by the addition of glycidol to yield a glyceryl agarose that contains aldehyde groups after oxidation by periodate. Proteins are covalently bound in the gel after electrophoresis by reaction of their amino groups with the aldehydes in the presence of the reducing agent, sodium cyanoborohydride. Further direct probing with antibodies can be carried out without the need for transfer to

another support (Shainoff, 1993). Composite gels of agarose or glyoxal agarose have also been prepared to provide differing degrees of sieving (Peacock and Dingman, 1968; Shainoff, 1993).

Although few proteins are as large as vWF, the evaluation of von Willebrand factor multimers illustrates an important clinical application of the use of agarose as a medium for electrophoresis. Assessment for the presence of the largest multimers is physiologically important for the diagnosis of von Willebrand disease and for selection of the most appropriate treatment (Rick, 2001). The original procedure for this analysis (see Alternate Protocol, with minor modifications) included glycidol in the agarose which served to aid in the fixation of the protein bands while the further washing and antibody identification steps were accomplished (Hoyer and Shainoff, 1980). It was subsequently found that diffusion of protein bands was not a limiting factor with vWF, and this immobilization step was eliminated from the method.

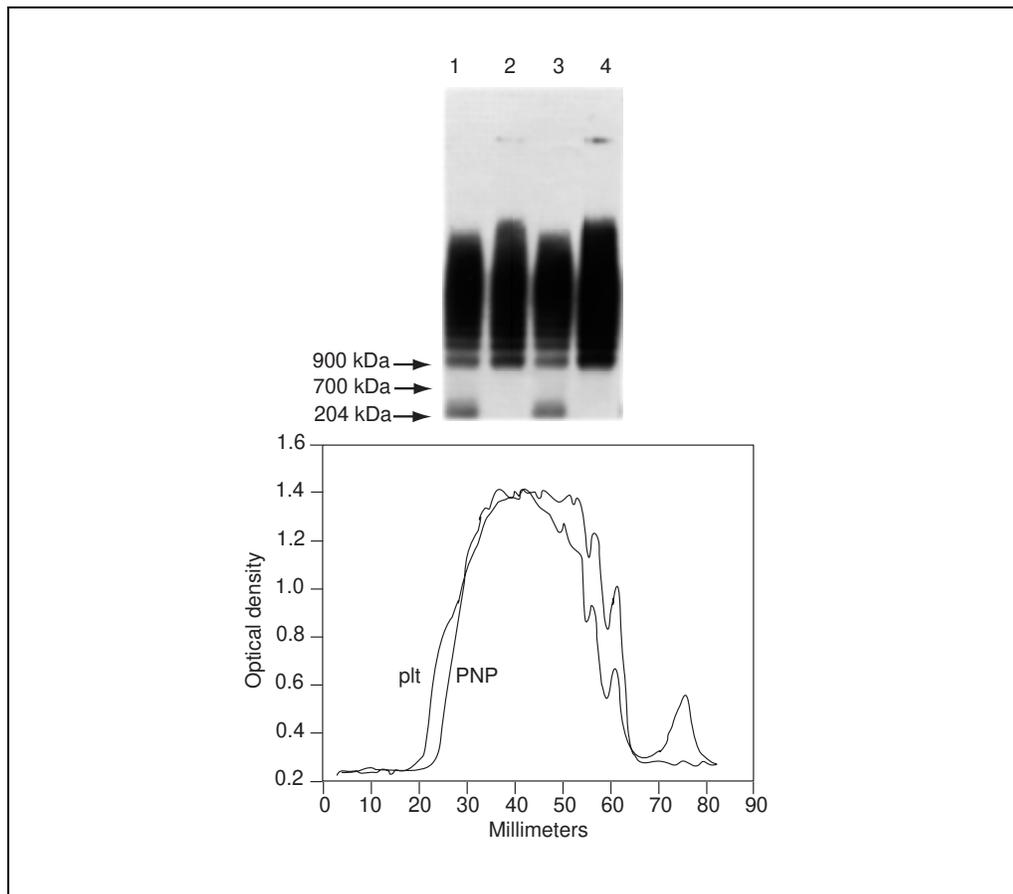


Figure 6.7.3 Luminograph of vWF multimers in pooled normal plasma (PNP; lanes 1 and 3) and vWF multimers extracted from platelets (plt; newly synthesized vWF; lanes 2 and 4). The densitometric tracing below shows the earlier “take-off” of the platelet vWF, indicating larger multimers. Adapted with permission from Krizek and Rick (2000).

Analysis of the distribution of von Willebrand factor multimers is also used to assess the function of an important protease that cleaves von Willebrand factor and decreases the prothrombotic “unusually high-molecular-weight” multimers of von Willebrand factor; these multimers are initially synthesized and secreted into the circulation, but are cleaved by the vWF protease (Krizek and Rick, 2001; Aronson, Krizek, and Rick, 2001).

Critical Parameters and Troubleshooting

It is important to maintain the temperature at 4°C during electrophoresis using the horizontal bed (see Basic Protocol). The blotting step must be carried out for a sufficient time to allow transfer of very high-molecular-weight proteins. Thorough washing after blocking buffer and antibody additions is important in both protocols. See Table 6.7.1 for troubleshooting agarose gel electrophoresis and immunoblotting.

Anticipated Results

The radiographs that result from the chemiluminescent and radioactive detection procedures show a wide distribution of multimer sizes of normal von Willebrand factor. In certain subtypes of von Willebrand disease (i.e., Type 2) there is a marked or modest decrease in the higher-molecular-weight multimers (Fig. 6.7.2). If newly synthesized von Willebrand factor is extracted from platelets, the unusually high-molecular-weight multimers are seen (Fig. 6.7.3). In samples that are incubated under conditions that activate the von Willebrand factor protease, a decrease in the high and intermediate sized multimers is seen (Fig. 6.7.4; Rick and Krizek, unpub. observ.).

Time Considerations

Both protocols should be started in the morning to allow sufficient time for electrophoresis. Horizontal electrophoresis and blotting can be completed within 48 hours: blotting is conveniently completed overnight and detec-

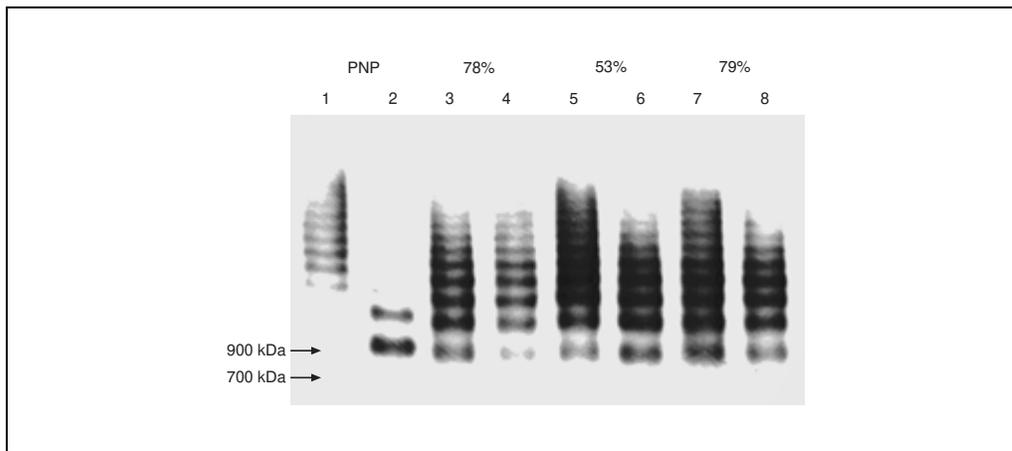


Figure 6.7.4 Luminograph of pooled normal plasma (PNP) showing normal vWF multimers (lane 1) and proteolysed multimers of normal vWF from PNP after exposure to conditions that activate the vWF protease (lane 2). Paired samples from patients with thrombotic thrombocytopenic purpura who have an inhibitor to the vWF protease are seen in lanes 3 to 8. Odd lanes contain plasma samples using conditions that do not activate the vWF protease, and even lanes contain the paired sample that was exposed to conditions that activate the protease. Very little proteolysis is observed in these patients' plasmas due to the presence of an inhibitor (even lanes). The numbers above the patient lanes indicate the retention of the high-molecular-weight multimers.

tion procedures can be completed the next day. The Alternate Protocol takes up to 5 or more days to obtain results, largely due to the time required for the incubation with antibody and development of the autoradiogram. Also, a radiolabeled antibody specific for the protein to be identified must be available.

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Key References

- Krizek and Rick, 2000. See above.
- This original paper of the procedure and use of immunoblotting and chemiluminescence for ararose gel electrophoresis provides the background and reasons for the development of this assay in the clinical laboratory setting.*
- Hoyer and Shainoff, 1980. See above.
- This paper outlines the original "in-gel" procedure for the electrophoresis of very high molecular weight proteins and provides examples of its usefulness in understanding the structure of von Willebrand factor.*
- Shainoff, 1993. See above.
- This reference provides general background and the rationale for the use of modified ararose for the separation and identification of (large) proteins by electrophoresis.*

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Fluorescence Detection of Glycoproteins in Gels and on Electroblots

The co-translational and post-translational covalent attachment of oligosaccharides to proteins is a common cellular event in eukaryotes, regulated by a variety of glycosidases and glycosyltransferases (Beeley, 1985; Reuter and Gabius, 1999; UNIT 15.2). Glycosylation profiles are dynamic, changing during development, differentiation, and disease. Glycosylation of proteins is critical to the adhesiveness of microorganisms and cells, cellular growth control, cell migration, tissue differentiation, and inflammatory reactions. Differences in glycosylation profiles are often used as a “barometer” to assess disease states. With the advent of proteomics, genome-wide protein analysis, there is renewed interest in the rapid and sensitive identification of glycoproteins by methods that do not require degradation of the protein component of the macromolecule (Packer et al., 1999; Hirabayashi et al., 2001). Until recently, there have been relatively few methods available for the direct analysis of glycans on proteins transferred to membranes and most especially of glycans on proteins within polyacrylamide gels (Packer et al., 1999; Koketsu and Linhardt, 2000; Raju, 2000). Such methods could readily be incorporated into integrated proteomics platforms that utilize automated gel stainers, image analysis workstations, robotic spot excision instruments, protein digestion work stations, and mass spectrometers (Patton, 2000a,b).

There are two principal approaches to the detection of glycoproteins in gels and on blots; reacting carbohydrate groups by periodate/Schiff’s base (PAS) chemistry and noncovalent binding of specific carbohydrate epitopes using lectin-based detection systems. The PAS method involves oxidation of carbohydrate groups, followed by conjugation with a chromogenic substrate (acid fuchsin, Alcian Blue), a fluorescent substrate (dansyl hydrazine, 8-aminonaphthalene-1, 3,6-trisulfonate, Pro-Q Emerald dye), biotin hydrazide, or digoxigenin hydrazide. Signal is detected directly in the case of the chromogenic or fluorescent conjugates and indirectly using enzyme conjugates of antibodies for bound digoxigenin or enzyme conjugates of streptavidin for bound biotin. Lectins permit detection of certain structural subclasses of glycoproteins by similar methods to those used in standard immunoblotting applications. Typically, lectin conjugates of biotin along with enzyme conjugates of streptavidin or direct conjugates of lectin and enzyme are utilized along with chromogenic, fluorogenic, or chemiluminescent substrates. Just as in immunoblotting, the most popular enzymes used to detect lectin or streptavidin are alkaline phosphatase and horseradish peroxidase.

This unit describes periodate/Schiff’s base and lectin methods for the detection of glycoproteins. The Pro-Q Emerald 300 glycoprotein detection method permits fluorescent direct detection of glycoproteins in gels (see Basic Protocol 1) or on blots (see Alternate Protocol) without the use of enzyme amplification systems. The method may also be used to detect lipopolysaccharides, constituents of the outer membrane surrounding gram-negative bacteria. The Pro-Q glycoprotein blot stain protocol for concanavalin A (see Basic Protocol 2) is suitable for the detection of glycoproteins containing α -mannopyranosyl and α -glucopyranosyl residues on blots using an alkaline phosphatase-based signal amplification system. Using different enzyme-lectin conjugates, such as alkaline phosphatase conjugates of wheat germ agglutinin or *Griffonia simplicifolia* lectin II (GS-II), the method can be adapted to the detection of other glycan structures present in glycoproteins.

Contributed by Wayne F. Patton

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**FLUORESCENT DETECTION OF GLYCOPROTEINS IN
POLYACRYLAMIDE GELS**

Pro-Q Emerald 300 Glycoprotein Gel Stain Kit provides a robust method for differentially staining glycosylated and non-glycosylated proteins in the same gel. The technique combines the green fluorescent Pro-Q Emerald 300 glycoprotein stain with the orange-red fluorescent SYPRO Ruby total protein gel stain.

Pro-Q Emerald 300 glycoprotein stain reacts with periodate-oxidized carbohydrate groups, creating a bright green-fluorescent signal on glycoproteins. Using this stain allows detection of <1 ng glycoprotein/band, depending upon the nature and the degree of glycosylation, making it 100-fold more sensitive than the standard periodic acid–Schiff base method using acidic fuchsin dye (rosaniline). The green-fluorescent signal from the Pro-Q Emerald 300 stain can be visualized using a standard 300-nm UV (UV-B) illumination source. The Pro-Q Emerald 488 Glycoprotein Gel Stain Kit is quite similar to the Pro-Q Emerald 300 Glycoprotein Gel Stain Kit, but it is optimized for use with gel scanners equipped with 470- to 488-nm lasers. The Pro-Q Emerald dye staining method is more reliable than mobility-shift assays using glycosidases since even glycoproteins that are not susceptible to deglycosylation with specific enzymes may readily be identified as glycoproteins.

After detecting glycoproteins with Pro-Q Emerald 300 dye, total protein profiles may be detected using SYPRO Ruby protein gel stain. SYPRO Ruby protein gel stain interacts noncovalently with basic amino acid residues in proteins. The stain is capable of detecting <1 ng of protein/band, making it at least as sensitive as the best silver staining procedures available. The orange-red fluorescent signal from SYPRO Ruby protein gel stain can be visualized using a standard 300-nm UV (UV-B) illumination source, or alternatively may be excited using 470- to 488-nm laser, gas discharge, or xenon arc sources.

Materials

- Protein sample of interest
- Fix solution (see recipe)
- Wash solution (see recipe)
- Pro-Q Emerald 300 Glycoprotein Gel Stain Kit (Molecular Probes) containing:
 - 50× Pro-Q Emerald 300 reagent, concentrate in DMF
 - Pro-Q Emerald 300 dilution buffer
 - Periodic acid (oxidizing reagent; see recipe)
 - CandyCane glycoprotein molecular weight standards (see recipe), sufficient volume for approximately 20 gel lanes
 - SYPRO Ruby protein gel stain
- Deionized, high quality water
- 10% (v/v) methanol or ethanol, spectroscopy grade (optional)
- 7% (v/v) glacial acetic acid (optional)
- Polystyrene staining dishes (e.g., a weighing boat for minigels or larger container for larger gels)
- Orbital shaker
- UV transilluminator
- Photographic camera or CCD camera and appropriate filters
- Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (UNIT 6.1)

Run gel

1. Prepare the protein samples of interest (e.g., crude protein isolates, cell lysates, serum, partially purified plasma membranes) for SDS-polyacrylamide gel electrophoresis.

Typically, the protein sample is diluted to ~10 to 100 µg/ml with 2× sample buffer, heated for 4 to 5 min to 95°C, and 5 to 10 µl of diluted sample is then applied per gel lane for 8 × 10-cm gels. Larger gels require proportionally more material.

For convenience, CandyCane glycoprotein molecular weight standards may also be applied to a lane or two. Typically, 2 µl of this standard is diluted in 6 µl of sample buffer and heated in the same manner as the samples to be characterized. These standards contain a mixture of glycosylated and non-glycosylated proteins ranging from 14 to 180 kDa in molecular weight. The standards serve as molecular weight markers and provide alternating bands as positive and negative controls for glycoprotein and total protein detection. Each protein is present at 0.5 mg/ml.

2. Separate proteins by SDS-polyacrylamide gel electrophoresis using standard methods (UNIT 6.1).

The staining procedure is optimized for gels that are 0.5- to 1- mm thick.

Fix gel

3. After electrophoresis, fix the gel by immersing it in 75 to 100 ml of fix solution in a polystyrene staining dish and incubating with gentle agitation (e.g., on an orbital shaker at 50 rpm) for 45 min at room temperature.
4. Wash the gel by incubating it in 50 ml wash solution with gentle agitation for 10 min at room temperature. Repeat wash one additional time.
5. Oxidize the gel in 25 ml periodic acid solution with gentle agitation for 30 min at room temperature.
6. Wash the gel in 50 ml wash solution with gentle agitation for 5 to 10 min at room temperature. Repeat this washing step two additional times.

Stain gel for glycoproteins

7. Prepare fresh Pro-Q Emerald 300 staining solution by diluting the 50× Pro-Q Emerald 300 concentrate reagent 50-fold into Pro-Q Emerald 300 dilution buffer.

For example, dilute 500 µl of 50× Pro-Q Emerald 300 reagent into 25 ml dilution buffer to make enough staining solution for one 8 × 10-cm gel.

8. Incubate the gel in subdued light in 25 ml of Pro-Q Emerald 300 staining solution (step 7) while gently agitating for 90 to 120 min.

The signal can be seen after ~20 min and maximum sensitivity is reached at ~120 min. Staining overnight is not recommended.

9. Wash the gel with 50 ml wash solution for 15 min at room temperature. Repeat this wash one additional time. Do not leave the gel in wash solution for >2 hr, as the staining signal will start to decrease.

10. Visualize the stain using a standard UV transilluminator.

The Pro-Q Emerald 300 stain has an excitation maximum at ~280 nm and an emission maximum near 530 nm. Stained glycoproteins can be visualized using a 300 nm UV-B transilluminator.

11. Document results before proceeding to the next step. Use a photographic camera or CCD camera and the appropriate filters to obtain the greatest sensitivity (see Fig. 6.8.1).

Pro-Q Emerald dye signal will fade after SYPRO Ruby dye staining.

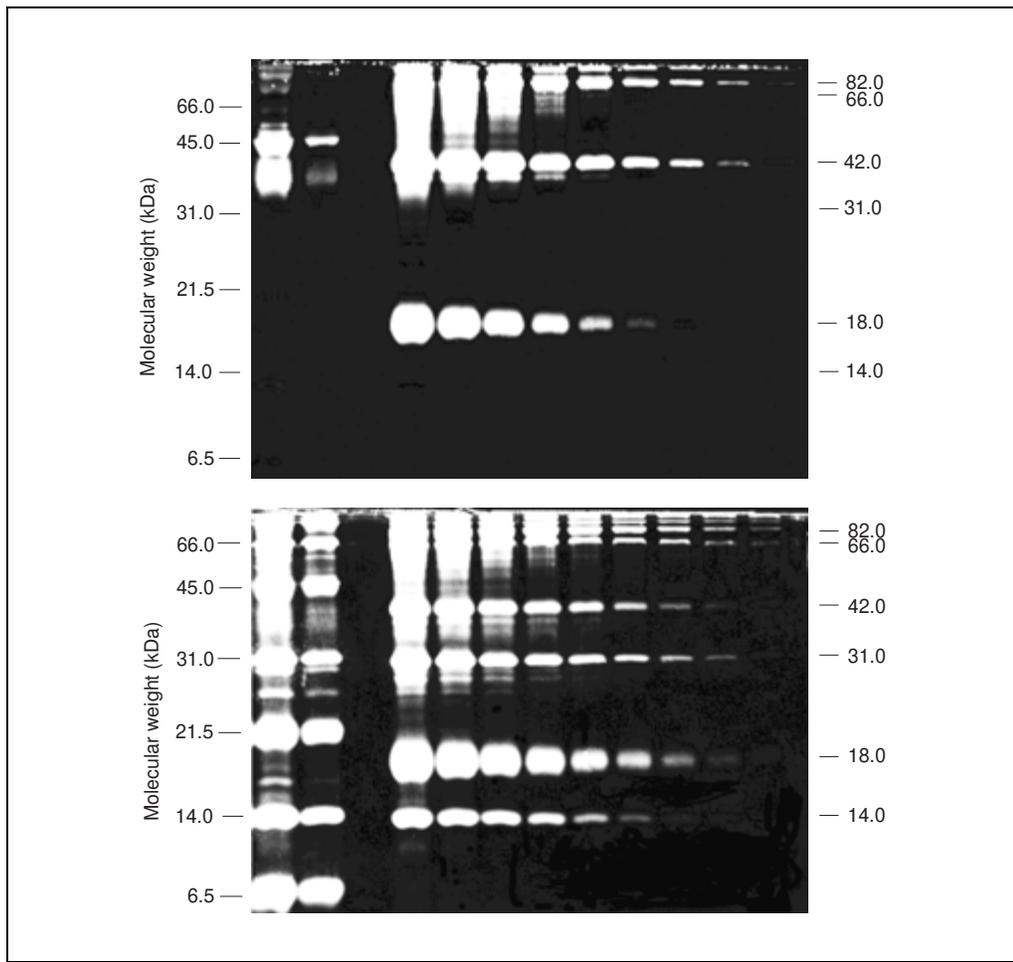


Figure 6.8.1 Sensitivity and specificity of glycoprotein detection in 13% SDS-polyacrylamide gels using Pro-Q Emerald 300 glycoprotein gel stain. **(A)** Detection of glycoproteins using Pro-Q Emerald 300 glycoprotein gel stain. **(B)** Detection of the total protein profile using SYPRO Ruby protein gel stain. Lanes 1 and 2, broad range molecular weight markers containing the 45-kDa glycoprotein ovalbumin, 1000 and 250 ng, respectively. Lane 3, blank. Lanes 4 to 12, CandyCane molecular-weight markers, a mixture of glycosylated and unglycosylated proteins (1000 to 3.9 ng, as two-fold serial dilutions). Gels were imaged using a Lumi-Imager F1 instrument (Roche Molecular Biochemicals). Both dyes were excited using the instrument's 300-nm UV-B transilluminator and images were captured using the instrument's cooled CCD camera. Pro-Q Emerald 300 dye signal was collected using the standard 520-nm band-pass emission filter. Gels were then stained with SYPRO Ruby protein gel stain and SYPRO Ruby dye signal was collected using the 600-nm band-pass emission filter provided with the instrument. Figure courtesy of Courtenay Hart, Molecular Probes.

Stain the gel for total protein

- In order to counter-stain non-glycosylated proteins in the sample, pour the SYPRO Ruby protein gel staining solution into a small, clean plastic dish.

For one or two standard-size mini-gels, use ~50 ml to 100 ml of staining solution; for larger gels, use 500 to 750 ml.

- Place the gel into the staining solution and gently agitate (e.g., on an orbital shaker at 50 rpm) at room temperature.

The staining time ranges from 90 min to 3 hr, depending upon the thickness and percentage of polyacrylamide in the gel. Specific staining can be seen in as little as 30 min. However, a minimum of 3 hr of staining is required for the maximum sensitivity and linearity. For convenience, gels may be left in the dye solution overnight or longer without over staining.

14. After staining, rinse the gel in water for 30 to 60 min to decrease background fluorescence.

Alternatively, to further decrease background fluorescence, the gel can be washed in a mixture of 10% methanol (or ethanol) and 7% acetic acid for 30 min instead of water. The gel may be monitored periodically using UV illumination to determine the level of background fluorescence.

15. Visualize the stain using an appropriate method (see Fig. 6.8.1).

The stained gel is best viewed on a standard 300-nm UV-B transilluminator; though stain will be visible using a 254-nm UV-C or 365-nm UV-A transilluminator. Gels may also be visualized using various laser scanners: 473-nm (SHG) laser; 488-nm argon-ion laser; or 532-nm (YAG) laser. Alternatively, use a xenon arc lamp, blue fluorescent light, or blue light-emitting diode (LED) source. Gels may be photographed by Polaroid or CCD camera. Use Polaroid 667 black-and-white print film and the SYPRO protein gel stain photographic filter (Molecular Probes).

FLUORESCENT DETECTION OF GLYCOPROTEINS ON ELECTROBLOT MEMBRANES

ALTERNATE PROTOCOL

Pro-Q Emerald 300 Glycoprotein Blot Stain Kit provides a robust method for differentially staining glycosylated and non-glycosylated proteins on the same electroblot. The technique combines the green fluorescent Pro-Q Emerald 300 glycoprotein stain with the orange-red fluorescent SYPRO Ruby total protein gel stain.

The Pro-Q Emerald 300 glycoprotein stain reacts with periodate-oxidized carbohydrate groups, creating a bright green-fluorescent signal on glycoproteins. Using this stain, allows detection of <1 ng glycoprotein/band, depending upon the nature and the degree of glycosylation, making it 100-fold more sensitive than the standard periodic acid–Schiff base method using acidic fuchsin dye (rosaniline). The green-fluorescent signal from Pro-Q Emerald 300 stain can be visualized using a standard 300-nm UV (UV-B) illumination source. The Pro-Q Emerald 488 Glycoprotein Blot Stain Kit is quite similar to the Pro-Q Emerald 300 Glycoprotein Gel Stain Kit, but is optimized for use with gel scanners equipped with 470- to 488-nm lasers. The staining method is more reliable than mobility-shift assays using glycosidases since even glycoproteins that are not susceptible to deglycosylation with specific enzymes may readily be identified as glycoproteins.

After detecting glycoproteins with Pro-Q Emerald 300 dye, total protein profiles may be detected using SYPRO Ruby protein blot stain. SYPRO Ruby protein blot stain interacts noncovalently with basic amino acid residues in proteins. The stain is capable of detecting <4 ng of protein/band, making it at least as sensitive as the best colloidal gold staining procedures available. The orange-red fluorescent signal from SYPRO Ruby protein blot stain can be visualized using a standard 300 nm UV (UV-B) illumination source or alternatively may be excited using 470- to 488-nm laser, gas discharge, or xenon arc sources.

Materials

- Protein sample of interest
- PVDF membrane
- Fix solution (see recipe)
- Wash solution (see recipe)
- Pro-Q Emerald 300 Glycoprotein Blot Stain Kit (Molecular Probes) containing:
 - 50× Pro-Q Emerald 300 reagent, concentrate in DMF
 - Pro-Q Emerald 300 dilution buffer
 - Periodic acid (oxidizing solution; see recipe)

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CandyCane glycoprotein molecular weight standards (see recipe), sufficient volume for ~20 gel lanes
SYPRO Ruby protein blot stain
Methanol, spectroscopy grade (optional)
Glacial acetic acid (optional)
95°C heat block
Polystyrene staining dishes (e.g., weighing boat for minigels or larger containers for larger gels)
Orbital shaker
UV epi-illuminator
Photographic camera or CCD camera and appropriate filters
Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (UNIT 6.1) and electroblotting (UNIT 6.2)

Run gel

1. Prepare the protein samples of interest (e.g., crude protein isolates, cell lysates, serum, partially purified plasma membranes) for SDS-polyacrylamide gel electrophoresis.

Typically, the protein sample is diluted to ~10 to 100 µg/ml with 2× sample buffer, heated for 4 to 5 min at 95°C, and then 5 to 10 µl of diluted sample is applied per gel lane for 8 × 10-cm gels. Larger gels require proportionally more material.

For convenience, CandyCane glycoprotein molecular weight standards may also be applied to a lane or two. Typically, 2 µl of this standard is diluted in 6 µl of sample buffer and heated in the same manner as the samples to be characterized. These standards contain a mixture of glycosylated and non-glycosylated proteins ranging from 14 to 180 kDa in molecular weight. The standards serve as molecular weight markers and as alternate bands of positive and negative controls for glycoprotein and total protein detection. Each protein is present at 0.5 mg/ml.

2. Separate proteins by SDS-polyacrylamide gel electrophoresis using standard methods (UNIT 6.1).

The procedure is optimized for gels that are 0.5- to 1-mm thick.

Prepare blot

3. After electrophoresis, transfer the proteins to PVDF membrane using standard electroblotting procedures (UNIT 6.2).

The use of nitrocellulose membranes is not recommended.

4. After transfer, fix the blot by immersing in 25 ml fix solution and incubate with gentle agitation (e.g., on an orbital shaker at 50 rpm) for 45 min at room temperature.
5. Wash the blot by incubating in 25 ml wash solution with gentle agitation for 10 min, room temperature. Repeat this wash step one additional time.
6. Oxidize the blot in 25 ml periodic acid solution with gentle agitation for 30 min.
7. Wash the blot in 25 ml wash solution with gentle agitation for 5 to 10 min. Repeat this washing step two additional times.

Visualize glycoproteins

8. Prepare fresh Pro-Q Emerald 300 staining solution by diluting the 50× Pro-Q Emerald 300 concentrate reagent 50-fold into Pro-Q Emerald 300 dilution buffer.

For example, dilute 500 µl of 50× Pro-Q Emerald 300 concentrate reagent into 25 ml of dilution buffer to make enough staining solution for one 8 × 10-cm gel.

9. Incubate the blot in the dark in 25 ml Pro-Q Emerald 300 staining solution (step 8) while gently agitating for 90 to 120 min, room temperature.

The signal can be seen after ~20 min and maximum sensitivity is reached at ~120 min. Staining overnight is not recommended.

10. Wash the blot with 25 ml wash solution for 15 min at room temperature. Repeat this wash one additional time. Do not leave the blot in wash solution for >2 hr, as the staining signal will start to decrease.

11. Allow the membrane to air dry.

12. Visualize the stain using a standard 300-nm UV epi-illuminator.

The Pro-Q Emerald 300 stain has an excitation maximum at ~280 nm and an emission maximum near 530 nm.

A UV transilluminator may also be used to visualize the glycoproteins, but this results in poorer detection sensitivity.

13. Document results before proceeding to the next step using a photographic camera or CCD camera with the appropriate filters to obtain the greatest sensitivity.

Pro-Q Emerald dye signal will fade after SYPRO Ruby dye staining.

Visualize total protein

14. In order to counter-stain non-glycosylated proteins in the sample, pour the SYPRO Ruby protein blot stain solution into a small, clean plastic dish.

For one or two standard-size mini-blot, use ~50 ml to 100 ml of staining solution; for larger blots, use 500 to 750 ml.

15. Place the air-dried blot face down onto the surface of the staining solution and gently agitate (e.g., on an orbital shaker at 50 rpm) for 15 min at room temperature.

16. After staining, rinse the blot in four changes of water for 1 min each to decrease background fluorescence.

17. Allow blots to air dry and visualize the stain using an appropriate method.

The stained blot is best viewed on a standard 300-nm UV epi-illuminator, though stain will be visible using a 254-nm UV-C or 365-nm UV-A epi-illuminator. Blots may also be visualized using various laser scanners: 473-nm (SHG) laser, 488-nm argon-ion laser, or 532-nm (YAG) laser. Alternatively, use a xenon arc lamp, blue fluorescent light, or blue light-emitting diode (LED) source.

Blots may be photographed by Polaroid or CCD camera. Use Polaroid 667 black-and-white print film and the SYPRO protein gel stain photographic filter (Molecular Probes). Exposure times vary with the intensity of the illumination source; for an f-stop of 4.5, ~1 to 3 sec should be required.

FLUORESCENT DETECTION OF GLYCOPROTEINS CONTAINING TERMINAL α -MANNOPYRANOSYL AND α -GLUCOPYRANOSYL RESIDUES ON ELECTROBLOT MEMBRANES

Lectins are sugar-binding proteins of nonimmune origin capable of agglutinating cells or precipitating glycoconjugates (Beeley, 1985). The specific interactions between labeled lectins and oligosaccharides form the basis of glycoprotein detection after separation by gel electrophoresis and transfer to membranes by electroblotting. Concanavalin A is a tetrameric protein, with each subunit containing a carbohydrate-binding site, a calcium ion-binding site, and a manganese-ion binding site. Concanavalin A binds specifically

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to α -D-mannopyranosyl and α -D-glucopyranosyl residues, with substitutions or modifications at the C-3, C-4, or C-6 positions of the ring structure leading to greatly diminished binding (Beeley, 1985).

The Pro-Q Glycoprotein Blot Stain Kit with concanavalin A utilizes alkaline phosphatase-conjugated concanavalin A along with the fluorogenic substrate DDAO phosphate [9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate] to detect glycoproteins on nitrocellulose and poly(vinylidene difluoride) (PVDF) membranes. The detection procedure is similar to that of standard western (immuno)blotting. DDAO phosphate is rapidly converted to the long wavelength, red-fluorescent product, DDAO. DDAO absorbs maximally at either 275 nm or 646 nm and emits maximally at 659 nm. Consequently, the blots may be imaged using standard UV epi-illumination or with a laser-based gel scanner equipped with appropriate excitation source. The enzymatic amplification step greatly enhances the signal, allowing low nanogram detection of glycoproteins, a sensitivity on par with chemiluminescence detection methods. Pro-Q Glycoprotein Blot Stain kits with wheat germ agglutinin or with *Griffonia simplicifolia* lectin II (GS-II) allow detection of *N*-acetylglucosamine and sialic acid residues or terminal *N*-acetylglucosamine residues, respectively. The detection procedures for these lectins are quite similar to the concanavalin A method, and the same fluorogenic substrate, DDAO-phosphate is used in the kits.

Materials

- Protein samples of interest
- PVDF membrane
- 50% methanol
- Wash solution II (see recipe)
- Blocking solution (see recipe)
- Pro-Q Glycoprotein Blot Stain Kit with Concanavalin A (Molecular Probes) containing:
 - Concanavalin A, alkaline phosphatase conjugate (Con A-AP) stock solution (see recipe)
 - DDAO phosphate stock solution (see recipe)
 - Dimethylformamide (DMF)
 - CandyCane glycoprotein molecular weight standards (see recipe), sufficient volume for ~20 gel lanes
- Incubation buffer (see recipe)
- 10 mM Tris/1 mM MgCl₂, pH 9.5
- Polystyrene staining dishes (e.g., weigh boat for minigel or larger container for larger gels)
- Plastic wrap
- UV epi-illumination and a digital or film camera, or a laser equipped with a 633-nm helium-neon laser or 635-nm diode laser source
- Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (UNIT 6.1), electroblotting procedures (UNIT 6.2), and SYPRO Ruby protein blot staining (see Alternate Protocol)

Run gel

1. Prepare the protein samples of interest (e.g., crude protein isolates, cell lysates, serum, partially purified plasma membranes) for SDS-polyacrylamide gel electrophoresis (UNIT 6.1).

Typically, the protein sample is diluted to ~10 to 100 μ g/ml with 2 \times sample buffer, heated for 4 to 5 min at 95°C, and 5 to 10 μ l of diluted sample is then applied per gel lane for 8 \times 10-cm gels. Larger gels require proportionally more material.

For convenience, CandyCane glycoprotein molecular weight standards may also be applied to a lane or two. Typically, 2 μ l of this standard is diluted in 6 μ l of sample buffer and heated in the same manner as the samples to be characterized. These standards contain a mixture of glycosylated and non-glycosylated proteins ranging from 14 to 180 kDa in molecular weight. The standards serve as molecular weight markers and as alternating bands of positive and negative controls for glycoprotein and total protein detection. Each protein is present at 0.5 mg/ml.

2. Separate proteins by SDS-polyacrylamide gel electrophoresis using standard methods (UNIT 6.1).

The procedure is optimized for gels that are 0.5- to 1-mm thick.

Blot proteins

3. After electrophoresis, transfer the proteins to PVDF membrane using standard electroblotting procedures (UNIT 6.2).

The use of nitrocellulose membranes is not recommended.

4. *Optional:* Stain blots with SYPRO Ruby protein blot stain at this point to visualize the total protein pattern and to verify that the blotting procedure was successful. Follow the staining procedure described in Alternate Protocol, steps 14 to 17.

Total protein staining must be performed prior to lectin blotting as the blocking mixture will produce very high background on the blot. Since SYPRO Ruby protein blot stain is washed off during the subsequent lectin blotting process, it is important to document staining results before continuing with the procedure.

5. If the PVDF blot is dry, briefly hydrate in 50% methanol and incubate in wash solution II for 10 min at room temperature. Repeat the wash step for a total of three washes.

Visualize glycoproteins

6. Incubate the blot in blocking solution for 1 to 2 hr at room temperature.
7. Briefly pellet any potential protein aggregates in the Con A-AP stock solution by microcentrifugation. Using the supernatant only, dilute the Con A-AP stock solution 2000-fold by adding 5 μ l to 10 ml of incubation buffer for a final concentration of 1 μ g/ml. Remove the blocking solution that the blot is immersed in and incubate the blot with Con A-AP solution for 1 hr at room temperature.
8. Remove the diluted Con A-AP solution and wash the blot in blocking solution four times for 10 min each at room temperature.
9. Perform two final washes in wash solution II for 5 min each at room temperature.
10. Dilute the DDAO phosphate stock solution 1000-fold into 10 mM Tris/1 mM MgCl₂, pH 9.5, for a final concentration of 1.25 μ M.

Approximately 1 ml of the DDAO phosphate staining solution will be needed for an 8 \times 10-cm blot. Note that DDAO phosphate is unstable when stored at room temperature as an aqueous solution. Always make up the DDAO phosphate staining solution just prior to use.

11. Incubate the blot in freshly prepared DDAO phosphate staining solution.

The staining step may be performed either face up or face down, depending on the configuration of the imaging instrumentation being used. If using UV epi-illumination or a laser scanner with a light source that illuminates from above the imaging bed, stain the blot face up. For laser scanners with light sources that illuminate the blot from below the imaging bed, stain the blot face down.

12. Using powder-free gloves, cut a piece of plastic wrap to the size of the blot. For face-up staining, place the blot on the plastic wrap and pipet 1 ml of DDAO phosphate staining solution onto the blot. For face-down staining, pipet 1 ml of the DDAO

phosphate staining solution onto the plastic wrap and lay the blot face down onto the solution, being careful not to trap air bubbles.

The time required for optimal staining must be determined empirically because the substrate turnover rate depends on the amount of glycoprotein on the blot. Generally, a 5- to 20-min incubation is sufficient, but overnight incubation is permissible. Do not wash the blot after staining as this will cause extensive loss of signal. The blot may be air-dried, however.

13. Visualize the fluorescent DDAO product using either UV epi-illumination and a digital or film camera, or using a laser equipped with a 633-nm helium-neon laser or 635-nm diode laser source. For UV epi-illumination, place the blot, signal side up, on a flat surface. For highest sensitivity and lowest background, use a UV-blocking filter, such as the SYPRO gel stain photographic filter. Long-pass filters with a cutoff at ~630 nm are ideal for CCD-cameras. For laser scanners, place the blot, signal side down, on the scanner bed. For highest sensitivity, match the light sources and filters of the instrument as closely as possible to the absorbance maximum (646 nm) and emission maximum (659 nm) of DDAO.
14. If desired, the Con A-AP complex can be stripped off of the blot and the blot reprobed with another lectin-AP complex or an antibody-AP complex. To strip, incubate the blot in stripping buffer for 40 min at 50°C with gentle agitation. Then, wash the blot in wash buffer two times for 5 min each at room temperature.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Blocking solution

50 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
150 mM NaCl
0.2% (v/v) Tween 20
0.25% (w/v) Mowiol 4-88 (Calbiochem or VWR)
Store up to 6 months at room temperature

The use of Mowiol 4-88 in the Blocking buffer is not essential, but does decrease background staining and improves detection sensitivity. As an alternative to Mowiol 4-88, 0.5% (w/v) bovine serum albumin or 4% (w/v) gelatin (high purity, e.g., TopBlock from Juro Supply) may be used.

CandyCane molecular weight standards

For a standard lane on an 8 × 10-cm gel, dilute 0.5 µl of the standards with 7.5 µl of 2× sample buffer (see recipe) and vortex. This will result in ~250 ng of each protein per lane, a sufficient amount for detection of the glycoproteins by the Pro-Q Emerald 300 stain. For large 16 × 18-cm gels, double the amount of standard and buffer used. Store up to 6 months at room temperature.

Con A-AP stock solution

Prepare a 2 mg/ml stock solution of Con A-AP by dissolving the vial contents in 250 µl deionized water and add 2 mM sodium azide. The stock solution is stable for at least 6 months when stored undiluted at 4°C. Do not freeze.

DDAO phosphate stock solution

Add 200 μ l DMF to the vial containing 250 μ g DDAO phosphate {[9H-(1,3-dichloro-9, 9-dimethylacridin-2-one-7-yl] phosphate, diammonium salt} to make 1.25 mg/ml stock solution. Store the stock solution at -20°C , desiccated and protected from light. When properly stored, the stock solution should be stable for at least 6 months. When the solution turns a blue color, the substrate has broken down and is no longer usable. Prepare the working solution fresh.

Fix solution

Prepare a solution of 50% methanol and 50% deionized water. Store up to 6 months at room temperature.

One 8 \times 10-cm gel will require ~100 ml of fix solution.

Incubation buffer

Prepare blocking solution (see recipe) with 1 mM CaCl_2 and 0.5 mM MgCl_2 . Store up to 6 months at room temperature.

Periodic acid solution

Add 250 ml of 3% (v/v) acetic acid to the bottle containing the periodic acid (oxidizing solution) and mix until completely dissolved. Store up to 6 months at room temperature.

Sample buffer, 2 \times

100 mM Tris \cdot Cl, pH 6.8 (*APPENDIX 2A*)

20% (v/v) glycerol

4% (w/v) sodium dodecyl sulfate

0.1% (w/v) bromophenol blue

Store up to 6 months at room temperature

Wash solution

Prepare a solution of 3% (v/v) glacial acetic acid in water. Store up to 6 months at room temperature.

One 8 \times 10-cm gel will require ~400 ml of wash solution.

Wash solution II

Prepare a solution of 50 mM Tris \cdot Cl, pH 7.5/150 mM NaCl. Store up to 6 months at room temperature.

COMMENTARY

Background Information

The analysis of protein glycosylation is readily accomplished by polyacrylamide gel electrophoresis (Koch and Smith, 1990; Packer et al., 1997; Taverna et al., 1998; Koketsu and Linhardt, 2000). However, relatively few high-sensitivity methods have been developed to reliably detect oligosaccharide residues covalently attached to proteins for visualization in polyacrylamide gels or on electroblot membranes (Packer et al., 1997; Packer et al., 1999; Koketsu and Lindhardt, 2000). Perhaps the most common procedure to visualize glycoproteins reported in the literature entails detection by periodic acid/Schiff (PAS) staining using the colorimetric acid fuchsin dye. A major limita-

tion of this method is that detection sensitivity is poorer than Coomassie Blue staining, rendering the technique obsolete for modern high-sensitivity proteomics investigations. Other methods in use include PAS-labeling with digoxigenin hydrazide followed by immunodetection with anti-digoxigenin antibody conjugated to alkaline phosphatase, or PAS-labeling with biotin hydrazide, followed by detection with horseradish peroxidase or alkaline phosphatase conjugated to streptavidin (Packer et al., 1995). Lectins are commonly employed to detect certain structural subclasses of glycoproteins by methods similar to those employed in immunoblotting (Koketsu and Lindhardt, 2000). All of these methods require that pro-

teins be electroblotted to membranes first and many glycoproteins transfer relatively poorly. In addition, detection of glycoproteins after electroblotting is very time consuming compared with direct detection in gels. A recently developed approach to the detection of glycoproteins relies upon the utilization of a new fluorescent hydrazide, Pro-Q Emerald 300 dye, that is affixed to glycoproteins using a standard PAS conjugation mechanism (Steinberg et al., 2001). The glycols present in glycoproteins are initially oxidized to aldehydes using periodic acid. The dye then reacts with the aldehydes on the glycoproteins to generate the fluorescent conjugate. A reduction step with sodium metabisulfite or sodium borohydride is not required to stabilize the resulting conjugate.

Critical Parameters

All stock solutions should be prepared using deionized water (dH₂O) having a resistance of at least 18 MΩ. All stock solutions may be stored for up to 6 months at room temperature, except when specifically indicated. Dilution of the DDAO phosphate stock solution or the Pro-Q Emerald 300 dye solution should be performed immediately prior to their use in the staining protocols. Both reagents are unstable when stored at room temperature as aqueous solutions. The staining methods outlined in this unit are highly sensitive and it is critical that all glassware and staining dishes be scrupulously clean. Gels and blots should never be touched or otherwise manipulated using bare hands. Always wear powder-free latex gloves when handling gels and blots during all staining procedures for the fluorescence detection of glycoproteins.

Troubleshooting

Should the detection sensitivity obtained using the cited fluorescence methods be suboptimal, there are two potential sources for the problem, either instrumental or chemical. With respect to the imaging instrument, it is important to clean the surface of the transilluminator after each use with deionized water and a soft cloth (e.g., cheesecloth). Otherwise, fluorescent dyes can accumulate on the glass surface and cause a high background fluorescence. The polyester backing on some pre-cast gels is highly fluorescent. For maximum sensitivity using a UV transilluminator, the gel should be placed polyacrylamide side down and an emission filter used to screen out the blue fluorescence of the plastic. For UV detection of fluorophores, a 300-nm UV-B transilluminator with

six 15-watt bulbs is recommended. Excitation with different UV light sources, such as a simple hand held UV lamp will not provide the same level of detection sensitivity as a full-fledged transilluminator. For all three procedures described in this unit, using a Polaroid camera and Polaroid 667 black-and-white print film, the highest sensitivity is achieved with a 490-nm long-pass filter, such as the SYPRO protein gel stain photographic filter (S-6656; Molecular Probes). Gels are typically photographed using an f-stop of 4.5 for 2 to 4 sec, using multiple 1-sec exposures. Using a CCD camera, images are best obtained by digitizing at ~1024 × 1024-pixels resolution with 12-, 14-, or 16-bit gray-scale levels per pixel. A 520-nm long-pass filter is suitable for visualizing Pro-Q Emerald dye, while a 580-nm long-pass filter is appropriate for detection of DDAO. A CCD camera-based image analysis system can gather quantitative information that will allow comparison of fluorescence intensities between different bands or spots. Using such a system, the Pro-Q Emerald dye and the DDAO dye have a linear dynamic range of three orders of magnitude.

A potential problem associated with the Pro-Q Emerald 300 glycoprotein gel stain is non-specific labeling of non-glycosylated proteins. The most common source of this problem is the presence of residual SDS in the polyacrylamide gel. Adding an extra fixation step to the procedure should prevent its occurrence. The author finds that an overnight fixation step for two-dimensional gels is advisable. When detecting glycans using Pro-Q Emerald 300 glycoprotein gel stain, it is prudent to run control gels in which the periodate oxidation step has been omitted. Similar precautions are advisable when evaluating results using other glycoprotein detection methods. This avoids erroneous interpretation of results arising from low levels of noncovalent binding of dye molecules or confusion arising from the inherent fluorescence of certain high-abundance proteins in the gel profile.

The most common problem encountered using the Pro-Q glycoprotein detection kit with concanavalin A is poor signal intensity. This is usually due to decomposition of the DDAO phosphate stock solution. When the stock solution appears by eye to be a blue color, the substrate has broken down and is no longer usable.

Table 6.8.1 Comparison of Commonly Used Glycoprotein Detection Methods for Polyacrylamide Gels and Electroblot Membranes

Detection method	Time required (hr)	Number of steps	Detection sensitivity		Assets (+) or liabilities (-)
			(1) α 1-acid glycoprotein (40% CHO) ^a		
			(2) glucose oxidase (12% CHO) ^a		
		(3) avidin (7% CHO) ^a			
			<i>Gels</i>	<i>Blots</i>	
Acid fuchsin sulfite (pararosaniline)	5-6	7	(1) 75 ng (2) 150 ng (3) 150 ng	20 ng 75 ng 75 ng	(+) short procedure (+) can use either on blots or in gels (-) poor sensitivity
Biotin hydrazide/streptavidin-HRP Luminol detection reagents	6	11	(1) na (2) na (3) na	18-37 ng 37 ng 150 ng	(-) signal fades over time, optimal image, 20-30 min (-) cannot save and reimage blots
Biotin hydrazide/streptavidin-alkaline phosphatase NBT/BCIP solution	5-6	11	(1) na (2) na (3) na	2 ng 5-9 ng 18-37 ng	(+) good sensitivity (+) can save and reimage blots (-) long procedure (-) cross reaction with carbonic anhydrase
Pro-Q Glycoprotein Detection Kit with Con-A alkaline phosphatase (see Basic Protocol 2)	4	5	(1) na (2) na (3) na	not detected <15.6 ng 15.6 ng	(+) can save and re-image blots (+) can strip and reprobe (+) can post-stain with total protein stains (+/-) stains specific subsets of glycoproteins (-) long procedure
Dansyl hydrazine	4	9	(1) 1.25-2.5 ng (2) 1.25-2.5 ng (3) 16-19 ng	not tested	(+) inexpensive (-) requires longer exposure for competitive brightness (-) low-level non-specific detection of unglycosylated proteins (-) requires hot, acidified DMSO
Digoxigenin-3-O-succinyl- ϵ -aminocaproic acid hydrazide/Anti-digoxigenin-alkaline phosphatase. Stain with NBT/x-phosphate	5-6	11	(1) na (2) na (3) na	2 ng 5-9 ng 18-37 ng	(+) good sensitivity (+) can save and reimage blots (-) long procedure (-) cross reaction with carbonic anhydrase
Pro-Q Emerald 300 Dye (see Basic Protocol 1 and Alternate Protocol)	2 (blots) 4 (gels)	7	(1) 300 pg (2) 300 pg (3) 1-2 ng	2 ng 18 ng 9 ng	(+) can use either on blots or in gels (+) great sensitivity (+) can save and re-image blots (+) short procedure (+) can counterstain unglycosylated proteins with SYPRO Ruby dye

^aAbbreviations: CHO, carbohydrate; na, not applicable.

Anticipated Results

The performance characteristics of the Pro-Q Emerald 300 Glycoprotein Gel Stain Kit, Pro-Q Emerald 300 Glycoprotein Blot Stain Kit, and Pro-Q Glycoprotein Detection Kit with Concanavalin A are summarized in Table 6.8.1 and contrasted with alternative glycoprotein detection technologies. The fluorescence-based methods permit detection of low-nanogram amounts of glycoprotein with a dynamic range of quantitation that encompasses three orders of magnitude of glycoprotein abundance. Pro-Q Emerald 300 dye may be used to detect a variety of glycoconjugates in addition to glycoproteins, such as bacterial lipopolysaccharides (LPS) and glycogen. Detection sensitivity for chondroitin 4-sulfate, however, is ~3000-fold poorer than glycogen or LPS, with limits of detection in the vicinity of 16 µg of applied material. This is not unexpected as glycosaminoglycans such as chondroitin sulfate, hyaluronic acid, and keratan sulfate are known to stain poorly by conventional PAS procedures. Concanavalin A specifically binds to nonsubstituted and 2-*O*-substituted α -mannosyl residues and thus detects fewer glycoproteins than the Pro-Q Emerald 300 dye. For example, α 1-acid glycoprotein is not detected by concanavalin A. Similarly, glycoproteins such as ovomucoid (28 kDa) and ovotransferrin (76 kDa) are not effectively detected by concanavalin A. The differences in staining specificity between the Pro-Q glycoprotein detection kit with concanavalin A, the Pro-Q glycoprotein detection kit with wheat germ agglutinin and the Pro-Q Emerald 300 glycoprotein stain kits can be exploited in defining structural features of glycans on glycoproteins.

Time Considerations

The time considerations and number of steps required to detect glycoproteins using the Pro-Q Emerald 300 Glycoprotein Gel Stain Kit, Pro-Q Emerald 300 Glycoprotein Blot Stain Kit, and Pro-Q Glycoprotein Detection Kit with Concanavalin A are summarized in Table 6.8.1 and contrasted with alternative glycoprotein detection technologies. The methods can be completed in ~2 to 4 hr and require 5 to 7 steps to complete. This compares favorably with other methods that may require as much as 6 hr and 11 steps to complete.

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Describes optimized methods for protein identification by matrix-assisted laser desorption time-of-flight mass spectrometry after staining gels with SYPRO Ruby dye.

Internet Resources

<http://www.cbs.dtu.dk/databases/OGLYCBASE/>

O-GLYCBASE; a database of 198 glycoprotein entries with experimentally verified O-glycosylation site information.

<http://www.glycosuite.com>

GlycoSuite; a relational database that curates information from the scientific literature on glycoprotein derived glycan structures, their biological sources, the references in which the glycan was described, and the methods used to determine the glycan structure.

<http://www.expasy.ch/tools/glycomod/>

GlycoMod; a software tool designed to find all possible compositions of a glycan structure from its experimentally determined mass.

<http://www.probes.com>

Molecular Probes commercial Web site containing information about fluorescence detection technologies, including glycoprotein, total protein, lipopolysaccharides, and nucleic acids.

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Gel electrophoresis has become a ubiquitous method in molecular biology for separating biomolecules. This prominence is the result of several factors, including the robustness, speed, and potentially high throughput of the technique. The results of this method are traditionally documented using silver halide–based photography followed by manual interpretation. While this remains an excellent method for qualitative documentation of single-gel results, digital capture offers a number of significant advantages when documentation requires quantitation and sophisticated analysis. Digital images of gel electropherograms can be obtained rapidly using an image-capture device, and the images can be easily manipulated using image analysis software.

REASONS FOR DIGITAL DOCUMENTATION AND ANALYSIS

There are several reasons to consider digital documentation and analysis of electrophoresis results. These justifications can usually be categorized into issues of ease of handling, accuracy, reproducibility, and cost.

Ease of Handling

A major advantage of the digital revolution has been in storage and retrieval of information. Storage in notebooks and filing cabinets previously meant that searching for specific data or experiments was a tedious manual process. With digital information, modern search engines can quickly find specific information in a fraction of the time usually required for a manual search. Making backup copies of non-digital data can be difficult, expensive, and time-consuming since it requires copying, re-typing, or photographic reproduction. Copies of digital data can be generated more easily and at reduced costs.

Manipulation of information is also easier when it is in a digital format. While the cut-and-paste analogy comes from physical documentation, it takes on a new perspective when applied digitally. Electrophoresis images can be resized, cropped, and inserted into reports. Data can be passed to spreadsheets and statistical packages for analysis and later insertion into notebooks and reports. These reports can be passed out via the Internet to colleagues throughout the world. A single individual can do all this in a few hours.

Digital analysis also provides an easier method for handling the data when comparing large numbers of results or large numbers of separate experiments. Research that requires comparing the banding patterns on 1000 gels containing 50 lanes each can be an undertaking of heroic proportions if the analysis is performed manually. Database software can dramatically speed the analysis and handle the more mundane tasks, leaving the researcher free to interpret the data.

Accuracy

The human eye is an extremely versatile measuring instrument. It can handle light intensities covering a range of nearly nine orders of magnitude and is sensitive to a fairly wide spectrum of light (Russ, 1995). Yet the eye cannot accurately and reproducibly quantitate density and patterns, nor can it deal with large numbers of bands or spots. Accuracy of measurement is a primary reason for using digital analysis on electrophoretically separated proteins and nucleic acids. Two categories of accuracy are key to digital analysis: positional accuracy, which is important for mobility determinations such as molecular weight, and quantitative accuracy.

Positional accuracy is based on both resolution of the recording medium and measuring accuracy. Silver halide–based recording has a theoretical resolution based on ~2000 imaging elements (silver grains) per inch. Measurement traditionally occurs using a ruler, with an accuracy of ~20 to 40 elements (50 to 100 elements per inch). In comparison, typical digital systems have 200 to 600 picture elements (pixels) per inch. The advantage that digital systems have is in measuring accuracy, which can occur at the level of a single imaging element.

Quantitative accuracy is also an issue. The amount of material represented by a band or spot is difficult to determine accurately from an image of a gel unless it is a digital image. On a digital image, the amount present is directly correlated with the derived volume of the band or spot—the volume is calculated using the intensity values of the pixels within the object.

Reproducibility

Any technique or measurement is only as good as its ability to be faithfully replicated. With software-defined routines, measurements

are performed in the same manner every time. Allowing the computer to do repetitive tasks and complicated calculations minimizes the chance for individual errors. This does not imply that such measurements are correct, just that they are reproducible. An incorrect routine or algorithm can also invalidate data.

Cost

A consideration when evaluating any laboratory method is cost. Digital electrophoresis analysis equipment can be expensive. In many cases, however, it offers the only method for achieving acceptable analysis performance. In other cases, equal performance can be achieved using silver halide technology. However, traditional photography can also be expensive when the costs of consumable supplies such as film and developers, as well as other expensive requirements such as developing tanks and dark rooms, are included. Often, digital methods can be a good choice when all costs are considered.

KEY TERMS FOR IMAGING

There are several specialized terms encountered during digital image analysis. The most commonly encountered are contrast, brightness, gamma, saturation, resolution, and dynamic range. They describe controls on how the light detectors report a range of light intensities. Below is a brief description of each.

Contrast

Contrast describes the slope of the light intensity response curve. An increase in the contrast increases the slope of the curve. The result is a more detailed display over a narrowed range of intensities with less detail in the remaining portions of the intensity range. This is depicted in Figure 6.9.1A and 6.9.1B, where a normal, unadjusted image and a contrast-adjusted image are displayed, respectively. The contrast was increased on midrange intensity values in Figure 6.9.1B to highlight band intensity differences at the expense of background information. Images with a narrow range of informative intensities can benefit from increasing the contrast, since that effectively increases the scale and improves detection of minor differences in intensity. Contrast settings should be lowered if information is being lost outside of the contrast range. For example, in Figure 6.9.1B, loss of background information between peaks indicates that this image should not be used for quantitation.

Brightness

While brightness can have many different definitions, only one will be considered here. Brightness shifts the light intensity response curve without changing its slope as is shown in Figure 6.9.1C. Another name for brightness is black level, since it is commonly used to control the number of black picture elements (pixels) in an image. Incorrect brightness levels can lead either to high background and potential image saturation or, as is illustrated in Figure 6.9.1C, to a total loss of background information and partial loss of band information.

Gamma

Nonlinear corrections are often applied to images to compensate for how the eye perceives changes in intensity, how display devices reproduce images, or both. The most common correction is an exponential one, with the exponent in the equation termed the gamma. A typical gamma value for camera-based systems is 0.45 to 0.50, and is illustrated in Figure 6.9.1D. This is a compromise value that compensates for the 2.2 to 2.5 gamma present in most video monitors and the print dynamics of most printers. Since it is a nonlinear correction, special care must be taken if quantitation is desired. Unless directed to by the manufacturer, gamma values other than 1.0 should be avoided when quantitating. More information on gamma correction can be found on Poynton's Gamma FAQ (www.inforamp.net/~poynton/Poynton-color.html).

Dynamic Range

Dynamic range describes the breadth of intensity values detectable by a system and is usually expressed in logarithmic terms such as orders of magnitude, decades, or optical density (OD) units. A large dynamic range is important when trying to quantitate over a wide range of concentrations. The most accurate quantitation occurs in the linear part of the dynamic range, which is usually not the complete dynamic range of the system. An additional consideration is the dynamic range of the visualization method. Many popular visualization methods have linear dynamic ranges of 1 to 2.5 orders of magnitude. An imaging system with greater dynamic range analyzing the results of such a visualization method will not improve the dynamic range.

Saturation

Saturation occurs when a detector or visualization method receives input levels beyond

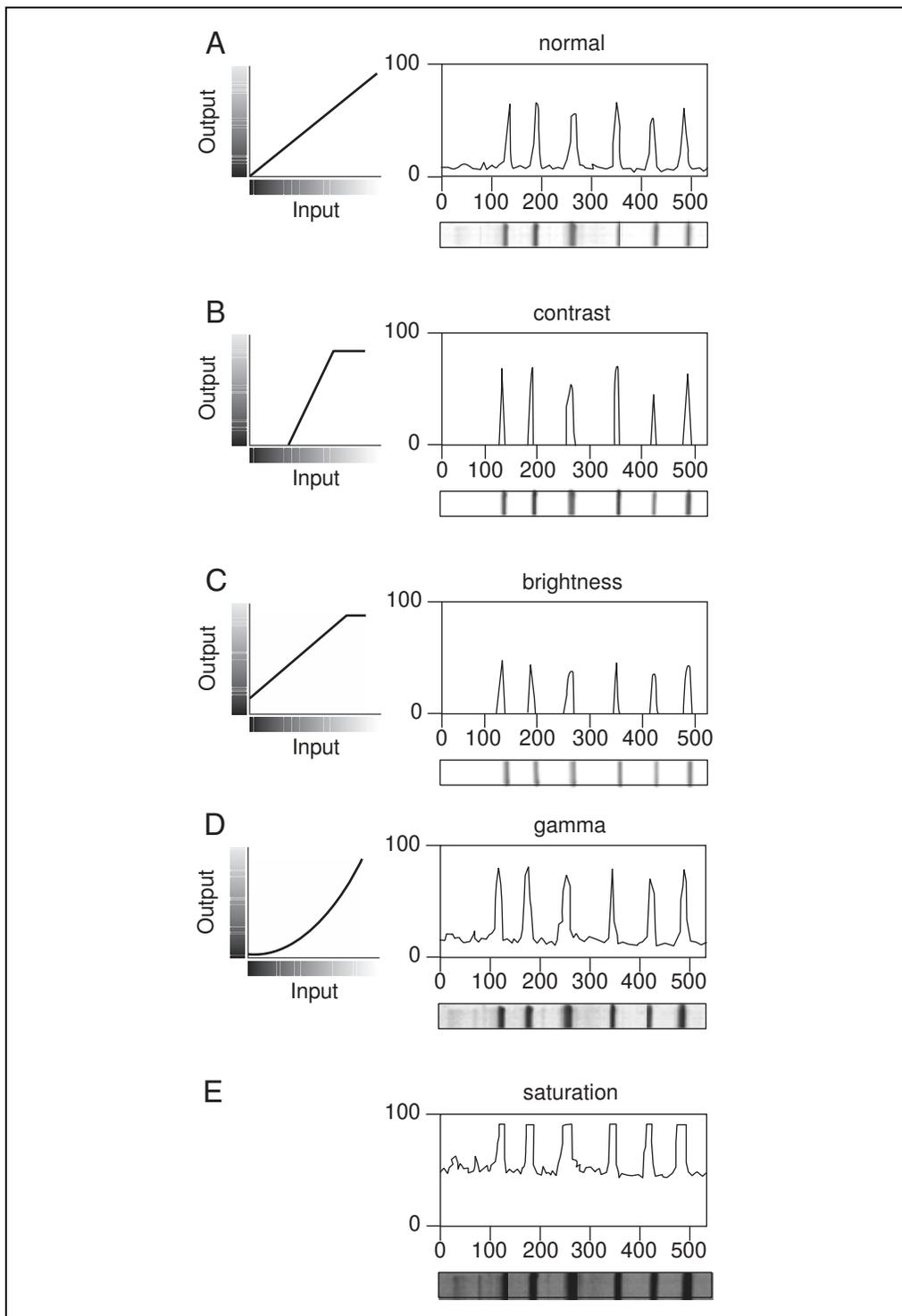


Figure 6.9.1 Examples of how altering image capture settings affects the image and the analysis. The graph on the left displays the light intensity response curve used for image capture while the image and resulting lane profile on the right display how the setting affects the image. The lane profile displays pixel position versus normalized pixel intensity (**A**). In this case, the output has not been altered, giving a straight line with a slope of 1 on the response curve. (**B**) The image acquisition was adjusted to increase the contrast of the displayed image. Although useful for images with a narrow range of informative intensity values, increasing the contrast can lead to a loss of low and high values. (**C**) Decreasing the brightness reduces peak values but also leads to a loss of the weak bands and original background. (**D**) Gamma adjusts raw data to appear more visually accurate. Note that this leads to a loss of fidelity between the adjusted image and the original. (**E**) Saturation indicates that the detector is reporting its maximum value or that the dynamic range for the visualization method has been exceeded.

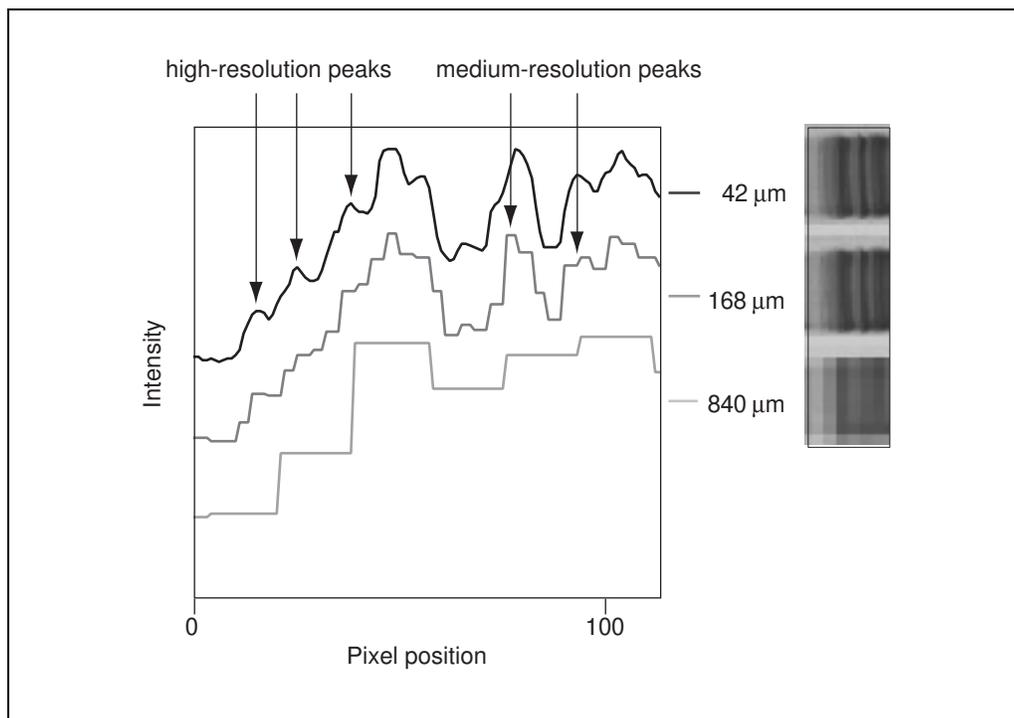


Figure 6.9.2 The effect of spatial resolution on the ability to detect closely spaced objects. Whole-cell protein lysates from *E. coli* were separated using SDS-PAGE and visualized with Coomassie blue staining. An image was captured at 42 μm (600 dpi), 168 μm (150 dpi), and 840 μm (30 dpi) from a segment of the lane, and a lane profile was generated for each image. The lane profiles have offset intensities to allow for comparison. Only major bands can be detected with the low-resolution image (840 μm); at higher resolutions more bands are detectable.

the maximum end of the dynamic range. This results in a loss of detail and quantitative information from those data points that are saturated. For fluorescent and luminescent samples, reduction in the sampling time can sometimes correct saturation problems. Optical density-based visualization techniques can also generate saturated images, as is illustrated in Figure 6.9.1E; this can sometimes be avoided with longer sampling times or increased detection source intensities. More often, it will be necessary to perform another electrophoresis with more dilute samples or to alter the visualization process to generate a less optically dense material.

Resolution

Resolution is the ability of a system to distinguish between two closely placed or similar objects. Three types of resolution are important for analysis—spatial resolution, intensity resolution, and technique-dependent resolution.

Spatial resolution is the ability to detect two closely placed objects in one-, two-, or three-dimensional space. It is most accurately described as the closest distance two objects can be placed and still be detected as separate ob-

jects. In practice, it is often defined nominally in terms of the number of detectors per unit area such as dots per inch (dpi) or the number of detectors present in total or in each dimension such as 512×512 (262,144 total detectors). Actual resolution is less than half the nominal resolution due to the need for two detectors for every resolvable object (one for the object and one for the separation space) and the effects of optical resolution. Figure 6.9.2 demonstrates how spatial resolution can affect detection of objects. The 42- μm resolution image allows detection of closely spaced bands, the 168- μm resolution image detects fewer bands, and the 840- μm resolution image detects only major bands. For instruments with on-line detection systems, a pseudo spatial resolution is often reported in units of time from the start of the separation or the time interval between two objects crossing the detection path.

Intensity resolution is the ability to identify small changes in intensity. It is a function of both the dynamic range of a detector and the number of potential values that detector can report. Greater dynamic range decreases the intensity resolution of a given detector. The number of potential values a detector reports is

described by its bit depth. An 8-bit detector can report 256 (2^8) different possible values, while a 12-bit detector can report 4,096 (2^{12}) values, and a 16-bit detector can report 65,536 (2^{16}) values. The higher the bit depth, the greater the intensity resolution.

Technique-dependent resolution directly affects the spatial and intensity resolution. Electrophoretic separation techniques that generate overlapping objects or that have object separation distances shorter than the spatial resolution will fail to provide reliable data. Many factors, including the amount of sample loaded, gel pore size, buffer constituents, and electrophoresis field strengths, can dramatically affect separation and resolution of biomolecules. Likewise, detection methods that can only generate a small range of discrete intensity values will not benefit from systems with improved intensity resolution.

IMAGE CAPTURE

Devices

Capturing digital images involves a detection beam or source, a sensor for that beam or source, and some method of assembling a two-dimensional image from the data generated. Most systems use a light source for detection. The light wavelengths used range from ultraviolet (UV) to infrared (IR) and can be broad spectrum or narrow wavelength. Broad-spectrum detection is more versatile since it can often be used for more than one detection wavelength. However, when compared to narrow-wavelength sources such as lasers, broad-spectrum detection suffers from reduced sensitivity and reduced dynamic range. While many types of light sensors have been used, including charge-coupled devices (CCDs), charge-injection devices (CIDs), and photon multiplier tubes (PMTs), technology advances in CCDs have led to their dominance. CCDs are semiconductor imaging devices that convert photons into charge. This charge is then read and converted into a digital format via an analog-to-digital converter (ADC).

The method of image assembly depends on the light source and detector geometry. One method is to capture the image all at once using a two-dimensionally arrayed CCD detector similar to the detectors found in digital and video cameras. Typically a camera-type sensor is paired with a light source that evenly illuminates the sample. This same sensor is often used with fluorescent and chemiluminescent detection methods, as its ability to detect light con-

tinuously over the entire sample reduces image capture times. Another method of image assembly is to capture the image a line at a time. This typically involves a linearly arrayed CCD scanning slowly across the sample in conjunction with the detection beam of light. The data from each line is then compiled into a composite image. Spatial resolution in this method can be significantly better on large-format samples than the resolution of a camera-based system. This method is also advantageous when OD-based detection is used, since the more focused light beam is usually of higher intensity and can penetrate denser material. A third method of image assembly is to use a point light source and single-element detector on each point on a sample. The image is then compiled from each point sampled. This method is slower than the others but can offer extremely high resolution and sensitivity. A fourth commonly encountered method is that of generating a pseudoimage of electrophoresis results through the use of a finish line type of detection system. This is comprised of a light source positioned at the bottom of the gel (i.e., the end opposite of the site of sample loading) and light detectors positioned next to each lane to detect the transmitted light or emitted fluorescence. A lane trace is generated using time on the x axis and light intensity on the y axis. The pseudoimage is then generated from this data (Sutherland et al., 1987).

Capture Process

Prior to image capture, electrophoretic separation and any visualization steps are performed. To calibrate the separation process, standards are usually run at the edges of the gel and often at internal positions. If quantitation of specific proteins or nucleic acids is to be performed, a dilution series of standards with similar properties to the experimental samples should also be included. After separation, the protein or nucleic acid is visualized if necessary. Visualization can include binding of a fluorochrome or chromophore such as Coomassie blue, precipitation of metal ions such as copper, silver, or gold, enzymatic reactions, and exposure of film or phosphor screens to radiant sources. These methods can be grouped based on the type of detection into optical density, fluorescence, chemiluminescence, and radioactivity. The suitability of popular detection devices with these methods is described in Table 6.9.1. Once visualization has occurred, image capture consists of the following steps: previewing the image while

Table 6.9.1 Compatibility of Popular Image-Capture Devices with Common Visualization Methods^a

Visualization method	Image-capture device				
	Silver halide photography	CCD camera	Desktop scanner	Storage phosphor	Fluorescent scanner
Optical density ^b	+	+	++	–	–
Fluorescence	+	+	–	–	++
Chemiluminescence	+	++	–	±	–
Radioactivity	+	–	–	++	–

^aThe device with the highest sensitivity and greatest dynamic range for a visualization method is marked with a ++, other devices that can detect this visualization method are indicated with a +, and devices that are not suitable for a visualization method are indicated with a –. A ± indicates that only some devices of this type can be used with this visualization method.

^bOptical density methods include Coomassie blue staining.

adjusting capture parameters, capturing the image, and saving the image for later analysis.

During the preview process, capture parameters are optimized for data content and for ease and rapidity of later processing steps. Typically, the first step is to place the sample so that when the image is captured, the rectangular edges of the gel are horizontal and vertical on the monitor and any lanes are either horizontal or vertical. Since band and spot detection will be much easier if the image is properly oriented, this eliminates the need to later rotate the image digitally. Image rotation is time-consuming and can result in spatial linearity errors (a change in the size and shape of objects in image) caused by rectangular image-capture device geometries. The next step for camera-based systems is to adjust magnification and to focus the sample image. For thicker samples, it might be necessary to reduce the aperture on camera-based systems to get a sufficient depth of field to focus the entire sample. Often at this point image imperfections—e.g., dust, liquid, or other foreign objects that will detract from later analysis—are detected, and they need to be removed. Next, image intensity is set. Within the area of interest on the image, band or spot peaks should have values less than the maximum saturated value, and the background should have nonzero values. This is usually accomplished through adjustment of the light-source intensity or the sensor signal integration. If the device allows precapture optimization of other parameters such as spatial resolution, contrast, brightness, gain, or gamma, they are adjusted next. Note that this only applies to controls that affect the response of the sensor or processing of the image prior to a data reduction step and not to controls that affect the

image at later stages. The latter process can enhance visualization of specific features but is best left to adjustments in look-up tables (LUTs) in later analysis steps rather than during image capture, since there is a risk of data loss during postacquisition image processing. LUTs are indexed palettes or tables where each index value corresponds to color or gray-scale intensity values present in an image. Many image analysis programs alter LUTs instead of image values directly, since it both is faster and does not change the original image data.

Once all the capture parameters are optimized, the image capture process is initiated. This might take less than a second for images captured with camera-based detectors and up to hours for scanning single-point detectors. When the image has been captured, it should be carefully examined for content. It should fully capture the area of interest and the parameters should have been set so that all necessary information is detectable. Furthermore, it should be in a form that will allow for easy analysis. Extra time spent optimizing the capture parameters will often result in a reduction in total image analysis time and in an increase in data quality. When the best possible image has been captured, it often contains information outside the area of interest. While this is unlikely to cause problems with later analysis, it is often advantageous to crop the image so that the only portion that is saved contains the area of interest. This reduces the amount of disk space necessary to store the image, and the image usually will load and analyze faster with the analysis software.

The last step in image capture is to save the image. Several options are available at this point, including choosing which location to

save the image at, what file type or format to use, and whether to use some form of compression.

The location where the image is saved is not as trivial a question as it might seem if the image will need to be transferred to another computer at some point. File sizes can easily exceed 15 megabytes on high-resolution images. This is a manageable size for hard drives but exceeds current floppy drive sizes by an order of magnitude. There are software utilities available that will subdivide files into disk-size chunks and then reassemble them at the next computer, but this is an inconvenient and slow method. If the computer used to help capture the image is connected to a network, the image files can easily be transferred this way or potentially saved on a central server. Alternatively, several types of high-capacity removable media are available (e.g., Zip or Jazz). This usually requires the installation of additional hardware onto two or more computers but does make backing up data easier.

Since image files can be very large, compression techniques are sometimes used to reduce disk space requirements. Compression algorithms use several methods, typically by replacing frequent or repetitive values or patterns with smaller reference values and by replacing pixel values with the smaller difference values describing the change in adjacent pixels. When the file is later decompressed, the compressed values are then replaced with the original information. Not all images compress equally, with simple images containing mostly repetitive motifs compressible by $\geq 90\%$, while complex images will benefit much less from compression. Because compression is a much slower method of saving files and will not benefit every file, compression is not used to save all files. Several different forms of compression are available but are separable into two main classes, lossless and lossy. Lossless methods faithfully and completely restore the image when it is decompressed (no loss of data) but offer only moderate file compression; compression values range from $\sim 10\%$ to 90% , depending on the image. Examples of lossless compression include Huffman coding (Huffman, 1952), RLE (Run Length Encoding), and LZW (Lempel, Ziv, and Welch; Welch, 1984). In comparison, lossy methods such as JPEG (Joint Photographic Experts Group), MPEG (Moving Picture Experts Group), or fractal compression schemes can reach compression values of $\geq 98\%$ (Russ, 1995). The trade-off is that not all

information from the original file is recovered during decompression. Lossy compression is sometimes necessary for applications with extremely large image files such as real time video capture, but it usually represents an unacceptable loss of data if used with electrophoresis image capture.

Many different file types have been developed to store digital images. Some of these file types are proprietary or hardware specific. For example, PICT is a Macintosh format and BMP is a PC-compatible format. Each file type has its own structure. Some types do not allow compression, for others it is optional, and for some it is mandatory. File types vary in the types of images they support, particularly in the number of colors or gray levels. Below is a brief description of a few of the more prevalent file types.

TIFF (Tagged-Image File Format) is one of the most commonly used formats. It is particularly versatile since it is an open format that can be modified for specific applications. One reason for its versatility is the ability to attach or tag data to the image. The tags can include information such as optical density calibration, resolution, experimenter, date of capture, and any other data that the application software supports. TIFF images can be monochrome, 4-, 8-, or 16-bit gray-scale, or one of many color-image formats. Compression is optional, with LZW, RLE, and JPEG often supported (Russ, 1995). Since TIFF is supported by both Macintosh and PC computers, it is a good choice for multiple-platform environments. The versatility of TIFF can also be a weakness. Since there are many different tagging schemes and since not all programs support all possible compression and color schemes, it is sometimes not possible for one program to access the information in a TIFF file generated by a different program.

GIF (Graphics Interchange Format) is a file format that is widely encountered on the Internet due to its compactness and standardization. Its compactness is attributable to a mandatory modified LZW compression. Another feature of GIF is the use of a LUT to index the values in the image. One interesting ability of GIF is that it supports storing multiple images within a single file. This can offer some advantages for applications such as time-lapse image capture. A GIF image can contain no more than 256 individual color or gray levels and does not support intensity resolutions higher than 8 bit. In addition, since the image

is implemented as a LUT, it also is not a true gray-scale image. Due to these limitations and others, alternative formats such as PNG have been developed to replace GIF.

PICT is a file format and graphics metafile language (it contains commands that can be played back to recreate an image) designed for the Apple Macintosh. It can contain both bitmap images and vector-based objects such as polygons and fonts. It supports a ≤ 256 -gray-level LUT, and monochrome images can be RLE compressed. Because it only offers a 256-gray-level LUT, it has the same weaknesses that GIF does with true gray-scale and high-intensity-resolution images. In addition, any vector objects in the image are difficult to translate on a PC since they are designed to be interpreted by Macintosh QuickDraw routines.

BMP is the native bitmap file format present on Windows-based PCs. It supports 2-, 16-, 256-, or 16-million-level images. With images of ≤ 256 gray levels, it implements a LUT, while the highest-resolution image is implemented directly. RLE compression is optional for 16- and 256-gray-level images. Since compression is prohibited on 16 million-gray-level images and there is no intermediate level supported beyond 256 levels, BMP is not a good choice for images with high-intensity resolution requirements.

ANALYSIS

Once the image has been captured, the data needs to be analyzed and distilled into information about the results of the electrophoresis experiment. Through the use of standards and experimenter input, this software-driven process can estimate mass and quantity of objects in an image and detect relationships between objects within one image and between similar images. The type of software used depends on the analysis to be performed. Images from single electrophoretic separations are examined by one-dimensional analysis software optimized for lane-based band detection. Images from two-dimensional electrophoresis are best handled by specific programs designed to detect spots and to assign two mobility values and a quantity value to the spot. After the initial characterization of bands and spots, comparisons are often made between bands or spots from different experiments through the use of database programs and matching algorithms.

Software for One-Dimensional Analysis

Lane positioning

For one-dimensional analysis, the first activity is to detect the lanes on the image. One of three different methods is commonly employed for this. For images with straight, well-defined lanes with a large number of bands, automatic lane-detection algorithms can quickly and accurately place the lanes. On images with very well-defined lanes, such as pseudoimages from finish-line type electrophoresis equipment, automated lane calling based on image position is possible. For images with "smiling," bent, or irregular lanes, manual positioning of the lanes is often the fastest and most accurate method of lane definition. Regardless of the method of identifying the lanes, the lane boundaries need to be carefully set for accurate quantitation and mass determinations. Lane widths should be wide enough so that the entire area of all bands in that lane are included, but they should not be so wide as to include bands from adjacent lanes. To accomplish this, curved or bent lanes might need to be used in order to follow the electrophoresis lane pattern. Lane length and position also must be adjusted as necessary so that all bands of interest are included. If mass determinations are necessary, the sample loading point should probably also be included in the lane or be the start of the lane. At this point, lines of equal mobility (often called R_f or iso-molecular-weight lines) are added to the image as necessary. These lines allow for correction of lane-to-lane deviations in the mobility of reference bands and generate more accurate measurements of mass. A similar form of correction is also possible for within-lane correction of mobilities. This correction is important for accurate detection and quantitation of closely spaced bands.

Band detection

Once the lanes have been defined, the bands present in each lane need to be detected. There are many methods for detecting bands. One method is to systematically scan the lane profile from one end to the other, identifying regions of local maxima as bands. Another common method is to use first- and second-order derivatives of the lane image or lane profile in order to find inflection points in the change of slope in pixel intensity values (Patton, 1995). Regardless of the method used, it is often necessary to alter the search parameters so that they perform reliably under a given experimental condition.

Typical search parameters include ones for detection sensitivity, smoothing, minimum inter-band gaps, and minimum or maximum band peak size. Smoothing reduces the number of bands detected due to noise in the image. A minimum interband gap is often used to avoid detection of false secondary bands on the shoulders of primary bands. Limitations on peak sizes, especially for within-lane comparison to the largest band's peak, can be a useful way to allow sensitive detection of bands in underloaded lanes without detecting false bands in overloaded lanes.

Band edges are often detected in addition to band peaks in order to further define bands or to quantitate band amounts. This can be accomplished by using local minima, derivatives of the lane profile, or fixed parameters such as image distances or a percentage of band peak height. The band edges can be applied as edges perpendicular to the long axis of the lane or as a contour of equal intensity circling the band. The perpendicular method is advantageous for bands with uneven distribution of material across the face of the band, while the latter method is better for "smiling" or misshapen bands.

Background subtraction

With nearly all electrophoresis procedures, the most informative images have a low level of signal intensity at each pixel that does not result from protein or nucleic acid. Instead, this background intensity is attributable to the gel medium, the visualization method, electronic noise, and other factors. Since this background tends to be nonuniformly distributed throughout the image, failure to subtract it can make band detection and quantitation less accurate. Many methods of background subtraction are possible. Sometimes it is possible to generate a second image under conditions that do not detect the protein or nucleic acid. The second image is then digitally subtracted from the data-containing image to remove the background. More often, background information must be obtained from a single image. If the background varies uniformly across the image, a line that crosses the variation can be defined at a point where no bands are present. The intensity values at each point on the line can be used as the background value for the pixels perpendicular to the line at that point. Commonly, background is also present as variations in intensity along the long axis within each lane. One simple method is to take the lowest point in the lane profile as the background. Another

method is to use an average value of the edge of each band as the background for that band. More complicated methods such as valley-to-valley and rolling-disk use local minima points in the lane profile to define a variable background along the length of the lane. Because there can be many different causes and distributions of background, no single method of background determination can be recommended for all experiments.

Characterization

Once lanes and bands have been detected, it is possible to interpret the mobility of the nucleic acid or protein bands. Depending on the method of electrophoretic separation, information on mass (length or size), pI, or relative mobility (R_f) can be inferred from mobility information. The mobility is characterized using a standard curve with internal standards of known properties. The type of curve depends on several parameters. By definition, with R_f -based separation, a linear first-order curve is used since it represents the linear relationship between mobility and R_f . Similarly, pI and mobility are generally linear in isoelectric focusing separations. For separations based on size, a curve generated from mobility versus the log of the molecular weight provides a relatively good fit as measured by the correlation coefficient (R^2). Several other curves have been suggested for size-based separations, including modified hyperbolic curves and curves of mobility versus (molecular weight)^{2/3} that have good correlation coefficients (Plikaytis et al., 1986). In some cases, no single curve equation can adequately represent the data, and methods of fitting smooth contiguous curves using only neighboring points, such as a Lagrange or spline fit (described in Hamming, 1973), are necessary. This is most common for size separations with a very large range of separation sizes and with nonlinear gradient gels. Care must be taken with multiple-curve techniques since they rely on only a few data points for any one part of the composite curve, and outlying data points can drastically affect the outcome.

For size and R_f determination, a uniform position must be found in each lane as a point from which to measure the mobility of each band. Many software analysis packages use the end of the lane as the measuring start point, so for them it is important to position each lane start point at an iso-molecular-weight or iso- R_f point. A convenient point is the well or sample-loading position since it is usually easily de-

teachable and at an equal mobility position in each lane. A consistent point on each band must also be chosen to measure mobility. A band's peak is easily defined in digital image analysis and is commonly used. Since peak positions are harder to detect visually than edges on silver halide images, the leading band edge is sometimes used when comparing digital results with silver halide-based results.

Once lanes and bands have been detected, it is also possible to quantify the amount or at least relative amount of nucleic acid or protein present in each band. The amount in a band is related to the sum total of the intensity values of each pixel subtracted by the background value for each pixel in a band. For absorptively detected bands, intensity values are converted to OD values. The total value that is calculated is equivalent to the volume of the band and can be directly compared to other bands that are within the linear range for the visualization method. If standards of known amounts are loaded onto the same gel, they can be used to generate a standard curve that converts band volume into standard units such as micrograms. For greatest accuracy, it is important to be able to generate multiple standard curves when using visualization methods, such as Coomassie blue staining, that are affected by band or spot composition.

Quantitation becomes more complicated when bands are not fully resolved. In this case, material from one band is contributing to the volume measurement of an adjacent band and vice versa. The simplest method for handling this is to partition into each band only the volume within its edges. Alternatively, a Gaussian curve can be fitted to each band and the volume contained within the curve used to estimate the amount of the band. Since most electrophoresis bands have a pronounced skew towards the leading edge of the band, modified Gaussian curves have also been used (Smith and Thomas, 1990). In either case, the curve-fitting process is calculation intensive and can significantly increase analysis times for images with many bands.

Software for Two-Dimensional Analysis

In two-dimensional analysis, the first-dimension separation is performed in a single column or lane and then a second separation is performed perpendicular to the first. The result after visualization is a rectangular image of up to 10,000 spots. The most common two-dimensional gel type is one in which protein is sepa-

rated first by apparent pI and second by molecular weight, although two-dimensional separation of nucleic acids is also possible. While many of the concepts and analysis techniques used with one-dimensional gels are applicable to two-dimensional gels, the complex nature of most two-dimensional gels requires somewhat different methodology. For example, spots are more difficult to detect since they are not conveniently arranged in lanes and can vary more in shape and overlap than bands. In addition, two-dimensional experiments usually require some method of comparing between two images, whereas one-dimensional images usually contain all of the information from an experiment.

Spot detection

Probably the most difficult aspect of two-dimensional analysis is efficient and accurate spot detection. If it is incorrectly done, it can lead to hours of manual editing. Due to the complexity and computational intensity of some algorithms, the detection process itself can last hours on relatively fast desktop computers. One theoretically effective but computationally intense method is to treat the image as essentially a three-dimensional image with spots treated as hills and background as valleys. A large number of Gaussian curves are then combined to describe the topology of the image. Many other methods make use of a digital-imaging technique known as filtering. In essence, filtering is a way to weight the value of a pixel and its neighbors in order to generate a new value for a pixel. By passing a filter across an image pixel by pixel, a secondary image is generated. Filters can be designed for many tasks, including sharpening an image or removing high-frequency noise. Filters can also be generated to help detect spots by making images that are first and second derivatives of the original image. The derivative images indicate inflection points in the intensity pattern and can be used to detect spot centers and edges. In a different method, called thresholding, filters can be used to detect the edges of objects. Instead of looking for inflection points, threshold filters identify intensities above a set level or at ratios between central and edge pixels above a set value. Since the edges on two-dimensional spots tend to be diffuse, sharpening filters are sometimes used prior to the thresholding filter. In some cases, multiple techniques are used to detect spots (Glasbey and Horgan, 1994).

Unlike one-dimensional detection, detection on images of two-dimensional experiments usually requires secondary processing to get acceptable performance. One example of a secondary process is to discard spots with sizes below a set minimum or above a set maximum. Another is to analyze spots that are oval for possible splitting into two spots. Even after secondary processing, it is likely that a small amount of manual editing will be necessary. When manually editing an image, care must be taken to use as objective criteria as possible, especially if two or more images are to be matched and spot volumes compared.

Characterization

In a two-dimensional system, determination of protein or nucleic acid mobility is complicated by the fact that there are two mobilities to account for and that the second-dimension separation tends to make estimation of the separation which occurred in the first dimension more difficult. One method for dealing with this is to have a series of markers in the sample that, after both separations are completed, are evenly distributed within the gel and image. It is also possible to estimate separation characteristics from calibration points located at the periphery of the gel. For example, distance measurements can be used to pass calibration data from the first dimension separation, and standards can be separated at the ends of the gel to calibrate for mobility in the second dimension. Regardless of the method used, in many instances, a series of related images will be examined and similar spots in each image will be matched. When this occurs, it is possible to calibrate one image and then pass the calibration information via the matches to the related images.

Quantity determination is similar in many regards to that which occurs in one-dimensional analysis, but there are some differences. If spot edges are detected, a simple method of determining spot volume is to take the sum of the intensity value of each pixel in the spot reduced by a background intensity value. Multiple Gaussian curves can also be fitted to the spot to approximate the volume (Garrels, 1989). More difficult is attempting to compensate for a skewed distribution in a size-separating dimension while trying to use a regular Gaussian fit for a pI separation, such as is encountered with the most common form of two-dimensional protein separations. The distribution of background makes quantitation more difficult in two-dimensional gels. There is no lane-dependent component, so it is nec-

essary to use other methods such as image stripes, finding local minimum values or using values derived from the spot edges to determine background values.

Matching

Matching is the process in which proteins or nucleic acids with similar separation properties are linked or clustered together. Matching can occur within one image or between multiple images as long as a frame of reference is established. Matching allows for comparisons between samples. It also makes annotation and data entry easier, since if one spot or band is matched to others and is characterized or annotated, this information is easily passed to all the other matches. An underlying assumption of matching is that objects with similar separation properties are actually similar. Care must be taken to confirm the identity of matched spots or bands by other methods on critical experiments.

A simple form of matching is to link bands or spots at similar positions on the gel images. This works well when separation and imaging conditions are uniform. This is very seldom the case, since slight differences in the electrophoresis, visualization, and imaging conditions across a gel and between gels generates incorrect matching with this method. Since bands on one-dimensional gels are relatively easy to calibrate for mobility, matching can occur along contours of equal mobility. This dramatically decreases but does not eliminate the variability in detecting similar bands. Much of the remaining variability can be attributed to calibration errors. This error can often be compensated for by allowing a small tolerance in mobility values in determining whether a band is matched or not. Because of the difficulties in calibrating mobility in two-dimensional gels, it is often more practical to use matched spots for calibrating mobility than vice versa. Spot matching between two two-dimensional images starts with finding a small number of landmark spots that are used as seeds for subsequent matches (Appel et al., 1991; Monardo et al., 1994). There are many methods for finding the landmarks in both images, including finding the highest-intensity spots, finding spots in unique clusters, and manual positioning. The most common procedure from this point is to derive a vector that describes the direction and extent of the path from one matched spot to the other when the two images are superimposed. The vector is used as the basis for finding more matches near the landmark matches. To allow

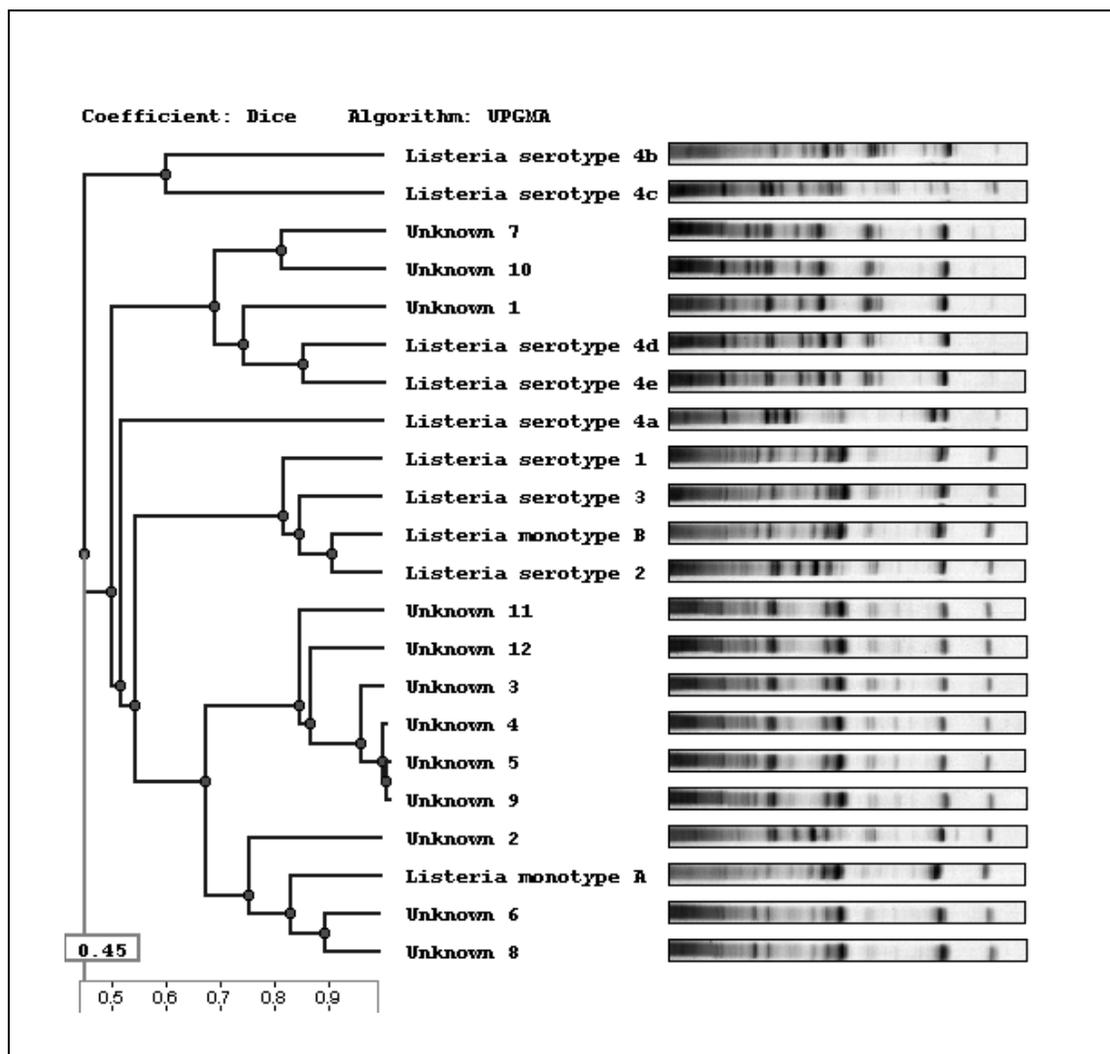


Figure 6.9.3 Example of a dendrogram generated from similarity data on band matching between lanes. DNA samples from 22 isolates of *Listeria* were subjected to Random Amplification of Polymorphic DNA (RAPD) analysis and the resulting electrophoresis image was analyzed with ImageMaster software (Amersham Pharmacia Biotech). Clustering was performed using the Dice coefficient with a tree structure based on the Unweighted Pair-Group Method using Arithmetic Averages (UPGMA). Similarity values between isolates can be determined by locating the node that connects the isolates and reading the value from the scale on the lower left edge of the dendrogram.

for error, the area within a small radius is searched extending from the end of the vector. Once another match is found, its vector is computed and used as the starting point for finding neighboring matches. From this progression, the entire gel is matched. If all vectors are displayed graphically when matching is complete, questionable matches can often be identified as vectors that are significantly different from neighboring vectors.

One specialized use of matching is as an estimator of the similarity and potential genetic relatedness of organisms. For example, on a one-dimensional gel image, a ratio of the matched to unmatched bands for each pairwise combination of lanes can be calculated. This

ratio can be used as an indicator of similarity, with values near 1 indicating a pair of highly similar lanes, and values near zero indicating very dissimilar lanes. Assuming that the contents of the lanes are valid samples of the originating organism's genetic makeup, the information on lane similarity can be converted to estimates of genetic similarity. A convenient way to display this similarity data graphically is to generate a dendrogram with similar objects close to each other and less similar objects more distantly placed. An example of such a dendrogram is presented in Figure 6.9.3, where samples from *Listeria* isolates are arranged based on banding pattern.

Databases

In many cases, image analysis is not the last step in the process. The image and analysis data need to be archived in a searchable format. There may be a need to analyze the data from multiple experiments conducted at different sites or in laboratories around the world. Bioinformatic links to diverse data sources might be desired to help develop a unified understanding of the biology behind particular phenomena. When these situations arise, database programs can be utilized to store, link, and search image analysis results.

As the number of images that are captured and analyzed grows, it becomes increasingly more difficult to find particular information from the large number of files that are stored. Relatively simple databases can be used if the major requirement is to find previously analyzed images and associated data. Such databases often display a miniaturized version of each image to aid in visual scanning for the file as well as simple searching for image-specific information such as date of analysis, file name, or other information that was entered at the time of image capture. More powerful database products are also available that can perform complex searches on data generated during the analysis. For an example, a search on a two-dimensional database might include finding proteins exhibiting a specific expression profile and having a molecular weight >20 kDa with a pI between 3 and 5 or 8 and 10 with an amount <50 ng in a series of experiments conducted <1 year ago. Such searches can quickly target potentially interesting molecules for further analysis.

With the increasing ease of transferring data through the Internet as well as local- and wide-area networks, it has become practical to quickly find and examine data from distant locations. Of course, great care must be taken to ensure that similar experimental conditions are employed, as otherwise the results will be difficult to compare. In this manner it is sometimes possible to dramatically increase the sample size and statistical accuracy as well as the probability of detecting rare events. In addition, if one data set is more completely characterized, this extra information can be extracted and applied to the other data set. For example if there is a band in common in two databases and there is sequence information for it in one database, that sequence information can be added to the other database. Currently most public electrophoresis database sites are two-dimensional protein databases. A list with links

to many of these Internet database sites can be found at <http://www-lmmb.ncifcrf.gov/EP/table2Ddatabases.html>.

With biological questions becoming more complicated and the answers to the questions often requiring information from a variety of sources, it is becoming increasingly important to be able to move easily between information sources. A relational type of database can help achieve this. Unlike a conventional database with a fixed arrangement of data, relational databases have links between related files that allow for easy movement from one file to another. Another approach to interconnecting electrophoresis data with data from other sources is to generate a series of hypertext links between data sets, similar to what occurs on the Internet. Selecting a specific link moves the search to the related network site and the related information. Regardless of the method, the end goal is similar. An example of what is possible: a researcher selects a protein spot on a two-dimensional gel image, which triggers accessing of related information on this protein. The protein sequence is accessed from mass spectroscopy analysis of the spot on a separate gel. The sequence of the gene and the cDNA that generated the protein is retrieved. The expression pattern of the gene in various tissues and conditions, as well as information on similar genes in other organisms, is incorporated. Citations and annotations to this are retrieved as well. All of this information is compiled automatically into an interactive report about the protein. From this report, the researcher can formulate a more refined hypothesis and plan the most appropriate experiments to test it.

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Russ, 1995. See above.

A general reference book on digital image capture and analysis.

Sutherland, J.C. 1993. Electronic imaging of electrophoretic gels and blots. *In Advances in Electrophoresis*, Vol. 6. (A. Chrambach, M.J. Dunn, and B.J. Radola, eds.) pp. 1-41. VCH Verlagsgesellschaft mbH, Weinheim, Germany.

Provides an overview of image capture with particular emphasis on types of capture equipment.

INTERNET RESOURCES

rsb.info.nih.gov/nih-image

NIH Image is free software that provides basic image analysis tools for the Macintosh.

<http://www.inforamp.net/~poynton/Poynton-color.html>

Contains an excellent description of gamma correction in the Gamma FAQ.

<http://www-Immb.ncifcrf.gov/EP/table2Ddatabases.html>

A list of links to many two-dimensional databases that are available via the Internet.

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CHAPTER 7

Protein Labeling and Immunoprecipitation

INTRODUCTION

The characterization of cellular proteins in complex mixtures, as well as analysis of their biosynthesis, processing, intracellular trafficking and degradation, generally requires that the proteins be labeled either intrinsically (*UNIT 7.1*) or extrinsically. The labeled proteins are then isolated by immunoprecipitation (*UNIT 7.2*) and analyzed using electrophoretic techniques (Chapter 6).

Intrinsic labeling refers to the incorporation of a labeled precursor into the protein during synthesis. The most common form of intrinsic labeling consists of supplying the labeled precursor to live cells so that it becomes incorporated into proteins by the cell's own biosynthetic machinery. This form of intrinsic labeling is referred to as metabolic or biosynthetic labeling. The complexity and fidelity of the cell's biosynthetic pathways does not allow much flexibility in the design of precursors suitable for metabolic labeling. For instance, chemical derivatization of amino acid precursors renders them extraneous to plasma membrane transporters and aminoacyl-tRNA synthetases. Incorporation of unnatural amino acids can also affect the folding and stability of the polypeptide chains. Thus, the choice of precursors is limited to those labeled with an uncommon isotope of one of its constituent atoms. Although stable heavy isotopes have been used to label precursors, by far the most common means of labeling amino acids entails substituting radionuclides such as ^{35}S , ^3H , or ^{14}C in place of their nonradioactive counterparts. Because isotopes have identical biochemical properties, these substitutions do not affect protein synthesis.

The first unit in this chapter (*UNIT 7.1*) presents a compilation of protocols for metabolic labeling of proteins in mammalian cells using radiolabeled amino acids. The preferred radiolabeled amino acid for this purpose is [^{35}S]methionine, although [^{35}S]cysteine or ^3H -labeled amino acids can also be used. One of the methods described in *UNIT 7.1* is referred to as pulse labeling, and consists of incubating the cells with the radiolabeled amino acid for short periods (10 to 30 min). Pulse labeling is used to label newly synthesized proteins. In many cases, the pulse is followed by a chase in which cells are further incubated with the unlabeled counterpart of the precursor used for labeling. Pulse-chase protocols allow study of the fate of proteins after synthesis. Another method described in this unit is referred to as long-term labeling, and consists of labeling cells for periods of 6 to 32 hr. Long-term labeling is generally used to study the properties of mature proteins or protein complexes, rather than their biogenesis.

Metabolically labeled proteins can be directly analyzed using electrophoretic techniques (*UNIT 6.1*). Most often, however, specific metabolically labeled proteins are isolated from other cellular proteins by immunoprecipitation. Even when metabolically labeled proteins are first separated by the physical methods described in Chapter 5, specific proteins ultimately have to be isolated by immunoprecipitation. Immunoprecipitation techniques exploit the ability of the immune system to produce specific antibodies to virtually any protein (see Chapter 16). In the most widely used immunoprecipitation protocols, antibodies are bound to protein A–agarose beads, after which the immobilized antibodies are incubated with a detergent extract of the cells. Specific proteins can then be rapidly separated from other proteins in the mixture by repeated washing of the beads. *UNIT 7.2*

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Protein Labeling
and Immuno-
precipitation

7.0.1

Supplement 15

describes a series of simple protocols for immunoprecipitation of proteins from cell extracts. Included in this unit are protocols for extracting both adherent and suspended animal cells under either nondenaturing or denaturing conditions. There is also a protocol for lysing yeast cells, which requires mechanical breakage of the cell wall. *UNIT 7.2* also includes procedures for binding antibodies to protein-agarose beads, incubating the antibody-bound beads with cell extracts, and washing the beads. Finally, the unit describes an immunoprecipitation-recapture protocol in which proteins are immunoprecipitated twice sequentially with either the same antibody or different antibodies. This type of protocol can be used to reduce the nonspecific background of the first immunoprecipitation or to analyze the composition and assembly of multiprotein complexes.

Proteins can be metabolically labeled using precursors other than amino acids—e.g., sulfate, lipids, carbohydrates, and phosphate, provided that the proteins are modified by the addition of these groups. *UNIT 7.3* describes protocols for both long-term and pulse-chase metabolic labeling using [³⁵S]sulfate. Use of these protocols allows radiolabeling of proteins that are sulfated on either tyrosine residues or carbohydrate moieties. *UNIT 7.4* describes protocols for metabolic labeling fatty-acylated proteins with [³H]palmitic acid or [³H]myristic acid. This unit also includes protocols for determining the type of linkage in which the fatty acid is attached and the identity of the fatty acid. *UNIT 7.5* deals with another type of lipid modification of proteins, namely prenylation by addition of farnesyl or geranylgeranyl groups. Metabolic labeling of prenylated proteins can be achieved by incubation with [³H]mevalonic acid, which is a precursor of prenyl groups. Prenylated proteins are often also carboxyl-methylated. This modification can be studied by metabolic labeling with [³H-methyl]methionine, a protocol for which is also included in this unit.

The yeast, *Saccharomyces cerevisiae*, is widely used as a model organism for the study of cellular processes that are common to all eukaryotic cells. Critical to these studies is the ability to label yeast proteins metabolically. Metabolic labeling and immunoprecipitation of yeast proteins can be achieved by modification of methods described for mammalian proteins (*UNITS 7.1 & 7.2*). *UNIT 7.6* describes protocols for metabolic labeling yeast proteins with ³⁵S-labeled amino acid mixtures and for mechanical disruption of the labeled yeast cells with glass beads (a variation of this method of yeast cell lysis is described in *UNIT 7.2*). In addition, *UNIT 7.6* includes protocols for preparation of spheroplasts from the radiolabeled yeast cells by enzymatic lysis of the cell wall, and for treatment of immunoprecipitated yeast proteins with endoglycosidase H. This unit also discusses criteria for metabolic labeling of yeast cells with precursors other than ³⁵S-labeled amino acid mixtures.

Another organism that is commonly used in biological research is the fruit fly, *Drosophila melanogaster*. Unlike *Saccharomyces cerevisiae*, *Drosophila* is a multicellular organism and possesses a body organization and function that is in many ways similar to that of higher eukaryotes. Powerful genetic methodologies have been developed that allow detailed analyses of embryonic development and function of organ systems. Studies at the cellular level in *Drosophila*, however, have lagged behind. Methods for metabolically labeling *Drosophila* cells in culture, in particular, are hard to find. In *UNIT 7.7* we present a protocol for metabolic labeling and immunoprecipitation using *Drosophila* S2M3 cells. The unit also contains a protocol for the growth and maintenance of S2M3 cells. Finally the Commentary provides references for preparation of primary cultures of *Drosophila* cells. The ability to culture *Drosophila* cells from wild-type and mutant flies allows analysis of the cellular roles of specific gene products.

The protocols described thus far describe labeling of proteins and protein conjugates by incubation of cells with radiolabeled precursors (i.e., intrinsic labeling). However, proteins can also be labeled, either in purified form or in mixtures, after extraction from the cells (i.e., extrinsic labeling). *UNIT 7.9* presents the first set of protocols in this chapter

dealing with extrinsic labeling of proteins. In our aerobic world, cellular proteins are constantly suffering oxidative damage by reactive oxygen species. Amino acid residues, such as histidine, lysine, arginine, proline, threonine, cytosine, tyrosine and aspartate, can all undergo oxidative modification within cells. Upon protein extraction from cells, modified amino acid residues can be detected and quantified by reaction with specific chemical reagents. The products of these reactions can then be analyzed by gel electrophoresis, mass spectrometry, or ELISA, either directly (as described in this unit) or following isolation of specific proteins by immunoprecipitation (*UNIT 7.2*).

Most of the protocols discussed to this point are based on intrinsic labeling of cellular proteins or protein conjugates. However, proteins can also be labeled extrinsically by chemical modification of specific amino acid residues. A commonly used method is the chemical modification of tyrosine residues with ¹²⁵I. Radioiodination can be used to label proteins on the cell surface and in isolated organelles, detergent extract, or soluble fractions as described in *UNIT 7.9*. Proteins can be labeled to high specific activities, generally with little or no loss of function. The risk of contamination by inhalation of volatile radioiodine, however, has led to the replacement of radioiodination by safer extrinsic labeling techniques such as biotinylation.

Juan S. Bonifacino

Metabolic Labeling with Amino Acids

Metabolic labeling techniques are used to study biosynthesis, processing, intracellular transport, secretion, degradation and physical-chemical properties of proteins. In this unit, protocols are described for metabolically labeling mammalian cells with radiolabeled amino acids (Table 7.1.1). Cells labeled using these procedures are suitable for analysis by immunoprecipitation (UNIT 7.2), characterization of cellular proteins (see Chapter 5), analysis of protein trafficking, and one- and two-dimensional gel electrophoresis (see Chapter 6). The first protocols describe pulse-labeling (10 to 30 min) of mammalian cells in suspension with [³⁵S]methionine (see Basic Protocol), and necessary modifications for adherent mammalian cells (see Alternate Protocol 1). Alternate protocols are also presented for pulse-chase analysis (see Alternate Protocol 2) and long-term labeling (“steady state,” 6 to 32 hr; see Alternate Protocol 3). This is followed by conditions for metabolic labeling of cells with radiolabeled amino acids other than [³⁵S]methionine (see Alternate Protocol 4). The degree of label incorporation can be determined by precipitation with trichloroacetic acid (TCA; see Support Protocol).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified, 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

Table 7.1.1 Radiolabeled Amino Acids Used in Metabolic Labeling of Proteins

Amino acid ^a	Frequency (%) ^b	Radioisotope	Specific activity (Ci/mmol)	Comments ^c
Leucine	10.4	³ H	5-190	E
Serine	8.1	³ H	5-40	T, I
Glutamic acid	7.3	³ H	15-80	T, I
Lysine	7.0	³ H	40-110	E
Alanine	7.0	³ H	10-85	T
Valine	6.2	³ H	10-65	E
Glycine	5.7	³ H	10-60	T, I
Threonine	5.6	³ H	5-25	E
Arginine	5.0	³ H	30-70	E
Aspartic acid	4.9	³ H	10-50	T, I
Proline	4.9	³ H	15-130	—
Glutamine	4.5	³ H	20-60	T, I
Phenylalanine	4.5	³ H	15-140	E
Tyrosine	3.6	³ H	15-60	—
Asparagine ^d	3.5	—	—	—
Cysteine	3.4	³⁵ S	>800	—
Isoleucine	2.9	³ H	30-140	E
Histidine	2.5	³ H	30-70	E
Methionine	1.8	³⁵ S	>800	E
Tryptophan	1.3	³ H	20-30	E

^aAll amino acids in this table are in the L configuration.

^bFrequency of amino acid residues in proteins, taken from Lathe (1985).

^cE, essential amino acids; T, amino acids that are modified by transamination (Coligan et al., 1983);

I, amino acids that are converted by cells to other amino acids.

^dAsparagine is difficult to label (Coligan et al., 1983).

SAFETY PRECAUTIONS FOR WORKING WITH ³⁵S-LABELED COMPOUNDS

When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

Solutions containing ³⁵S-labeled compounds have been found to release volatile radioactive substances (Meisenhelder and Hunter, 1988). In addition to the usual safety practices followed when handling radioactive materials (see *APPENDIX 1D*), some extra precautions should be taken when using ³⁵S-labeled amino acids:

1. Vials containing ³⁵S-labeled compounds should always be handled in a designated fume hood equipped with an activated charcoal filter. This includes thawing the solution, opening the vial, and adding the radiolabeled amino acid to the labeling medium. Before opening, vials should be vented with a needle attached to a syringe packed with activated charcoal. Avoid using tissue culture hoods for this purpose as they are likely to become contaminated.
2. Minimize exposure of ³⁵S-containing solutions to the air.
3. Use baths, incubators, and centrifuges designated for work with radioactive materials. Place a tray containing a layer of activated charcoal in the CO₂ incubator to reduce the amount of ³⁵S-labeled compounds released to the air during cell labeling. Alternatively, filters impregnated with activated carbon (β-Safe, Schleicher & Schuell) can be attached to the covers of tissue culture dishes.
4. Monitor areas used during labeling by conducting wipe tests.
5. Dispose of solid and liquid ³⁵S waste quickly and with appropriate precautions.

BASIC PROTOCOL

PULSE-LABELING OF CELLS IN SUSPENSION WITH [³⁵S]METHIONINE

Pulse-labeling of proteins is performed by incubating cells for short periods (≤30 min) in culture medium containing a radiolabeled amino acid. The conditions described below are optimized for labeling times of 10 to 30 min. The same protocol is used for labeling cells for up to 6 hr, although a smaller number of cells and/or a larger amount of labeling medium should be used for optimal results. Very short pulses (<10 min) may require special conditions that are discussed elsewhere (see *Critical Parameters*). For labeling times >6 hr, see *Alternate Protocol 3*. [³⁵S]methionine is the radiolabeled amino acid of choice for metabolic labeling of proteins because of its high specific activity (>800 Ci/mmol) and ease of detection. A potential disadvantage of [³⁵S]methionine is its low abundance in proteins (~1.8% of the average amino acid composition; see *Table 7.1.1*). For proteins that contain little (e.g., only one) or no methionine, other radiolabeled amino acids should be used (see *Alternate Protocol 4*).

Materials

[³⁵S]L-Methionine (>800 Ci/mmol) or [³⁵S]-labeled protein hydrolyzate (>1000 Ci/mmol)

Pulse-labeling medium (see recipe), warmed to 37°C

Cell suspension (e.g., Jurkat, RBL, K562, BW5147, T and B cell hybridomas), grown in a humidified, 37°C, 5% CO₂ incubator or prepared from tissues (e.g., lymphocytes, *UNIT 2.2*)

PBS (*APPENDIX 2A*), ice cold

Vacuum aspirator with trap for liquid radioactive waste

Additional reagents and equipment for TCA precipitation (optional; see Support Protocol)

1. Thaw [³⁵S]methionine at room temperature and prepare a 0.1 to 0.2 mCi/ml working solution in prewarmed (37°C) pulse-labeling medium.

CAUTION: Volatile ³⁵S-containing compounds can be released during the labeling procedure (refer to Safety Precautions and APPENDIX 1D). Keep [³⁵S]methionine-containing medium in a tightly capped tube in a 37°C water bath until use. Do not let it sit >60 min at 37°C.

2. Harvest $0.5\text{--}2 \times 10^7$ cells in suspension by centrifuging 5 min at $300 \times g$, room temperature.
3. Wash cells with ~10 ml prewarmed pulse-labeling medium. Centrifuge 5 min at $300 \times g$, room temperature, and aspirate supernatant. Resuspend cells by gently tapping the bottom of the tubes and repeat wash.
4. Resuspend cells at 5×10^6 cells/ml in prewarmed pulse-labeling medium and incubate 15 min in a 37°C water bath to deplete intracellular pools of methionine. Invert tubes periodically to resuspend cells.
5. Centrifuge cells 5 min at $300 \times g$, room temperature, and aspirate supernatant.
6. Resuspend cells in a fresh 15-ml centrifuge tube, using 2 ml [³⁵S]methionine working solution (step 1). Cap tubes tightly. Incubate cells 10 to 30 min (see Critical Parameters) in a 37°C water bath, resuspending frequently by gentle inversion of the tubes.

Alternatively, use a rotator placed in a 37°C incubator.

7. Centrifuge cells 5 min at $300 \times g$, 4°C, and aspirate supernatant. Resuspend with gentle swirling in 10 ml ice-cold PBS and repeat centrifugation.

CAUTION: The medium and wash are radioactive—follow applicable safety regulations for disposal.

Because of the short times and high concentrations of radiolabeled amino acids employed, pulse-labeling may not result in complete depletion of label from the medium. If this is the case, the labeling mixture can be reused. Collect the labeling medium containing [³⁵S]methionine or ³⁵S-labeled protein hydrolyzate and filter carefully through a 0.45- μm filter unit. Estimate the percentage of unincorporated radioactivity by scintillation counting aliquots of the labeling mixture before and after labeling the cells. Store the labeling mixture up to 2 months frozen at -20°C .

8. *Optional:* Determine amount of label incorporation by trichloroacetic acid (TCA) precipitation (see Support Protocol 1).

Labeled cells are now ready for the desired processing and analysis. If cell pellets cannot be processed immediately, they can be kept on ice for a few hours or frozen at -80°C for several days. Thaw frozen cell pellets on ice before analysis. In most cases, freezing cells will not affect the biochemical properties of the proteins, but freezing and thawing cell extracts after solubilization with detergents (UNIT 7.2) can cause dissociation of multisubunit complexes or proteolysis of labeled proteins.

PULSE-LABELING OF ADHERENT CELLS WITH [³⁵S]METHIONINE

Labeling adherent cells is essentially the same as described for cells in suspension except that cells are labeled while attached to a dish. As with suspended cells, adherent cells are pulse-labeled 10 to 30 min (see Basic Protocol introduction). Alternatively, some adherent cells can be detached from plates by incubation for 10 min at 37°C with 10 mM EDTA in PBS (*APPENDIX 2A*) and labeled as a suspension (see Basic Protocol). This is particularly advantageous when labeling cells for pulse-chase experiments, because it simplifies handling of multiple samples and allows more uniform labeling of the cells.

Additional Materials (also see *Basic Protocol*)

Adherent cells (e.g., HeLa, NRK, M1, COS-1, CV-1, or fibroblasts or endothelial cells in primary culture; *UNITS 2.1 & 2.3*)

100-mm tissue culture dishes

1. Grow adherent cells to 80% to 90% confluency in 100-mm tissue culture dishes.

Depending on the cell type, a confluent 100-mm dish will contain $0.5\text{--}2 \times 10^7$ cells.

2. Prepare [³⁵S]methionine working solution as described (see Basic Protocol, step 1).
3. Aspirate culture medium from the dishes and wash twice by gently swirling with 10 ml prewarmed (37°C) pulse-labeling medium, aspirating medium after each wash.
4. Add 5 ml prewarmed pulse-labeling medium and incubate 15 min in a humidified, 37°C, 5% CO₂ incubator to deplete intracellular pools of methionine.
5. Remove medium from cells, add 2 ml [³⁵S]methionine working solution (from step 2), and incubate 10 to 30 min in a CO₂ incubator.

If necessary, as little as 1 ml of labeling medium per plate can be used. It is critical, however, that the plates sit on a perfectly horizontal surface during incubation to avoid drying of the cell monolayer.

6. Remove medium from cells. Wash once with 10 ml ice-cold PBS and remove PBS.

CAUTION: *The medium and wash are radioactive—follow applicable safety regulations for disposal.*

If significant detachment of cells occurs during labeling, it may be necessary to scrape the cells carefully in the [³⁵S]methionine-containing medium and transfer the suspension to a 15-ml centrifuge tube before centrifuging and washing with PBS.

7. Add 10 ml ice-cold PBS and scrape cells carefully with either a disposable plastic scraper or a rubber policeman.
8. Transfer the suspension to a 15-ml centrifuge tube, centrifuge 5 min at 300 × g, 4°C, and discard supernatant.
9. *Optional:* Determine amount of label incorporation by trichloroacetic acid (TCA) precipitation (see Support Protocol 1).

Labeled cells are now ready for the desired processing and analysis (see Basic Protocol, step 8 annotation, if cells are not to be used immediately).

PULSE-CHASE LABELING OF CELLS WITH [³⁵S]METHIONINE

Pulse-chase protocols are used to analyze time-dependent processes, such as posttranslational modification, transport, secretion, or degradation of newly synthesized proteins. Cells in suspension or adherent cells are pulse-labeled with [³⁵S]methionine (see Basic Protocol and Alternate Protocol 1), after which they are incubated (chased) in complete medium containing excess unlabeled methionine, as described below.

Additional Materials (also see Basic Protocol and Alternate Protocol 1)

Chase medium (see recipe), 37°C

1. Prepare $0.5\text{--}2 \times 10^7$ cells per sample per time point, and pulse-label 10 to 30 min with 2 ml of 0.1 to 0.2 mCi/ml [³⁵S]methionine per $0.5\text{--}2 \times 10^7$ cells (see Basic Protocol, steps 1 to 6, or see Alternate Protocol 1, steps 1 to 5).
2. Remove the [³⁵S]methionine working solution, wash once with 10 ml 37°C chase medium, and add 10 ml 37°C chase medium.

Rapid termination of the labeling reaction can be achieved by adding two times the volume of chase medium containing excess unlabeled methionine (15 mg/liter) directly to the labeling mixture.

Final concentration of cells in suspension should be 2×10^6 cells/ml for chases ≤ 2 hr, or 0.5×10^6 cells/ml for chases > 2 hr. For adherent cells, add 10 ml/100-mm dish.

3. Incubate for the desired time at 37°C. Incubate cell suspensions with rotation in tightly capped tubes. Incubate adherent cells in a CO₂ incubator.
- 4a. *For cells in suspension:* Collect cells by centrifuging 5 min at $300 \times g$, 4°C.

The supernatant can be discarded or can be collected for analysis of proteins that are secreted or shed into the medium.

- 4b. *For adherent cells:* Scrape off adherent cells and transfer to 15-ml centrifuge tubes. Centrifuge 5 min at $300 \times g$, 4°C, and either save or discard the supernatant as in step 4a.
5. *Optional:* Determine amount of label incorporation by trichloroacetic acid (TCA) precipitation (see Support Protocol 1).

Labeled cells are now ready for the desired processing and analysis (see Basic Protocol, step 8 annotation, if cells are not to be used immediately).

LONG-TERM LABELING OF CELLS WITH [³⁵S]METHIONINE

Long-term labeling refers to continuous metabolic labeling of cells for periods of 6 to 32 hr. Long-term labeling is particularly advantageous when studying proteins that are synthesized at low rates. It is also used to accumulate mature labeled proteins, rather than biosynthetic precursors, for characterization of their properties. Steady-state labeling is a form of long-term labeling in which cells are incubated with the radiolabeled amino acid until the rates of synthesis and degradation of the radiolabeled proteins are equal. Steady-state labeling allows calculation of the stoichiometry of subunits within a protein complex, provided that the primary structure of the subunits is known. In these procedures, unlabeled methionine is added to the medium to maintain cell viability and to sustain incorporation of label for the duration of the experiment. The amount of unlabeled methionine added depends on factors such as the length of the labeling period and the cell density. Media containing between 5% and 20% the normal amount of unlabeled methionine are generally used. The conditions described below are suitable for overnight (~16 hr) labeling of cells in suspension or adherent cells.

**ALTERNATE
PROTOCOL 2**

**ALTERNATE
PROTOCOL 3**

**Protein Labeling
and Immuno-
precipitation**

7.1.5

Additional Materials (also see *Basic Protocol and Alternate Protocol 1*)

Long-term labeling medium (see recipe), warmed to 37°C
75-cm² tissue culture flask

1. Prepare a 0.02 to 0.1 mCi/ml [³⁵S]methionine working solution in prewarmed (37°C) long-term labeling medium (see *Basic Protocol*, step 1).

For cell suspensions

- 2a. Prepare and wash cells once with prewarmed long-term labeling medium (see *Basic Protocol*, steps 2 and 3).
- 3a. Resuspend 0.5–2 × 10⁷ cells in 25 ml of 0.02 to 0.1 mCi/ml [³⁵S]methionine working solution and transfer to a 75-cm² tissue culture flask.

For adherent cells

- 2b. Grow 0.5–2 × 10⁷ adherent cells in a 75-cm² tissue culture flask (80% to 90% confluency) in a CO₂ incubator. Wash once with prewarmed long-term labeling medium (see *Alternate Protocol 1*, step 3).
- 3b. Add 25 ml of 0.02 to 0.1 mCi/ml [³⁵S]methionine working solution to each 75-cm² flask.
4. Tighten caps to prevent release of volatile ³⁵S-labeled compounds. Incubate 16 hr in a CO₂ incubator.
5. Wash cells and determine incorporation (see *Basic Protocol*, steps 7 and 8, or *Alternate Protocol 1*, steps 6 to 9).

*Labeled cells are now ready for the desired processing and analysis (see *Basic Protocol*, step 8 annotation, if cells are not to be used immediately).*

**ALTERNATE
PROTOCOL 4**

METABOLIC LABELING WITH OTHER RADIOLABELED AMINO ACIDS

In some instances, it may be necessary to label proteins with radiolabeled amino acids other than [³⁵S]methionine—e.g., when the proteins have a low methionine content or have no methionine residues at all. In these cases, the best choices are [³⁵S]cysteine or [³H]leucine.

Cysteine residues are more abundant in proteins than are methionine residues (3.4% versus 1.8%; Table 7.1.1), although cysteine is less stable. Formulations of [³⁵S]cysteine that have high specific activity (>800 Ci/mmol) can be obtained from several companies. Leucine has the advantage of being the most frequent amino acid in proteins (10.4%; Table 7.1.1) and specific activities of [³H]leucine are the highest among ³H-labeled amino acids (up to 190 Ci/mmol). If proteins are known to be rich in a particular amino acid or if special methods are to be performed (e.g., radiochemical sequencing or multiple labeling), ³H-labeled amino acids other than [³H]leucine can be used.

Label cells with these amino acids as described for [³⁵S]methionine in the previous protocols. Substitute [³⁵S]cysteine or ³H-labeled amino acids in the labeling medium. Use labeling media lacking cysteine or any other respective amino acid.

CAUTION: Solutions containing [³⁵S]cysteine release volatile ³⁵S-labeled compounds (refer to Safety Precautions).

TCA PRECIPITATION TO DETERMINE LABEL INCORPORATION

In metabolic labeling experiments, it is often useful to monitor the incorporation of radioactivity into total cellular proteins. This can easily be achieved by precipitation with trichloroacetic acid (TCA) using BSA as a carrier protein. This protocol can be used at the end of the labeling procedures in each protocol.

Materials

Labeled cell suspension (see Basic Protocol or Alternate Protocols 1 to 4)
BSA/ NaN_3 : 1 mg/ml BSA containing 0.02% (w/v) sodium azide (NaN_3)
10% (w/v) TCA solution (see recipe), ice cold
Ethanol

Filtration apparatus attached to a vacuum line
2.5-cm glass microfiber filter disks (Whatman GF/C)

CAUTION: TCA is extremely caustic. Protect eyes and avoid contact with skin when preparing and handling TCA solutions.

1. Add 10 to 20 μl of a labeled cell suspension to 0.1 ml BSA/ NaN_3 . Place on ice.
2. Add 1 ml ice-cold 10% (w/v) TCA solution. Vortex vigorously and incubate 30 min on ice.
3. Filter the suspension onto 2.5-cm glass microfiber filter disks in a filtration apparatus under vacuum.
4. Wash the disks twice with 5 ml ice-cold 10% (w/v) TCA solution and twice with ethanol. Air dry 30 min.

CAUTION: The wash fluids should be handled as mixed chemical/radioactive waste—follow applicable safety regulations for disposal (see APPENDIX 1D).

5. Spot the same volume of the radiolabeled cell suspension used in step 1 (10 to 20 μl) on a glass microfiber disk. Air dry.

This disk will be used to measure the total amount of radiolabeled amino acid in the cell suspension.

6. Transfer disks from steps 4 and 5 to 20-ml scintillation vials, add 5 ml scintillation fluid, and measure the radioactivity in a scintillation counter.
7. Calculate the ratio of TCA-precipitable label to total radioactivity (i.e., the ratio of sample radioactivity in step 4 to the total radioactivity in step 5).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

NOTE: Use sterile tissue culture technique to prepare these reagents.

Chase medium

Pulse-labeling medium (see recipe) containing 15 mg/liter unlabeled methionine or equivalent excess amount of other amino acid used for radiolabeling. Store up to 2 weeks at 4°C.

Long-term labeling medium (90% methionine-free medium)

Mix 9 vol pulse-labeling medium (see recipe), lacking methionine or other appropriate amino acid, with 1 vol chase medium (see recipe) containing the same amino acid. Store up to 2 weeks at 4°C.

Pulse-labeling medium

Use supplemented Dulbecco's modified Eagle medium (DMEM; see APPENDIX 2A but omit nonessential amino acids) or RPMI 1640, each lacking methionine or other specific amino acid, but containing 10% (v/v) FBS (dialyzed overnight against saline solution to remove unlabeled amino acids). Add 25 mM HEPES buffer (with pH adjusted to 7.4 with NaOH). Store up to 2 weeks at 4°C.

The amino acid used to label cells is omitted from this medium (e.g., if [³⁵S]methionine is employed, methionine-free DMEM or RPMI 1640 must be used). Specific amino acid-free media can be obtained from several tissue culture media suppliers, or can be prepared (see APPENDIX 2B) from amino acid-free medium by adding individual amino acid components, omitting the one that will be used to label. A kit for the preparation of such media is available (Select-Amine from Life Technologies).

Trichloroacetic acid (TCA) solution, 10% (w/v)

Prepare a 100% (w/v) TCA stock solution by dissolving the entire contents of a newly opened TCA bottle in water (e.g., dissolve the contents of a 500-g bottle of TCA in sufficient water to yield a final volume of 500 ml). Store up to 1 year at 4°C. Prepare 10% (w/v) TCA by dilution and store up to 3 months at 4°C.

CAUTION: TCA is extremely caustic. Protect eyes and avoid contact with skin when preparing and handling TCA solutions.

COMMENTARY

Background Information

Metabolic labeling of cellular proteins is achieved by placing cells in a nutritional medium containing all components necessary for growth of cells in culture, except for one amino acid that is substituted by its radiolabeled form. The radiolabeled amino acids are transported across the plasma membrane by carrier-mediated systems and, once in the cytosol, are loaded onto tRNA molecules before being incorporated into newly synthesized proteins.

Because metabolic labeling techniques use the metabolic machinery of the cell to incorporate radiolabeled amino acids, there are limitations on the type of radiolabeled amino acids that can be employed. The list of potential precursors is restricted to L-amino acids normally found in proteins, in which one or more atoms are substituted by a radioisotope (Table 7.1.1). Sulfuric amino acids, such as methionine and cysteine, are conveniently labeled with ³⁵S. Most other amino acids can be obtained labeled with ³H.

Critical Parameters

The protocols in this unit should be considered as models from which more specific methods can be designed. The conditions described here have been optimized for radiolabeling proteins that are expressed at low to moderate levels (10⁴ to 10⁵ copies per cell) and assume that the labeled cells will be used for immunoprecipitation (UNIT 7.2). This level of expression

is characteristic of most endogenous membrane proteins, luminal organellar proteins, signal transduction proteins, and transcription factors. More abundant proteins, such as cytoskeletal proteins or proteins expressed by infection or transfection, can be labeled with less radiolabeled amino acid and/or fewer cells. The choice of a particular labeling protocol and its modification will be aided by careful consideration of a number of parameters that influence the incorporation of radiolabeled amino acids into proteins, as discussed below.

Selection of amino acid label

Purified [³⁵S]methionine of high specific activity (>800 Ci/mmol) can be obtained from several suppliers of radiolabeled amino acids. Protein hydrolyzates of *Escherichia coli* grown in the presence of [³⁵SO₄]²⁻ (e.g., Tran³⁵S-label from ICN Biomedicals or Expre³⁵S³⁵S from NEN Life Sciences) can be used as substitutes for [³⁵S]methionine in metabolic labeling techniques. These preparations contain ³⁵S distributed among several different compounds. In a typical batch, ~70% of the radioactivity will be present as [³⁵S]methionine and ~15% as [³⁵S]cysteine; the remainder are other ³⁵S-labeled compounds. Labeling with these radioactive protein hydrolyzates in methionine-free medium will result in incorporation of label only in methionine residues; use of methionine- and cysteine-free medium will result in labeling of both methionine and cysteine residues.

Purified [³⁵S]methionine should be used whenever certainty of labeling with only [³⁵S]methionine is required. Another reason for using purified [³⁵S]methionine is that it tends to be more stable and emit less volatile decomposition products than ³⁵S-labeled protein hydrolyzates. In most cases, however, the relatively inexpensive radioactive protein hydrolyzates (~1/3 the cost of [³⁵S]methionine) can be used instead of purified [³⁵S]methionine. [³⁵S]methionine preparations should be stored frozen at -80°C. Under these conditions, they are stable for at least 1 month; the half-life of ³⁵S is 88 days.

For proteins with one or no methionine residues but several cysteine residues, labeling with [³⁵S]cysteine (available with specific activities >800 Ci/mmol) is a good option. ³⁵S-labeled protein hydrolyzates are not good sources of [³⁵S]cysteine for radiolabeling because only ~15% of the ³⁵S-labeled compounds are [³⁵S]cysteine.

Purified [³H]leucine (available with specific activities of up to 190 Ci/mmol) is a good alternative to ³⁵S-labeled amino acids. The half-life of ³H is ~12 years. The specific activities of other ³H-labeled amino acids range between 5 and 140 Ci/mmol (Table 7.1.1). Several problems can arise when using certain ³H-labeled amino acids due to their participation in metabolic pathways. The following problems must be considered when using tritiated amino acids for metabolic labeling of proteins.

Nonessential versus essential amino acids. Cells are able to synthesize nonessential amino acids from other compounds. If the radiolabeled amino acids used are nonessential, their specific activity will be reduced by dilution with the endogenously synthesized amino acids. Therefore, essential amino acids (E in Table 7.1.1) should be favored for metabolic labeling.

Transamination. The α -amino groups of many amino acids can be removed in reactions catalyzed by transaminases (T in Table 7.1.1). Deamination of the radiolabeled amino acids can be prevented by addition of the transaminase inhibitor (aminoxy)acetic acid during starvation and labeling of the cells (Coligan et al., 1983).

Interconversion. Certain radiolabeled amino acids can be converted by cells into other amino acids (I in Table 7.1.1). This problem is of particular importance in methods used to determine the amino acid composition or sequence of radiolabeled proteins.

Labeling time

The experimental purpose, the turnover rate of the protein of interest, and the viability of the cells should all be considered when determining the length of time for labeling. If the purpose of the experiment is to identify or characterize a protein precursor, pulse-labeling (10 to 30 min) must be used (see Basic Protocol and Alternate Protocol 1). If the protein of interest has a low turnover rate or if it is necessary to accumulate a labeled mature product, a protocol for long-term labeling (6 to 32 hr) is more appropriate (see Alternate Protocol 3). If the biosynthesis, posttranslational modification, intracellular transport, or fate of newly synthesized proteins is being analyzed, a pulse-chase protocol should be used (see Alternate Protocol 2).

The turnover rate of the protein of interest is an important parameter to be considered when determining the labeling time. Proteins with a high turnover rate are optimally labeled for short times. Longer times will result in increased labeling of other cellular proteins, causing a decrease in the relative abundance of the labeled protein of interest in the cell lysate. This could result in increased detection of non-specific proteins in immunoprecipitates (see UNIT 7.2). Conversely, proteins that turn over slowly should be labeled for longer times.

Incorporation of radiolabeled amino acids into proteins is directly proportional to their length of labeling time for a certain period, after which it tends to plateau. When all the limiting amino acid is consumed, protein synthesis ceases. The length of the initial phase of linear incorporation will vary with the concentration of the labeling amino acid and the density and metabolic activity of the cells.

Concentration and specific activity of the radiolabeled amino acid

Because radiolabeled amino acids are used in limiting amounts, their incorporation into proteins is directly proportional to their concentration in the labeling medium. If very short pulses are required, the concentration of labeled amino acid can be increased to compensate for the shorter labeling time. The incorporation of radiolabeled amino acids is also directly proportional to their specific activity. Addition of unlabeled amino acids, as is required in long-term labeling protocols, will result in reduced incorporation over short periods.

Cell density

At low cell densities, the amount of labeled protein synthesized is directly proportional to the cell concentration. At high densities, however, incorporation will increase nonlinearly. Very concentrated cell samples ($>5 \times 10^7$ cells/ml) can only be labeled for short periods (≤ 5 min) as rapid loss of metabolic activity and cell viability will occur due to acidification of the medium and accumulation of toxic metabolites.

Conditions for very short pulses

Very short pulses (2 to 10 min) are required to study posttranslational modifications that occur shortly after synthesis. This includes folding, disulfide bond formation, and early carbohydrate modifications of newly synthesized proteins. Proteins that are rapidly degraded after synthesis are also best studied with very short pulses. The protocol for very short pulses is similar to the Basic Protocol for pulse-labeling, with the following modifications: (1) the pulse-labeling medium contains higher concentration of radiolabeled amino acid (up to 1 mCi/ml), (2) labeling is stopped by addition of ice-cold PBS containing 20 mM freshly prepared *N*-ethylmaleimide (NEM) to prevent oxidation of free sulfhydryls, and (3) if a chase is necessary, 1 mM cycloheximide is added to the chase medium to quickly stop the elongation of nascent polypeptide chains. For additional details on very short pulses, see Braakman et al. (1991).

Temperature and pH

Unless otherwise required for special conditions (e.g., heat shock, temperature-sensitive mutants), metabolic labeling should be performed at 37°C. For this reason, it is important that the labeling medium be warmed to 37°C before adding it to the cells. A dramatic reduction in incorporation occurs at room temperature. It is also important that the pH of the labeling medium be ~ 7.4 .

Anticipated Results

A typical incorporation of labeled amino acid precursor after a 30-min pulse under the conditions described in this unit (see Basic Protocol and Alternate Protocols 1 and 2) is 5% to 20%. In the case of long-term labeling, the incorporation typically reaches 30% to 60%. If the labeled cells are used for immunoprecipitation (UNIT 7.2), followed by SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3), specific bands should be visible within two hours to two months of exposure.

Time Considerations

It takes 1 to 2 hr to prepare cells and materials for labeling. The actual labeling time depends upon the protocol chosen—pulse-labeling takes 10 to 30 min and long-term labeling takes 6 to 32 hr. Washing and processing cells may take an additional 1.5 hr.

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- Meisenhelder, J. and Hunter, T. 1988. Radioactive protein labelling techniques. *Nature* 335:120.

Key Reference

Coligan et al., 1983. See above.

Contains a detailed description of conditions used to metabolically label proteins with different radio-labeled amino acids.

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Immunoprecipitation is a technique in which an antigen is isolated by binding to a specific antibody attached to a sedimentable matrix. The source of antigen for immunoprecipitation can be unlabeled cells or tissues, metabolically or extrinsically labeled cells (UNIT 7.1), subcellular fractions from either unlabeled or labeled cells (see Chapter 3), or in vitro–translated proteins (UNIT 11.2). Immunoprecipitation is also used to analyze protein fractions separated by other biochemical techniques such as gel filtration or sedimentation on density gradients (UNIT 5.3). Either polyclonal or monoclonal antibodies from various animal species can be used in immunoprecipitation protocols. Antibodies can be bound noncovalently to immunoadsorbents such as protein A– or protein G–agarose, or can be coupled covalently to a solid-phase matrix.

Immunoprecipitation protocols consist of several stages (Fig. 7.2.1; see Basic Protocol 1). In stage 1, the antigen is solubilized by one of several techniques for lysing cells. Soluble and membrane-associated antigens can be released from cells grown either in suspension culture (see Basic Protocol 1) or as a monolayer on tissue culture dishes (see Alternate Protocol 1) with nondenaturing detergents. Cells can also be lysed under denaturing conditions (see Alternate Protocol 2). Soluble antigens can also be extracted by mechanical disruption of cells in the absence of detergents (see Alternate Protocol 3). All of these lysis procedures are suitable for extracting antigens from animal cells. Yeast cells require disruption of their cell wall in order to allow extraction of the antigens (see Alternate Protocol 4). In stage 2, a specific antibody is attached, either noncovalently or covalently, to a sedimentable, solid-phase matrix to allow separation by low-speed centrifugation. This unit presents the noncovalent attachment of antibody to protein A– or protein G–agarose beads (see Basic Protocol 1). Stage 3 consists of incubating the solubilized antigen from stage 1 with the immobilized antibody from stage 2, followed by extensive washing to remove unbound proteins (see Basic Protocol 1). Immunoprecipitated antigens can be dissociated from antibodies and reprecipitated by a protocol referred to as “immunoprecipitation-recapture” (see Basic Protocol 2). This protocol can be used with the same antibody for further purification of the antigen, or with a second antibody to identify components of multisubunit complexes or to study protein-protein interactions (Fig. 7.2.2). Immunoprecipitated antigens can be analyzed by one-dimensional electrophoresis (UNIT 6.1), two-dimensional electrophoresis, or immunoblotting (UNIT 6.2). In some cases, immunoprecipitates can be used for structural or functional analyses of the isolated antigens. Immunoprecipitates can also be used as sources of immunogens for production of monoclonal or polyclonal antibodies.

IMMUNOPRECIPITATION USING CELLS IN SUSPENSION LYSED WITH A NONDENATURING DETERGENT SOLUTION

BASIC PROTOCOL 1

In this protocol, unlabeled or labeled cells in suspension are extracted by incubation in nondenaturing lysis buffer containing the nonionic detergent Triton X-100 (steps 1 to 7). This procedure results in the release of both soluble and membrane proteins; however, many cytoskeletal and nuclear proteins, as well as a fraction of membrane proteins, are not efficiently extracted under these conditions (see UNIT 5.1). The procedure allows immunoprecipitation with antibodies to epitopes that are exposed in native proteins.

For immunoprecipitation, a specific antibody is immobilized on a sedimentable, solid-phase matrix (steps 8 to 14). Although there are many ways to attach antibodies to matrices (see Commentary), the most commonly used methods rely on the property of immunoglobulins to bind *Staphylococcus aureus* protein A, or protein G from group G *Streptococcus* (Table 7.2.1). The best results are obtained by binding antibodies to protein A or

Protein Labeling and Immuno- precipitation

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7.2.1

protein G that is covalently coupled to agarose beads. In this protocol, Sepharose beads are used (Sepharose is a more stable, cross-linked form of agarose). Immunoprecipitation is most often carried out using rabbit polyclonal or mouse monoclonal antibodies, which, with some exceptions (e.g., mouse IgG1), bind well to protein A (Table 7.2.1). Antibodies that do not bind to protein A–agarose can be adsorbed to protein G–agarose (Table 7.2.1) using exactly the same protocol. For optimal time management, incubation of antibodies with protein A–agarose can be carried out either before or during lysis of the cells.

The final stage in immunoprecipitation is combining the cell lysate with the antibody-conjugated beads and isolating the antigen (steps 18 to 26). This can be preceded by an optional preclearing step in which the lysate is absorbed with either “empty” protein A–agarose beads or with an irrelevant antibody bound to protein A–agarose (steps 15 to

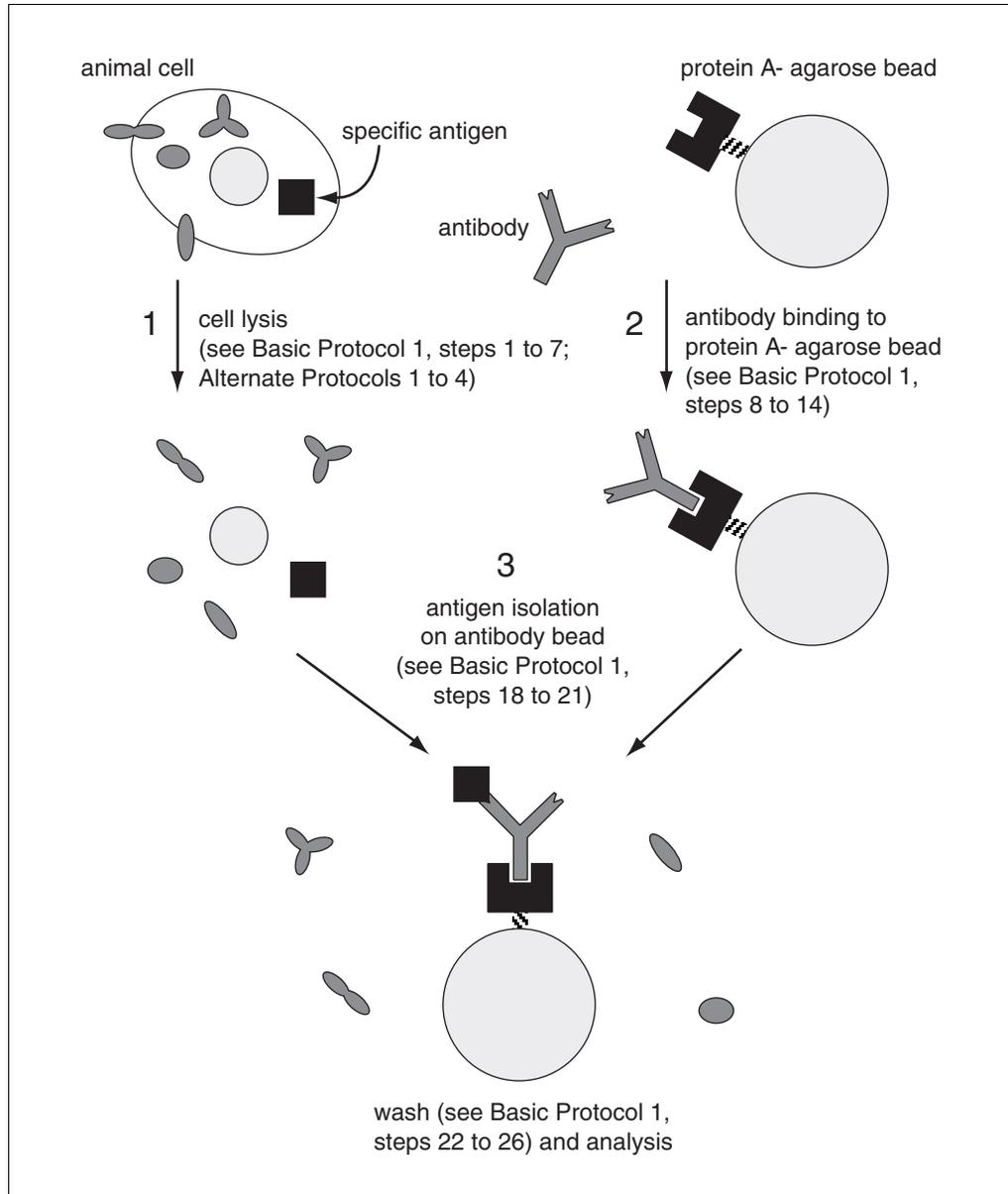


Figure 7.2.1 Schematic representation of the stages of a typical immunoprecipitation protocol. (1) Cell lysis: antigens are solubilized by extraction of the cells in the presence or absence of detergents. To increase specificity, the cell lysate can be precleared with protein A–agarose beads (steps 15 to 17, not shown). (2) Antibody immobilization: a specific antibody is bound to protein A–agarose beads. (3) Antigen capture: the solubilized antigen is isolated on antibody-conjugated beads.

17). The need for preclearing depends on the specific experimental system being studied and the quality of the antibody reagents. The protocol described below incorporates a preclearing step using protein A–agarose. Protein fractions separated by techniques such as gel filtration or sedimentation on sucrose gradients (*UNIT 5.3*) can be used in place of the cell lysate at this stage. After binding the antigen to the antibody-conjugated beads, the unbound proteins are removed by successive washing and sedimentation steps.

Materials

Unlabeled or labeled cells in suspension (*UNIT 7.1*)

PBS (*APPENDIX 2A*), ice cold

Nondenaturing lysis buffer (see recipe), ice cold

50% (v/v) protein A–Sepharose bead (Sigma, Pharmacia Biotech) slurry in PBS containing 0.1% (w/v) BSA and 0.01% (w/v) sodium azide (NaN₃)

Table 7.2.1 Binding of Antibodies to Protein A and Protein G^{a,b,c}

Antibody	Protein A binding	Protein G binding ^d
<i>Monoclonal antibodies^e</i>		
Human IgG1	++	++
Human IgG2	++	++
Human IgG3	–	++
Human IgG4	++	++
Mouse IgG1	+	++
Mouse IgG2a	++	++
Mouse IgG2b	++	++
Mouse IgG3	++	++
Rat IgG1	+	+
Rat IgG2a	–	++
Rat IgG2b	–	++
Rat IgG2c	++	++
<i>Polyclonal antibodies</i>		
Chicken	–	–
Donkey	–	++
Goat	+	++
Guinea pig	++	+
Hamster	+	++
Human	++	++
Monkey	++	++
Mouse	++	++
Rabbit	++	++
Rat	+	+
Sheep	+	++

^a++, moderate to strong binding; +, weak binding; –, no binding.

^bA hybrid protein A/G molecule that combines the features of protein A and protein G, coupled to a solid-phase matrix, is available from Pierce.

^cInformation from Harlow and Lane (1988), and from Pharmacia Biotech, Pierce, and Jackson Immunoresearch.

^dNative protein G binds albumin from several animal species. Recombinant variants of protein G have been engineered for better binding to rat, mouse, and guinea pig IgG, as well as for avoiding binding to serum albumin.

^eProtein A binds some IgM, IgA, and IgE antibodies in addition to IgG, whereas protein G binds only IgG.

Specific polyclonal antibody (antiserum or affinity-purified immunoglobulin) or monoclonal antibody (ascites, culture supernatant, or purified immunoglobulin)
Control antibody of same type as specific antibody (e.g., preimmune serum or purified irrelevant immunoglobulin for specific polyclonal antibody; irrelevant ascites, culture supernatant, or purified immunoglobulin for specific monoclonal antibody; see Critical Parameters)

10% (w/v) BSA (APPENDIX 2A)

Wash buffer (see recipe), ice cold

Microcentrifuge with fixed-angle rotor (Eppendorf 5415C or equivalent)

Tube rotator (capable of end-over-end inversion)

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer (also see APPENDIX 1D).

NOTE: All solutions should be ice cold and procedures should be carried out at 4°C or on ice.

Prepare cell lysate

1. Collect cells in suspension by centrifuging 5 min at $400 \times g$, 4°C, in a 15- or 50-ml capped conical tube. Place tube on ice.

Approximately $0.5\text{--}2 \times 10^7$ cells are required to yield 1 ml lysate, which is generally used for each immunoprecipitation.

Labeled cells are likely to have been pelleted earlier as part of the labeling procedure. If the cells are frozen, they should be thawed on ice before solubilization.

2. Aspirate supernatant with a Pasteur pipet attached to a vacuum trap.

CAUTION: *Dispose of radioactive materials following applicable safety regulations (APPENDIX 1D).*

3. Resuspend cells gently by tapping the bottom of the tube. Rinse cells twice with ice-cold PBS as in steps 1 and 2, using the same volume of PBS as in the initial culture.

4. Add 1 ml ice-cold nondenaturing lysis buffer per $\sim 0.5\text{--}2 \times 10^7$ cells and resuspend pellet by gentle agitation for 3 sec with a vortex mixer set at medium speed.

Do not shake vigorously as this could result in loss of material or protein denaturation due to foaming.

5. Keep suspension on ice 15 to 30 min and transfer to a 1.5-ml conical microcentrifuge tube.

Tubes can have flip-top or screw caps. Screw-capped tubes are preferred because they are less likely to open accidentally during subsequent procedures. They are also recommended for work with radioactivity.

6. Clear the lysate by microcentrifuging 15 min at $16,000 \times g$ (maximum speed), 4°C.

Centrifugation can be carried out in a microcentrifuge placed in a cold room or in a refrigerated microcentrifuge. Take precautions to ensure that the 4°C temperature is maintained during the spin (e.g., use a fixed-angle rotor with a lid, as the aerodynamics of this type of rotor reduces generation of heat by friction). If it is necessary to reduce background, the lysate can be spun for 1 hr at $100,000 \times g$ in an ultracentrifuge.

7. Transfer the supernatant to a fresh microcentrifuge tube using an adjustable pipet fitted with a disposable tip. Do not disturb the pellet, and leave the last 20 to 40 μl of supernatant in the centrifuge tube. Keep the cleared lysate on ice until preclearing (step 15) or addition of antibody beads (step 18).

NOTE: Resuspension of even a small amount of sedimented material will result in high nonspecific background due to carryover into the immunoprecipitation steps. A cloudy layer of lipids floating on top of the supernatant will not adversely affect the results of the immunoprecipitation.

When the lysate is highly radioactive—as is the case for metabolically labeled cells—the use of tips with aerosol barriers is recommended to reduce the risk of contaminating internal components of the pipet.

Cell extracts can be frozen at -70°C until used for immunoprecipitation. However, it is preferable to lyse the cells immediately before immunoprecipitation in order to avoid protein degradation or dissociation of protein complexes. If possible, freeze the cell pellet from step 3 rather than the supernatant from step 7.

Prepare antibody-conjugated beads

8. In a 1.5-ml conical microcentrifuge tube, combine 30 μl of 50% protein A–Sepharose bead slurry, 0.5 ml ice-cold PBS, and the following quantity of specific antibody (select one):

- 1 to 5 μl polyclonal antiserum
- 1 μg affinity-purified polyclonal antibody
- 0.2 to 1 μl ascitic fluid containing monoclonal antibody
- 1 μg purified monoclonal antibody
- 20 to 100 μl hybridoma culture supernatant containing monoclonal antibody.

The quantities of antibody suggested are rough estimates based on the expected amount of specific antibodies in each preparation. Quantities can be increased or decreased, depending on the quality of the antibody preparation (see Commentary).

Substitute protein G for protein A if antibodies are of a species or subclass that does not bind to protein A (see Table 7.2.1).

If the same antibody will be used to immunoprecipitate multiple samples (e.g., samples from a pulse-chase experiment; UNIT 7.1), the quantities indicated above can be increased proportionally to the number of samples and incubated in a 15-ml capped conical tube. In this case, the beads should be divided into aliquots just prior to the addition of the cleared cell lysate (step 18).

Antibody-conjugated beads can be prepared prior to preparation of the cell lysate (steps 1 to 7), in order to minimize the time that the cell extract is kept on ice.

9. Set up a nonspecific immunoprecipitation control in a 1.5-ml conical microcentrifuge tube by incubating 30 μl of 50% protein A–Sepharose bead slurry, 0.5 ml ice-cold PBS, and the appropriate control antibody (select one):

- 1 to 5 μl preimmune serum as a control for a polyclonal antiserum
- 1 μg purified irrelevant polyclonal antibody (an antibody to an epitope that is not present in the cell lysate) as a control for a purified polyclonal antibody
- 0.2 to 1 μl ascitic fluid containing irrelevant monoclonal antibody (an antibody to an epitope that is not present in the cell lysate and of the same species and immunoglobulin subclass as the specific antibody) as a control for an ascitic fluid containing specific monoclonal antibody

- 1 μg purified irrelevant monoclonal antibody as a control for a purified monoclonal antibody
- 20 to 100 μl hybridoma culture supernatant containing irrelevant monoclonal antibody as a control for a hybridoma culture supernatant containing specific monoclonal antibody

The amount of irrelevant antibody should match that of the specific antibody and the antibody should be from the same species as the specific antibody.

10. Mix suspensions thoroughly. Tumble incubation mixtures end over end ≥ 1 hr at 4°C in a tube rotator.

Addition of 0.01% (w/v) Triton X-100 may facilitate mixing of the suspension during tumbling. Incubations can be carried out for as long as 24 hr. This allows preparation of the antibody-conjugated beads prior to immunoprecipitation.

11. Microcentrifuge 2 sec at $16,000 \times g$ (maximum speed), 4°C .
12. Aspirate the supernatant (containing unbound antibodies) using a fine-tipped Pasteur pipet connected to a vacuum aspirator.
13. Add 1 ml nondenaturing lysis buffer and resuspend the beads by inverting the tube 3 or 4 times.

For lysates prepared with detergents (this protocol and see Alternate Protocols 1 and 2), use 1 ml nondenaturing lysis buffer; for lysates prepared by mechanical disruption (see Alternate Protocol 3), use detergent-free lysis buffer (see recipe).

Use of a repeat pipettor is recommended when processing multiple samples.

14. Wash by repeating steps 11 to 13, and then steps 11 and 12 once more.

At this point the beads have been washed twice with lysis buffer and are ready to be used for immunoprecipitation. Antibody-bound beads can be stored up to 6 hr at 4°C until used.

Preclear lysate (optional)

15. In a microcentrifuge tube, combine 1 ml cell lysate (from step 7) and 30 μl of 50% protein A–Sepharose bead slurry.

The purpose of this step is to remove from the lysate proteins that bind to protein A–Sepharose, as well as pieces of insoluble material that may have been carried over from previous steps. If the lysate was prepared from cells expressing immunoglobulins—such as spleen cells or cultured B cells—the preclearing step should be repeated at least 3 times to ensure complete removal of endogenous immunoglobulins.

If cell lysates were frozen and thawed, they should be microcentrifuged 15 min at $16,000 \times g$ (maximum speed), 4°C , before the preclearing step.

16. Tumble end over end 30 min at 4°C in a tube rotator.
17. Microcentrifuge 5 min at $16,000 \times g$ (maximum speed), 4°C .

Immunoprecipitate

18. Add 10 μl of 10% BSA to the tube containing specific antibody bound to protein A–Sepharose beads (step 14), and transfer to this tube the entire volume of cleared lysate (from step 7 or 17). If a nonspecific immunoprecipitation control is performed, divide lysate in two ~ 0.4 -ml aliquots, one for the specific antibody and the other for the nonspecific control.

In order to avoid carryover of beads with precleared material, leave 20 to 40 μl of supernatant on top of the pellets in the preclearing tubes. Discard beads and remaining supernatant. The BSA quenches nonspecific binding to the antibody-conjugated beads during incubation with the cell lysate.

19. Incubate 1 to 2 hr at 4°C while mixing end over end in a tube rotator.

Samples can be incubated overnight, although there is an increased risk of protein degradation, dissociation of multiprotein complexes, or formation of protein aggregates.

20. Microcentrifuge 5 sec at 16,000 × g (maximum speed), 4°C.

21. Aspirate the supernatant (containing unbound proteins) using a fine-tipped Pasteur pipet connected to a vacuum aspirator.

The supernatant can be kept up to 8 hr at 4°C or up to 1 month at –70°C for sequential immunoprecipitation of other antigens or for analysis of total proteins. To reuse lysate, remove the supernatant carefully with an adjustable pipet fitted with a disposable tip. Before reprecipitation, preabsorb the lysate with protein A–Sepharose (as in steps 15 to 17) to remove antibodies that may have dissociated during the first immunoprecipitation.

CAUTION: *Dispose of radioactive materials following applicable safety regulations.*

22. Add 1 ml ice-cold wash buffer, cap the tubes, and resuspend the beads by inverting the tube 3 or 4 times.

Use of a repeat pipettor is recommended when processing multiple samples.

23. Microcentrifuge 2 sec at 16,000 × g (maximum speed), 4°C.

24. Aspirate the supernatant, leaving ~20 µl supernatant on top of the beads.

25. Wash beads three more times (steps 22 to 24).

Total wash time (steps 22 to 26) should be ~30 min, keeping the samples on ice for 3 to 5 min between washes if necessary (see Critical Parameters).

26. Wash beads once more using 1 ml ice-cold PBS and aspirate supernatant completely with a drawn-out Pasteur pipet.

The final product should be 15 µl of settled beads containing bound antigen.

Immunoprecipitates can either be processed immediately or frozen at –20°C for later analysis. For subsequent analysis of the isolated proteins prior to electrophoresis (e.g., comparison of the electrophoretic mobility of the antigen with or without treatment with glycosidases), samples can be divided into two or more aliquots after addition of PBS. Transfer aliquots of the bead suspension to fresh tubes, centrifuge and aspirate as in the previous steps. Immunoprecipitates can be analyzed by one-dimensional electrophoresis (UNIT 6.1), two-dimensional electrophoresis, or immunoblotting (UNIT 6.2).

IMMUNOPRECIPITATION USING ADHERENT CELLS LYSED WITH A NONDENATURING DETERGENT SOLUTION

ALTERNATE PROTOCOL 1

Immunoprecipitation using adherent cells can be performed in the same manner as with nonadherent cells (see Basic Protocol 1). This protocol is essentially similar to steps 1 to 5 of Basic Protocol 1, but describes modifications necessary for using the same non-denaturing detergent solution to lyse cells attached to tissue culture plates. It is preferable to use cells grown on plates rather than in flasks, because the cell monolayer is more easily accessible.

Additional Materials (also see Basic Protocol 1)

Unlabeled or labeled cells grown as a monolayer on a tissue culture plate (UNIT 7.1)

NOTE: All solutions should be ice cold and procedures should be carried out at 4°C or on ice.

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1. Rinse cells attached to a tissue culture plate twice with ice-cold PBS. Remove the PBS by aspiration with a Pasteur pipet attached to a vacuum trap.

CAUTION: *Dispose of radioactive materials following applicable safety regulations.*

2. Place the tissue culture plate on ice.
3. Add ice-cold nondenaturing lysis buffer to the tissue culture plate.

Use 1 ml lysis buffer for an 80% to 90% confluent 100-mm-diameter tissue culture plate. Depending on the cell type, a confluent 100-mm dish will contain $0.5\text{--}2 \times 10^7$ cells. For other plate sizes, adjust volume of lysis buffer according to the surface area of the plate.

4. Scrape the cells off the plate with a rubber policeman, and transfer the suspension to a 1.5-ml conical microcentrifuge tube using an adjustable pipettor fitted with a disposable tip. Vortex gently for 3 sec and keep tubes on ice for 15 to 30 min.

Tubes can have flip-top or screw caps. Screw-capped tubes are preferred because they are less likely to open accidentally during subsequent procedures. They are also recommended for work with radioactivity.

5. Clear the lysate and perform immunoprecipitation (see Basic Protocol 1, steps 6 to 26).

ALTERNATE PROTOCOL 2

IMMUNOPRECIPITATION USING CELLS LYSED WITH DETERGENT UNDER DENATURING CONDITIONS

If epitopes of native proteins are not accessible to antibodies, or if the antigen cannot be extracted from the cell with nonionic detergents, cells should be solubilized under denaturing conditions. This protocol is based on that for nondenaturing conditions (see Basic Protocol 1, steps 1 to 7), with the following modifications. Denaturation is achieved by heating the cells in a denaturing lysis buffer that contains an ionic detergent such as SDS or Sarkosyl (*N*-lauroylsarcosine). The denaturing lysis buffer also contains DNase I to digest DNA released from the nucleus. Prior to immunoprecipitation, the denatured protein extract is diluted 10-fold with nondenaturing lysis buffer, which contains Triton X-100; this step protects the antigen-antibody interaction from interference by the ionic detergent. Immunoprecipitation is performed as described (see Basic Protocol 1).

The following protocol is described for cells in suspension culture, although it can be adapted for adherent cells (see Alternate Protocol 1). Only antibodies that react with denatured proteins can be used to immunoprecipitate proteins solubilized by this protocol.

Additional Materials (also see Basic Protocol 1)

Denaturing lysis buffer (see recipe)
Heating block set at 95°C (Eppendorf Thermomixer 5436 or equivalent)
25-G needle attached to 1-ml syringe

1. Collect cells in suspension culture (see Basic Protocol 1, steps 1 to 3). Place tubes on ice.
2. Add 100 μ l denaturing lysis buffer per $\sim 0.5\text{--}2 \times 10^7$ cells in the pellet.
3. Resuspend the cells by vortexing vigorously 2 to 3 sec at maximum speed. Transfer suspension to a 1.5-ml conical microcentrifuge tube.

The suspension may be very viscous due to release of nuclear DNA.

Tubes can have flip-top or screw caps. Screw-capped tubes are preferred because they are less likely to open accidentally during subsequent procedures. They are also recommended for work with radioactivity.

4. Heat samples 5 min at 95°C in a heating block.
5. Dilute the suspension with 0.9 ml nondenaturing lysis buffer. Mix gently.

The excess 1% Triton X-100 in the nondenaturing lysis buffer sequesters SDS into Triton X-100 micelles.
6. Shear DNA by passing the suspension five to ten times through a 25-G needle attached to a 1-ml syringe.

If the DNA is not digested by DNase I in the denaturing lysis buffer or thoroughly sheared mechanically, it will interfere with the separation of pellet and supernatant after centrifugation. Repeat mechanical disruption until the viscosity is reduced to manageable levels.
7. Incubate 5 min on ice.
8. Clear the lysate and perform immunoprecipitation (see Basic Protocol 1, steps 6 to 26).

IMMUNOPRECIPITATION USING CELLS LYSED WITHOUT DETERGENT

ALTERNATE PROTOCOL 3

Immunoprecipitation of proteins that are already soluble within cells (e.g., cytosolic or luminal organellar proteins) may not require the use of detergents. Instead, cells can be mechanically disrupted by repeated passage through a needle, and soluble proteins can be separated from insoluble material by centrifugation. The following protocol describes lysis of cells in a PBS-based detergent-free lysis buffer. Other buffer formulations may be used for specific proteins.

Additional Materials (also see Basic Protocol 1)

- Detergent-free lysis buffer (see recipe)
- 25-G needle attached to 3-ml syringe

NOTE: All solutions should be ice-cold and procedures should be carried out at 4°C or on ice.

1. Collect and wash cells in suspension (see Basic Protocol 1, steps 1 to 3).
2. Add 1 ml of ice-cold detergent-free lysis buffer per $\sim 0.5\text{--}2 \times 10^7$ cells in a pellet.
3. Resuspend the cells by gentle agitation for 3 sec with a vortex mixer set at medium speed.
4. Break cells by passing the suspension 15 to 20 times through a 25-G needle attached to a 3-ml syringe.

Extrusion of the cell suspension from the syringe should be rapid, although care should be exercised to prevent splashing and excessive foaming. Cell breakage can be checked under a bright-field or phase-contrast microscope. Repeat procedure until >90% cells are broken.

It is helpful to check ahead of time whether the cells can be broken in this way. If the cells are particularly resistant to mechanical breakage, they can be swollen for 10 min at 4°C with a hypotonic solution containing 10 mM Tris-Cl, pH 7.4 (APPENDIX 2A) before mechanical disruption.

5. Clear the lysate and perform immunoprecipitation (Basic Protocol 1, steps 6 to 26).

IMMUNOPRECIPITATION USING YEAST CELLS DISRUPTED WITH GLASS BEADS

Unlike animal cells, yeast cells have an extremely resistant, detergent-insoluble cell wall. To allow extraction of cellular antigens, the cell wall needs to be broken by mechanical, enzymatic, or chemical means. The most commonly used procedure consists of vigorous vortexing of the yeast suspension with glass beads. The breakage can be done in the presence or absence of detergent, as previously described for animal cells (see Basic Protocol 1, Alternate Protocol 2, and Alternate Protocol 3). The protocol described below is suitable for mechanical disruption of most yeast species, including *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. A protocol for metabolic labeling for yeast has been described by Franzusoff et al. (1991).

Additional Materials (also see Basic Protocol 1)

Unlabeled or radiolabeled yeast cells

Lysis buffer, ice cold: nondenaturing, denaturing, or detergent-free lysis buffer (see recipes)

Glass beads (acid-washed, 425- to 600- μm diameter; Sigma)

NOTE: All solutions should be ice-cold and procedures should be carried out at 4°C or on ice.

1. Collect 10 ml of yeast culture at 1 OD₆₀₀ per immunoprecipitation sample, and centrifuge 5 min at 4000 \times g, 4°C. Place tube on ice.
2. Remove supernatant by aspiration with a Pasteur pipet attached to a vacuum trap.

CAUTION: Dispose of radioactive materials following applicable safety regulations.

3. Loosen pellet by vortexing vigorously for 10 sec. Rinse cells twice with ice-cold distilled water as in steps 1 and 2.

Radiolabeled yeast cells are likely to have been pelleted earlier as part of the labeling procedure. If the pellets are frozen, they should be thawed on ice prior to cell disruption.

4. Add 3 vol ice-cold lysis buffer and 3 vol at 1 OD₆₀₀ glass beads per volume of pelleted yeast cells.

Use nondenaturing lysis buffer or detergent-free lysis buffer as required for the antigen under study. If the experiment requires denaturation of the antigen, the procedure can be adapted (see Alternate Protocol 2 for higher eukaryotic cells); however, the yeast cells must be broken with glass beads before heating the sample at 95°C.

5. Shake cells by vortexing vigorously at maximum speed for four 30-sec periods, keeping the cells on ice for 30 sec between the periods.

Check cell breakage under a bright-field or phase-contrast microscope. It is helpful to check ahead of time if the cells can be broken in this way.

6. Remove the yeast cell lysate from the beads using a pipettor with a disposable tip. Transfer to a fresh tube.
7. Add 4 vol (see step 4) lysis buffer to the glass beads, vortex for 2 sec, and combine this supernatant with the lysate from step 6.
8. Clear the lysate and perform immunoprecipitation (see Basic Protocol 1, steps 6 to 26).

IMMUNOPRECIPITATION-RECAPTURE

Once an antigen has been isolated by immunoprecipitation, it can be dissociated from the beads and reimmunoprecipitated (“recaptured”) with either the same antibody used in the first immunoprecipitation or with a different antibody (Fig. 7.2.2). Immunoprecipitation-recapture with the same antibody allows identification of a specific antigen in cases where the first immunoprecipitation contains too many bands to allow unambiguous identifica-

**BASIC
PROTOCOL 2**

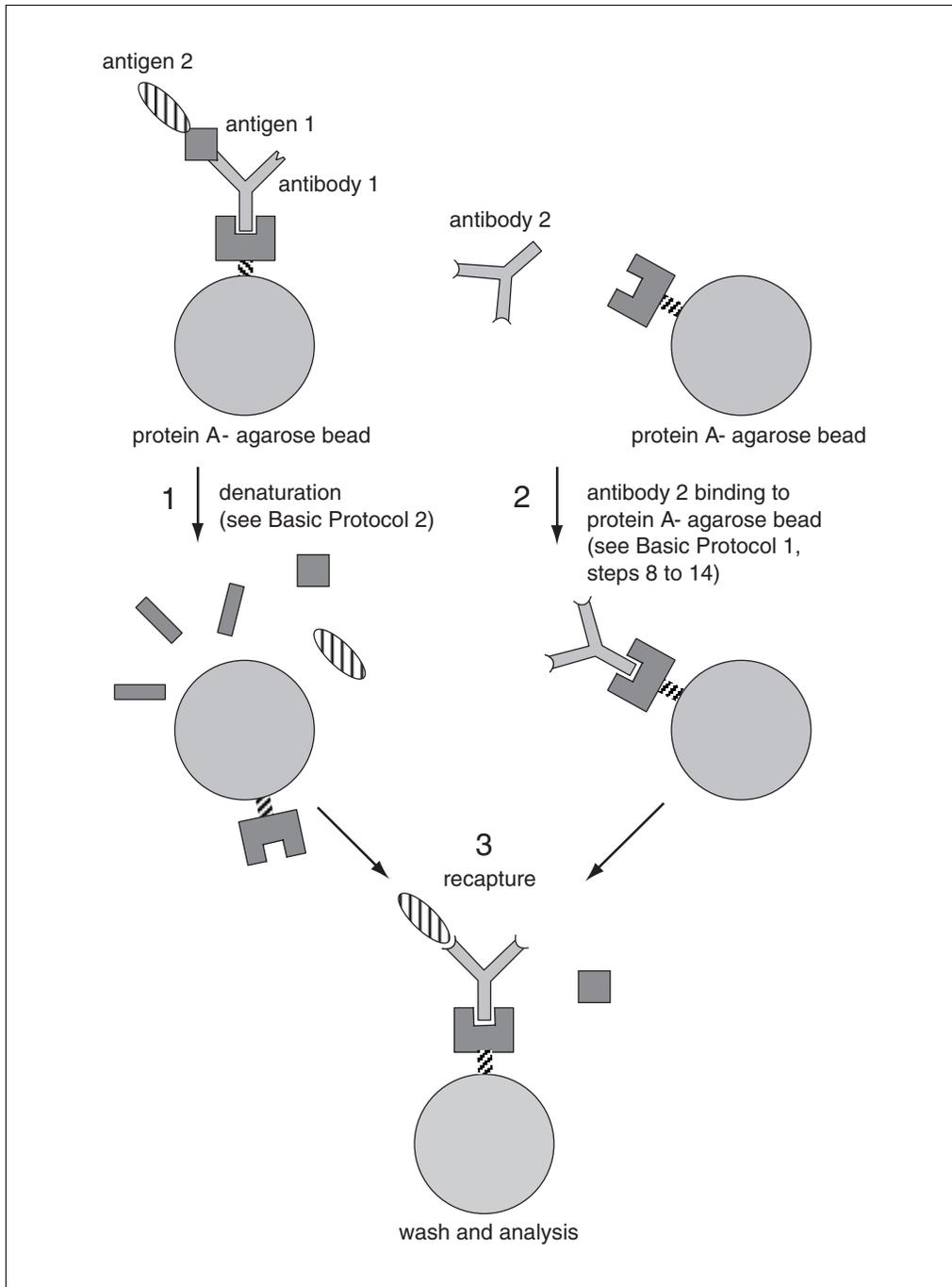


Figure 7.2.2 Scheme showing the stages of immunoprecipitation-recapture. (1) Dissociation and denaturation of the antigen: an antigen immunoprecipitated with antibody 1 bound to protein A-agarose beads is dissociated and denatured by heating in the presence of SDS and DTT. (2) Immobilization of the second antibody: antibody 2 is bound to protein A-agarose beads. (3) Recapture: the denatured antigen (striped oval) is recaptured on antibody 2 bound to protein A-agarose beads. Alternatively, antibody 1 can be used again for further purification of the original antigen (square).

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tion. By using a different antibody in the second immunoprecipitation, immunoprecipitation-recapture can be used to analyze the subunit composition of multi-protein complexes (Fig. 7.2.3). The feasibility of this approach depends on the ability of the second antibody to recognize denatured antigens.

Dissociation of the antigen from the beads is achieved by denaturation of antigen-antibody-bead complexes at high temperature in the presence of SDS and DTT. Prior to recapture, the SDS is diluted in a solution containing Triton X-100, and the DTT is neutralized with excess iodoacetamide. Recapture is then performed as in the first immunoprecipitation (see Basic Protocol 1, step 26).

Materials

Elution buffer (see recipe)

Beads containing bound antigen (see Basic Protocol 1, step 26)

10% (w/v) BSA (APPENDIX 2A)

Nondenaturing lysis buffer (see recipe)

Heating block set at 95°C (Eppendorf Thermomixer 5436 or equivalent)

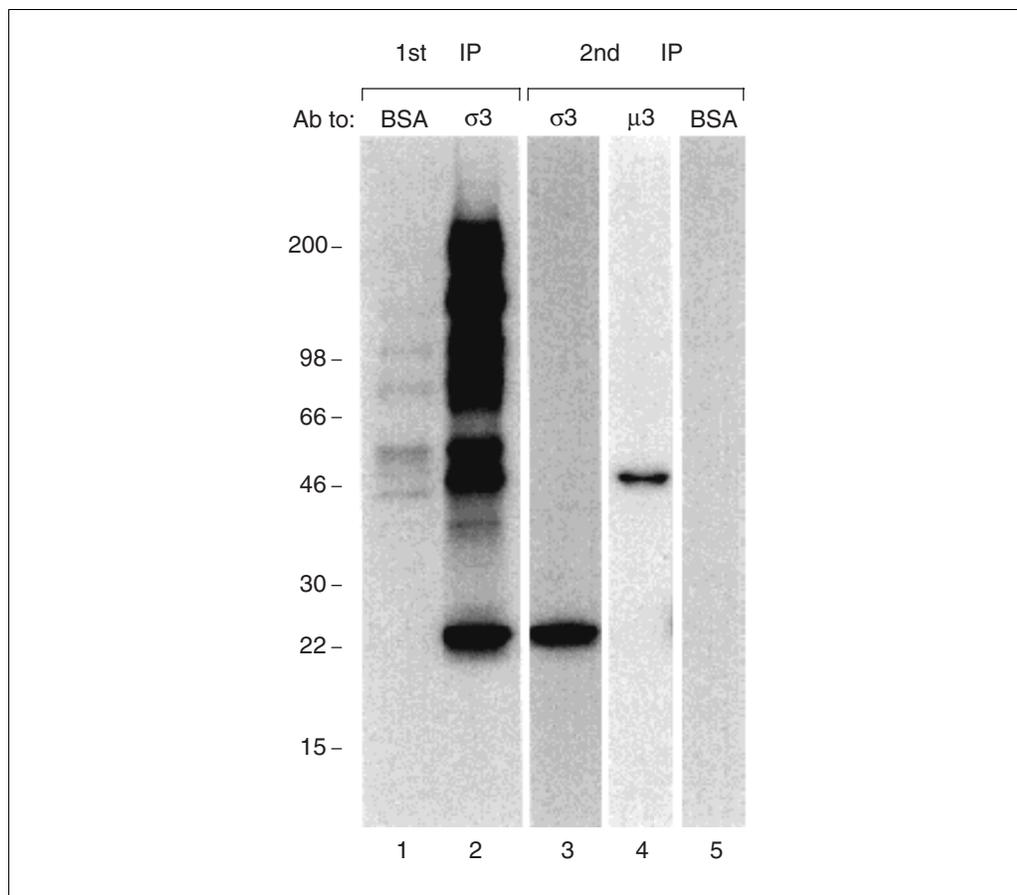


Figure 7.2.3 Example of an immunoprecipitation-recapture experiment. Human M1 fibroblasts were labeled overnight with [³⁵S]methionine (UNIT 7.1) and extracted with nondenaturing lysis buffer (see Basic Protocol 1). The cell extract was then subjected to immunoprecipitation with antibodies to BSA (irrelevant antibody control; lane 1) and to the AP-3 adaptor (σ 3; lane 2), a protein complex involved in protein sorting. Notice the presence of several specific bands in lane 2. The AP-3 immunoprecipitate was denatured as described in Basic Protocol 2 and individual components of the AP-3 complex were recaptured with antibodies to two of its subunits: σ 3 (M_r ~22,000; lane 3) and μ 3 (M_r ~47,000; lane 4). An immunoprecipitation with an antibody to BSA was also performed as a nonspecific control (lane 5). The amount of immunoprecipitate loaded on lanes 1 and 2 is $\sim 1/10$ the amount loaded on lanes 3 to 5. Notice the presence of single bands in lanes 3 and 4. The positions of M_r standards (expressed as $10^{-3} \times M_r$) are shown at left. IP, immunoprecipitation.

1. Add 50 μ l elution buffer to 15 μ l beads containing bound antigen. Mix by vortexing.
The DTT in the elution buffer reduces disulfide bonds in the antigen and the antibody, and the SDS contributes to the unfolding of polypeptide chains.
2. Incubate 5 min at room temperature and 5 min at 95°C in a heating block. Cool tubes to room temperature.
3. Add 10 μ l of 10% BSA. Mix by gentle vortexing.
BSA is added to prevent adsorption of antigen to the tube, and to quench nonspecific binding to antibody-conjugated beads.
4. Add 1 ml nondenaturing lysis buffer.
The iodoacetamide in the nondenaturing lysis buffer reacts with the DTT and prevents it from reducing the antibody used in the recapture steps. The presence of PMSF and leupeptin in the buffer is not necessary at this step.
5. Incubate 10 min at room temperature.
6. Clear the lysate and perform second immunoprecipitation (see Basic Protocol 1, steps 6 to 26).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Denaturing lysis buffer

1% (w/v) SDS

50 mM Tris-Cl, pH 7.4 (APPENDIX 2A)

5 mM EDTA (APPENDIX 2A)

Store up to 1 week at room temperature (SDS precipitates at 4°C)

Add the following fresh before use:

10 mM dithiothreitol (DTT, from powder)

1 mM PMSF (APPENDIX 2A)

2 μ g/ml leupeptin (store 10 mg/ml stock in H₂O up to 6 months at -20°C)

15 U/ml DNase I (store 15,000 U/ml stock solution up to 2 years at -20°C)

1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), added fresh from a 0.1 M stock solution in H₂O, can be used in place of PMSF. AEBSF stock can be stored up to 1 year at -20°C.

Detergent-free lysis buffer

PBS (APPENDIX 2A) containing:

5 mM EDTA (APPENDIX 2A)

0.02% (w/v) sodium azide

Store up to 6 months at 4°C

Immediately before use add:

10 mM iodoacetamide (from powder)

1 mM PMSF (APPENDIX 2A)

2 μ g/ml leupeptin (store 10 mg/ml stock in H₂O up to 6 months at -20°C)

1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), added fresh from a 0.1 M stock solution in H₂O, can be used in place of PMSF. AEBSF stock can be stored up to 1 year at -20°C.

Elution buffer

1% (w/v) SDS
100 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
Store up to 1 week at room temperature
10 mM dithiothreitol (DTT, add fresh from powder before use)

Nondenaturing lysis buffer

1% (w/v) Triton X-100
50 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
300 mM NaCl
5 mM EDTA (APPENDIX 2A)
0.02% (w/v) sodium azide
Store up to 6 months at 4°C
Immediately before use add:
10 mM iodoacetamide (from powder)
1 mM PMSF (APPENDIX 2A)
2 µg/ml leupeptin (store 10 mg/ml stock in H₂O up to 6 months at -20°C)
1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), added fresh from a 0.1 M stock solution in H₂O, can be used in place of PMSF. AEBSF stock can be stored up to 1 year at -20°C.

Wash buffer

0.1% (w/v) Triton X-100
50 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
300 mM NaCl
5 mM EDTA (APPENDIX 2A)
0.02% (w/v) sodium azide
Store up to 6 months at 4°C

COMMENTARY

Background Information

The use of antibodies for immunoprecipitation has its origin in the *precipitin* reaction (Nisonoff, 1984). The term precipitin refers to the spontaneous precipitation of antigen-antibody complexes formed by interaction of certain polyclonal antibodies with their antigens. The precipitation arises from formation of large networks of antigen-antibody complexes, due to the bivalent or polyvalent nature of immunoglobulins and to the presence of two or more epitopes in some antigens. This phenomenon was quickly exploited to isolate antigens from protein mixtures; however, its use remained limited to antibodies and antigens that were capable of multivalent interaction. In addition, the efficiency of precipitate formation was highly dependent on the concentrations of antibody and antigen. Thus, the precipitin reaction was not generally applicable as a method for immunoprecipitation.

A significant improvement was the use of secondary anti-immunoglobulin reagents (generally anti-immunoglobulin serum) to cross-link the primary antibodies, thus promoting the

formation of a precipitating network. Protocols based on the use of cross-linking secondary antibodies are still used in immunoprecipitation and are reputed to give very low backgrounds (Springer, 1996).

In the 1970s, immunoprecipitation became widely applicable to the study of cellular antigens as a result of several technological advances. A critical development was the introduction of methods for the production of monoclonal antibodies (Köhler and Milstein, 1975). The ability to produce unlimited amounts of antibodies with specificity against virtually any cellular antigen had a profound impact in many areas of biology and medicine. The fact that preparation of monoclonal antibodies did not require prior purification of the antigens accelerated the characterization of cellular proteins and organelles, a process in which immunoprecipitation protocols played a major role. To this day, monoclonal antibodies produced in mice or rats continue to be among the most useful tools in cell biology.

Another important development was the discovery of bacterial Fc receptors, proteins found

on the surface of bacteria that have the property of binding a wide range of immunoglobulins. Two of the most widely used bacterial Fc receptors are protein A from *Staphylococcus aureus* and protein G from group G streptococci. Protein A and protein G bind both polyclonal and monoclonal antibodies belonging to different subclasses and from different animal species (Table 7.2.1). Protein A was initially used to adsorb immunoglobulins as part of fixed, killed *Staphylococcus aureus* particles. Both protein A and protein G are now produced in large quantities by recombinant DNA procedures and are available coupled to solid-phase matrices such as agarose. In most cases, the binding of polyclonal or monoclonal antibodies to immobilized protein A (or G) avoids the need to use a secondary antibody to precipitate antigen-antibody complexes. Because of their broad specificity and ease of use, protein A-agarose and protein G-agarose (and related products) are the state-of-the-art reagents for the isolation of soluble antigen-antibody complexes in immunoprecipitation protocols.

Recent progress in the field of antibody engineering (reviewed by Rapley, 1995; Irving et al., 1996) promises to make antibody production a less time-consuming and haphazard process. Antibody fragments with high affinity for specific antigens can now be selected from phage display antibody libraries. Selected recombinant antibodies can then be produced in large quantities in *Escherichia coli*. Techniques have been developed for producing antibodies in soluble, secreted form. Affinity tags are added to the recombinant antibody molecules to facilitate purification, detection, and use in procedures such as immunoprecipitation. While attractive in principle, the production of recombinant antibodies has been plagued by technical difficulties that so far have limited their widespread use in cell biology. However, as technical problems are overcome, recombinant techniques will progressively replace immunization of animals as a way of producing antibodies for immunoprecipitation and for other applications.

Critical Parameters

Extraction of antigens

Isolation of cellular antigens by immunoprecipitation requires extraction of the cells so that the antigens are available for binding to specific antibodies, and are in a physical form that allows separation from other cellular components. Extraction with nondenaturing deter-

gents such as Triton X-100 (see Basic Protocol 1 and Alternate Protocol 1) or in the absence of detergent (see Alternate Protocol 3) allows immunoprecipitation with antibodies to epitopes that are exposed on native proteins. Other nondenaturing detergents such as Nonidet P-40, CHAPS, digitonin, or octyl glucoside are also appropriate for extraction of native proteins (UNIT 5.1). Some of these detergents (e.g., digitonin) preserve weak protein-protein interactions better than Triton X-100. If the antigen is part of a complex that is insoluble in nondenaturing detergents (e.g., cytoskeletal structures, chromatin, membrane “rafts”) or if the epitope is hidden within the folded structure of the protein, extraction under denaturing conditions is indicated (see Alternate Protocol 2).

The number of cells necessary to detect an immunoprecipitated antigen depends on the cellular abundance of the antigen and on the efficiency of radiolabeling. The protocols for radiolabeling (UNIT 7.1) and immunoprecipitation described in this book are appropriate for detection of antigens that are present at low to moderate levels (10,000 to 100,000 copies per cell), as is the case for most endogenous integral membrane proteins, signal transduction proteins, and transcription factors. For more abundant antigens, such as cytoskeletal and secretory proteins or proteins that are expressed by viral infection or transfection, the quantity of radiolabeled cells used in the immunoprecipitation can be reduced accordingly.

Production of antibodies

Immunoprecipitation can be carried out using either polyclonal or monoclonal antibodies (see discussion of selection below). Polyclonal antibodies are most often prepared by immunizing rabbits, although polyclonal antibodies produced in mice, guinea pigs, goats, sheep, and other animals, are also suitable for immunoprecipitation. Antigens used for polyclonal antibody production can be whole proteins purified from cells or tissues, or can be whole or partial proteins produced in bacteria or insect cells by recombinant DNA procedures. Another useful procedure is to immunize animals with peptides conjugated to a carrier protein. Production of polyclonal antibodies to recombinant proteins and peptides has become the most commonly used approach to obtain specific probes for immunoprecipitation and other immunochemical techniques, because it does not require purification of protein antigens from their native sources. The only requirement for making these antibodies is knowledge of the

sequence of a protein, information which is now relatively easy to obtain as a result of cDNA and genomic DNA sequencing projects. Polyclonal antibodies can be used for immunoprecipitation as whole serum, ammonium sulfate-precipitated immunoglobulin fractions, or affinity-purified immunoglobulins. Although all of these forms are suitable for immunoprecipitation, affinity-purified antibodies often give lower backgrounds and are more specific.

Most monoclonal antibodies are produced in mice or rats. The sources of antigen for monoclonal antibody production are the same as those for production of polyclonal antibodies, namely, proteins isolated from cells or tissues, recombinant proteins or protein fragments, and peptides. A significant advantage of monoclonal antibodies is that antigens do not need to be purified to serve as immunogens, as long as the screening method is specific for the antigen. Another advantage is the unlimited supply of monoclonal antibodies afforded by the ability to grow hybridomas in culture or in ascitic fluid. Many monoclonal antibodies can now be produced from hybridomas deposited in cell banks or are directly available from companies. Ascitic fluid, cell culture supernatant, and purified antibodies are all suitable sources of monoclonal antibodies for immunoprecipitation. Ascitic fluid and purified antibodies should be used when a high antibody titer is important. Cell culture supernatants have lower antibody titers, but tend to give cleaner immunoprecipitations than ascitic fluids due to the lack of contaminating antibodies.

Selection of antibodies: Polyclonal versus monoclonal

What type of antibody is best for immunoprecipitation? There is no simple answer to this question, as the outcome of both polyclonal and monoclonal antibody production protocols is still difficult to predict. Polyclonal antibodies to whole proteins (native or recombinant) have the advantage that they frequently recognize multiple epitopes on the target antigen, enabling them to generate large, multivalent immune complexes. Formation of these antigen-antibody networks enhances the avidity of the interactions and increases the efficiency of immunoprecipitation. Because these antibodies recognize several epitopes, there is a better chance that at least one epitope will be exposed on the surface of a solubilized protein and thus be available for interaction with antibodies. Thus, the likelihood of success is higher. These properties can be a disadvantage, though, as

some polyvalent antibodies can cross-react with epitopes on other proteins, resulting in higher backgrounds and possible misidentification of antigens. By being directed to a short peptide sequence, anti-peptide polyclonal antibodies are less likely to cross-react with other proteins. However, their usefulness is dependent on whether the chosen sequence turns out to be a good immunogen in practice, as well as on whether this particular epitope is available for interaction with the antibody under the conditions used for immunoprecipitation.

Unfractionated antisera are often suitable for immunoprecipitation. However, there is a risk that serum proteins other than the antibody will bind nonspecifically to the immunoadsorbent, and in turn bind proteins in the lysate that are unrelated to the antigen. For instance, transferrin can bind nonspecifically to immunoadsorbents, potentially leading to the isolation of the transferrin receptor as a contaminant (Harford, 1984). Polyclonal antisera can also contain antibodies to other antigens (e.g., viruses, bacteria) to which the animal may have been exposed, and these antibodies can also cross-react with cellular proteins during immunoprecipitation. Affinity-purified antibodies are a better alternative when antisera do not yield clean immunoprecipitations. Affinity-purification can lead to loss of high-affinity or low-affinity antibodies; however, the higher specificity of affinity-purified antibodies generally makes them “cleaner” reagents for immunoprecipitation.

The specificity, high titer, and limitless supply of the best immunoprecipitating monoclonal antibodies are unmatched by those of polyclonal antibodies. However, not all monoclonal antibodies are useful for immunoprecipitation. Low-affinity monoclonal antibodies can perform acceptably in immunofluorescence microscopy protocols (UNIT 4.3) but may not be capable of holding on to the antigen during the repeated washes required in immunoprecipitation protocols. The use of ascitic fluid has the same potential pitfalls as the use of polyclonal antisera, as ascites may also contain endogenous antibodies to other antigens and proteins such as transferrin that can bind to other proteins in the lysate.

In conclusion, an informed empirical approach is recommended in order to select the best antibody for immunoprecipitation. In general, it is advisable to generate and/or test several antibodies to a particular antigen in order to find at least one that will perform well in immunoprecipitation protocols.

Antibody titer

The importance of using the right amount of antibody for immunoprecipitation cannot be overemphasized. This is especially the case for quantitative immunoprecipitation studies, in which the antibody should be in excess of the specific antigen. For instance, in pulse-chase analyses of protein degradation or secretion (UNIT 7.1), it is critical to use sufficient antibody to deplete the antigen from the cell lysate. This is particularly important for antigens that are expressed at high levels, a common occurrence with the growing use of high-yield protein expression systems such as vaccinia virus or replicating plasmids in COS cells. Consider for example a protein that is expressed at high levels inside the cell, and of which only a small fraction is secreted into the medium. If limiting amounts of antibody are used in a pulse-chase analysis of this protein, the proportion of protein secreted into the medium will be grossly overestimated, because the limiting antibody will bind only a small proportion of the cell-associated protein and a much higher proportion of the secreted protein. The same considerations apply to degradation studies. Thus, it is extremely important in quantitative studies to ensure that the antibody is in excess of the antigen in the cell samples. This can be ascertained by performing sequential immunoprecipitations of the samples (see Basic Protocol 1, annotation to step 21). If the second immunoprecipitation yields only a small amount of the antigen relative to that isolated in the first immunoprecipitation (<10%), then the antibody titer is appropriate. If, on the other hand, the amount of antigen isolated in the second immunoprecipitation is >10%, either more antibody or less antigen should be used.

Too much antibody can also be a problem, as nonspecific immunoprecipitation tends to increase with increasing amounts of immunoglobulins bound to the beads. Thus, titration of the antibody used for immunoprecipitation is strongly advised.

Immunoabsorbent

If cost is not an overriding issue, the use of protein A- or protein G-agarose is recommended for routine immunoprecipitation. Protein A- or protein G-agarose beads (or equivalent products) have a very high capacity for antibody binding (up to 10 to 20 mg of antibody per milliliter of gel). Both protein A and protein G bind a wide range of immunoglobulins (Table 7.2.1). Backgrounds from nonspecifically bound proteins are generally low. Protein A-

and protein G-agarose beads are also stable and easy to sediment by low-speed centrifugation. A potential disadvantage, in addition to their cost, is that some polyclonal or monoclonal antibodies bind weakly or not at all to protein A or protein G (Table 7.2.1). This problem can be solved by using an intermediate rabbit antibody to the immunoglobulin of interest. For example, a goat polyclonal antibody can be indirectly bound to protein A-agarose by first incubating the protein A-agarose beads with a rabbit anti-goat immunoglobulin, and then incubating the beads with the goat polyclonal antibody. Anti-immunoglobulin antibodies (e.g., rabbit anti-goat immunoglobulins) coupled covalently to agarose can also be used for indirect immunoprecipitation in place of protein A- or protein G-agarose.

Fixed *Staphylococcus aureus* particles (Pansorbin) can be used as a less expensive alternative to protein A-agarose. They have a lower capacity, can give higher backgrounds, and take longer to sediment. However, they work quite well in many cases. In order to establish if they are appropriate for a particular experimental setup, conduct a preliminary comparison of the efficiency of protein A-agarose with *Staphylococcus aureus* particles as immunoabsorbent.

Specific antibodies coupled covalently to various affinity matrices can also be used for direct immunoprecipitation of antigens. After binding to protein A-agarose, antibodies can be cross-linked with dimethylpimelimidate (Gersten and Marchalonis, 1978). Purified antibodies can also be coupled directly to derivatized matrices such as CNBr-activated Sepharose (Springer, 1996). This latter approach avoids having to bind the antibody to protein A-agarose. Covalently bound antibodies should be used when elution of immunoglobulins from the beads complicates further analyses of the complexes. This is the case when proteins in immunoprecipitates are analyzed by one- or two-dimensional gel electrophoresis (UNIT 6.1) followed by Coomassie blue or silver staining, or are used for microsequencing. Also, the released immunoglobulins could interfere with detection of some antigens by immunoblotting (UNIT 6.2) following immunoprecipitation.

Nonspecific controls

For correct interpretation of immunoprecipitation results, it is critical to include appropriate nonspecific controls along with the specific samples. One type of control consists of setting up an incubation with an irrelevant antibody in the same biochemical form as the

experimental antibody (e.g., serum, ascites, affinity-purified immunoglobulin, antibody bound to protein A–agarose or directly conjugated to agarose), and belonging to the same species and immunoglobulin subclass as the experimental antibody (e.g., rabbit antiserum, mouse IgG2a). For an antiserum, the best control is preimmune serum (serum from the same animal obtained before immunization). Nonimmune serum from the same species is an acceptable substitute for preimmune serum in some cases. “No-antibody” controls are not appropriate because they do not account for nonspecific binding of proteins to immunoglobulins. In immunoprecipitation-recapture experiments, control immunoprecipitations with irrelevant antibodies should be performed for both the first and second immunoprecipitation steps (Fig. 7.2.3). Another type of control is to perform an immunoprecipitation from cells that do not express a specific antigen in parallel with immunoprecipitation of the antigen-expressing cells. For instance, untransfected cells are a perfect control for transfected cells. In yeast cells, null mutants that do not express a specific antigen are an ideal control for wild-type cells.

Order of stages

In the immunoprecipitation protocols described in this unit, the antibody is prebound to protein A–agarose before addition to the cell lysate containing the antigen. This differs from other protocols in which the free antibody is first added to the lysate and the antigen-antibody complexes are then collected by addition of the immunoabsorbent. Although both procedures can give good results, the authors prefer the protocols described here because this method allows better control of the amount of antibody bound to the immunoabsorbent. Prebinding antibodies to the immunoabsorbent beads allows removal of unbound antibodies. The presence of unbound antibodies in the incubation mixture could otherwise result in decreased recovery of the antigen on the immunoabsorbent beads. Another advantage of the prebinding procedure is that most proteins other than the immunoglobulin in the antibody sample (e.g., serum proteins) are removed from the beads and do not come in contact with the cell lysate. This eliminates potential adverse effects of these proteins on isolation of the antigen.

Washing

The five washes described (see Basic Protocol 1; four with wash buffer and one with PBS)

are sufficient for maximal removal of unbound proteins; additional washes are unlikely to decrease the background any further. The last wash with PBS removes the Triton X-100 that can lead to decreased resolution on SDS-PAGE. It also removes other components of the wash buffer that could interfere with enzymatic treatment of immunoprecipitates. It is not advisable to complete all the washes quickly (e.g., in 5 min), because this may not allow enough time for included proteins to diffuse out of the gel matrix. Instead, beads should be washed over ~30 min, which may require keeping the samples on ice for periods of 3 to 5 min between washes. In order to reduce nonspecific bands, samples can be subjected to an additional wash with wash buffer containing 0.1% (w/v) SDS, or with a mixture of 0.1% (w/v) SDS and 0.1% (w/v) sodium deoxycholate (Fig. 7.2.4). This wash should be done between the fourth wash and the wash with PBS.

Troubleshooting

Two of the most common problems encountered in immunoprecipitation of metabolically labeled proteins are failure to detect specific antigens in the immunoprecipitates, and high background of nonspecifically bound proteins for antigens that were radiolabeled *in vivo* and analyzed by SDS-PAGE (UNIT 6.1) followed by autoradiography or fluorography (UNIT 6.3). When immunoprecipitates are analyzed by immunoblotting (UNIT 6.2), an additional problem may be the detection of immunoprecipitating antibody bands in the blots (Table 7.2.2).

Anticipated Results

For antigens that are present at >10,000 copies per cell, the radiolabeling and immunoprecipitation protocols described in this book would be expected to result in the detection of one or more bands corresponding to the specific antigen and associated proteins in the electrophoretograms. Specific bands should not be present in control immunoprecipitations done with irrelevant antibodies. If antigens are labeled with [³⁵S]methionine (UNIT 7.1), specific bands should be visible within 2 hr to 2 months of exposure. Due to the relatively low yield of the immunoprecipitation-recapture procedure (<10% of that of a single immunoprecipitation step), detection of specific bands is likely to require longer exposure times. This may turn out to be problematic due to the radioactive decay of ³⁵S (half-life = 88 days). In immunoprecipitation-recapture experiments in which the antibodies used for the first and second

immunoprecipitation steps recognize different antigens (e.g., for the study of protein-protein interactions; Fig. 7.2.2), it is advisable to include in the second immunoprecipitation step a positive control with either the antibody used for the first immunoprecipitation (if the antibody recognizes both the native and denatured forms of the antigen) or a different antibody with specificity for the same antigen (Fig. 7.2.3, lane 3).

Time Considerations

Preparation of cell extracts (Basic Protocol 1 and Alternate Protocols 1 to 4) takes 1 to 3 hr

to complete, and isolation of the antigen on antibody-conjugated beads takes 2 to 3 hr. Binding antibodies to immunoabsorbent beads can be done prior to or simultaneously with preparation of the cell extracts and also takes 1 to 3 hr. Therefore, the whole immunoprecipitation procedure can be completed in 1 day. Immunoprecipitates can be analyzed immediately (e.g., resolved by SDS-PAGE) or frozen and analyzed another day. Immunoprecipitation-recapture experiments (Basic Protocol 2) require an additional 1 to 2 hr to denature and prepare the antigen for immunoprecipitation, and 2 to 3 hr to isolate the antigen. Completion

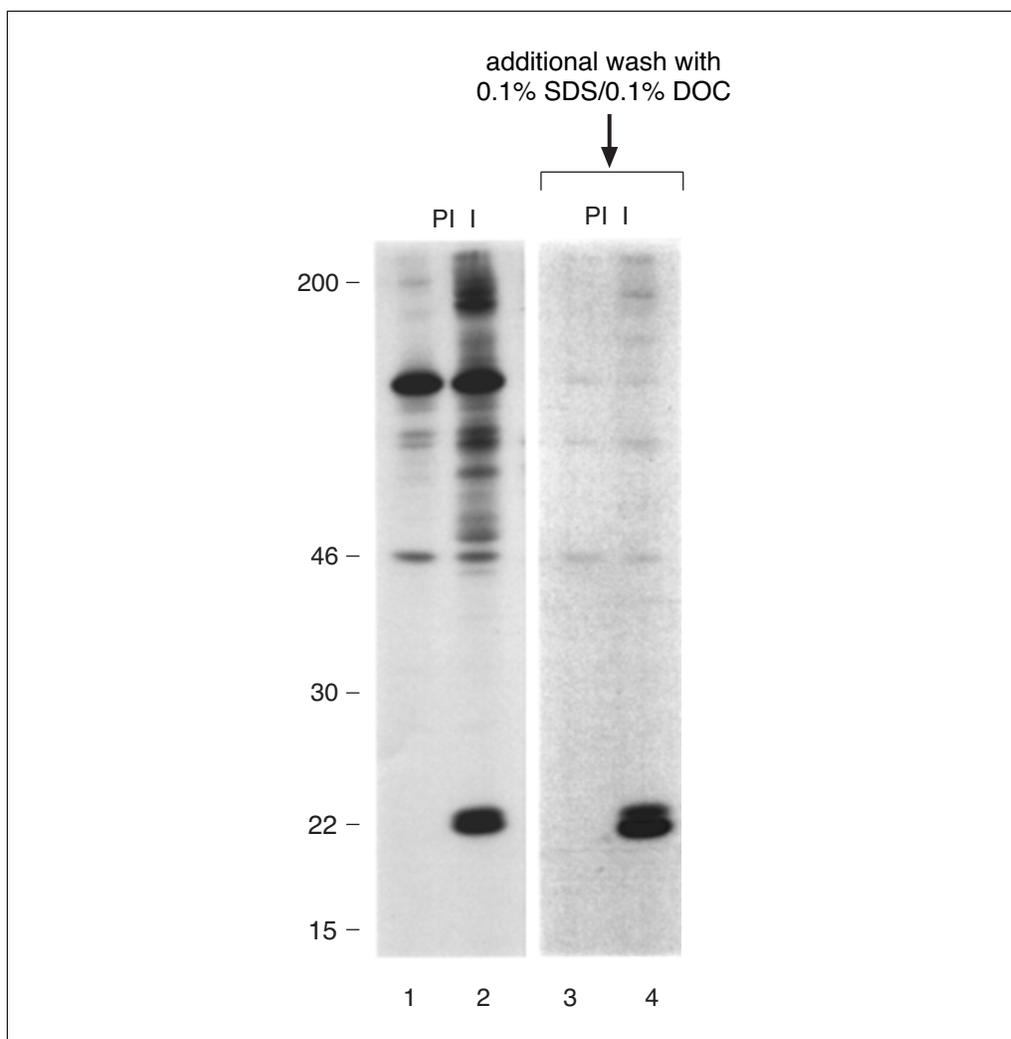


Figure 7.2.4 Lowering background by washing with SDS and sodium deoxycholate (DOC). In this experiment, BW5147 cells (mouse thymoma) labeled with [³⁵S]methionine for 1 hr were extracted with nondenaturing lysis buffer (see Basic Protocol 1). The extracts were subjected to immunoprecipitation with protein A-agarose beads incubated with either preimmune (PI) or immune (I) serum from a rabbit immunized with the ribosomal protein L17 (doublet at $M_r \sim 22,000$). Lanes 1 and 2 correspond to immunoprecipitates obtained using the protocols described in this unit. Notice the presence of nonspecific bands and/or associated proteins in lane 2. Lanes 3 and 4 correspond to beads that were washed an additional time with 0.1% (w/v) SDS and 0.1% (w/v) DOC. Notice the disappearance of most of the nonspecific bands and/or associated proteins. The positions of M_r standards (expressed as $10^{-3} \times M_r$) are shown at left.

Table 7.2.2 Troubleshooting Guide for Immunoprecipitation

Problem	Cause	Solution
<i>No specific radiolabeled antigen band</i>		
Gel is completely blank after prolonged autoradiographic exposure	Poorly labeled cells: too little radiolabeled precursor, too few cells labeled, lysis/loss of cells during labeling, too much cold amino acid in labeling mix, wrong labeling temperature	Check incorporation of label by TCA precipitation (<i>UNIT 7.1</i>); troubleshoot the labeling procedure
Only nonspecific bands present	Antigen does not contain the amino acid used for labeling	Label cells with another radiolabeled amino acid, or for glycoproteins, with tritiated sugar
	Antigen expressed at very low levels	Substitute cells known to express higher levels of antigens as detected by other methods; transfect cells for higher expression
	Protein has high turnover rate and is not well labeled by long-term labeling	Use pulse labeling
	Protein has a low turnover rate and is not well labeled by short-term labeling	Use long-term labeling
	Protein is not extracted by lysis buffer used to solubilize cells	Solublize with a different nondenaturing detergent or under denaturing conditions
	Antigen is not extracted with Triton X-100 at 4°C	Extract with Triton X-100 at 37°C or use another detergent
	Antibody is nonprecipitating	Identify and use antibody that precipitates antigen
	Epitope is not exposed in native antigen	Extract cells under denaturing conditions
	Antibody does not recognize denatured antigen	Extract cells under nondenaturing conditions
	Antibody does not bind to immunoadsorbent	Use a different immunoadsorbent (<i>Table 7.2.1</i>); use intermediate antibody
	Antigen is degraded during immunoprecipitation	Ensure that fresh protease inhibitors are present
<i>High background of nonspecific bands</i>		
Isolated lanes on gel with high background	Random carryover of detergent-insoluble proteins	Remove supernatant immediately after centrifugation, leaving a small amount with pellet; if resuspension occurs, recentrifuge
High background in all lanes	Incomplete washing	Cap tubes and invert several times during washes
	Poorly radiolabeled protein	Optimize duration of labeling to maximize signal-to-noise
	Incomplete removal of detergent-insoluble proteins	Centrifuge lysate 1 hr at 100,000 × g
	Insufficient unlabeled protein to quench nonspecific binding	Increase concentration of BSA

continued

Table 7.2.2 Troubleshooting Guide for Immunoprecipitation, continued

Problem	Cause	Solution
	Antibody contains aggregates	Microcentrifuge antibody 15 min at maximum speed before binding to beads
	Antibody solution contains nonspecific antibodies	Use affinity-purified antibodies; absorb antibody with acetone extract of cultured cells that do not express antigen; for yeast cells, absorb antibody with null mutant cells
	Too much antibody	Use less antibody
	Incomplete preclearing	Preclear with irrelevant antibody of same species of origin and immunoglobulin subclass bound to immunoadsorbent
	Nonspecifically immunoprecipitated proteins	Fractionate cell lysate (e.g., ammonium sulfate precipitation, lectin absorption, or gel filtration) prior to immunoprecipitation; after washes in wash buffer, wash beads once with 0.1% SDS in wash buffer or 0.1% SDS/0.1% sodium deoxycholate
<i>Immunoprecipitating antibody detected in immunoblots</i>		
Complete immunoglobulin or heavy and/or light chains visible in immunoblot	Protein A conjugate or secondary antibody recognizes immunoprecipitating antibody	Use antibody coupled covalently to solid-phase matrix for immunoprecipitation; probe blots with primary antibody from a different species and the appropriate secondary antibody specific for immunoblotting primary antibody

of an entire immunoprecipitation-recapture experiment requires a very long workday. Alternatively, samples can be frozen after the first immunoprecipitation, and the elution and recapture can be carried out another day.

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**Protein Labeling
and Immuno-
precipitation**

7.2.21

Metabolic Labeling with Sulfate

UNIT 7.3

Many newly synthesized secretory or transmembrane proteins are posttranslationally modified as they traverse the secretory pathway (UNIT 15.1). Posttranslational modifications can be exploited to determine where a protein normally resides in a cell or to follow its transport through the cell. One of these modifications is the addition of sulfate either to the protein backbone on tyrosine residues or to carbohydrate side chains. Sulfation of proteins on tyrosine occurs in the lumen of the *trans*-Golgi network (TGN; Niehrs and Huttner, 1990) through the action of the enzyme tyrosylprotein sulfotransferase (TPST). Sulfation of carbohydrate side chains on proteins also occurs in the later compartments of the Golgi complex, mediated by a variety of sulfotransferases (for recent review see Bowman and Bertozzi, 1999). The xylose side chains of proteoglycans can also be sulfated. Thus, sulfation can potentially be used to study the transport of a variety of molecules as long as they (1) are substrates for the sulfotransferases and (2) are resident in or pass through the compartment(s) that contains the sulfotransferases.

Incorporation of radioactive sulfate into sulfated proteins and proteoglycans is a useful tool to study protein/proteoglycan transport from the Golgi complex. It provides greater resolution of the kinetics of transport than does metabolic labeling in the endoplasmic reticulum using radiolabeled amino acids (see UNIT 7.1). This unit describes how to label mammalian cells in culture with radioactive sulfate to determine whether the protein or proteoglycan of interest is a substrate for sulfotransferases and can be labeled with [³⁵S]sulfate.

This unit describes a long-term labeling protocol for adherent monolayer cells in culture (see Basic Protocol 1), which ensures that a high specific activity is achieved for subsequent analysis. Modification of this protocol to provide a pulse-chase protocol (see Basic Protocol 2) is achieved by shorter labeling times (the pulse), followed by a quenching of the label with excess cold sulfate (the chase).

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see UNIT 7.1 and APPENDIX 1D)

NOTE: All solutions used for the labeling protocols are tissue culture-grade reagents. Sterility of the media, label, and cultures is important for long-term labeling procedures.

NOTE: All culture incubations should be performed in a humidified, 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

LONG-TERM [³⁵S]SULFATE LABELING OF MONOLAYER CELLS IN CULTURE

The most useful way to initially determine if the molecule of interest is sulfated is to label the cells to steady state using a lengthy labeling period—e.g., a time equivalent to at least one cell cycle period. This ensures that all the proteins within the cell that can be labeled with sulfate are labeled and provides the highest signal for starting the analysis. Cell cultures ~70% to 80% confluent are required along with the appropriate labeling medium and CO₂ incubators. All washing steps are performed at room temperature in a sterile cabinet.

**BASIC
PROTOCOL 1**

**Protein Labeling
and Immuno-
precipitation**

7.3.1

Contributed by Sharon A. Tooze

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Supplement 4

Materials

Dialyzed serum (see recipe)
200 mM L-glutamine in H₂O
Sulfate-free DMEM (see recipe)
Sodium [³⁵S]sulfate (>1000 Ci/mmol)
Cells of interest, cultured to 70% to 80% confluence
DPBS (APPENDIX 2A), 4°C

Additional reagents and equipment for immunoprecipitation of proteins (UNIT 7.2)
or for SDS-PAGE (UNIT 6.1) and fluorography (UNIT 6.3)

NOTE: The labeling medium is a modified formula for DMEM, which the author has found works best. Other media, such as RPMI, have not been used as successfully.

NOTE: All solutions for overnight labeling should be prepared and used under aseptic conditions.

NOTE: The size of the cultures used depends on the amount of the protein of interest present in the cells and the techniques used for subsequent analysis.

1. Prepare depletion medium by adding one-tenth the normal concentration of dialyzed serum and 4 mM glutamine to sulfate-free DMEM.

Prepare enough medium to wash cultures once and for the addition of label. The amount of wash and labeling medium required depends on the size of the culture. Typically a 35-mm-diameter dish requires 2.0 ml and a 90-mm-diameter dish requires 10 ml.

The addition of glutamine before each experiment allows the sulfate-free DMEM to be stored up to 6 months.

2. Prepare labeling medium by removing the appropriate amount of depletion medium to a plastic tube and adding sodium [³⁵S]sulfate to 0.2 to 0.5 mCi/ml.
3. Warm both media to 37°C in a water bath.
4. Gently wash cells once with depletion medium. Remove wash thoroughly and add labeling medium.
5. Ensure that cells are well covered, return them to the CO₂ incubator, and incubate overnight (≥16 hr).
6. Remove dishes from incubator and place on ice. Remove labeling medium and save.
7. Wash cells three times with ice-cold DPBS
8. Lyse in an appropriate solution and analyze by immunoprecipitation (UNIT 7.2) followed by SDS-PAGE (UNIT 6.1) and fluorography (UNIT 6.3). Use a lysis buffer that is optimal for solubilizing the cells and protein of interest.

If the protein or proteoglycan of interest is secreted it maybe possible to detect it in the labeling medium. Adjust the medium with salts, buffer, and detergent to that present in the lysis conditions used for immunoprecipitation and proceed as in UNIT 7.2.

After such a labeling experiment, it is worthwhile analyzing the total lysate and an aliquot of the medium by SDS-PAGE (UNIT 6.1). The molecule of interest may be highly sulfated and readily detectable without prior enrichment, even if it is a relatively minor fraction of the total protein.

PULSE-CHASE [³⁵S]SULFATE LABELING OF MONOLAYER CELLS IN CULTURE

Once the relative abundance of a protein is known and it can be easily detected after an overnight labeling with [³⁵S]sulfate, it is possible to use the radioactive sulfate in a pulse-chase protocol. This is done by labeling the protein for a short period of time (e.g., a 4- to 5-min pulse). The short time period is required to ensure that the labeled protein is still in the TGN. Further incubation (the chase) is done at 37°C after the addition of excess unlabeled sulfate to stop the incorporation of radioactive sulfate. The chase can be carried out for any length of time; the position of the radiolabeled proteins in the cell will be a function of the chase time. This procedure is invaluable if the protein or proteoglycan is thought to be secreted. If the protein or proteoglycan is thought to localize to a post-Golgi compartment, the protocol can be combined with cell fractionation to identify the intracellular compartment. The very short pulse provides a very accurate starting point for the analysis, and the kinetics of transport or secretion of the protein can be easily obtained.

Depending on the experiment it will be necessary to prepare several identical cultures: a dish of cells is required for each time point, including a zero time point, which is defined as the end of the pulse. A typical time course may have five or six chase time points (for example, 5, 10, 20, 40, and 80 min).

Materials

Cultured cells of interest, ~70% to 80% confluent
Dialyzed serum (see recipe)
200 mM L-glutamine in H₂O
Sulfate-free DMEM (see recipe)
Sodium [³⁵S]sulfate (>1000 Ci/mmol)
DPBS (APPENDIX 2A), 4°C
Chase medium (see recipe)
Platform rocker

Additional reagents and equipment for immunoprecipitation of proteins (UNIT 7.2), SDS-PAGE (UNIT 6.1), and fluorography (UNIT 6.3)

1. Label dishes of cultured cells on bottom with appropriate time or sample number and organize them on an easily movable tray. Return to incubator.

It is important to be organized before the experiment begins as the manipulations must be carried out as quickly as possible during the time course.

2. Prepare ~4 vol depletion medium for each culture dish by adding dialyzed serum to 1/100th the normal concentration and 4 mM glutamine to sulfate-free DMEM.

For each time point, enough depletion medium will be needed to make the labeling medium, wash each culture twice, and incubate once for the depletion step. For washes and depletion, use 2.0 ml for a 35-mm-diameter dish and 10 ml for a 90-mm-diameter dish. For labeling medium, use 1.0 ml for a 35-mm dish and 2.5 ml for a 90-mm dish. A reduced volume of labeling medium is used to save on the cost of the label.

The addition of glutamine before each experiment allows the sulfate-free DMEM to be stored up to 6 months.

3. Prepare the labeling medium required for all dishes as one batch. Transfer the amount required for each dish, multiplied by the number of dishes, to a plastic tube and add [³⁵S]sulfate to give 5 mCi/ml.
4. Warm both media to 37°C in a water bath.

5. Gently wash all dishes twice with depletion medium. Thoroughly remove second wash and add another aliquot of depletion medium. Incubate 20 min in the incubator.

The depletion step reduces the intracellular pool of sulfate to allow the highest specific activity of incorporated radioactive sulfate to be achieved.

6. Remove depletion medium from all dishes and quickly add equal aliquots of labeling medium to each culture. Start timer. Ensure that cells are well covered, place on a platform rocker in the 37°C CO₂ incubator, and incubate for 4 min.

The culture dishes should be rocked gently during the labeling period as the volume of medium used is not sufficient to cover cells.

This 4-min incubation is effectively a 2-min pulse because 2 min is required for the [³⁵S]sulfate to be converted to the activated sulfate donor (PAPS, see Commentary).

7. Remove all dishes from incubator and quickly place the time-zero dish on ice. Remove labeling medium from the time-zero dish and save. Add ice-cold DPBS to that dish and keep on ice. Remove label from remaining dishes at room temperature, discard, add chase medium at twice the volume of the labeling medium, and return to the incubator but do not rock.
8. At predetermined time points, remove the appropriate dish from the incubator, place immediately on ice, remove and save medium, and add ice-cold DPBS. Keep dishes on ice. Repeat until all the dishes are on ice and the chase protocol is complete.
9. Wash all dishes three times with ice-cold DPBS.
10. Lyse in an appropriate solution and immunoprecipitate (UNIT 7.2). Use a lysis buffer that is optimal for solubilizing the cells and protein of interest.
11. If the protein/proteoglycan of interest is secreted, adjust the medium saved from the zero time point and each chase time point by adding salts, buffer, and detergent to the conditions required for immunoprecipitation, and proceed as in UNIT 7.2.

CAUTION: Remember that the time-zero medium contains the radioactive-sulfate solution and should be treated with appropriate precautions.

12. Analyze immunoprecipitates by SDS-PAGE (UNIT 6.1) and fluorography (UNIT 6.3).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Chase medium

Use Dulbecco's minimal essential medium (DMEM; as purchased from supplier) or another growth medium. Use $\frac{1}{100}$ th the normal serum concentrations to avoid problems with serum proteins during the analysis. Add 160 mM Na₂SO₄ (filter sterilized through a 0.2- μ m filter) to a final concentration of 1.6 mM. Prepare fresh for each experiment.

Dialyzed serum

Dialyze serum overnight against DPBS (APPENDIX 2A) at 4°C. Use 100 times the serum volume for the dialysis, and change the solution once during dialysis. Sterile filter after dialysis and store in 1- or 2-ml aliquots in sterile tubes for up to 1 year at -20°C. After thawing, store dialyzed serum up to 1 month at 4°C.

Low–Met/Cys amino acid mixture, 10×

For 1 liter:

0.84 g L-arginine-HCl

0.063 g L-cystine

0.30 g glycine

0.42 g L-histidine-HCl

1.05 g L-isoleucine

1.05 g L-leucine

1.46 g L-lysine-HCl

0.03 g L-methionine

0.66 g L-phenylalanine

0.42 g L-serine

0.95 g L-threonine

0.16 g L-tryptophan

0.94 g L-valine

Mix into sterile Milli-Q-purified water and adjust volume to 1 liter

Store up to 6 months at -20°C

Having low methionine and cystine improves the sulfate incorporation by reducing the amount of sulfated essential amino acids that can be broken down, thereby diluting the radioactive pool of sulfate. Methionine and cystine at one-tenth the normal amount is usually sufficient to maintain protein synthesis and keep the cells healthy.

Tyrosine is added to the dilute sulfate-free DMEM because it is insoluble at 10× the normal concentration.

Do not add antibiotics as some (e.g., streptomycin) are sulfate salts.

Sulfate-free Dulbecco's minimal essential medium (DMEM)

For one liter:

265.00 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

0.10 mg $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$

400.00 mg KCl

168.75 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$

6400.00 mg NaCl

125.00 mg $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$

5 ml 20% (w/v) glucose

10 ml 11.0 mg/ml sodium pyruvate, added dropwise

3 ml 0.5% (w/v) phenol red

49.3 ml 7.5% (w/v) NaHCO_3

72.0 mg tyrosine

40 ml 100× MEM vitamins (Sigma)

100 ml 10× low–Met/Cys amino acid mixture (see recipe)

Combine all ingredients except phenol red in 900 ml Milli-Q-purified water, cover, and stir to dissolve for several hours at room temperature or overnight at 4°C . Add phenol red and adjust pH to 7.2 with 1 M NaOH. Adjust volume to 1 liter. Sterile filter with 0.2- μm filter. Store up to 6 months at 4°C in the dark.

Use plastic containers or glassware that have not been washed with detergent to prepare media.

Sodium pyruvate is added dropwise to avoid precipitation.

COMMENTARY

Background Information

Posttranslational modifications of proteins and proteoglycans can be exploited to follow the route of the modified molecules through the secretory pathway. Sulfation on both carbohydrates and tyrosine residues is particularly useful for studying events in the later compartments of the secretory pathway—i.e., those after the *trans*-Golgi—and to study secretion. A short pulse of sulfate (5 min or less) can be used to label the proteins or proteoglycans in the compartment where the sulfotransferase is resident. Incubation with excess sulfate starts the chase of the sulfated molecules out of the Golgi complex to their final destination. Thus, the major advantage of using sulfation to follow proteins through the cell is the ability to label the molecules late in the secretory pathway, providing a sharply defined cohort of labeled molecules as the starting point for subsequent analysis.

Sulfation of carbohydrate chains on proteins and proteoglycans in the lumen of the Golgi complex is catalyzed by a variety of different sulfotransferases, some of which are only known as activities, while others have been extensively characterized, cloned, and sequenced. A co-substrate for the sulfotransferases is 3'-phosphoadenosine-5'-phosphosulfate (PAPS), which is synthesized in the cytosol and translocated across the Golgi membrane (for a comprehensive explanation see Huttner and Baeuerle, 1988). Sulfation of carbohydrates on proteins can occur on the N- or O-glycan chains (see *UNIT 15.2*), while sulfation of proteoglycans occurs on the glycosaminoglycan moiety. It is possible to determine the nature of the sulfated N- or O-glycan by using a variety of endoglycosidases that cleave the N- or O-linked oligosaccharide chains, such as endoglycosidase H (endo H; see *UNIT 15.2*). There are several classes of sulfated proteoglycans, the most common being keratan sulfate, heparan sulfate, heparin, chondroitin sulfate, and dermatan sulfate. Determining whether a sulfated molecule is a proteoglycan is more complex, as there is a limited range of available endoglycosidases, heparinase being the most commonly used. For the best methods to approach the analysis of more complex sulfated molecules, see Linhardt (1994).

The analysis of sulfated tyrosine residues in the protein backbone is more tractable. Posttranslational modification by sulfation on tyrosine residues is catalyzed by the enzyme tyro-

sylprotein sulfotransferase (Beisswanger et al., 1998) and occurs by transfer of the activated donor PAPS to the tyrosine residue. Sulfation of tyrosine residues, although widespread, is highly selective. The consensus features of the substrate recognition sequence in the protein backbone have been defined (Huttner and Baeuerle, 1988), allowing one to predict whether the protein of interest may be sulfated. If it is likely that the protein is sulfated on tyrosine residues, this can be confirmed by first demonstrating that the sulfated protein is resistant to endoglycosidases, and then chemically treating the protein with Ba(OH)₂, which allows the identification of Tyr-SO₄ by thin-layer chromatography (Huttner, 1984). A recent paper elegantly demonstrated just this approach (Farzan et al., 1999). This paper also demonstrated the usefulness of the drug chlorate, which inhibits the first enzyme (ATP-sulfurylase) in the biosynthesis of PAPS, and thereby inhibits the sulfation of both proteins and proteoglycans (Baeuerle and Huttner, 1986).

An interesting application of these approaches is to use the tyrosine sulfation consensus sequence to insert a sulfation site as a method of tagging secretory proteins to follow their movement out of the Golgi complex. This is particularly useful if the protein is not posttranslationally modified by N- or O-linked glycosylation. An example of this approach was pioneered by Niehrs et al. (1992).

Critical Parameters and Troubleshooting

The most important consideration is optimization of sulfate incorporation into the molecule to be analyzed. The efficiency of sulfation is the most variable parameter and relies on maintaining the cells in a medium that is compatible with continued protein synthesis and with sulfate labeling conditions. During the incubation with [³⁵S]sulfate, a series of reactions in the cytosol converts the [³⁵S]sulfate into [³⁵S]PAPS. Thus, it is important to reduce the concentration of endogenous PAPS to increase the specific activity of the radiolabeled PAPS. In some cell types this can be done easily by preincubation with sulfate-free medium, and is enhanced by reducing the methionine and cysteine (Yanigashita et al., 1989).

If the suggested medium (DMEM) is not compatible with the cell types being used, it is possible to substitute other media prepared without sulfate, or even to substitute DPBS for

the labeling medium. However, if the suggested labeling medium is not suitable, pilot experiments should be done on total extracts to test the efficiency of [³⁵S]sulfate incorporation. It has been observed that some media (such as RPMI) do not allow efficient sulfate incorporation. Suitable media can be easily tested by incubating the cells for long-term labeling (see Basic Protocol 1) in the test medium, then measuring the incorporation of ³⁵S into proteins. One simple method to do this is to precipitate the proteins with trichloroacetic acid and then count the insoluble precipitate (see UNIT 7.1). The goal is to optimize the ³⁵S incorporation per microgram of protein.

Anticipated Results

The result of labeling with [³⁵S]sulfate should be a protein or proteoglycan that is radioactively labeled to allow easy detection.

Time Considerations

Four elements affect the time required to complete this type of experiment. These include the length of time required to (1) seed and culture cells to 70% to 80% confluence, (2) label overnight and prepare the sample (2 days total), (3) immunoprecipitate, and (4) expose the radioactive sample for detection. Parameters 1 through 3 should be known by the investigator before starting the experiment. Parameter 4 will depend on the specific activity of the [³⁵S]PAPS in the cell and the resulting incorporation of sulfate. Low levels of incorporation may result in exposure times exceeding a week.

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Metabolic Labeling with Fatty Acids

UNIT 7.4

Protein acylation is the covalent attachment of fatty acids to a protein; the most commonly added fatty acids are myristate (14:0) and palmitate (16:0). First, radiolabeled fatty acids are used to label eukaryotic cells (see Basic Protocol 1). The radiolabeled material produced can then be analyzed by various methods: the type of fatty acid linkage can be determined (see Basic Protocol 2), the nature of the protein-bound label can be determined to check for interconversion (see Basic Protocol 3), and the protein-bound fatty acid can be identified (see Basic Protocol 4).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1D*).

BIOSYNTHETIC LABELING WITH FATTY ACIDS

To identify proteins that are modified with fatty acid groups, cultured cells are incubated first in medium containing sodium pyruvate, which acts as a source of acetyl-CoA and minimizes interconversion of the fatty acid to other metabolites, and then with [³H]fatty acids. Fatty acids tritiated at positions 9 and 10 provide the best combination of high specific activity and detectability for in vitro labeling, and because the tritium label is far removed from the carboxyl end where β-oxidation occurs, reincorporation of label is minimized.

Materials

Cells for culture

Complete tissue culture medium appropriate for cells

Labeling medium: complete tissue culture medium containing the relevant dialyzed serum and 5 mM sodium pyruvate, 37°C

5 to 10 μCi/μl [9,10(*n*)-³H]fatty acid, e.g., [9,10(*n*)-³H]palmitic acid or [9,10(*n*)-³H]myristic acid (30 to 60 Ci/mmol; Amersham International, American Radiolabeled Chemicals, or NEN Research Products) in ethanol PBS, pH 7.2 (*APPENDIX 2A*), ice-cold

1% (w/v) SDS or SDS sample buffer (for SDS-PAGE, when using adherent or nonadherent cells respectively; *UNIT 6.1*) or RIPA lysis buffer (for immunoprecipitation; see recipe)

5× SDS sample buffer (see recipe)

Cell scrapers

Nitrogen gas

Additional reagents and equipment for culturing cells (*UNIT 1.1*), immunoprecipitation (*UNIT 7.2*), SDS-PAGE (*UNIT 6.1*), treating a gel with sodium salicylate (*UNIT 6.3*), and fluorography (*UNIT 6.3*)

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

**BASIC
PROTOCOL 1**

**Protein Labeling
and Immuno-
precipitation**

7.4.1

Contributed by Caroline S. Jackson and Anthony I. Magee

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Supplement 5

1. On the day before the labeling experiment, split the cells into fresh complete tissue culture medium.

Set up the cells at two split ratios; then choose the culture closest to 70% to 80% confluency for labeling.

2. The next day, replace the medium with a minimum volume of 37°C labeling medium. Incubate 1 hr.

Cells in suspension should be used at a cell density of 10^6 to 10^7 cells/ml. For adherent cells that are 70% to 80% confluent, the minimum amount of medium necessary to cover the dish—e.g., 1.5 ml for 60-mm dishes and 3 ml for 100-mm dishes—should be used.

3. Add 5 to 10 $\mu\text{Ci}/\mu\text{l}$ [9,10(*n*)- ^3H]fatty acid to a concentration of 50 to 500 $\mu\text{Ci}/\text{ml}$. Incubate up to 24 hr.

Cells vary in the rate and extent of incorporation (see Critical Parameters), so both the amount of label and the duration of incubation need to be optimized. Labeling cells overnight in the presence of 200 $\mu\text{Ci}/\text{ml}$ [^3H]fatty acid will maximize the chances of detecting labeled proteins. The amount of label and/or time of incubation can then be reduced if good incorporation of label is achieved, or increased if poor incorporation is attained.

Short labeling times (e.g., pulses on the order of minutes up to 2 hr) require amounts of label at the higher end of the indicated range. In this case, uptake is relatively low and the medium plus label can be reused one or more times. The level of label in the medium can be monitored by scintillation counting. For longer incubations the interconversion of fatty acids becomes a greater problem, and the protein-bound fatty acid label should be analyzed (see Basic Protocols 3 and 4).

If the [^3H]fatty acid is not supplied in ethanol or if the concentration is too low, remove the solvent by blowing nitrogen over the solution in its original container until dry and redissolve the label in ethanol at a concentration of 5 to 10 $\mu\text{Ci}/\mu\text{l}$. Do not transfer into another container or evaporate the solvent in a plastic container, as this will cause a significant loss of label that will adhere to the side of the container.

For adherent cells:

- 4a. Place the dish on ice and aspirate the medium. Wash the cells twice with ice-cold PBS and lyse the cells by adding 1% SDS for SDS-PAGE (UNIT 6.1) or RIPA lysis buffer for immunoprecipitation (UNIT 7.2), using 100 μl of 1% SDS for a 60-mm dish or 300 μl for a 100-mm dish, or 1 ml RIPA lysis buffer.

CAUTION: Radioactive medium and washes must be disposed of appropriately.

- 5a. Using a cell scraper, remove the lysed cells from the dish and transfer them to a 1.5-ml microcentrifuge tube. Add 20 μl lysate to 5 μl of 5 \times SDS-PAGE sample buffer. Use all of lysate for immunoprecipitation. Resuspend immunoprecipitate in 20 μl SDS sample buffer.

For SDS-PAGE, use DTT at a final concentration ≤ 20 mM, and do not boil the samples, but incubate them only 3 min at 80°C; this is necessary because the thioester linkage of the fatty acid is susceptible to cleavage.

For nonadherent cells:

- 4b. Microcentrifuge the cell suspension 1 min at 6000 rpm, 4°C, to pellet the cells. Decant the supernatant and wash the cell pellet once by resuspending it in 1 ml ice-cold PBS and centrifuging again.
- 5b. Lyse the cells by resuspending the cell pellet in 100 μl SDS-PAGE sample buffer for discontinuous SDS-PAGE (UNIT 6.1) or 1 ml RIPA lysis buffer for immunoprecipitation (UNIT 7.2) for 10^6 to 10^7 cells. Resuspend immunoprecipitate in 20 μl SDS sample buffer.

CAUTION: *Radioactive medium and washes must be disposed of appropriately.*

For analysis of total protein-bound fatty acid label, lyse the cells in 100 μ l 1% SDS.

For SDS-PAGE, use DTT at a final concentration ≤ 20 mM, and do not boil the samples, but incubate them only 3 min at 80°C; this is necessary because the thioester linkage of the fatty acid is susceptible to cleavage.

6. Analyze whole-cell lysate or immunoprecipitate on an SDS-PAGE minigel, using 20 μ l lysate per lane. Store remaining lysate at -20°C .
7. Treat the gel with sodium salicylate (UNIT 6.3). Using preflashed film, fluorograph the gel (UNIT 6.3) at -70°C .

Typical exposure times are overnight to 1 month.

Alternatively, the gel may be treated with DMSO/2,5-diphenyloxazole (DMSO/PPO) instead of sodium salicylate (see Magee et al., 1995).

ANALYSIS OF FATTY ACID LINKAGE TO PROTEIN

To determine the type of linkage by which the [^3H]fatty acid is attached to the protein (i.e., thioester, oxyester, or amide linkage), the fatty acid is selectively cleaved from the protein. The most convenient method is to run replicate lanes on an SDS-PAGE gel, cut the lanes apart, and analyze each lane separately. Thioesters and amides are by far the most common types of linkage; oxyesters are extremely rare.

Materials

Lysate or immunoprecipitate from [^3H]fatty acid-labeled cells (see Basic Protocol 1, step 6)

0.2 M potassium hydroxide (KOH) in methanol

Methanol

1 M hydroxylamine-HCl, titrated to pH 7.5 with NaOH

1 M Tris-Cl, pH 7.5 (APPENDIX 2A)

Additional reagents and equipment for SDS-PAGE (UNIT 6.1), treating a gel with sodium salicylate (UNIT 6.3), and fluorography (UNIT 7.2)

1. Run an SDS-PAGE gel (UNIT 6.1) using 20 μ l lysate or immunoprecipitate from [^3H]fatty acid-labeled cells in each of four lanes.
2. Cut the four lanes apart and transfer each lane to a 15-ml tube containing one of the following solutions:

0.2 M KOH in methanol

Methanol

1 M hydroxylamine-HCl

1 M Tris-Cl, pH 7.5.

Incubate 1 hr at room temperature with shaking.

The 0.2 M KOH in methanol will cleave thio- and oxyesters, but not amides; 1 M hydroxylamine-HCl will rapidly cleave thioesters but will cleave oxyesters only poorly, and will not cleave amides. Methanol and 1 M Tris-Cl serve as controls.

3. Wash each gel strip three times, 5 min each time, with water. Treat the strips with sodium salicylate and fluorograph using preflashed film at -70°C (UNIT 6.3).

Typical exposure times are overnight to 1 month.

BASIC PROTOCOL 2

Protein Labeling and Immuno- precipitation

7.4.3

Alternatively, the gel may be treated with DMSO/PPO instead of sodium salicylate (see Magee et al., 1995).

Cleavage is measured as a reduction in the fluorographic signal compared to those for controls, and can be quantitated by densitometric scanning of the lane or scintillation counting of excised bands. Bands with fatty acids linked to the protein by thioesters will be missing or greatly reduced in lanes treated with 0.2 M KOH in methanol and 1 M hydroxylamine-HCl; oxysters will be greatly reduced or missing in the lane treated with 0.2 M KOH in methanol and may be slightly reduced in the lane treated with 1 M hydroxylamine-HCl; and amide linkages will not be affected by any of these treatments, so that proteins with amide-linked fatty acids will appear in all four lanes.

BASIC PROTOCOL 3

ANALYSIS OF TOTAL PROTEIN-BOUND FATTY ACID LABEL IN CELL EXTRACT

Due to problems of interconversion of fatty acids by β -oxidation and chain elongation and of reincorporation of label into other metabolic precursors, the protein-bound label derived from [^3H]fatty acids should be analyzed, especially for experiments with long labeling incubations. This protocol is used to determine how much of the label has been converted into other fatty acids or metabolites during the incubation; a different procedure must be used to determine whether the fatty acid on the protein of interest is different from that added during labeling (see Basic Protocol 4).

Materials

0.1 M HCl/acetone, -20°C
Lysate from [^3H]fatty acid-labeled cells in 1% SDS (see Basic Protocol 1, step 4a or 5b)
1% (w/v) SDS
2:1 (v/v) chloroform/methanol
Diethyl ether
6 M HCl (concentrated HCl diluted 1:1 with H_2O)
Hexane
5 to 10 $\mu\text{Ci}/\mu\text{l}$ [$9,10(n)-^3\text{H}$]fatty acid standards (30 to 60 Ci/mmol; Amersham International, American Radiolabeled Chemicals, or NEN Research Products) in ethanol
90:10 (v/v) acetonitrile/acetic acid
EN 3 HANCE spray (NEN Research Products)
15-ml polypropylene centrifuge tubes
Mistral 3000i benchtop centrifuge with swing-out four-bucket rotor or equivalent
Nitrogen gas
30-ml thick-walled Teflon container with an air-tight screw top
110 $^\circ\text{C}$ oven
Thin-layer chromatography tank
RP18 thin-layer chromatography plate (e.g., Merck)
Kodak XAR-5 film, preflashed

Precipitate protein

1. Add 5 vol of 0.1 M HCl/acetone to 100 μl lysate from [^3H]fatty acid-labeled cells in 1% SDS in a 15-ml polypropylene tube. Incubate ≥ 1 hr at -20°C .

This will precipitate the protein.

2. Centrifuge 10 min at $1500 \times g$ (1000 rpm in Mistral 3000i swing-out rotor), 4°C , to pellet the precipitate. Remove the supernatant and allow the pellet to air dry gently.

Remove free label

3. Dissolve the pellet in a minimum volume of 1% SDS and transfer to a 1.5-ml microcentrifuge tube. Add 5 vol of 0.1 M HCl/acetone. Incubate ≥ 1 hr at -20°C .
4. Repeat steps 2 and 3.

These precipitation steps concentrate the protein and remove much of the SDS and free label.

5. Add 500 μl of 2:1 chloroform/methanol and vortex. Centrifuge 10 min at 1000 rpm, 4°C , and remove the supernatant. Repeat this step at least three times until no more free label is extracted into the organic solvent, as determined by scintillation counting of the supernatant.
6. Add 100 μl diethyl ether to the pellet and vortex. Centrifuge 10 min at 1000 rpm, 4°C , and decant the supernatant. Dry the pellet by placing the microcentrifuge tube under a gentle stream of nitrogen.
7. Place the tube into a 30-ml thick-walled Teflon container with an air-tight screw top containing 1 ml of 6 M HCl. Flush the tube and container with nitrogen. Close the lid tightly and incubate in an oven 16 hr at 110°C .

This hydrolyzes the fatty acids from the protein.

Extract hydrolyzed fatty acids

8. Extract the contents of the tube twice with 0.5 ml hexane and pool the extracts. Dissolve the residue in 0.5 ml of 1% SDS. Determine the radioactivity in the hexane extracts and in the residue.

Fatty acids will be extracted into hexane, while label incorporated into sugars and amino acids will be mainly in the hexane-insoluble residue.

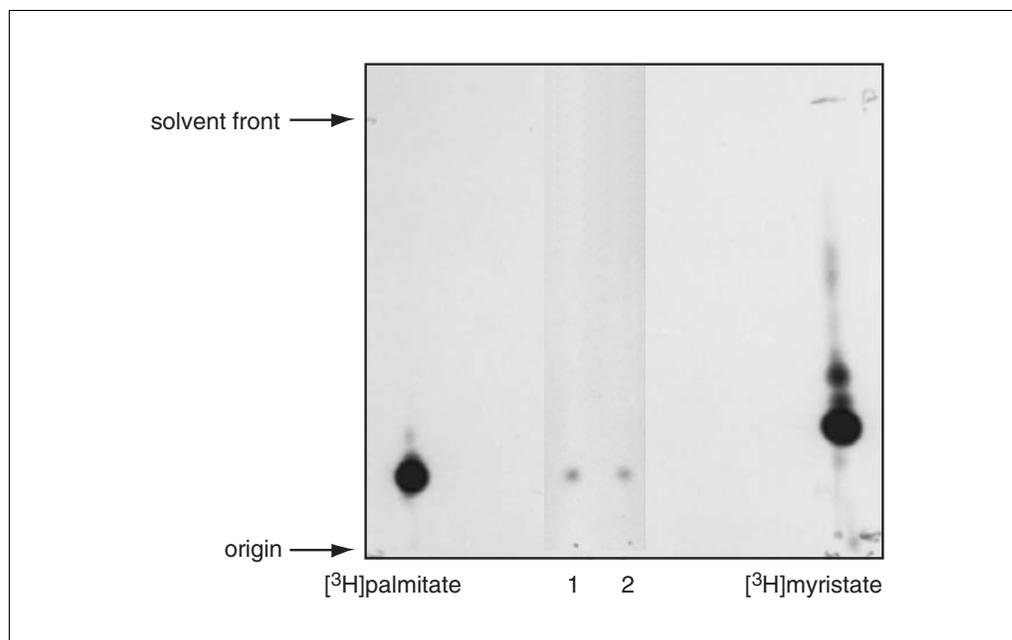


Figure 7.4.1 Fluorogram of thin-layer chromatography plate showing analysis of acylated nerve growth factor (NGF) receptor. Outside lanes, migration of 0.5 μCi $[^3\text{H}]$ palmitate and $[^3\text{H}]$ myristate standards. Lane 1, NGF receptor immunoprecipitated from cells labeled with $[^3\text{H}]$ palmitic acid. Lane 2, NGF receptor immunoprecipitated from cells labeled with $[^3\text{H}]$ myristic acid. Although the cells were labeled with different fatty acids, the protein was labeled with palmitic acid due to chain elongation of $[^3\text{H}]$ myristic acid to $[^3\text{H}]$ palmitic acid by the cells. Exposure for standards, 1 week; exposure for lanes 1 and 2, 1 month.

9. Evaporate the hexane extracts just to dryness with a gentle stream of nitrogen. Dissolve in 2 to 5 μl of 2:1 chloroform/methanol.

It is important not to overdry the sample because it may then be difficult to dissolve.

Identify fatty acids

10. Preequilibrate a thin-layer chromatography tank with 90:10 acetonitrile/acetic acid for 15 min.
11. Spot resuspended hexane extract onto an RP18 thin-layer chromatography plate. Dilute 1 μl [9,10(*n*)- ^3H]fatty acid standards in ethanol to give 1 $\mu\text{Ci}/\mu\text{l}$ and spot 0.5 μl in parallel lanes. Develop the plate in 90:10 acetonitrile/acetic acid. Air dry the plate.
12. Detect the radioactivity by spraying the plate with EN 3 HANCE spray and exposing it to preflashed Kodak XAR-5 film overnight at -70°C . Identify the fatty acids.

See Figure 7.4.1 for an example of a typical fluorogram.

BASIC PROTOCOL 4

ANALYSIS OF FATTY ACID LABEL IDENTITY

This protocol is used to identify the labeled fatty acid(s) associated with a specific protein band on an SDS-PAGE gel. Following electrophoresis, the band of interest is located either by comparison with molecular weight standards or more accurately by fluorography of a sodium salicylate-treated gel (see UNIT 6.3). DMSO/PPO-treated gels cannot be used. The labeled material is analyzed by thin-layer chromatography.

Materials

SDS-PAGE gel of lysate from [^3H]fatty acid-labeled cells
Additional reagents and equipment for analysis of protein-bound label (see Basic Protocol 3)

1. Excise the band(s) of interest from a wet or dried (fluorographed) SDS-PAGE gel. Wash three times with shaking, 5 min each, with 0.5 ml water.

If the gel is fluorographed it should be treated with sodium salicylate (UNIT 6.3), not DMSO/PPO solution. The dried gel piece will rehydrate and the salicylate will be washed out during the washes.

2. Place the gel piece in a 1.5-ml microcentrifuge tube and lyophilize.
3. Hydrolyze the fatty acids in the band and identify them by thin-layer chromatography (see Basic Protocol 3, steps 7 to 12).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

RIPA (RadioImmunoPrecipitation Assay) lysis buffer

1% (w/w) Nonidet P-40 (NP-40)
1% (w/v) sodium deoxycholate
0.1% (w/v) SDS
0.15 M NaCl
0.01 M sodium phosphate, pH 7.2 (APPENDIX 2A)
2 mM EDTA (APPENDIX 2A)
10 mM sodium fluoride

0.2 mM sodium vanadate added fresh from 0.2 M stock solution

100 U/ml aprotinin (Trasylol, Pentex/Miles)

Store buffer without vanadate at 4°C up to 1 year

Sodium vanadate stock solution can be stored in plastic at room temperature.

SDS sample buffer (for discontinuous systems), 5×

3.125 ml 1 M Tris·Cl, pH 6.8 (0.313 M final)

1 g SDS (10% final)

5 mg bromphenol blue (0.05% final)

5 ml glycerol (50% final)

H₂O to 10 ml

Store at room temperature

Add DTT to appropriate concentration (see Basic Protocol 1, annotations under steps 5a and 5b) just before use

Warm the solution before use because it tends to solidify.

COMMENTARY

Background Information

The two most common acyl groups that modify proteins are 14-carbon myristic acid and 16-carbon palmitic acid (Fig. 7.4.2), and they occur both on different and on overlapping sets of proteins. By increasing the hydrophobicity of the protein, these fatty acid moieties can play a role in localization of the protein to the membrane and sometimes to specific types of membrane structures—e.g., glycolipid-enriched domains (lipid rafts) or caveolae (Rodgers et al., 1994). Identifying the type of acylation of a protein and determining whether the level of modification can be affected by stimuli can provide more information on the mechanisms of action of proteins involved in signaling pathways.

Fatty acids are used in labeling cells *in vitro* because they will diffuse across the plasma membrane and then be converted to acyl-CoA by the action of the enzyme acyl-CoA synthetase. This activated form of the fatty acid is

the substrate for protein-acyl transferase activities that transfer the acyl group to the protein. Tritiated fatty acids are most commonly used in biosynthetic labeling of proteins, but fluorescent analogs of fatty acids have been used to study palmitoylation of rhodopsin (Moench et al., 1994a,b), and [ω -¹²⁵I]iodo-fatty acids have been used to study myristoylation of v-src (Peseckis et al., 1993).

A wide variety of proteins are myristoylated, including viral structural proteins and many proteins involved in cell signaling, such as the α subunits of trimeric G proteins and the Src family of tyrosine kinases (Resh, 1994; Wedegaertner et al., 1995). Myristoylation most commonly occurs cotranslationally via an amide linkage to an NH₂-terminal glycine residue, and is an irreversible modification. Myristoylation is dependent on the removal of the initiator methionine and has been shown to occur by the time the nascent polypeptide is 100 amino acids long. Inhibitors of protein synthesis will

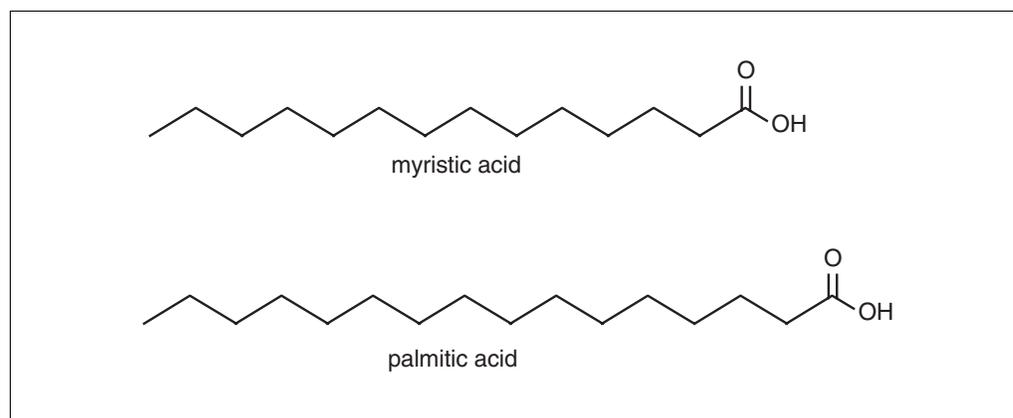


Figure 7.4.2 Structures of myristic and palmitic acids.

therefore block myristoylation. The enzyme responsible for NH₂-terminal myristoylation, *N*-myristoyl transferase (NMT), was first isolated from *Saccharomyces cerevisiae*; both the yeast and human homologs have been well characterized and show different protein substrate specificities (Gordon et al., 1991). Substrate specificity of yeast NMT is determined by recognition of a sterically unhindered NH₂-terminal glycine followed by an amino acid sequence that conforms to the following criteria: an uncharged residue or proline at position 2; any amino acid at positions 3 and 4; serine (more common), alanine, or threonine at position 5; no proline at position 6; and, preferably, basic residues at positions 7 and 8. Residues C-terminal to this region appear to be important in recognition of the α subunits of trimeric G proteins and the Src family of tyrosine kinases (Glover et al., 1988; Gordon et al., 1991).

The NMT enzymatic reaction proceeds by formation of a myristoyl-CoA-enzyme complex, subsequent binding of the peptide, transfer of the myristate moiety to the peptide, release of CoA, and release of the myristoylated peptide (Rudnick et al., 1991). Several assays have been developed for this enzyme (e.g., King and Sharma, 1991; Rudnick et al., 1992; French et al., 1994). Inhibitors of myristoylation include 2-hydroxymyristic acid, which is converted in vivo to 2-hydroxymyristoyl-CoA, a potent inhibitor of NMT. Other inhibitors of acylation are fatty acid analogs and other compounds that inhibit acyl CoA synthetase and therefore block the conversion of fatty acids to acyl CoA; the latter include α -bromopalmitate (DeGrella and Light, 1980) and the triacsin family of antibiotics (Tomoda et al., 1987). The endogenous pool of fatty acid can be depleted by incubation with cerulenin, an antibiotic that inhibits de novo fatty acid biosynthesis.

Myristoylation of a protein can be necessary for its activity, e.g., the transforming activity of Src (Kamps et al., 1986). Myristoylation alone may not be sufficient for a protein to be localized to the membrane; for this, further lipid modification, such as palmitoylation or cooperative interaction with protein sequences, is required (Resh, 1994).

Palmitoylation more accurately referred to as *S*-acylation, (with and without myristoylation) occurs on many signaling molecules, including rhodopsin, α -subunits of G proteins, and Src-family tyrosine kinases. *S*-acylation is a post-translational event occurring via a thioester linkage to a cysteine residue, and where it occurs with NH₂-terminal myristoyla-

tion, the myristoylation is usually a prerequisite for *S*-acylation. This may be because the enzyme responsible for *S*-acylation recognizes the myristoylated protein or, more likely, because myristoylation brings the protein to the correct cell location for *S*-acylation to occur. *S*-acylation is also found in conjunction with isoprenoid modification at the C-terminus of proteins belonging to the Ras superfamily; it is responsible for the localization of these proteins to the membrane (Newman and Magee, 1993). This *S*-acylation is dependent on prior modification of the protein by isoprenoid. G protein subunits such as the α_1 subfamily and members of the Src family of tyrosine kinases have an NH₂-terminal amino acid sequence of Met-Gly-Cys, where the initiator methionine is removed and replaced by myristate and the cysteine is *S*-acylated (Resh, 1994). *S*-acylation is a reversible modification and has been shown to be dynamic in vivo, with the level of *S*-acylation changing in response to various stimuli such as receptor activation, insulin, and growth factors (James and Olsen, 1989; Jochen et al., 1991; Wedegaertner et al., 1995). This phenomenon is thought to play a role in switching on or off signaling molecules by altering either the localization of the molecules or their presentation to other signaling molecules with which they interact. Deacylation of proteins is catalyzed by the activity of protein-*S*-acyl thioesterase (Camp and Hoffman, 1993). Protein-*S*-acyltransferase activities have been reported by several groups, but attempts to purify these to homogeneity have so far been unsuccessful. In fact, the possibility that *S*-acylation is nonenzymatic has recently been raised (Dunphy and Linder, 1998).

Analysis of the type of fatty acid linkage present should always be performed. Post-translational myristoylation via a thioester linkage has been found in platelets (Muszbek and Laposata, 1993). The term "palmitoylation" is not strictly accurate because other long-chain fatty acids, such as stearate (18:0) and oleate (18:1), can also be thioesterified to proteins; "*S*-acylation" is now becoming more commonly used to describe this modification. The acylating activity seems to be relatively unspecific for chain length and degree of unsaturation, and utilizes acyl-CoAs partly in proportion to their abundance in the cell—hence the predominance of palmitate. This and the potential for interconversion of labeled fatty acids necessitate analysis of the chain length of the attached label. Other methods can be used to identify the type of fatty acid attached to the

protein, depending on the availability of appropriate equipment; these include gas chromatography, reversed-phase HPLC, and mass spectroscopy (Aitken, 1992). Structural and sequence analysis of the acylated protein can be performed using mass spectrometry (Aitken, 1992).

Further information about the cellular localization of acylated proteins can be found by extraction of the cell lysate in the presence of either Triton X-114 or X-100. Extraction with Triton X-114, which has the property of phase separation at 30°C, can distinguish between hydrophobic and hydrophilic proteins (Aitken, 1992). Extraction of membranes with Triton X-100 at 4° and 37°C can identify proteins that are associated with glycolipid cholesterol-enriched lipid rafts (Rodgers et al., 1994).

Critical Parameters and Troubleshooting

Cells should be subconfluent; it is recommended that the cells be plated at two split ratios so the culture closest to 70% to 80% confluency can be selected to be used for labeling.

The pH of all solutions should be ≤ 7.5 in order to avoid hydrolysis of labile thioesters; the high pH of SDS-PAGE buffers does not seem to be a problem when using minigels, where the running times are relatively short. Dithiothreitol (DTT) or 2-mercaptoethanol should be used with care, as these can also cleave thioester bonds; for SDS-PAGE a maximum concentration of 20 mM DTT should be used and samples should not be boiled but incubated only 3 min at 80°C.

The use of short labeling times (especially for palmitate labeling) will reduce reincorporation of the label.

The ability to detect myristoylation and/or *S*-acylation of a protein using these methods will depend on the ability of the cells to take up radiolabeled compounds and incorporate them into metabolic precursors; the pool sizes of endogenous fatty acids and fatty acyl-CoA esters; the expression level of the protein-myristoyl transferase and supposed protein-*S*-acyl transferase; the abundance, rate of synthesis, and turnover of the protein(s) and modification(s) of interest; and the efficiency of antibodies for immunoprecipitation.

Anticipated Results

Typically, it is possible to detect myristoylated proteins using fluorographic exposure times ranging from <1 week for high-level expression of protein in transformed cells (e.g.,

Lck in LSTRA cells) to 1 to 4 weeks for a well-expressed endogenous protein.

Time Considerations

In vitro labeling experiments require 2 to 3 days for growing and labeling the cells. Harvesting the cells, preparing the lysate for SDS-PAGE (with or without prior immunoprecipitation) and SDS-PAGE require 1 to 2 days plus time for fluorography.

Linkage analysis of the labeled proteins takes 1 to 2 hr after the gel has been run. Analysis of the label in cell lysates requires 1 day to precipitate the protein, remove free label, and hydrolyze the sample. A second day is required to extract the label and perform thin-layer chromatography, and fluorography requires an overnight exposure. Analysis of the protein-bound label in gel bands takes ~3 days after fluorography (dried gel) and a similar amount of time from a wet gel, except the analysis begins after the gel has been run.

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A compilation of methods used in studying lipid modification of proteins.

Contributed by Caroline S. Jackson and
Anthony I. Magee
National Institute for Medical Research
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Metabolic Labeling of Prenyl and Carboxyl-Methyl Groups

UNIT 7.5

In the last fifteen years or so, several post-translational modifications of proteins with lipid moieties have been discovered. Analysis of fatty acylation is covered in *UNIT 7.4*. This unit describes methods for analysis of prenylation and the carboxyl-methylation that often accompanies it. The two prenyl groups that have been found attached to proteins—farnesyl (C15) and geranylgeranyl (C20)—are both derived from intermediates in the isoprenoid biosynthetic pathway that utilizes mevalonic acid. In the protocols described here, radiolabeled mevalonate is used to label these intermediates in intact cells (see Basic Protocol 1); the labeled intermediates then become incorporated into proteins. Carboxyl-methylation of C-terminal prenylated cysteine residues often occurs subsequent to prenylation. Methods are given for radiolabeling of the methyl group with [³H-methyl]-methionine, that is converted intracellularly into *S*-adenosylmethionine (see Basic Protocol 2). Three recent publications (Casey, 1990; Hooper and Turner, 1992; Casey and Buss, 1995) contain a number of methods related to analysis of lipid modification of proteins.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1D*).

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

PRENYLATION OF PROTEINS IN CULTURED CELLS

To radiolabel intermediates in the isoprenoid biosynthetic pathway, cultured cells are incubated with [³H]mevalonic acid, which is the 6-carbon precursor to isopentenyl diphosphate. Isopentenyl diphosphate (5-carbon) is polymerized in a stepwise fashion to yield farnesyl (15-carbon) and geranylgeranyl (20-carbon) diphosphates, which are the known precursors of protein-bound prenyl groups. To maximize incorporation of exogenous [³H]mevalonate, the endogenous pool of unlabeled mevalonate is usually depleted by preincubation with an inhibitor of hydroxymethyl glutaryl-coenzyme A (HMG-CoA) reductase, the enzyme responsible for production of mevalonate from hydroxymethyl glutarate. Medium containing dialyzed serum is also used to reduce the endogenous pool.

Materials

Cells

Complete tissue culture medium appropriate for the cells, 37°C

Complete tissue culture medium containing dialyzed serum, 37°C

10 mM mevinolin (see recipe)

1 μCi/μl *R*-[5-³H]mevalonic acid (10 to 40 Ci/mmol; DuPont NEN or American Radiolabeled Chemicals)

PBS, pH 7.2 (*APPENDIX 2A*) ice-cold

1× SDS-PAGE sample buffer (*UNIT 6.1*) or RIPA lysis buffer for immunoprecipitation (*UNIT 7.4*)

Nitrogen gas

**BASIC
PROTOCOL 1**

**Protein Labeling
and Immuno-
precipitation**

Contributed by Anthony I. Magee

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7.5.1

Supplement 5

Additional reagents and equipment for immunoprecipitation (UNIT 7.2), SDS-PAGE (UNIT 6.1), treating a gel with sodium salicylate (UNIT 6.3), and fluorography (UNIT 6.3)

1. On the day before the labeling experiment, split the cells into complete tissue culture medium.

Set up cells at two split ratios and choose the culture closest to 70% to 80% confluency for labeling. The cultures should contain 10^6 to 10^7 cells.

2. On the day of the experiment, replace the culture medium with a minimum volume of prewarmed complete tissue culture medium containing dialyzed serum. Add 10 mM mevinolin to a final concentration of 50 μ M. Preincubate the cells in this medium ≥ 60 min.

Mevinolin is an inhibitor of HMG-CoA reductase. Alternative HMG-CoA reductase inhibitors are compactin/mevastatin (Sigma) and pravastatin (Pravachol, Bristol-Meyers Squibb).

3. Transfer enough 1 μ Ci/ μ l R-[5- 3 H]mevalonic acid to label all samples at a concentration of 50 to 200 μ Ci/ml into a microcentrifuge tube. If necessary, remove organic solvent by exposing the tube to a gentle stream of nitrogen, but do not dry the mevalonic acid completely.

The volume of the [3 H]mevalonic acid can be made up with sterile water to facilitate accurate pipetting.

Alternatively, the organic solvent may be removed in a Speedvac evaporator.

The mevalonic acid must not be heated above room temperature because it is unstable.

4. Add the partially dried [3 H]mevalonic acid at the desired concentration to the culture medium. Incubate up to 24 hr.

The most appropriate concentration of label and duration of incubation should be experimentally determined for each cell type. Mevalonic acid and mevinolin tend to cyclize to form the acetone (see Troubleshooting). If this is suspected, the lactone can be hydrolyzed (see recipe for mevalonic acid in Reagents and Solutions).

5. Place the cells on ice and aspirate the medium. Wash the cells twice with ice-cold PBS.

CAUTION: Radioactive medium and washes must be disposed of appropriately.

6. Lyse cells in the appropriate solution for further processing—e.g., for a 50-mm dish containing 4×10^6 cells, add 0.2 ml of 1 \times SDS-PAGE sample buffer (UNIT 6.1) or 1 ml RIPA lysis buffer for immunoprecipitation (UNIT 7.2).

Use the entire lysate for immunoprecipitation.

7. Analyze the lysate or immunoprecipitate by SDS-PAGE (UNIT 6.1).

Use 25 μ l of the whole cell lysate for a minigel or 50 μ l for a standard gel; load all of the immunoprecipitate after solubilizing it in 25 or 50 μ l of 1 \times SDS-PAGE sample buffer.

8. Treat the gel with sodium salicylate and fluorograph the gels at -70°C using preflashed film (UNIT 6.3).

Typical exposure times are 1 week to 1 month, although exposures up to 3 months may sometimes be required (see Time Considerations).

Alternatively, the gel may be treated with DMSO/2,5-diphenyloxazole (DMSO/PPO) instead of sodium salicylate (see Magee et al., 1995), or with other commercially available water-soluble fluors such as Enlightening (DuPont NEN) or Amplify (Amersham).

CARBOXYL-METHYLATION OF PROTEINS IN CULTURED CELLS

Prenylation at C-terminal CXXX and CXC motifs is followed by carboxyl-methylation of the α -carboxyl group directly or after removal of XXX residues. This is performed by a membrane-associated methyl transferase that uses the universal methyl group donor *S*-adenosylmethionine (SAM). The methyl group of SAM can be radiolabeled in vitro by [³H-methyl]methionine, and it is then transferred onto the α -carboxyl group as an alkali-labile ester. This can be distinguished from alkali-stable methylations such as lysyl N-methylation by treating the sample with sodium hydroxide, which releases [³H]methanol. The released volatile [³H]methanol is collected by distillation into scintillation fluid and counted (Chelsky et al., 1985; Gutierrez et al., 1989). Labeled methionine will also be incorporated into the peptide backbone and is alkali-stable, thus providing an internal standard that can be used to calculate the stoichiometry of carboxyl-methylation. The length of the labeling incubation required to get an acceptable signal depends inversely on the expression level of the protein under study.

Materials

Cells

Complete tissue culture medium (CM) appropriate for the cells

Methionine-free tissue culture medium containing dialyzed serum (MFM)

95:5 (v/v) MFM/complete culture medium with dialyzed serum (95:5 MFM/CM)

1 μ Ci/ μ l L-[³H-methyl]methionine (~80 Ci/mmol, Amersham)

PBS, pH 7.2 (APPENDIX 2A), ice-cold

1 \times SDS-PAGE sample buffer (UNIT 6.1) or RIPA lysis buffer (UNIT 7.4)

1 M NaOH

1 M HCl

Warm room or incubator at 37°C

Pre flashed film (UNIT 6.3)

Additional reagents and equipment for immunoprecipitation (UNIT 7.2), treating a gel with sodium solicylate (UNIT 6.3), SDS-PAGE (UNIT 6.1), and fluorography (UNIT 6.3)

Label the cells

1. On the day before the labeling experiment, split the cells into fresh complete tissue culture medium.

Set up cells at two split ratios and choose the culture closest to 70% to 80% confluency for labeling. The cultures should contain 10^6 to 10^7 cells.

For labeling times ≤ 8 hr

- 2a. On the day of the experiment, replace the culture medium with the minimum volume MFM, e.g., 3 ml for a 100-mm tissue culture dish. Incubate 1 hr.
- 3a. Add 200 μ Ci [³H-methyl]methionine per milliliter MFM. Incubate up to 8 hr.

For labeling times of 8 to 24 hr

- 2b. On the day of the experiment, replace the culture medium with the minimum volume 95:5 MFM/CM, e.g., 3 ml for a 100-mm tissue culture dish. Incubate 1 hr.

- 3b. Add 50 μCi [^3H -methyl]methionine per milliliter 95:5 MFM/CM. Incubate 8 to 24 hr.
4. After labeling is complete, aspirate the medium and wash the cells twice with ice-cold PBS.

CAUTION: *Labeling and wash media are radioactive.*

5. Lyse in the appropriate solution for further processing—e.g., for a 50-mm dish containing 4×10^6 cells, add 0.2 ml 1 \times SDS-PAGE sample buffer (UNIT 6.1) or 1 ml RIPA lysis buffer for immunoprecipitation (UNIT 7.2).

Separate and fluorograph the samples

6. Analyze the immunoprecipitate or lysate by SDS-PAGE (UNIT 6.1).

Use 25 μl whole-cell lysate for a minigel or 50 μl for a standard gel. Use the whole immunoprecipitate solubilized in 25 or 50 μl of 1 \times SDS-PAGE sample buffer.

The α -carboxyl-methyl esters are stable at the moderately alkaline pH of the standard Laemmli gel system, whereas methyl esters of the side-chain carboxyls of aspartate and glutamate are quite labile.

7. Treat the gel 20 min with sodium salicylate (UNIT 6.3) and immediately dry gel at 60°C on a backing of filter paper.

The gel must not be dried at too high a temperature.

Alternatively, the gel may be treated with DMSO/PPO instead of sodium salicylate (see Magee et al., 1995) or with other commercially available water-soluble fluors, such as Enlightening (Dupont NEN) or Amplify (Amersham).

8. Using radioactive ink, mark the filter paper to note the alignment of the gel. Fluorograph the gel at -70°C , using preflashed film. Expose the film for 1 week to 1 month.

An inexpensive radioactive ink can be made by washing out old radiolabel vials with a small volume (e.g., 0.1 ml) of an aqueous solution containing 0.01% (w/v) bromphenol blue.

9. Using the alignment marks, align the film exactly with the gel. Excise the radioactive band(s) of interest with a scalpel.

Release the alkali-labile label

10. Place each gel piece into a separate 1.5-ml microcentrifuge tube. Carefully lower the open tube into a scintillation vial that contains enough scintillation fluid to come about half way up the side of the tube.

11. Add enough 1 M NaOH into the tube to cover the gel piece (usually 100 to 200 μl). Immediately cap the scintillation vial, leaving the tube open inside. Incubate overnight in a 37°C warm room or 37°C incubator.

During this incubation, radioactive methanol is hydrolytically released and distilled into the scintillation fluid.

12. The next day, uncap the vial and carefully remove the microcentrifuge tube, taking care not to spill any of the contents into the vial. Recap the vial.

This scintillation vial contains the alkali-labile [^3H]methanol.

Analyze the sample for alkali-stable label

13. Neutralize the NaOH in the microcentrifuge tube by adding an equal volume of 1 M HCl. Transfer the contents of the tube to a new scintillation vial.

This scintillation vial contains the alkali-stable [³H]label which represents [³H]methionine incorporated into the peptide backbone. However, alkali-stable methyl groups cannot be distinguished from backbone methionine in this way, so it is essential to have good reason to believe that other methyl groups are not present before alkali-stable counts can be used to calculate stoichiometry of carboxyl-methylation.

14. Add a volume of scintillation fluid to this vial equal to that used for the alkali-labile sample (step 12).
15. Count both samples in a scintillation counter using the tritium channel.

Calculate the methylation stoichiometry

16. Calculate the methylation stoichiometry as:

$$\text{stoichiometry} = \frac{\text{alkali-labile cpm} \times \text{number of methionine residues in protein}}{\text{alkali-stable cpm}}$$

This calculation can only be made if the methionine content of the protein is known. Also the possibility that the initiator methionine might be removed must be taken into account. This can be predicted from the primary sequence (Aitken, 1992).

REAGENTS AND SOLUTIONS

Use deionized or distilled in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Mevalonic acid, 100 mM

Hydrolyze mevalonic acid lactone (Sigma) to the free acid (Kita et al., 1980) by dissolving 30 mg mevalonic acid lactone in 1.8 ml ethanol at 55°C. Add 0.9 ml of 0.6 M NaOH and 18 ml water. Incubate 30 min at room temperature. Adjust to pH 8 with HCl and dilute to 100 mM. Store indefinitely at –20°C.

Mevinolin, 10 mM

Dissolve 90 mg mevinolin in 1.8 ml ethanol at 55°C. Add 0.9 ml of 0.6 M NaOH and 18 ml distilled water. Incubate 30 min at room temperature. Adjust to pH 8 with HCl and dilute to 10 mM. Store indefinitely at –20°C in 500- μ l aliquots.

Mevinolin, also called Lovastatin and Mevacore, is available from Merck or Biomol Research Labs (see SUPPLIERS APPENDIX; Alberts, 1988). It is supplied as the lactone and must be hydrolyzed to the free acid as described in the recipe for mevalonic acid above (Kita et al., 1980).

COMMENTARY

Background Information

Prenylation of proteins is a widespread post-translational modification that occurs at C-terminal motifs of the type CXXX, CXC, or CC (where C is cysteine and X is any amino acid). Recent reviews give a thorough description of this field (Clarke, 1992; Giannakouros et al., 1993). Either the 15-carbon isoprenoid farnesol or the 20-carbon isoprenoid geranylgeraniol (see Fig. 7.5.1) can be added, depending on the exact sequence at the C-terminus. CX₁X₂X₃ motifs where X₃ is a small residue (e.g., methionine, serine, or alanine), are farnesylated; whereas if X₃ is large and hydrophobic (e.g., leucine or phenylalanine), they are ger-

anylgeranylated. The nature of the X₁ and X₂ residues also influences the ability of the motif to become prenylated such that some apparent CXXX sequences are not modified. The CXC and CC motifs are also geranylgeranylated on both cysteines. The prenyl moiety is attached by a thioether linkage to the sulphhydryl group of cysteine; this is an extremely stable linkage that is irreversible in vivo, but it can be chemically cleaved in vitro to allow analysis of the chain length of the attached isoprenoid (Farnsworth et al., 1990).

Prenylation occurs on otherwise cytosolic soluble proteins and is catalyzed by a ubiquitous family of at least three soluble heterodi-

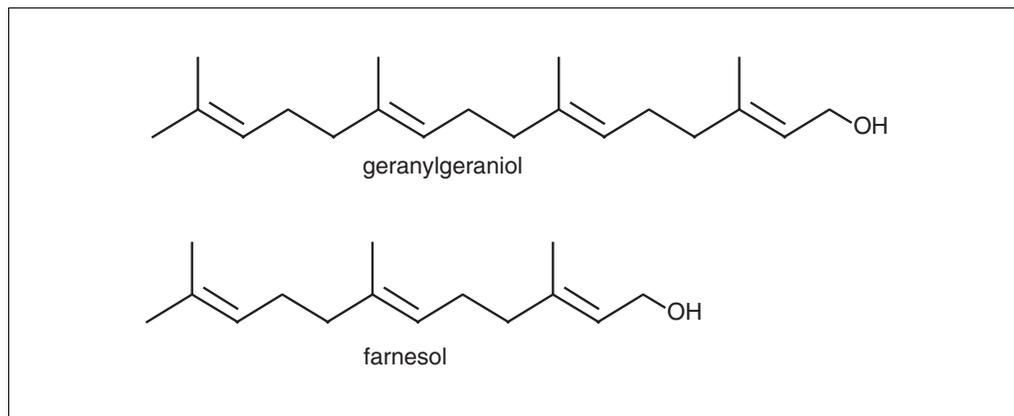


Figure 7.5.1 Structures of isoprenoid groups used in post-translational modification.

meric enzymes: farnesyl transferase, geranylgeranyl transferase I (active on CXXX motifs) and geranylgeranyl transferase II (active on CXC and CC motifs). In addition, geranylgeranyl transferase II has a third associated subunit called Rab escort protein that is required for activity. These enzymes utilize the activated diphosphates of farnesol and geranylgeraniol that are normal intermediates in the isoprenoid biosynthetic pathway, that leads to many important end products such as steroids, ubiquinone, and dolichols (Goldstein and Brown, 1990). Mevalonic acid is the precursor of these polyisoprenyl diphosphates and is readily taken up by most cells in culture. Hence radioactive mevalonate can be used to radiolabel prenyl chains biosynthetically. To maximize incorporation, endogenous mevalonate is usually depleted by preincubation of cells with an inhibitor of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme responsible for mevalonate synthesis. Both the ability of cells to take up exogenous mevalonate and the sensitivity to HMG-CoA reductase inhibitors can vary enormously and needs to be experimentally tested (e.g., the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*; Giannakouros et al., 1992).

Subsequent to and dependent on prenylation, other modifications can take place. CXXX motifs are proteolyzed to remove the XXX residues by a membrane-associated endoprotease; then they are carboxyl-methylated on the free C-terminal α -carboxyl by a membrane-bound carboxyl-methyl transferase. This methyl ester group is chemically relatively labile; it is sensitive to mild alkali, although it is not as labile as the side-chain methyl esters of aspartate and glutamate, which can hydrolyze even during electrophoresis in mildly alkaline buffers such as are used in the standard Laemmli SDS-PAGE system (Laemmli, 1970).

This lability is used experimentally as a test for the C-terminal α -carboxyl-methyl ester (Chelsky et al., 1985; Gutierrez et al., 1989). CXC motifs become doubly geranylgeranylated and are therefore also carboxyl-methylated because the methyl transferase recognizes a C-terminal prenylated cysteine. CC motifs, on the other hand, are not carboxyl-methylated. Many prenylated proteins also become palmitoylated (*S*-acylated) on nearby upstream cysteines. *S*-acylation is discussed in *UNIT 7.4*.

The primary function of prenylation is in membrane localization of substrate proteins that would otherwise be cytosolic because they are usually lacking in hydrophobic peptide sequences (Parenti and Magee, 1995). To perform this localization function, prenyl groups generally act in cooperation with themselves (e.g., for the doubly geranylgeranylated CXC proteins), with other modifications such palmitate moieties, or with peptide sequences. For example, the prenylated CXXX sequences of Ras proteins must cooperate with palmitoylation sites (in the case of H-ras, N-ras and K[A]-ras) or a polybasic region (as in K[B]-ras), which probably interacts with acidic membrane phospholipids (Hancock et al., 1991; Newman and Magee, 1993). These cooperating signals specify not only general membrane binding, as might be expected for a hydrophobic lipid moiety, but also targeting to specific subcellular destinations. The mechanism of this intracellular targeting is at present obscure.

Membrane association of prenylated proteins is frequently reversible in vivo. This is achieved in several ways (Newman et al., 1992). Both *S*-acylation and carboxyl-methylation can be dynamic in vivo, thus allowing modulation of the hydrophobicity and/or charge of the C-terminus. Phosphorylation of residues near the C-terminus will change the charge, reducing the interaction with acidic

phospholipid head groups. Thirdly, many prenylated proteins are removed from membranes by sequestration of their prenylated domains by binding proteins that mask the hydrophobic group and form soluble 1:1 complexes. These mechanisms allow prenylated proteins to move between subcellular sites, which is often an essential part of their function.

Critical Parameters and Troubleshooting

All of the labeling protocols in this unit that use cultured cells perform optimally when subconfluent cells are used. Certainly cells that are fully confluent should not be used. To ensure that cells at an appropriate density are available, it is helpful set up cultures at two different split ratios and use the ones that are most suitable. Occasionally a cell line is encountered that labels very poorly. Increasing the amount of radiolabel and/or the duration of labeling may lead to a satisfactory result. In the case of [³H]mevalonic acid labeling, the amount of mevinolin used and the duration of pretreatment can be increased. [¹⁴C]mevalonic acid has been used by some workers for radiolabeling prenyl moieties. Although tritium has a lower intrinsic detectability than ¹⁴C due to its weaker emission, the author nevertheless finds ³H is more sensitive because tritiated compounds can generally be obtained at much higher specific activities than ¹⁴C derivatives, and that more than compensates for the weaker emission. For [³H-methyl]methionine labeling, it may help to reduce the concentration of unlabeled methionine in the medium to zero and to increase the duration of pretreatment.

It is always useful to include an aliquot of the total cell lysate on the SDS-PAGE gel to assess the efficiency of labeling. Low levels of tritium require the highest sensitivity of fluorography for most rapid detection. The author has found that treating the gel with 2,5-diphenyloxazole in dimethylsulfoxide is the most sensitive method (Laskey, 1980; Magee et al., 1995). However, this method cannot be used if further processing of the gel for prenyl group analysis or alkaline release of carboxyl-methyl groups is to be performed. In these cases, fluorography is best performed after treating the gel with the water-soluble fluor salicylic acid (Chamberlain, 1979) or a commercial equivalent; this method is approximately half as sensitive.

Mevalonic acid and mevinolin tend to cyclize to form the lactone. If this is suspected (e.g., as indicated by a fall-off in labeling effi-

ciency), the lactone can be hydrolyzed by the method of Kita et al. (1980) as described in the recipes (see Reagents and Solutions). Lipid substrates should never be dried to completion in plastic tubes because they are often irreversibly adsorbed to the surface.

The ability to detect prenylation and/or carboxyl-methylation of any given protein using these methods will depend on a number of factors: the ability of the cells to take up radiolabeled compounds and incorporate them into metabolic precursors; the sensitivity of the cells to hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors; the pool sizes of endogenous mevalonate, prenyl diphosphates, and *S*-adenosylmethionine (SAM); the expression level of the protein prenyl transferases and carboxyl-methyl transferase; the abundance, rate of synthesis, and turnover of the protein(s) and modification(s) of interest; and for immunoprecipitation, the efficiency of the antibodies.

Anticipated Results

Typically it is possible to detect prenylated proteins using fluorographic exposure times of one to a few days for transfected cDNAs expressed at high levels, e.g., in COS cells, or in vitro translated mRNAs, 2 or 3 weeks for well-expressed endogenous proteins, or up to 3 months for endogenous proteins expressed at low levels or in cells that incorporate mevalonate poorly.

Similar exposure times are required for carboxyl-methylated proteins. However, because much of the radiolabel incorporated into these proteins is in the form of backbone methionine, the exposure time to detect the protein by fluorography may not be a good guide to the sensitivity of detection for alkali-labile methyl groups. This can be predicted if the primary sequence of the protein and therefore, its methionine content is known; it clearly becomes more of a problem with larger proteins and those with many methionines.

Time Considerations

Labeling experiments using cultured cells typically require ~3 days to grow and label the cells. Harvesting and analyzing the cells by SDS-PAGE (with or without immunoprecipitation) take an additional 2 days, plus the time required for fluorography. After the fluorogram is developed, it takes 2 to 4 hr plus an overnight incubation to perform the analysis for alkali-labile and alkali-stable label, and the samples can be counted the next day.

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Key References

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These references contain compilations of methods for studying lipid modifications of proteins.

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Metabolic Labeling and Immunoprecipitation of Yeast Proteins

Saccharomyces cerevisiae, hereafter referred to as yeast, has long been an important model system for studying basic cellular processes. An essential component of many of these studies has been the ability to pulse-label newly synthesized proteins and to follow the fate of a specific protein during a chase period by immunoprecipitation (UNIT 7.2) and SDS-PAGE (UNIT 6.1). Because yeast can assimilate inorganic sulfate into methionine and cysteine (Marzluf, 1997), it is possible to label yeast cells with $^{35}\text{SO}_4^{2-}$. However, for this purpose most researchers use a mixture of [^{35}S]methionine and [^{35}S]cysteine, which equilibrates more directly into the intracellular pools of charged aminoacyl-tRNAs. A cellular hydrolysate from *E. coli* labeled with $^{35}\text{SO}_4^{2-}$, marketed as TRAN ^{35}S -LABEL (ICN) or EXPRE $^{35}\text{S}^{35}\text{S}$ (NEN), works well as a source of [^{35}S] amino acids for metabolically labeling yeast proteins (see Basic Protocol).

Because yeast have a rigid cell wall, lysing the labeled cells in order to immunoprecipitate a specific protein poses an additional challenge. Mechanical shearing is the simplest and least expensive method for lysing yeast. This is done by vigorously vortexing samples in the presence of glass beads. Alternatively, one can remove the cell wall enzymatically using a β -glucanase preparation marketed as Zymolyase 100T (Seikagako Kogyo), Oxalyticase (Enzogenetics), or yeast lytic enzyme (ICN). The resulting spheroplasts can then be lysed by reducing the osmotic support (see Alternate Protocol). Both procedures are described below.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by the local radiation safety officer (see also APPENDIX 1D).

LABELING AND IMMUNOPRECIPITATING YEAST PROTEINS

The proteins of yeast are metabolically labeled using an *E. coli* hydrolysate as the source of [^{35}S]amino acids. After labeling, the cells are lysed using glass beads, and the protein(s) of interest are immunoprecipitated using specific antibody and protein A-Sepharose. Then the immunoprecipitates are analyzed by SDS-PAGE (UNIT 6.1) and fluorography (UNIT 6.3).

Materials

- Yeast
- Minimal medium containing 2% (w/v) glucose (SD medium), without agar (UNIT 1.6)
- Methionine-free SD medium (UNIT 1.6)
- [^{35}S]-protein hydrolyzate labeling mix (>1000 Ci/mmol)
- 50 \times chase solution (see recipe)
- 50% (w/v) trichloroacetic acid (TCA)
- Acetone, ice cold
- SDS/urea buffer (see recipe)
- Detergent IP buffer (see recipe)
- Antibody specific for protein of interest
- Preimmune serum for a negative control
- Protein A-Sepharose (see recipe)
- Detergent/urea buffer (see recipe)

BASIC PROTOCOL

Protein Labeling and Immuno- precipitation

7.6.1

50 mM Tris-Cl, pH 7.5/1% (w/v) SDS
1× (w/v) SDS sample buffer (*APPENDIX 2A*)
125-ml culture flask
Disposable 1.7-ml centrifuge tubes
0.1- to 0.25-mm glass beads (Glen Mills)
Ready-Caps and vials (Beckman)
SpeedVac evaporator
Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*)

Grow cells

1. Inoculate a single yeast colony from a plate into 25 ml of SD medium contained in a 125-ml culture flask. Use the appropriate medium supplements necessary to support growth of strains carrying auxotrophic mutations and to maintain selection for any plasmids. Shake vigorously (250 rpm) in a 30°C incubator overnight.

It is usually best to inoculate the culture in the morning to ensure that there will be an adequate number of cells for the next day.

2. Use a spectrophotometer to measure the OD₆₀₀ of a 1:4 or 1:10 dilution (in water) of the overnight culture.

Since turbidity or light scattering is being measured in the spectrophotometer rather than absorbance, it is best to read in the 0.05 to 0.5 range to achieve a linear response to cell density. A culture with an OD₆₀₀ reading of 1.0 (corrected for dilution) is considered to have a cell concentration of 1 OD₆₀₀/ml, which is equivalent to $\sim 1 \times 10^7$ cells/ml for most strains.

3. Proceed to step 5 if the overnight culture is still in log phase (has not exceeded an OD₆₀₀ of 1.0) and a sufficient number of cells are available for labeling.

The number of cells required for a pulse-chase experiment depends on the expression level of the protein of interest and has to be determined empirically. However, a total of 8 OD₆₀₀ are required for a typical pulse-chase experiment in which 2 OD₆₀₀ of cells are harvested at each of four time points.

4. For overnight cultures exceeding an OD₆₀₀/ml of 1.0, dilute to 0.25 OD₆₀₀ in the same medium (25 to 50 ml) and grow for 4 to 5 hr as described above.

The cells will usually double only once in this time period when cultured in minimal medium.

5. Measure and record the OD₆₀₀ of the culture and pellet the yeast by centrifuging 5 min at 5000 × g. Discard the spent medium and resuspend the cells in SD medium (methionine-free) to a concentration of 5 OD₆₀₀/ml.

6. Determine the number of cells required for the experiment and transfer an appropriate culture volume to a disposable centrifuge tube (i.e., Falcon 2059 or 2070).

The culture should occupy <10% the total volume of the tube.

Label cells

7. Preincubate the cells at the desired temperature (typically 30°C) for 15 min with vigorous shaking (250 rpm). To initiate the labeling period, add [³⁵S]methionine and cysteine to a final concentration of 125 μCi/ml (or use 25 μCi/OD₆₀₀ if a different concentration of cells is used). Incubate 5 to 15 min.

8. To initiate the chase, add 50× chase solution to a 1× final concentration. At the desired chase times (e.g., 0, 5, 15 and 45 min), transfer equal aliquots (100 to 400 μl) from

the culture to properly labeled microcentrifuge tubes sitting on ice containing 0.25 vol 50% (w/v) TCA (final TCA concentration equals 10%) to terminate the chase.

CAUTION: TCA is extremely caustic. Protect eyes and avoid contact with skin when preparing and handling TCA solutions.

The cells can be stored in 10% TCA in a refrigerator for several days or processed immediately for immunoprecipitation.

Lyse cells

9. After at least 15 min in TCA, pellet the cells 10 min in a microcentrifuge at full speed. Aspirate the supernatant into a radioactive waste trap and wash the pellet with ~1.0 ml of ice-cold acetone. Microcentrifuge for 4 min at full speed and aspirate the acetone into the radioactive waste trap. Repeat the acetone wash.

CAUTION: The supernatants and washes should be handled as mixed chemical/radioactive wastes; follow applicable safety regulations for disposal (also see APPENDIX 1D).

Individually immersing the bottom of the tubes in a bath sonicator and then vortexing will help disperse the pellets.

10. Dry the acetone-washed TCA pellets in a SpeedVac evaporator, then add 100 μ l of SDS/urea buffer and let sit for at least 15 min at room temperature.
11. Add glass beads (0.1- to 0.25-mm) to 80% to 90% (v/v) of the sample volume and vortex 1 min.

Care should be taken here because glass beads adhering around the lip of the tube will prevent the cap from closing tightly. Use a razor blade to cut the bottom off of a microcentrifuge tube and use this as a "funnel" for adding the glass beads. A 0.1-ml capacity Coors porcelain spatula (Fisher, VWR) works well to deliver the appropriate volume of glass beads.

12. Heat the samples 4 min at 95°C, then vortex again for 15 to 30 sec.
13. Add 900 μ l of detergent IP buffer, vortex and put the samples on ice for \geq 10 min.
14. Microcentrifuge the samples 15 min at full speed and transfer 0.85 ml of the supernatants (lysate) to fresh tubes being careful not to disturb the glass beads or any insoluble material pelleted on top of the glass beads.
15. To determine the incorporation of ^{35}S into protein, spot 5 μ l of each supernatant into a Beckman Ready-Cap, transfer the caps to vials and measure the radioactivity in a scintillation counter.

Alternatively, measure incorporation of radioactivity by TCA precipitation (UNIT 7.1).

Immunoprecipitate protein

16. Add antiserum (usually 1 to 2 μ l per OD of labeled cells) and 75 to 100 μ l protein A-Sepharose suspension to the supernatants. Put the tubes in a rack and lay the rack on its side and rock the samples for 4 hr to overnight in a cold room. Agitate sufficiently to keep the protein A-Sepharose in suspension.

Alternatively, use antibody pre-bound to protein A-Sepharose (see UNIT 7.2).

17. Pellet the immune complexes by microcentrifuging 30 sec at 3000 \times g and transfer the supernatants to fresh tubes.

Do not discard these supernatants as it is often desirable to immunoprecipitate a second protein from these lysates.

18. Wash the pellets twice with 1 ml of detergent/urea buffer, then once with detergent IP buffer. Aspirate each supernatant from the washes into the radioactive waste trap.
19. (*Optional*) To immunoprecipitate the lysates with a second antibody, clear any remaining antigen-antibody complexes by adding 75 μ l of protein A–Sepharose and rocking the samples for 2 hr in the cold as above. Microcentrifuge 10 min at full speed and transfer the supernatants to fresh tubes. Use the cleared lysate to immunoprecipitate with a second antibody.
20. Resuspend the pellets in 100 μ l of 50 mM Tris-Cl, pH 7.5/1% SDS and heat at 95°C for 4 min. Repeat steps 13 to 18, omitting step 15. Dry the final pellet for 5 to 10 min under vacuum.

A second immunoprecipitation (“recapture”) step is usually required to achieve a “clean” immunoprecipitation.
21. Resuspend the pellets in 50 μ l of 1 \times SDS sample buffer and heat at 95°C for 4 min. Subject half of the sample to SDS-PAGE (*UNIT 6.1*). Save the remainder of the samples (at 4°C) in case the electrophoresis has to be done again. Process gels for fluorography or phosphor imaging (*UNIT 6.3*).

ALTERNATE PROTOCOL

MAKING YEAST SPHEROPLASTS

Proteins secreted from yeast are usually retained in the periplasmic space, which lies between the plasma membrane and the cell wall. The contents of the periplasmic space can be separated from the cells by enzymatically removing the cell wall and pelleting the resulting spheroplasts. Secreted proteins will remain in the supernatant. In addition, if one wishes to perform native immunoprecipitations or fractionate labeled yeast cells, it is necessary to remove the cell wall in order to gently lyse the cells by reducing the osmotic support.

Additional Materials (also see Basic Protocol)

Radiolabeled yeast cells
 2 \times spheroplast/stop solution (see recipe)
 Bovine serum albumin (BSA), fatty acid–free Fraction V
 10 mg/ml Zymolyase 100T (Seikagako Kogyo)

1. Grow and label yeast cells (see Basic Protocol, steps 1 to 7).
2. Chase the cells, substituting 1 vol 2 \times spheroplast/stop solution for the 50% (w/v) TCA (see Basic Protocol, step 8). Add BSA to 1 mg/ml to act as a carrier if secreted proteins are going to be TCA precipitated from the periplasmic space fraction.
3. Keep samples on ice until all the chase points are collected.
4. Add 2 μ l of 10 mg/ml Zymolyase 100T (ICN) per OD₆₀₀ of cells in each sample and incubate 30 min at 30°C with gentle agitation.
5. Pellet cells by microcentrifuging 5 min at 5000 \times g and transfer the supernatants to fresh tubes.
6. To monitor the spheroplasting reaction, take 200 μ l of a 10 OD₆₀₀/ml suspension of unlabeled cells in 1 \times spheroplast/stop buffer, split the sample equally and add Zymolyase to one of the tubes. After incubating 30 min at 30°C, remove 25 μ l from each tube and dilute to 1 ml with water. Read the OD₆₀₀.

The untreated sample should give an OD₆₀₀ reading of ~0.25, but the treated sample should lyse when diluted in water and give an OD₆₀₀ reading that is 5% to 10% that of the untreated sample (~0.01 to 0.025). If the OD₆₀₀ reading of the treated sample is \geq 10% of the untreated sample, add more Zymolyase to the samples and continue the incubation.

Process yeast spheroplasts for immunoprecipitation or subcellular fractionation

- 7a. *For TCA precipitation followed by immunoprecipitation:* Add 0.25 vol of 50% TCA to the supernatants and 0.5 ml of 10% (w/v) TCA to the cell pellets to precipitate proteins. Proceed from this point to step 9 of the Basic Protocol.
- 7b. *For immunoprecipitation without TCA precipitation:* Dissolve cell pellets in 100 μ l SDS-urea buffer, then heat at 95°C for 4 min to lyse cells and avoid TCA precipitation and glass-bead lysis steps. Proceed from this point to step 13 of the Basic Protocol.
- 7c. *For native immunoprecipitation:* Resuspend the cell pellets in 1 ml nondenaturing lysis buffer (UNIT 7.2). Perform native immunoprecipitation from the lysates as described in UNIT 7.2.
- 7d. *For subcellular fractionation:* Lyse the spheroplasts by resuspending the cell pellet in low-osmotic-strength buffer (e.g., 25 mM Tris·Cl, pH 7.2/0.1 M sorbitol) and fractionate to enrich specific organelles (Graham et al., 1994).

ENDO H TREATMENT OF IMMUNOPRECIPITATES

N-linked oligosaccharides on yeast glycoproteins are sensitive to endoglycosidase H (endo H) regardless of the extent of Golgi modification. Thus, endo H treatment can be used to determine if an immunoprecipitated protein is modified with N-linked oligosaccharides (also see UNIT 15.2). Add 64 μ l of freshly prepared SDS-2ME solution (0.2% w/v SDS/1% w/v 2-mercaptoethanol) to dried immunoprecipitates (Basic Protocol, step 17) and heat 4 min at 95°C. Add 16 μ l of 250 mM sodium citrate pH 5.5 buffer to each sample. Mix and centrifuge briefly. Transfer 40 μ l of each sample to a fresh tube, then add 0.5 mU of Endo H (0.5 μ l) to one portion and incubate each pair of samples (+ and – endo H) overnight in a 37°C air incubator. Then add 13.3 μ l of 4 \times SDS sample buffer (APPENDIX 2A) to each tube, heat 4 min at 95°C and electrophorese the samples in an SDS-polyacrylamide gel (UNIT 6.1).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Chase solution, 50 \times

50 mM methionine

5 mM cysteine

Filter sterilize and store up to 1 year at –20°C in small aliquots

Detergent IP buffer

50 mM Tris·Cl, pH 7.5

0.1 mM EDTA

150 mM NaCl

0.5% (w/v) polyoxyethylenesorbitan monolaurate (Tween 20)

Store up to 3 months at 4°C

Detergent/urea buffer

100 mM Tris·Cl, pH 7.5

200 mM NaCl

0.5% (w/v) polyoxyethylenesorbitan monolaurate (Tween 20)

2 M urea

Store up to 3 months at 4°C

SUPPORT PROTOCOL

Protein Labeling and Immuno- precipitation

7.6.5

Protein A–Sepharose

Swell 0.4 g protein A–Sepharose CL-4B (Pharmacia) in 11.2 ml of 10 mM Tris-Cl (pH 7.5)/1 mM NaN_3 /1 mg/ml bovine serum albumin for 2 hr to overnight at 4°C. Aspirate the supernatant from the settled beads and add fresh buffer to the original volume. Store up to 3 months at 4°C.

SDS/urea buffer

50 mM Tris-Cl, pH 7.5 (*APPENDIX 2A*)
1 mM EDTA
1% (w/v) SDS
6 M urea
Store up to 1 month at room temperature

Spheroplast/stop solution, 2×

2 M sorbitol
50 mM Tris-Cl, pH 7.5 (*APPENDIX 2A*)
40 mM NaN_3
40 mM NaF
Store up to 6 months at room temperature
Add 20 mM DTT from 1 M stock solution just before use

COMMENTARY

Background Information

Radiolabeling of proteins, lipids, and carbohydrates

Incorporation of ^{35}S into newly synthesized yeast protein can be achieved by labeling cells with either $^{35}\text{SO}_4^{2-}$ or a mixture of [^{35}S]methionine and [^{35}S]cysteine. While $^{35}\text{SO}_4^{2-}$ is less expensive and is primarily incorporated into protein in yeast (and not other macromolecules such as polysaccharides), there are drawbacks to its use. For example, standard minimal medium contains a high concentration of cold sulfate, which obviously competes with the isotope for incorporation into protein and also represses the synthesis of the high-affinity sulfate permeases (Cherest et al., 1997). Thus, efficient uptake of $^{35}\text{SO}_4^{2-}$ requires growing the cells in a specially prepared low-sulfate medium (LSM) to induce expression of the permeases. LSM is SD medium where chloride salts are used in place of sulfate salts. LSM is supplemented with 100 μM $(\text{NH}_4)_2\text{SO}_4$ for growing cells and 25 μM $(\text{NH}_4)_2\text{SO}_4$ for labeling. Even with these precautions, a relatively high concentration of $^{35}\text{SO}_4^{2-}$ (250 to 500 $\mu\text{Ci}/\text{ml}$) is needed to achieve an adequate incorporation of radiolabels into protein. Therefore, labeling cells with [^{35}S]amino acids has become the method of choice because standard minimal media (SD, methionine-free) can be used, and it is not necessary to

starve the cells for sulfate prior to labeling. Care should be taken when working with small proteins or peptides to check if methionine or cysteine residues are present in the sequence. If not, it is possible to label cells with [^3H]leucine (or other amino acid) in minimal medium (SD, leucine-free).

The methods described in this unit can also be used to study the phosphorylation of proteins *in vivo*. Cells can be labeled with $^{32}\text{PO}_4^{3-}$ in a low-phosphate medium in which the phosphate salts in LSM are replaced with the appropriate chloride salts and then supplemented with 50 μM potassium phosphate. Cells are typically labeled 20 to 60 min at 100 $\mu\text{Ci}/\text{OD}_{600}$ (Reneke et al., 1988).

A substantial amount of $^{32}\text{PO}_4^{3-}$ is incorporated into phospholipid to the extent that cells can be labeled in standard minimal medium if one wishes to examine the bulk cellular phospholipid content. Cells are grown for six generations in minimal medium (supplemented with 0.1 mM inositol and 1 mM choline) containing 10 $\mu\text{Ci}/\text{ml}$ $^{32}\text{PO}_4^{3-}$ for steady-state determinations, or pulse-labeled 10 to 60 min at 50 $\mu\text{Ci}/\text{ml}$ (Klig et al., 1985). After labeling, the lipids are extracted in a polar extraction solvent and separated by two-dimensional paper chromatography (Steiner and Lester, 1972).

Yeast glycoproteins can also be radiolabeled with [$2\text{-}^3\text{H}$]mannose, which is specifically incorporated into O- and N-linked oli-

gosaccharides as well as a small percentage of lipid-linked products. The label is not incorporated into other compounds because catabolism of mannose, specifically the conversion of mannose-6-phosphate to fructose-6-phosphate, results in the removal of the label as tritiated water (Huffaker and Robbins, 1982). It is also helpful to grow and label the cells in minimal medium containing 1% galactose as the carbon source rather than glucose. Galactose will not compete with mannose for entry into the cell. Cells can be labeled continuously during growth at 0.1 to 10 $\mu\text{Ci/ml}$ or pulse-labeled at 100 to 500 $\mu\text{Ci/ml}$ with the cells at 20 $\text{OD}_{600}/\text{ml}$ (Byrd et al., 1982).

Labeling conditions

The medium that yeast are grown in, as well as other conditions such as temperature and the stage of growth, can have a dramatic influence on the expression of specific genes. For example, if one wishes to examine the protein encoded by a galactose-regulated gene in a pulse-chase experiment, it is essential to culture the cells in galactose rather than glucose as the carbon source. In addition, many genes involved in fermenting carbon sources other than glucose are repressed when cells are grown in glucose. To derepress these genes, shift cells to medium containing 0.1% glucose for 1 hr prior to labeling or simply culture the cells using a different carbon source. However, labeling is typically done in synthetic minimal medium containing glucose, and it is preferable to grow the cells in the same medium used during the labeling period to avoid inducing changes in cellular metabolism. Another important consideration is that cells in the logarithmic stage of growth incorporate substantially more ^{35}S into protein than stationary phase cells.

One of the advantages of working with yeast is the availability of a large number of mutants that exhibit a defect in some cellular process. For temperature-sensitive mutants, it is often desirable to incubate cells at the nonpermissive temperature prior to labeling in order to establish the mutant phenotype. The length of this incubation period depends on the nature of the mutation carried by the strain and usually has to be determined empirically. In the case of the *sec18-1* mutant, for example, protein transport is blocked almost immediately upon shifting to the nonpermissive temperature and the cells remain viable for ≥ 2 hr (Graham and Emr, 1991). As

a control, it is important to label wild-type cells that have been subjected to the same temperature shifts to be certain that the mutation and not the change in temperature is the cause of any effects being observed.

Critical Parameters and Troubleshooting

Yeast colonies used to inoculate the overnight culture should be < 2 weeks old, particularly if they were grown on a minimal plate in order to select for a plasmid carried by the strain. It is preferable that the overnight culture does not grow to stationary phase because many strains can take several hours to start doubling again when diluted in minimal medium. However, 4 to 5 hr of incubation after diluting in fresh medium is usually sufficient for stationary phase cells to start growing. Poor growth of some strains in minimal medium can result in low incorporation of ^{35}S amino acids into protein ($< 5 \times 10^6$ cpm/ OD_{600}). In this case, add 0.2% (w/v) Difco yeast extract to the growth (steps 1 to 3) and chase (step 8) media. If yeast extract is used, it is essential to wash the yeast twice with sterile water (~ 30 ml) before resuspending the cells in labeling medium lacking yeast extract (steps 4 to 7). It is also very important to use clean glassware and centrifuge tubes that are free of detergent at all steps. Poor incorporation of ^{35}S in labeling experiments is usually solved by carefully remaking the medium. Old (> 1 month) or improperly stored [^{35}S] amino acids can also contribute to labeling problems.

In some cases, a biological process may not go to completion during the chase period. For example, some fraction of a protein that is normally completely secreted in 15 min may remain inside the cell even after a 60-min chase. This may indicate that the cells are being depleted of energy or some other critical nutrient. To remedy this problem, add yeast extract and additional glucose to a final concentration of 0.2% and 2% (w/v), respectively, to the chase (step 8).

Some integral membrane proteins will aggregate when the cell lysates are heated to 95°C in SDS-urea buffer. In this case, the protein will migrate near the top of the resolving gel or even remain in the stacking gel during electrophoresis. To help prevent this from occurring, heat the samples at 50°C for 5 min or 37°C for 10 min (steps 12, 20, and 21). These conditions do not inactivate cellu-

lar proteases as efficiently as the 95°C incubation, so addition of protease inhibitors may be necessary if degradation products are apparent on the autoradiograms.

The quality of the data produced by the procedures described here is primarily dependent on the quality of the antiserum and the expression level of the proteins being examined. Perhaps the most critical parameter is at step 14 of the Basic Protocol, to carefully transfer the supernatant from the clearing centrifugation step without carrying over any insoluble material. In addition, the “double” immunoprecipitation protocol (step 20), while not essential, usually provides immunoprecipitated proteins that are free of background bands. However, there are additional steps that can be taken to “clean up” immunoprecipitations (UNIT 7.2).

1. After step 15, add 75 μ l of protein A–Sepharose to the supernatants, rock the samples for 1 hr in the cold room and microcentrifuge 10 min at full speed. Then transfer the supernatants to fresh tubes and proceed to step 16.

2. If a strain is available that does not express the protein of interest (e.g., a strain carrying a complete disruption of the gene encoding the protein of interest), it is possible to prepare a cold-competing cell lysate to add to the immunoprecipitations. To do this, pellet the unlabeled cells and resuspend them at 250 OD₆₀₀/ml in SDS/urea buffer. Add glass beads until they occupy ~80% of the sample volume, vortex for 1 min and then heat 4 min at 95°C. Add 9 vol of detergent IP buffer, mix well by vortexing, and centrifuge 10 min at 10,000 \times g. The cold-competing cell lysate can then be added in place of the detergent IP buffer at steps 13 and 20 of the Basic Protocol.

3. Several pathogenic fungi have cell wall carbohydrate structures that are antigenic and are similar to those of *Saccharomyces cerevisiae*. Thus, animal serum can carry antibodies that cross-react with yeast glycoproteins causing background problems in immunoprecipitates. This problem is usually apparent in pre-immune control immunoprecipitations. Yeast mannan (Sigma) can be added at 0.5 mg/ml at steps 13 and 20 to help reduce this background. Using affinity purified antibodies may also help to ameliorate this potential problem.

4. Some investigators wash the immunoprecipitates an additional time (step 17) in

1% (v/v) 2-mercaptoethanol to reduce background. Likewise, the concentration of detergent, SDS, or urea can be increased in the wash buffer to provide greater stringency. However, depending on the antibodies being used, these steps can cause significant loss of the protein of interest from the immunoprecipitates.

Anticipated Results

The basic labeling procedure should result in the incorporation of 0.5 to 4×10^7 cpm/OD₆₀₀. Specific bands should be visible anywhere from 2 hr to 2 weeks after exposure of dried gels to X-ray film. At this level of incorporation, a protein that constitutes ~0.1% of cellular protein should be visible in 5 to 40 hr on the autoradiogram.

Time Considerations

Typically, 15 to 30 min are required on day 1 for inoculation of cultures. Cell labeling and preparation of cell lysates for immunoprecipitation requires ~4 hr on day 2, and the initial antibody incubations are usually performed overnight (step 16). The first wash steps require 30 to 90 min on the morning of the third day (step 17), and the second antibody incubations are for 4 to 7 hr (step 20). The second wash steps and gel loading requires 30 to 90 min. With this regimen, samples are electrophoresed overnight and the gel is processed the morning of the fourth day. Depending on the extent of labeling and the expression of the target protein(s), fluorography can take hours or weeks.

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Metabolic Labeling and Immunoprecipitation of *Drosophila* Proteins

UNIT 7.7

Cultured cells derived from the fruit fly, *Drosophila melanogaster*, provide a powerful experimental system for the biochemical and cell biological study of protein function. Given the great availability of mutant flies, the ability to culture cells from such mutants permits large amounts of cellular material of homogeneous genotype to be obtained for investigation. This unit describes a method for metabolic labeling and immunoprecipitation of proteins from cultured *Drosophila* cells (see Basic Protocol). The specific methods presented here work well for the wild-type S2M3 cell line, which was adapted from Schneider S2 cells to grow in Shields and Sang M3 medium. The Support Protocol describes the procedure for growth and maintenance of these cells. When combined with pulse-chase approaches and subcellular fractionation, this method permits the investigation of such questions as the turnover and cellular localization of specific proteins.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

LABELING AND IMMUNOPRECIPITATION OF PROTEINS FROM *DROSOPHILA* S2M3 CELLS

**BASIC
PROTOCOL**

Wild-type S2M3 cells can be labeled using either purified [³⁵S]methionine or an ³⁵S-labeled *E. coli* hydrolysate as a source for radioactive amino acids. Following metabolic labeling, the cells are gently solubilized with detergent and the protein of interest is immunoprecipitated using specific antibodies and protein A–Sepharose (UNIT 7.2). The precipitated proteins can then be analyzed by SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3).

Materials

- Wild-type *Drosophila* S2M3 cells (ATCC CRL 1963) growing in culture in M3 medium (see Support Protocol)
- M3 growth medium (see recipe)
- M3 labeling medium (see recipe)
- [³⁵S]L-Methionine (>800 Ci/mmol) or ³⁵S-labeled *E. coli* protein hydrolysate (e.g., TRAN³⁵S-LABEL, EXPRE³⁵S³⁵S; ICN Biomedicals; >1000 Ci/mmol)
- Activated charcoal
- PBS (APPENDIX 2A), ice-cold
- Cell lysis buffer (see recipe)
- 1:1 (v/v) protein A–Sepharose bead slurry in PBS (store up to 24 hr at 4°C)
- Specific antibody against protein of interest
- IP wash buffer: 0.1% (v/v) Triton X-100 in PBS (APPENDIX 2A) 1× SDS sample buffer (APPENDIX 2A)
- 35-mm tissue culture dishes
- Large sealed container (e.g., Tupperware)
- Cell scrapers
- End-over-end rotator
- Additional reagents and equipment for culturing *Drosophila* cells (see Support Protocol)

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see UNIT 7.1 and APPENDIX 1D).

**Protein Labeling
and Immuno-
precipitation**

7.7.1

Metabolically label cells

1. Grow cells to ~60% to 70% confluence in 35-mm tissue culture dishes at 25°C in *Drosophila* M3 growth medium (see Support Protocol).

The doubling time for these cells is ~24 to 26 hr.

These cells do not require a humidified CO₂ atmosphere for growth and can be cultured in a cool enclosed space such as a bench cabinet or drawer.

If cells are to be transfected, adjust plating such that they are at the proper confluence at the time of labeling.

2. Rinse cells briefly with two changes of 1 ml each of M3 labeling medium. Then incubate cells with 1 ml fresh M3 labeling medium for 30 min to 1 hr to deplete intracellular methionine pools.
3. Thaw [³⁵S]methionine or ³⁵S-labeled *E. coli* hydrolysate and prepare a 100 to 200 μCi/ml labeling solution in M3 labeling medium.

CAUTION: Open stock vials of ³⁵S-labeled amino acids in a fume hood, since volatile ³⁵S-containing compounds can be released.

4. Remove M3 labeling medium from cells and replace with 0.5 ml of [³⁵S]methionine labeling solution. Incubate cells 30 min to 2 hr at 25°C in a large sealed container containing a moistened paper towel. Place a 35-mm petri dish that contains a small amount of activated charcoal into the sealed container to absorb any released ³⁵S-containing compounds.

Alternatively, a filter that is impregnated with activated charcoal can be used.

The time of labeling can be shortened to 5 to 10 min to accommodate pulse-chase protocols. However, the concentration of radioactive amino acids should be increased to 0.5 to 1 mCi/ml and the chase medium should contain 1 g/liter yeast hydrolysate, a 10-fold excess of unlabeled methionine, and 10% undialyzed FBS.

5. Remove [³⁵S]methionine labeling solution and rinse cells three to four times with 1 ml of ice-cold PBS. Combine the labeling solution and the washes in a 10-ml screw-cap centrifuge tube and centrifuge 5 min at 300 × g, 4°C, to collect any cells that may have detached during the labeling period. Remove the supernatant.

CAUTION: Discard the used labeling solution, all of the wash solutions, and all contaminated disposable material as radioactive waste following the established guidelines set by the local radiation safety officer.

6. Add 2 to 3 ml of ice-cold PBS and scrape cells with a disposable cell scraper. Transfer cell suspension to a 10-ml screw-cap conical centrifuge tube. Combine with the cells collected from the labeling solution.
7. Centrifuge pooled cells for 5 min at 300 × g, 4°C, and discard supernatant.

Lyse cells and perform immunoprecipitation

8. Add 0.5 ml of ice-cold cell lysis buffer to each tube and incubate in ice/water slurry for 20 min.
9. Microcentrifuge 1 min at maximum speed, 4°C, to bring down insoluble material.
10. Transfer supernatant to a fresh tube and add 0.5 ml of additional cell lysis buffer.
11. Add 50 μl of a 1:1 protein A–Sepharose/PBS slurry and place tubes at 4°C on an end-over-end rotator for 0.5 to 1 hr to help “preclear” the supernatant and minimize nonspecific binding of cellular proteins to the protein A–Sepharose beads.

The protein A–Sepharose should be washed prior to use by resuspending 50 μ l, the beads should then be resuspended in PBS at 1:1, in 1 ml of PBS and centrifuging 1 min at maximum speed, 4°C.

12. Microcentrifuge 1 min at maximum speed, 4°C, to bring down the beads, and transfer the supernatant to a fresh tube.
13. Add 50 μ l of fresh 1:1 protein A–Sepharose suspension and add the appropriate amount of specific antibody against the protein of interest.

The amount of antibody used will vary for each specific antibody preparation, but a good rule of thumb is to use a small excess of antibody to ensure a more quantitative recovery of the desired protein.

14. Incubate the tube at 4°C on an end-over-end rotator for 1 to 2 hr and pellet the immune complexes by microcentrifuging 1 min at maximum speed, 4°C.
15. Discard supernatant and wash the immune complexes by resuspending the beads in 1 ml of IP wash buffer and microcentrifuging again at maximum speed, 4°C. Repeat two more times.
16. Resuspend the immune complexes in 50 μ l of 1 \times SDS-PAGE sample buffer and process for SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3).

GROWTH AND MAINTENANCE OF CULTURED *DROSOPHILA* S2M3 CELLS

SUPPORT PROTOCOL

Most *Drosophila* cells, including S2M3 cells, are conveniently cultured at 22°C to 25°C in tightly capped 25-cm² culture flasks and do not require a regulated CO₂ atmosphere. Thus, these cells can be cultured in a dry, cool place (e.g., a bench cabinet), provided that the temperature remains stable. Subculturing is accomplished by dislodging the cells from the flask by passing a gentle stream of medium over the cells with a pipet and transferring the cells to a new flask at the desired split ratio.

Materials

Drosophila S2M3 cells (ATCC CRL 1963)
M3 growth medium (see recipe)
25-cm² (T-25) tissue culture flasks with plug-seal caps

1. Aspirate off the old medium from a culture grown in a 25-cm² flask in a sterile tissue culture hood.
2. Tilt the flask and pass a gentle stream of medium (~2 to 3 ml) over the cells using a sterile cotton-plugged 5¹/₄-in. Pasteur pipet fitted with a rubber bulb.

*Most *Drosophila* cell lines adhere loosely to the growth substrate and thus do not require treatment with trypsin to dislodge them from the substrate. Indeed, the use of trypsin should be avoided since many lines are sensitive to trypsin.*

Cotton-plugged pipets can be sterilized by autoclaving them in square metal canisters and are an inexpensive alternative to using disposable plastic pipets.

3. Pipet the cells up and down, gently, to disperse them into a homogeneous suspension.
4. Transfer cells at a split ratio of 1:3 to 1:10 into a new 25-cm² flask that contains 3 ml of fresh M3 growth medium warmed to room temperature.

*S2M3 cells, like most *Drosophila* cells, should not be subcultured at high split ratios since they do not grow well when sparsely plated.*

5. Tightly seal the flasks and place in a dry, cool place (e.g., a bench cabinet) where the temperature is fairly constant at 22° to 25°C.

Protein Labeling and Immuno- precipitation

7.7.3

Cells can also be plated into 35-mm-diameter dishes containing 1.5 to 2 ml of M3 medium for individual experiments; however, the outsides of these dishes should be sealed with Parafilm.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Cell lysis buffer

1% (v/v) Triton X-100
50 mM Tris-Cl, pH 7.4 (APPENDIX 2A)
300 mM NaCl
5 mM EDTA
20 mM iodoacetamide
Store up to 6 months at 4°C
Just before use, add the following protease inhibitors:
100 µg/ml PMSF
1 µg/ml aprotinin

M3 growth medium

Shields and Sang M3 insect medium (Sigma-Aldrich) without *L*-glutamine and with potassium bicarbonate
10% (v/v) fetal bovine serum (FBS)
100 U/ml penicillin
100 µg/ml streptomycin
Store up to 2 to 3 months at 4°C

M3 labeling medium

Prepare Shields and Sang M3 insect medium without *L*-glutamine, with potassium bicarbonate lacking methionine, cysteine, and yeast hydrolysate (Sigma-Aldrich). Supplement the medium with 100 U/ml penicillin and 100 mg/ml streptomycin. This medium can be supplemented with 10% FBS that has been dialyzed overnight against saline to remove unlabeled amino acids. Store up to 6 months to 1 year at -20°C

COMMENTARY

Background Information

Since the late 1960s, roughly 100 continuous cell lines have been established from both wild-type and mutant *Drosophila* flies. Refer to Echalier (1997) for an excellent and comprehensive treatise on *Drosophila* cell culture and for specific methods to establish cell lines from *Drosophila* flies. Of the many wild-type cell lines described, the most commonly used lines are Echalier and Ohanessian's Kc cell line (Echalier and Ohanessian, 1970) and Schneider's S1, S2, and S3 cell lines (Schneider, 1972). Both the Kc line and the S2 line are available from ATCC. Both of these continuous cell lines were established from early *Drosophila* embryos, as were most continuous *Drosophila* cell lines described thus far. Both wild-type and mutant *Drosophila* cells have been used to investigate such processes as

insect hormone responses, cell adhesion, and EGF signaling.

Metabolic labeling and immunoprecipitation methods have been used to study protein function in a variety of cultured *Drosophila* cell lines, often in conjunction with transfection studies. As an example, Zak and Shilo (1990) utilized pulse-chase labeling and immunoprecipitation to study biosynthesis of the *Drosophila* EGF receptor homolog, DER. Buzin and Petersen (1982) profiled the pattern of protein expression induced during heat shock using metabolic labeling with [³⁵S]methionine and two-dimensional electrophoresis in Kc cells.

Critical Parameters and Troubleshooting

Three important factors must be considered when designing metabolic labeling experi-

ments for cultured *Drosophila* cells: composition of culture medium, growth properties of the cells, and methods of transfection.

The three most commonly used media for culturing *Drosophila* cells are Echalier-Ohanessian's D22 medium (Kc cells), Schneider's medium (S1, S2, and S3 cells), and Shields and Sang M3 medium (S2M3 cells, Shields' G1 line, Sang's *shibire* S4 line). Both Schneider's medium and Shields and Sang M3 medium are commercially available from Sigma-Aldrich; Schneider's medium is also available from Life Technologies. A methionine-, leucine-, and yeast extract-free formulation of M3 medium is also available from Sigma-Aldrich, which is useful for metabolic labeling. Most *Drosophila* media are supplemented with 5% to 20% fetal bovine serum. Since most formulations of *Drosophila* culture media contain either yeast extract or lactalbumin hydrolysate, it is important to omit these during metabolic labeling since they provide a source of unlabeled amino acids that could reduce labeling efficiency. All of these media formulations can be conveniently made in the laboratory (see Echalier, 1997), and thus can be prepared with these supplements omitted in addition to the particular amino acid to be labeled.

Another important consideration is the growth conditions of *Drosophila* cells. Almost none of the established cell lines require a regulated CO₂ atmosphere, and almost all grow optimally at 22°C to 25°C. Thus, most *Drosophila* cells can be conveniently cultured literally on the bench top. However, caution must be exercised to ensure that the temperature does not rise much above 27°C, since the *Drosophila* heat-shock response is induced at temperatures of 30° to 37°C (see Echalier, 1997). During the heat-shock response, the synthesis of most proteins is repressed in favor of heat-shock proteins (Buzin and Petersen, 1982). Also, many *Drosophila* cells adhere loosely to the growth substrate and often detach and grow in suspension. Thus, any floating cells should also be collected from the labeling medium during metabolic labeling experiments. Finally, one should avoid sparse plating (e.g., >1:20 split ratio), since *Drosophila* cells do not grow well at low cell densities.

Drosophila cells can be transfected using a modified calcium phosphate coprecipitation method (UNIT 15.5), lipid-based transfection reagents, and electroporation. The most widely used method of transfection is a modified calcium phosphate method where *N,N*-bis(2-hy-

droxyethyl)-2-aminoethanesulfonic acid (BES) is substituted for HEPES as a buffering agent (Chen and Okayama, 1987). 2× BES-buffered saline (2× BBS) contains 50 mM BES, pH 6.95, 280 mM NaCl, and 1.5 mM Na₂HPO₄. It is filter-sterilized and stored at -20°C. This change gives an ~10-fold higher transfection efficiency (Cherbas et al., 1994). Lipofectin-mediated transfection has also been shown to work well for cultured Kc and S2 cells (Echalier, 1997). Finally, electroporation works well for Kc167 cells (Cherbas et al., 1994). With regard to expression vectors, two commonly used promoters for efficient expression of proteins in *Drosophila* cells are the actin5C promoter (Jaynes and O'Farrell, 1988) and the metallothionein promoter (Bunch et al., 1988). Proteins are efficiently expressed in many cells when their transcripts are driven by the activity of these promoters. Three expression vectors, which utilize these promoters, are commercially available from Invitrogen. They are useful for expressing proteins in *Drosophila* cells; pMT/V5-His A,B,C (Metallothionein promoter); pMT/BiP/V5-His A,B,C (metallothionein promoter); pAc5.1/V5-His A,B,C (actin5C promoter).

Finally, proper controls should always be utilized to assess the specificity of each antibody used for immunoprecipitation. If using polyclonal antiserum, preimmune serum should be used on a duplicate sample to assess the specificity of immunoprecipitation. If using monoclonal antibodies, an irrelevant antibody should be used from the same species as the specific antibody, to assess nonspecific binding. Also, a mock immunoprecipitation, where the primary antibody is omitted, should be included to control for nonspecific binding to the protein A-Sepharose beads. The primary antibody should be titrated to optimize the amount used for immunoprecipitation such that it is present only in slight excess, to minimize nonspecific binding.

Anticipated Results

In a typical experiment, using a 30-min to 1-hr labeling period as described (see Basic Protocol), clearly visible bands result on X-ray film after 16 hr to 24 hr exposure following SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3). The relative transient transfection efficiency using the modified calcium phosphate method ranges from 10% to 20%. Thus, immunoprecipitation of transiently transfected proteins is variable and should be optimized for each specific case. This protocol should also

work equally well for labeling and immunoprecipitation of endogenous proteins.

Time Considerations

It usually requires 1.5 to 3 hr to prepare cells and perform the labeling, depending on the time used for labeling. Approximately 30 min is required to prepare cell extracts and 2 to 4 hr is required for immunoprecipitation. Washing and processing may require an additional 1 hr.

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Key Reference

Echalier, G. 1997. See above.

An excellent and comprehensive description of Drosophila cell culture protocols that includes methods for generating both primary and continuous cell lines, media formulations, and experimental uses of Drosophila cells.

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Metabolic Labeling of Glycoproteins with Radioactive Sugars

Most proteins synthesized in the secretory apparatus of mammalian cells are glycosylated. These glycoproteins are components of secretory and endocytic organelles, including the endoplasmic reticulum, Golgi complex, plasma membrane, endosomes, and lysosomes. These glycoproteins are also important constituents of the extracellular space. The glycans of these molecules include *N*-linked chains, which are linked to asparagine residues, and *O*-linked chains, which are linked to serine and threonine residues. The latter group includes short mucin-type chains and large glycosaminoglycan chains.

For glycoproteins from cultured cells, structural studies of glycoprotein glycans are best carried out on radioactive molecules prepared by metabolic labeling. This unit describes two methods for the preparation of metabolically labeled glycoconjugates from cultured cells. The first describes pulse-chase labeling (see Basic Protocol). Typically, cells are labeled in a pulse incubation of 5 to 30 min, followed by a chase incubation of up to several hours. Molecules labeled in this way are suitable for initial studies to characterize the types of glycans on a glycoprotein and to gather information about their biosynthesis. As an alternative, a method for long-term labeling is also described (see Alternate Protocol). Because the cells are labeled over one or more generations, uniform labeling of all the sugar residues in a molecule can be achieved, with each residue of a particular type being labeled to the same specific activity. This uniform labeling is important for detailed structural studies; however, only glycoproteins with mature glycans can be prepared using this protocol, so it provides little information about biosynthesis.

The unit also contains support protocols for the liberation of *N*- (see Support Protocol 1) and *O*-linked (see Support Protocol 2) glycans from glycoproteins. These protocols can be used to prepare labeled glycans for characterization and detailed structural analysis.

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by your institution's Radiation Safety Officer (also see *UNIT 7.1* and *APPENDIX 1D*).

PULSE-CHASE LABELING WITH RADIOACTIVE PRECURSORS

It is important to optimize labeling conditions to obtain maximum incorporation of the radioactive precursor. The radioactive precursor must be chosen with care. First, only some sugars—e.g., galactose (Gal), glucose (Glc), glucosamine (GlcNH₂), and mannose (Man)—are incorporated with high efficiency. Others are incorporated poorly because cells do not have pathways for their efficient uptake and utilization. Second, many sugar precursors undergo extensive metabolism within cells. This metabolism may result in the loss of radioactivity from the precursor or conversion to molecules that cannot be incorporated into glycans. The precursor may also be converted into other sugars that are incorporated, resulting in glycans with several different types of radioactive residues. Consequently, choosing an appropriate radioactive precursor will help to ensure that glycans with the label in the desired sugar residues are obtained. In addition, the labeling conditions should be chosen to optimize precursor incorporation into the glycoproteins of interest. For sugars, this is usually achieved by labeling in medium with reduced glucose concentration. Similarly, for sulfate and phosphate labeling, medium deficient in these compounds is used. Finally, it is necessary to make sure that the precursor is

BASIC PROTOCOL

Protein Labeling and Immuno- precipitation

7.8.1

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incorporated throughout the labeling period and to ensure that the cells remain healthy. These issues are discussed in detail elsewhere in the unit (see Commentary).

Metabolic labeling may be carried out either in a tissue culture incubator with a CO₂/air atmosphere or in an air atmosphere. In the former case, bicarbonate-buffered medium is used. In the latter, HEPES-buffered medium is employed, which is usually more convenient for short labelings. In addition, because incubations of radioactive samples do not need to be carried out in a tissue culture facility, use of HEPES-buffered medium limits the amount of lab space that must be certified for use with radioactive materials.

Materials

Growth medium: tissue culture medium supplemented with dialyzed serum (see recipe; use the serum concentration required for growth of the cultured cells being studied)

Tissue culture cells

Labeling medium (see recipe)

Radioactive precursor: [³⁵S]sulfate, [³²P]orthophosphate, or sugar labeled with either ³H or ¹⁴C

Phosphate-buffered saline (PBS; APPENDIX 2A), ice cold

Additional reagents and equipment for experiments optimizing label incorporation (see Critical Parameters)

1. Perform small-scale preliminary experiments to determine incubation conditions that give optimal incorporation of label as described (see Critical Parameters). Based on these experiments, choose the appropriate: (1) radioactive precursor, (2) number of cells, (3) labeling medium, (4) volume of labeling medium, (5) concentration of radioactive precursor, and (6) labeling time.
2. Prepare tissue culture cells in either subconfluent monolayers by plating cells in growth medium at high density 1 to 2 days prior to the experiment, or suspensions.
Suggested plating densities are given in Table 7.8.1.
3. Prepare labeling medium from sterile stocks and warm to 37°C.

For most experiments with radioactive sugars, this will be Glc-free MEM with nonessential amino acids supplemented with ~0.1 mg/ml Glc and 2% to 5% (v/v) dialyzed serum. For labeling with sulfate and phosphate, use medium deficient in unlabeled precursor. Prepare enough medium for washing and labeling the cells with radioactive precursor (Table 7.8.1).

Table 7.8.1 Parameters for Plating and Labeling Cultured Cells

Culture	Cell number ^a	Wash	Label ^b	Chase ^c
<i>Cell monolayers</i>				
16 mm (24-well plate)	1.4 × 10 ⁵	0.2 ml	0.1 ml	0.2 to 0.4 ml
35 mm	6 × 10 ⁵	0.5 ml	0.3 ml	0.5 to 1.5 ml
60 mm	1.5 × 10 ⁶	1 ml	0.7	1 to 3 ml
100 mm	4 × 10 ⁶	3 ml	2 ml	3 to 10 ml
<i>Cell suspensions^d</i>	Not applicable	5 × 10 ⁶ /ml	1.3 × 10 ⁷ /ml	0.5 to 2 × 10 ⁶ /ml

^aFor the Basic Protocol, cell monolayers are plated at the indicated densities 1 to 2 days prior to labeling. The density may have to be adjusted for individual cell lines. When long chases are used the cell density should be reduced. For the long labeling periods required for the Alternate Protocol, lower cell numbers should also be used.

^bThese volumes are suggested for experiments with labeling times of 1 hr or less, such as those of the Basic Protocol. Larger volumes must be used for longer pulse labelings and for the long incubations required in the Alternate Protocol.

^cFor chase incubations, the smaller volumes can be used for short chases. For longer incubations, larger volumes will be required to furnish enough nutrients for the cells.

^dFor cell suspensions, use the indicated cell densities to calculate the appropriate volumes.

4. Prepare a stock of radioactive precursor in warm labeling medium.

Many sugars are supplied in ethanol solution. If this is the case, remove the appropriate volume of solution from the source vial and transfer to a test tube. Place the tube in a beaker of warm water (30° to 40°C) and dry the ethanol under a stream of nitrogen. Dissolve the radioactive sugar in the appropriate volume of labeling medium (Table 7.8.1) and warm to 37°C. For short labeling incubations, this solution need not be sterile. For longer incubations, filter sterilize using a disposable plastic syringe and filter.

Best results are usually obtained with precursor of the highest available specific activity. For ³H-labeled sugars, this is usually 30 to 60 Ci/mmol (1.1 to 2.2 TBq/mmol).

See Critical Parameters, Optimizing labeling conditions, for a discussion of the rationale behind the choice of medium.

5a. *For cell monolayers:* Aspirate the medium and rinse the plate twice with labeling medium.

Suggested volumes are provided in Table 7.8.1.

5b. *For suspension cells:* Centrifuge the cells 5 min at 200 × g, room temperature. Aspirate the supernatant and suspend in warm labeling medium (Table 7.8.1). Repeat once and centrifuge.

6. Add the labeling medium containing the radioactive precursor. For cell suspensions, suspend the cells in the medium. Incubate at the appropriate temperature in a CO₂/air or air atmosphere, depending whether the medium is buffered with bicarbonate or HEPES, respectively.

The incubation time should have been determined previously from pilot experiments (see Commentary and step 1).

7. Remove the labeling medium, saving an aliquot for analysis of precursor utilization, if desired.

It may be possible to reuse this solution to label another cell sample if the supply of nutrients and radioactive precursor are not exhausted during the incubation. In this case, the solution may be used immediately or stored frozen.

8. (*Optional*) If a chase incubation is to be performed, wash the cells once in warm growth medium containing normal levels of Glc. Add warm growth medium and incubate at 37°C for the desired time. Remove the medium, saving it for sample isolation if the glycoprotein of interest is secreted. Chill the cells on ice, wash them several times with ice-cold phosphate-buffered saline (PBS), and harvest.

This growth medium can contain either HEPES or bicarbonate as a buffer, depending on whether or not the chase incubations will be done in a CO₂/air atmosphere. Table 7.8.1 lists suggested volumes.

For studies of glycan biosynthesis samples will usually be collected at several chase times.

In most cases, the glycoprotein of interest is isolated by immunoprecipitation (UNIT 7.2).

LONG-TERM LABELING WITH RADIOACTIVE PRECURSOR

This protocol is useful for obtaining molecules with uniformly-labeled glycans, where all the sugar residues of a particular type are labeled to the same specific activity. This uniform labeling is crucial if the glycans are to be used for detailed structural studies. An additional advantage of this technique is that cells can be labeled with precursors that are taken up poorly by cultured cells, namely fucose (Fuc), mannosamine (ManN), xylose (Xyl), N-acetylglucosamine (GlcNAc), N-acetylmannosamine (ManNAc), and sialic acids. As described above (see Basic Protocol), it is important to optimize the labeling

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conditions to obtain maximum incorporation and to ensure that the cells are healthy (see Commentary). The labeling may be carried out in either bicarbonate-buffered medium in a CO₂-containing atmosphere or in HEPES-buffered medium in air.

Additional Materials (also see *Basic Protocol*)

Disposable plastic syringe and 0.2- μ m syringe filter

1. Carry out small-scale preliminary experiments to optimize the incorporation of label (see Critical Parameters; also see *Basic Protocol*, step 1).
2. In growth medium, prepare cell suspensions, or prepare subconfluent monolayers by plating cells 1 to 2 days prior to the experiment.

The cells must be sparse enough that they will survive the entire labeling period. Suggested plating densities are given in Table 7.8.1.

3. Prepare a stock of radioactive precursor in warm growth medium as described (see *Basic Protocol*, step 4). Sterilize using a disposable plastic syringe and 0.2- μ m syringe filter.
- 4a. *For monolayer cells:* Aspirate the growth medium and replace with medium containing radioactive precursor.
- 4b. *For suspension cells:* Centrifuge cells 5 min at 200 \times g, room temperature. Aspirate the supernatant and suspend in labeling medium containing radioactive precursor.

Suggested volumes are given in Table 7.8.1.

5. Incubate 12 to 48 hr at the appropriate temperature in a CO₂/air or air atmosphere, depending whether HEPES or bicarbonate is the buffer in the labeling medium.

The incubation time should have been determined previously from pilot experiments (see Commentary and step 1).

6. Remove the labeling medium and save if the glycoprotein of interest is secreted. Otherwise, if required, save an aliquot for further analysis.
7. For isolation of cellular glycoproteins, chill the cells by placing on ice. Wash the cells several times with ice-cold PBS and harvest.

In most cases the glycoprotein of interest will be isolated by immunoprecipitation (UNIT 7.2).

**SUPPORT
PROTOCOL 1**

**ENZYMATIC RELEASE OF N-LINKED GLYCANS FROM
GLYCOPROTEINS**

This protocol can be used to prepare *N*-linked glycans from labeled glycoproteins using peptide *N*-glycosidase F, an enzyme that cleaves intact *N*-linked glycans from polypeptides. All mammalian *N*-linked glycans are substrates for this enzyme (Tarentino et al., 1985; Chu, 1986). Since the enzyme will not use native glycoproteins as substrates, the glycoprotein substrates are first denatured with SDS. Excess nonionic detergent (e.g., NP-40) is added prior to digestion with the enzyme to prevent denaturation of the glycosidase.

Materials

Sample (e.g., see *Basic Protocol* or *Alternate Protocol*)
1% (w/v) SDS/0.1 M EDTA/0.5 M 2-mercaptoethanol
200 mM sodium phosphate, pH 8.6 (*APPENDIX 2A*)
10% (w/v) NP-40

**Metabolic
Labeling of
Glycoproteins
with Radioactive
Sugars**

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Peptide *N*-glycosidase F (PNGase F, *N*-glycanase) from *F. meningosepticum* or recombinant enzyme expressed in *E. coli* (Glyko or Roche Diagnostics)

Additional reagents and equipment for immunoprecipitation (UNIT 7.2)

1. Immunoprecipitate the protein of interest from the sample (UNIT 7.2).

The protein of interest may be collected on a solid support, such as agarose beads. Alternatively, proteins can be precipitated from medium or cell lysates as described below (see Support Protocol 2, steps 1 to 3).

Lysate from 1×10^7 cells can be processed using this protocol.

2. Add 20 μ l of 1% (w/v) SDS/0.1 M EDTA/0.5 M 2-mercaptoethanol to the immunoprecipitate, or to the pellet of precipitated protein, in microcentrifuge tubes. Mix well and heat 5 min at 95°C. Cool to room temperature and microcentrifuge briefly at maximum speed to collect the liquid at the bottom of the tube.
3. Add 20 μ l of 200 mM sodium phosphate, pH 8.6, 15 μ l of 10% (w/v) NP-40, and 25 μ l water. Mix.
4. Add 2 U of peptide *N*-glycosidase F. Incubate 18 hr at 37°C.
5. Mix and microcentrifuge briefly at maximum speed to collect the liquid at the bottom of the tube.

This solution contains free glycans. These can be characterized by chromatography or subjected to detailed structural analyses. The glycans may be frozen at -20°C and stored indefinitely; their lifetime is limited only by the decay of the radioisotope.

RELEASE OF *O*-LINKED GLYCANS FROM GLYCOPROTEINS

Incubation of glycoproteins with alkaline sodium borohydride cleaves *O*-linked glycans from glycoproteins and reduces them to the corresponding oligosaccharide alcohols (Carlson, 1968). These released glycans are suitable for structural analysis. The protocol can be performed on culture medium, cell lysates, or immunoprecipitated proteins attached to a solid support (e.g., agarose beads).

Materials

Sample (e.g., see Basic Protocol or Alternate Protocol)
20% (w/v) trichloroacetic acid (i.e., 20 g TCA in 89.4 ml H₂O), ice cold
80% (v/v) aqueous acetone, ice cold
1 M NaBH₄/50 mM NaOH
4 M acetic acid
Cation exchange resin: AG 50-X8 (Bio-Rad), H⁺ form
Methanol
Nitrogen gas
Disposable chromatography column (e.g., Bio-Rad Poly-Prep columns)
Glass test tubes
Lyophilizer or SpeedVac evaporator

NOTE: Steps 1 to 3 apply only for whole cells or cell extracts. For immunoprecipitates on a solid support, begin at step 4.

1. Precipitate proteins from the sample in microcentrifuge tubes by adding an equal volume of ice-cold 20% (w/v) trichloroacetic acid and incubating 1 hr on ice. Microcentrifuge 10 min at $14,000 \times g$, 4°C. Discard the supernatant.

If the sample contains a nonionic detergent such as NP-40, it will precipitate at this step.

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2. Add 200 μl ice-cold 80% (v/v) aqueous acetone. Vortex, microcentrifuge, and discard the supernatant. Repeat.

This step will extract the trichloroacetic acid and nonionic detergent from the sample.

CAUTION: The wastes from immunoprecipitation are mixed chemical wastes and should be handled appropriately (see Radiation Safety Officer and APPENDIX 1D).

3. Let the tubes air dry at room temperature to remove most of the acetone. Take care not to let the sample dry completely.
4. Add 200 μl of 1 M NaBH_4 /50 mM NaOH. Incubate 16 hr at 45°C. Vortex several times during the incubation.

For immunoprecipitates, the NaBH_4 solution is added to the pellet of immunoprecipitated glycoprotein on a solid support.

5. Add 16.6 μl of 4 M acetic acid to destroy excess NaBH_4 .

The sample may foam as hydrogen gas is released from decomposition of the NaBH_4 .

6. Prepare 0.9 ml cation exchange resin in disposable columns. Rinse with 3 ml water.
7. Load each sample onto a column and collect the flowthrough in glass test tubes. Rinse the column with 0.9 ml water, pooling this with the flowthrough.
8. Dry the samples using a lyophilizer or Speed-Vac evaporator.
9. Dissolve each residue in 50 μl methanol. Place the tubes in warm water and evaporate the methanol under a stream of nitrogen. Repeat this step 3 more times.

This treatment converts the boric acid derived from the NaBH_4 to methyl borate, which is removed by evaporation.

10. Dissolve the glycans in water and transfer the liquid to a microcentrifuge tube.

This solution contains free glycans. These can be characterized by chromatography or subjected to detailed structural analyses. The glycans may be frozen at -20°C and stored indefinitely. Their lifetime is limited by the decay of the radioisotope.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Dialyzed serum

Place serum in dialysis tubing and dialyze fetal bovine serum against a 20-fold excess of 0.15 M NaCl at 4°C with stirring (APPENDIX 3D). Change the dialysis buffer after 12 and 24 hr. Remove the serum from the dialysis bag and filter sterilize into sterile containers. Store up to 1 month at 4°C or up to 1 year at -80°C .

Labeling medium

Formulas for media deficient in glucose, sulfate, or phosphate are given in Table 7.8.2. These are used for labeling with radioactive sugars, sulfate, or phosphate, respectively. To prepare these media, add the liquid components to water (80% of the final volume). Add the dry components and stir until dissolved. Use either 25 mM sodium bicarbonate or 20 mM HEPES as a buffer, depending on whether or not the medium will be used in the presence of CO_2 . Because the calcium and magnesium salts can form insoluble precipitates, add them last after the other salts have dissolved. Antibiotic and antimycotic agents (e.g., penicillin and streptomycin) can be added from liquid concentrates if desired. Adjust the pH to 7.1 to 7.2

Table 7.8.2 Deficient MEM with Nonessential Amino Acids for Incorporation of Sugar, Phosphate, or Sulfate Precursors

Ingredient	Glucose free	Phosphate free	Sulfate free
NaCl	3.4 g	3.4 g	3.4 g
KCl	0.2 g	0.2 g	0.2 g
NaH ₂ PO ₄ ·2 H ₂ O	79 mg	—	79 mg
CaCl ₂ ·2 H ₂ O	0.13 g	0.13 g	0.13 g
MgSO ₄ ·7 H ₂ O	0.1 g	0.1 g	—
MgCl ₂ ·6 H ₂ O	—	—	0.1 g
Phenol Red	5 mg	5 mg	5 mg
MEM 100× vitamins	5 ml	5 ml	5 ml
MEM 100 × nonessential amino acids	5 ml	5 ml	5 ml
MEM 50× amino acids (with L-glutamine)	10 ml	10 ml	10 ml
D-glucose	—	0.5 g	0.5 g
<i>Buffer^a</i>			
NaHCO ₃	1.1 g	1.1 g	1.1 g
HEPES	2.38 g	2.38 g	2.38 g

^aUse sodium bicarbonate if incubations will be performed in the presence of CO₂; otherwise, use HEPES.

with 1 M HCl or 1 M NaOH and add water to bring the volume to 500 ml. Because this medium is prepared from separate components, the initial pH may be farther from the desired value than for most powdered media. Sterilize by filtration into sterile 100-ml bottles using a 0.2- μ m bottle-top filter. Store up to 3 months at 4°C.

Labeling with radioactive precursor is usually carried out in medium with a reduced concentration of the precursor and related compounds. Some deficient media can be purchased from suppliers. These media are also readily prepared from standard chemicals and liquid concentrates of MEM components, which are available from most suppliers of tissue culture media (e.g., Life Technologies, Sigma-Aldrich). Other reagents should be tissue-culture grade.

COMMENTARY

Background Information

The glycans are important constituents of glycoproteins and proteoglycans. They provide important functional groups of mature molecules. The glycans are also important in glycoprotein folding and in glycoprotein transport along the secretory and endocytic paths. Glycoprotein glycans are synthesized in a complex process that begins in the endoplasmic reticulum and ends in the Golgi complex (Kornfeld and Kornfeld, 1985; Lidholt, 1997; Perez-Vilar and Hill, 1999). The biosynthesis of these glycans is not only interesting in its own right, it has also been instrumental in understanding the compartmentation and function of the secretory pathway (see *UNITS 15.1 & 15.2*).

Initial studies of the glycans on a glycoprotein of interest are usually performed by study-

ing molecules metabolically labeled in the protein backbone with radioactive amino acids (e.g., [³⁵S]methionine; *UNIT 7.1*). Typically a protein is labeled in a pulse-chase protocol. The protein is then immunoprecipitated (*UNIT 7.2*) and the size of the protein is monitored by SDS gel electrophoresis (*UNITS 6.1 & 15.2*). As the glycans are assembled during transport through the ER and Golgi, the apparent size of the glycoprotein increases on the gel. Additional information may be obtained using several modifications of this experiment.

First, treatment of cells with inhibitors of glycosylation provides information about the nature of the glycans. For example, tunicamycin, which inhibits the synthesis of *N*-linked glycans, has been widely used to test whether these glycans are added to proteins (Elbein, 1987).

Second, glycoproteins may be treated with specific endo- and exoglycosidases after they are isolated from the cells (UNIT 15.2). For example, the endoglycosidase peptide *N*-glycanase, which cleaves *N*-linked glycans from the peptide backbone can be used to test for the presence of these structures. Endoglycosidase H is also used widely because it cleaves immature *N*-glycans but not mature complex-type structures. Similarly, exoglycosidases, including neuraminidases, galactosidases, and mannosidases can be used both separately and in combination to test for the presence of sialic acid, Gal, and Man residues at the nonreducing ends of glycans. In all these cases, the effects of the treatments are assessed by comparing the mobility of treated and untreated samples by gel electrophoresis (UNIT 15.2). Some of these procedures can also be carried out on unlabeled proteins detected by immunoblotting with a specific antibody (UNIT 6.2).

Although much can be learned about protein glycosylation by this approach, these methods cannot provide information about the details of glycan structure. This analysis requires molecules with radioactively-labeled glycans.

Critical Parameters

Choosing a radioactive precursor

The choice of a radioactive label is determined by many issues. Obviously, the label must be a precursor of one of the sugar residues in the glycan of interest. The complex pathways of sugar uptake and metabolism and their relation to glycoprotein synthesis must also be considered (Fig. 7.8.1).

First, the position of the sugar in the glycan should be considered. If the goal is to study the maturation of a glycan during biosynthesis, sugars close to the reducing end of the structure are the desirable label, because these will be added first. All subsequent glycans in the pathway will be labeled. Conversely sugars that are added late in the pathway will only be found in mature structures.

Second, the ability of cells to take up and incorporate radioactive precursors must be considered. Some precursors are taken up rather poorly. These include fucose, ManN, Xyl, GlcNAc, ManNAc and sialic acids. Other sugars are taken up efficiently. This group includes Gal, Man, and GlcN; however, their uptake and incorporation is competitive with unlabeled Glc in the medium. Consequently, the incorporation of sugars in this group can be significantly improved by labeling in medium with

reduced Glc. On the other hand, labeling in Glc-free medium must be avoided because Glc starvation can affect the synthesis of sugar precursors by the cells, which results in the synthesis of glycoproteins with altered glycans (Rearick et al., 1981).

Third, the conversion of intermediates within the cells must be considered carefully. This problem can be a serious one for sugar labeling because many precursors undergo extensive metabolism within mammalian cells. The metabolic pathways are summarized in Figure 7.8.1. For example, labeled GlcN is incorporated into glycoproteins as labeled GlcNAc, GalNAc, and sialic acids. This situation is different from the one encountered when cells are labeled with amino acids. In the latter case, the essential amino acids are effective precursors (e.g., ³⁵S-methionine) because these compounds cannot be synthesized and cells carry out only limited conversions. In some cases the problem of sugar metabolism can be overcome by choosing precursors whose radioactivity is lost upon conversion to other sugars. For example [2-³H]Man is incorporated into glycans primarily as Man, with a small fraction as Fuc. The other pathway for metabolism of this sugar produces unlabeled fructose 6-phosphate, with loss of the radioactivity as ³H₂O. Specific labeling has also been obtained using cultured cell lines mutated in sugar metabolism. For example, the Chinese hamster ovary (CHO) cell mutant ld1D is defective in UDP-Gal epimerase (Kingsley et al., 1986), which interconverts both the UDP-Glc/UDP-Gal and UDP-GlcNAc/UDP-GalNAc pairs. As a consequence, radioactive Gal and GalN are specific labels in these cells because they are not converted to other sugars and because *de novo* synthesis is blocked. They are incorporated with high specific activity as Gal and GalNAc, respectively.

Optimizing labeling conditions

Obtaining the maximum incorporation into the glycoprotein of interest requires optimizing the conditions used for metabolic labeling. The optimal conditions will vary depending on which protocol is used (see Basic Protocol and Alternate Protocol). The conditions used will depend on the abundance of the glycoprotein that is being studied. Labeled glycans from abundant glycoproteins can be prepared in adequate amounts from small cultures. Conversely, glycoproteins of low abundance will require larger cultures, larger amounts of label, and

Basic Protocol, it is advisable to use these precursors in medium with reduced Glc (0.1 mg/ml). Lowering the Glc concentration further is not advisable because Glc starvation can affect glycan synthesis (Rearick et al., 1981). Serum that has been dialyzed to remove low-molecular-weight constituents should also be used. Preparation of dialyzed serum and medium with reduced Glc is described in Reagents and Solutions. The long-term labeling in the Alternate Protocol is usually carried out in complete medium to provide sufficient nutrients.

The cells must not exhaust the supply of Glc or other nutrients in the medium. As a rule of thumb for determining the nutrient supply in the medium, the author estimates that a confluent culture ($\sim 1 \times 10^6$ cells/ml) in normal medium (1 g/liter Glc) will exhaust its Glc in ~ 24 hr. This can be used to estimate the length of time available for cells under labeling conditions taking into account the increased cell concentration and reduced Glc concentration in the medium.

4. *Concentration of radioactive precursor.* Small-scale labeling can be carried out to determine the optimal concentration and specific activity (Diaz and Varki, 1995). Radioactive precursor is added to all cultures. In addition, cultures are supplemented with increasing concentrations of unlabeled precursor. The incorporation of radioactivity is determined and plotted against the total concentration of precursor. At low concentrations, incorporation will not depend strongly on concentration. At higher concentrations, incorporation will decrease linearly with concentration. The break point on the curve is the concentration that is saturating for uptake and incorporation. Choose a concentration 1.5- to 2-fold higher than this for incorporation.

5. *Labeling time.* Labeling time is determined by several factors. For pulse labeling (see Basic Protocol) the labeling time must be short enough to generate a homogeneous cohort of labeled molecules at the end of the pulse; however, longer labelings will yield greater incorporation. Consequently, the goal is to select the longest labeling time that still yields a set of molecules with similar glycans in a single cellular location. Most glycoproteins leave the ER within 10 to 40 min of synthesis and traverse the Golgi within a few minutes. The overall time required to traverse the secretory pathway is 30 to 90 min. These times can be used as a guide for choosing pulse labeling times. For long-term labeling (see Alternate Protocol), the

turnover rate of the glycoprotein under study is a key factor. Since most cellular proteins have lifetimes of 12 to 24 hr, this limits the yield in long labelings, when the degradation of labeled protein approaches the synthesis of new molecules.

Small-scale pilot experiments should be performed to find optimal labeling conditions. The incorporation should increase with time, demonstrating an adequate supply of nutrients and radioactive precursor. For the Basic Protocol, glycan structure should be monitored to demonstrate that a homogeneous cohort of molecules is generated at the end of the pulse. Finally, the survival of the cells should be monitored by microscopy, trypan blue exclusion, and growth (*UNIT 1.1*) after returning to normal medium to ensure that the cells survive under the labeling conditions. Once optimal conditions have been defined, the amount of labeled glycoprotein recovered should be used to determine how to scale up conditions for preparative experiments.

Anticipated Results

The amount of labeled protein depends on the planned experiments. If the protein will be analyzed by electrophoresis, then only 200 to 1000 cpm/sample is required. If structural studies will be carried out, larger amounts (1×10^3 to 1×10^4 cpm) are required. The amount of labeled protein recovered will depend on the issues discussed in the preceding section. In addition to these factors, the choice of radioisotope is important. Because of their longer half lives, ^3H - and ^{14}C -labeled compounds have lower specific activity than ^{35}S and ^{32}P that are used to label proteins and nucleic acids. As an example, $\sim 1 \times 10^4$ cpm were recovered in labeled transferrin receptor when 5×10^6 K562 human leukemia cells were labeled with 150 μCi [$2\text{-}^3\text{H}$]mannose for 45 min in 375 μl (Snider and Rogers, 1986).

As in all pulse-labeling experiments, the incorporation of radioactivity into products occurs with a lag time, while the radioactive label enters intracellular precursor pools. The turnover time of these pools determines the lag time that precedes incorporation into the product of interest. This turnover will also determine the continued incorporation at the beginning of the chase as radioactivity in the precursor pools is incorporated into product. This issue can be an important one for labeling glycans. For example, during the incorporation of [^3H]Man into *N*-linked glycans, the mannose is transferred via GDP-Man and dolichol-linked intermedi-

ates. Because the turnover time of these pools is 5 to 10 min in mammalian cells, there is a significant lag time before incorporation begins. In addition, incorporation continues to increase for 5 to 10 min after the beginning of a chase.

Time Considerations

Pulse-chase experiments take up to 24 hr, depending on the length of the chase. In a typical experiment, the pulse-chase labeling can be completed, cells can be lysed, and immunoprecipitation of the desired protein can be begun in a single day. Ten to twenty samples can be labeled and processed at once by one person. If very short pulses and chases are performed, it may not be possible to perform the required manipulation on large numbers of samples. In this case, it may be necessary to do the labeling on two smaller sets of samples. Long-term labeling experiments require 12 to 48 hr. Release of N- and O-linked glycans each requires ~24 hr. Groups of 10 to 20 samples can be handled easily by one person.

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An excellent discussion of monosaccharide incorporation, with methods for determining the specific activity of incorporated label.

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Analysis of Oxidative Modification of Proteins

UNIT 7.9

Reactions between protein molecules and reactive oxygen species (ROS) often lead to the modification of certain amino acid residues such as histidine, lysine, arginine, proline, and threonine, forming carbonyl derivatives. Carbonylation of proteins has thus often been employed for the quantification of generalized protein oxidation. Besides carbonylation, other types of oxidative damage that have been investigated in depth are the modifications of cysteine, tyrosine, and aspartate, or asparagine residues. Except for cysteine residues, whose oxidation is often determined by the loss of protein thiol groups, quantification of oxidative damage to tyrosine, and aspartate residues is usually carried out by the measurement of specific oxidation products such as dityrosine, nitrotyrosine (when nitrogen species are the oxidants), and isoaspartate.

This unit provides a variety of protocols for the determination of the protein oxidation products indicated above. A method for the quantification of protein carbonyls using spectrophotometric determination with a carbonyl specific reagent, 2,4-dinitrophenylhydrazine (DNPH), is described (see Basic Protocol 1), as are the details of immunoblot detection using anti-DNP antibodies to detect the carbonylation of specific proteins (see Support Protocol 1). Radiolabeling detection of total and specific proteins using tritiated sodium borohydride, another quantification method, is also included (see Basic Protocol 2 and Support Protocol 2) in this unit. Procedures are outlined for the selective determination of protein thiol groups radiolabeled with [¹⁴C] iodoacetamide followed by gel electrophoresis (see Basic Protocol 3). Procedures for dityrosine measurement by GC/MS are described (see Basic Protocol 4), as are preparation of dityrosine standards and nitrotyrosine detection by competitive ELISA (see Support Protocols 3 and 4). Methods for the detection of isoaspartate formation in proteins/peptides, analyzed by a methyl transfer reaction catalyzed by protein-L-isoaspartyl methyltransferase (PIMT), using [³H]methyl-S-adenosyl-L-methionine (SAM) as the methyl donor are given (see Basic Protocol 5), and the details of the different buffers needed for the electrophoretic separation of proteins containing isoaspartate residues are described (see Support Protocol 5).

NOTE: Tissue collection and preparation must be carried out in buffers supplemented with antioxidants, such as 100 μM diethylenetriaminepentaacetic acid (DTPA) and 1 mM butylated hydroxytoluene (BHT). The use of antioxidant buffers may be especially critical for the detection of trace amounts of oxidized products formed during aging or under oxidative stress. In addition, all buffers should be bubbled with nitrogen before use.

SPECTROPHOTOMETRIC QUANTITATION OF PROTEIN CARBONYLS USING 2,4-DINITROPHENYLHYDRAZINE

**BASIC
PROTOCOL 1**

Protein carbonyl groups can specifically react with 2,4 dinitrophenylhydrazine (DNPH) to generate protein conjugated hydrazones (protein-DNP) which have a peak absorbance around 360 nm. The use of DNPH thus provides an index for the quantification of protein carbonyl content in protein mixtures or purified proteins.

**Protein Labeling
and Immuno-
precipitation**

7.9.1

Materials

DNPH solution (see recipe)
Protein solution
2 M HCl
20% (v/v) trichloroacetic acid solution, ice-cold (TCA; see recipe)
1:1 (v/v) ethanol/ethyl acetate
0.2% (w/v) SDS/20 mM Tris·Cl, pH 6.8 (APPENDIX 2A)
Bicinchoninic acid protein assay kit (BCA; Pierce Co.)
Bovine serum albumin (BSA)

Benchtop centrifuge
Branson 2200 sonicator

1. Add 200 μ l of DNPH solution to 1 ml of protein solution. Incubate the mixture at room temperature for 60 min. Prepare a blank by adding 200 μ l of 2 M HCl without DNPH to one sample. Incubate under the same conditions.

For tissue samples, start with 0.5 to 1.0 mg/ml protein. If higher protein concentrations are used for DNPH treatment, it will be very hard to dissolve the pellet after washing off the free DNPH (see Critical Parameters and Troubleshooting).

2. Add 1.2 ml of 20% trichloroacetic acid (TCA) solution to the DNPH-treated protein solution and blank, then incubate on ice 10 min. Centrifuge the sample in a benchtop centrifuge for 10 min at 750 to 1000 \times g, room temperature.
3. Wash the pellet by adding 3 ml 1:1 (v/v) ethanol/ethyl acetate, followed by centrifugation in a benchtop centrifuge for 10 min at 750 to 1000 \times g at room temperature. Sonicate at full power, room temperature, in a Branson 2200 sonicator until the pellet is completely broken up.
4. Repeat step 3 twice. Solubilize the final pellet in 1 ml 0.2% (w/v) SDS/20 mM Tris·Cl, pH 6.8.

If the carbonyl content of lipoproteins is to be determined, use a denaturing buffer of 3% (w/v) SDS/150 mM sodium phosphate buffer, pH 6.8, to dissolve the final pellet (see Critical Parameters and Troubleshooting).

5. After the pellet is completely dissolved, pipet 100 μ l of the protein solution for protein assay using the BCA kit. Use bovine serum albumin (BSA) as the protein standard.

Initially, 6 M guanidine-HCl was used to dissolve the protein pellet, but samples dissolved in such a high concentration of guanidine-HCl are not suitable for further analysis by other techniques such as polyacrylamide gel electrophoresis (SDS-PAGE, UNIT 6.1).

6. Scan the sample in a spectrophotometer from 320 nm to 450 nm. Use the peak absorbency around 360 nm to calculate the carbonyl content. Use the protein samples treated with HCl, but not with DNPH, as blanks.

The extinction coefficient (ϵ) for DNPH is 22,000 $M^{-1}cm^{-1}$. Protein carbonyl content (nmol/mg protein) = (absorbance \times 10⁶/22,000)/mg protein = absorbance \times 45.45/mg protein.

7. Identify specific carbonylated proteins by SDS-PAGE (UNIT 6.1) and immunoblotting (see Support Protocol 1, also see UNIT 6.2).

IMMUNOBLOT DETECTION OF PROTEIN CARBONYLS

To identify specific proteins that are carbonylated, DNP-treated proteins can be further separated by gel electrophoresis according to standard techniques. Once the proteins are electrotransferred from the gel to a PVDF membrane, protein-bound DNP in individual proteins can be probed by the use of anti-DNP antibodies (also see *UNIT 6.2*).

Materials

DNP-treated proteins (Basic Protocol 1)
5% (w/v) nonfat dry milk in Tris-buffered saline with Tween-20 (TBST; see recipe)
Primary antibody (anti-DNP antibody; Sigma)
Secondary antibody: may be horseradish peroxidase-conjugated; select on the basis of nature of primary antibody
Tris-buffered saline with and without Tween-20 (TBS and TBST; see recipe)
ECL detection solution (Amersham)

Minigel electrophoresis unit and transfer unit (Bio-Rad; also see recipe for minigel recipes in *UNIT 6.1*)
Immobilon-P membranes (Millipore)
UV-transparent plastic wrap
X-ray film

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), staining gels with Coomassie blue (*UNIT 6.6*), and electroblotting proteins onto membranes (*UNIT 6.2*)

1. Separate DNP-treated proteins by SDS-PAGE in a minigel electrophoresis unit (*UNIT 6.1*).

DNP-treated protein samples dissolved in 0.2% SDS/20 mM Tris buffer, pH 6.8 can be directly mixed with SDS-PAGE loading buffer and analyzed by SDS-PAGE.

*Two gels are usually run simultaneously, one for protein staining by Coomassie blue (*UNIT 6.6*), and the other for immunoblot detection.*

2. Electroblot proteins from polyacrylamide gel to Immobilon-P membrane using a transfer unit per manufacturer's instructions.

*For an efficient transfer, perform tank transfer rather than semidry transfer (see *UNIT 6.2* for additional details).*

3. Block the membrane by immersing it in 5% (w/v) nonfat dry milk TBST for at least 1 hr at room temperature.
4. Wash the membrane with at least 50 ml TBST for 10 min. Repeat twice.
5. Immerse the membrane in primary antibody solution for at least 1 hr.

*Primary antibody solution is prepared by diluting the primary antibody in TBST solution containing 0.2% (w/v) BSA. Dilution of anti-DNP antibody is highly variable. A range of 1:1,000 to 1:10,000 is recommended (see *Critical Parameters and Troubleshooting*). Overnight incubation at 4°C usually gives better results. Do not reuse the antibody.*

6. Wash the membrane with at least 50 ml TBST three times, each time for 10 min.
7. Incubate the membrane with secondary antibody at room temperature for at least 3 hr.

*Antibody is diluted in TBST solution containing 0.2% (w/v) BSA. Secondary antibody dilution usually ranges between 1:10,000 and 1:25,000 (see *Critical Parameters and Troubleshooting*). Do not reuse the antibody.*

8. Wash the membrane three times with at least 50 ml TBST, each time for 10 min.
9. Wash the membrane twice with at least 50 ml TBS (no Tween 20), each time for 5 min.

10. Immerse the membrane with ECL detection solution for 1 min at room temperature.

ECL detection solution comes with the enhanced chemiluminescence (ECL) detection kit from Amersham.

11. Drain off excess detection solution and wrap membrane in plastic wrap. Gently smooth out any air bubbles. Immediately expose the membrane to X-ray film.

Film is usually exposed to the membrane for 0.5 to 2 min.

During immunoblot detection of protein carbonyls, it is preferable to include both a positive and a negative control on the SDS-PAGE. A positive control can be BSA oxidized by an oxidant such as hypochlorite followed by DNPH treatment as described (see Basic Protocol 1). A negative control is usually the sample not treated with DNPH. The inclusion of a positive control will indicate whether the anti-DNP antibody reacts properly, while the inclusion of a negative control will identify any proteins that cross-react with anti-DNP antibody. Occurrence of a cross-reaction between a protein and the antibody indicates that immunoblotting is not DNP-dependent for this particular protein. In such a case, anti-DNP antibodies from a different source may have to be tried.

BASIC PROTOCOL 2

QUANTITATION OF PROTEIN CARBONYLS DERIVATIZED WITH TRITIATED SODIUM BOROHYDRIDE

When protein concentration is a limiting factor, or when the studied proteins, such as heme-containing proteins, have absorption maxima around 360 nm, protein carbonyls cannot be determined by the use of DNPH. In such cases, tritiated sodium borohydride, which is able to convert protein carbonyl groups into protein-bound ethanol groups, can be used. Because of the introduction of tritium onto oxidized proteins, the method provides a quantitative measurement of protein carbonyl content.

Materials

Protein solution
3 M Tris·Cl, pH 8.6 (APPENDIX 2A)
0.5 M EDTA, pH 8.0 (APPENDIX 2A)
[³H]NaBH₄ working solution (see recipe)
2 M HCl
20% (v/v) trichloroacetic acid solution, ice-cold (TCA; see recipe)
1:1 (v/v) ethanol/ethyl acetate
0.2% (w/v) SDS/20 mM Tris·Cl, pH 6.8 (APPENDIX 2A)
0.5% (w/v) SDS/0.1 M NaOH
BCA protein assay kit (Pierce)
Scintisafe Plus 50% cocktail (Fisher Scientific.)

Benchtop centrifuge
Scintillation vials

CAUTION: Perform all incubations in a hood as tritium gas may be released during the reaction. When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see UNIT 7.1 and APPENDIX 1D).

1. Prepare a 1-ml reaction mixture containing 86 mM Tris·Cl, pH 8.6 (add from 3 M stock), 0.86 mM EDTA (add from 5 M stock), 50 mM tritiated sodium borohydride (add from working solution described in Reagents and Solutions), and 0.5 to 1.0 mg of protein.

86 mM Tris buffer, pH 8.6 is used as the incubation buffer, as the authors have found that if phosphate buffer is used, proteins can be degraded during incubation.

EDTA is included in the reaction mixture so that any potential carbonyl-independent labeling can be prevented.

2. Incubate 30 min at 37°C. Add 200 µl of 2 M HCl to stop the reaction. Add an equal volume of 20% TCA (final concentration 10%) to precipitate the proteins.

HCl also serves to render the alkaline reaction mixture acidic; otherwise, 10% TCA (final concentration) would not be sufficient to precipitate all proteins.

3. Keep the solution on ice for 10 min and then centrifuge 10 min at 750 to 1000 × g, 4°C, in a benchtop centrifuge.
4. Wash the protein pellet with 1:1 ethanol/ethyl acetate at least three times.
5. Dissolve the final pellet in 1 ml 0.5% SDS/0.1 M NaOH. Determine protein concentration for each sample using a BCA protein kit.

Determination of protein concentration is necessary because the recovery after washing with ethanol/ethyl acetate is rarely 100% (see Critical Parameters and Troubleshooting).

6. Count the radioactivity of each sample by adding 4 ml Scintisafe Plus 50% cocktail to the sample and counting on a scintillation counter, or quantitate by electrophoretic separation and excision of a specific protein band (see Support Protocol 2).

For the determination of the carbonyl content in a protein mixture, the sample can be counted right after the addition of scintillation cocktail. For the quantitation of the carbonyl content of a specific individual protein, the protein has to be separated from other proteins by means of SDS-PAGE, then processed as described in Support Protocol 2.

GEL ELECTROPHORETIC QUANTITATION OF PROTEIN CARBONYLS DERIVATIZED WITH TRITIATED SODIUM BOROHYDRIDE

SUPPORT PROTOCOL 2

To quantitate the carbonyl content of a specific protein in a protein mixture, as identified by immunoblot technique, protein samples derivatized with tritiated sodium borohydride can be separated by SDS-polyacrylamide gel electrophoresis (UNIT 6.4). The target protein band on the gel can then be excised and dissolved in 30% hydrogen peroxide, and incorporated radioactivity measured by the use of a scintillation counter.

Additional Materials (also see Basic Protocol 2)

Tritiated protein sample (see Basic Protocol 2)
30% (v/v) hydrogen peroxide

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and staining gels with Coomassie blue R-250 (UNIT 6.6).

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see UNIT 7.1 and APPENDIX 1D).

1. Label protein samples with [³H]NaBH₄ (see Basic Protocol 2). Mix the labeled protein sample with SDS-PAGE loading buffer.

TCA precipitation and washing with organic solvent is not needed.

2. Heat the sample 5 min at 95°C. Separate the tritiated protein sample by SDS-PAGE.
3. Stain the gel with Coomassie blue R-250 (UNIT 6.6) and destain until the bands can be clearly visualized.
4. Excise each band with a razor blade.

Protein Labeling and Immuno- precipitation

7.9.5

- Put the excised gel bands in scintillation vials; incubate for 2 hr at 60°C to dry the gel slices.
- Add 1 ml of 30% hydrogen peroxide to each vial and incubate for 24 hr at 60°C.
- Add 4 ml Scintisafe Plus 50% cocktail and keep the vials at 4°C for at least 12 hr.
Use Scintisafe Plus 50% cocktail because it is specifically designed for gel slices with solubilizers such as hydrogen peroxide. Other cocktails, such as Scintiverse BD, become emulsive after mixing with the solubilized gels, which can decrease counting efficiency.
- Count radioactivity with a scintillation counter.

GEL ELECTROPHORETIC ANALYSIS OF PROTEIN THIOL GROUPS LABELED WITH [¹⁴C] IODOACETAMIDE

To identify specific proteins in a tissue sample or a protein mixture exhibiting loss of thiol groups due to oxidative damage, the thiols can be labeled with [¹⁴C] iodoacetamide and analyzed by gel electrophoresis. The gels, after being dried onto filter papers, can be autoradiographed. Loss of thiol groups is inversely correlated with radioactive intensity shown by autoradiography (UNIT 6.3). Oxidized proteins thus identified can be further quantitated by the use of liquid scintillation counting technique, exactly as described in Support Protocol 2 of this unit.

Materials

- Protein sample
- 1% (w/v) SDS/0.6 mM Tris·Cl buffer, pH 8.6 (APPENDIX 2A)
- 2-mercaptoethanol, neat
- Nitrogen gas
- 500 mM [¹⁴C] iodoacetamide, 1 μCi/ml (Amersham)
- 500 mM nonradiolabeled iodoacetamide
- SDS-PAGE gels for Bio-Rad Mini gel system (see recipe):
 - 10% resolving gel
 - 4% stacking gel
- 10% (v/v) trichloroacetic acid (see recipe)
- Whatman 3MM filter paper
- X-ray film

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and staining gels with Coomassie blue R-250 (UNIT 6.1).

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see UNIT 7.1 and APPENDIX 1D).

- Dissolve protein sample (up to 1 mg) in 1 ml of 1% SDS/0.6 M Tris·Cl, pH 8.6.
The inclusion of 1% SDS is meant to completely denature the protein and expose buried thiol groups.
- Add 10 μl of 2-mercaptoethanol and incubate under nitrogen gas for 3 hr at room temperature.
- Add 100 μl colorless 500 mM [¹⁴C]iodoacetamide solution to a final concentration of 1 μCi/ml, using 500 mM freshly prepared, nonradiolabeled iodoacetamide, in distilled water, to dilute the [¹⁴C]iodoacetamide to the proper level of radioactivity. Incubate at 37°C for 30 min with constant gentle agitation.

CAUTION: Iodoacetamide is intrinsically unstable in light, especially in solution; reactions should therefore be carried out in the dark.

Iodoacetamide solutions must be colorless. A yellow color indicates the presence of iodine, which will quickly oxidize protein thiol groups and prevent labeling. Free iodine can also modify tyrosine residues.

The use of nonradiolabeled iodoacetamide will ensure an excess of the reagent over total thiol groups and a complete labeling.

4. Resolve proteins by SDS-PAGE with a 10% separating gel and 4% stacking gel using the Laemmli gel system (UNIT 6.1). After proteins are adequately separated, fix the gel in 10% TCA for 60 min. Stain with Coomassie blue R-250 and destain (UNIT 6.6).

The sample incubation prior to loading should be done at 95°C for 5 min.

With method described in this protocol, actual radioactivity counts can be obtained for any given samples, which can be used for statistical data analysis. A phosphor imager may not be appropriate in terms of quantitation, as it only gives the relative band intensity and thus may not be able to detect slight differences between samples.

- 5a. *For unknown protein samples:* Dry gels onto Whatman 3MM filter paper and expose to X-ray film for up to two weeks at -70°C.

To identify target proteins, purify the protein using two-dimensional gel electrophoresis (UNIT 6.4) and perform N-terminal microsequencing, followed by a computer database search.

- 5b. *Given target proteins:* Proceed to Support Protocol 2, step 4.

QUANTIFICATION OF PROTEIN DITYROSINE RESIDUES BY MASS SPECTROMETRY

BASIC PROTOCOL 4

In this protocol, trace amounts of dityrosine residues formed in proteins due to oxidative damage are first released by acid hydrolysis. The released dityrosine residues are then analyzed by mass spectrometry following derivatization with heptafluorobutyric anhydride/ethyl acetate. The assay entails the use of radiolabeled dityrosine as the internal standard in order to be quantitative.

Materials

Tissue sample
o,o'-dityrosine internal standards, labeled and unlabeled (see Support Protocol 3)
Nitrogen gas
6 M HCl/1% (v/v) benzoic acid/1% (v/v) phenol
Argon
10% and 0.1% (v/v) TCA solution (see recipe)
50 mM NaHPO₄/100 μM diethylenetriamine pentaacetic acid (DTPA), pH 7.4
25% methanol
1:3 (v/v) HCl/*n*-propyl alcohol
1:4 (v/v) pentafluoropropionic anhydride/ethyl acetate
Ethyl acetate
n-propanol
0.1% (w/v) trifluoroacetic acid (TFA)
Supelclean SPE reversed-phase C-18 column (Supelco)
Hewlett Packard 5890 gas chromatography equipped with a 12-m DB-1 capillary column interfaced with Hewlett-Packard 5988A mass spectrometer

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see UNIT 7.1 and APPENDIX 1D).

Protein Labeling and Immuno- precipitation

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1. Dry tissue sample under nitrogen and add 50 pmol ^{13}C -labeled *o,o'*-dityrosine as an internal standard.
2. Hydrolyze the sample in a tube flushed with an inert gas (i.e., argon) at 110°C for 24 hr in 0.5 ml of 6 M HCl/1% benzoic acid/1% phenol.
3. Supplement the sample with 50 μl 10% (v/v) TCA solution and pass over a reversed-phase C-18 column that has been previously washed with 12 ml of 50 mM NaHPO_4 /100 μM diethylenetriamine pentaacetic acid (DTPA), pH 7.4, followed by 12 ml of 0.1% TFA.
4. Elute amino acids with 25% methanol and dry under vacuum for derivatization.

Dityrosine is stable to acid hydrolysis and ~80% of the internal standard should be recovered from the C-18 column using this procedure.

5. Convert amino acids to carboxylic acid esters by the addition of 200 μl of 1:3 (v/v) concentrated HCl/*n*-propyl alcohol. Heat for 10 min at 65°C.
6. Add 50 μl of 1:4 (v/v) pentafluoropropionic anhydride/ethyl acetate to prepare pentafluoropropionyl derivatives of the amino acids. Heat at 65°C for 30 min.
7. Dry the derivatized samples under nitrogen and redissolve in 50 μl of ethyl acetate.
8. Analyze 1 μl of the sample using a gas chromatograph interfaced with a mass spectrometer with extended mass range. Set the injector and ion source temperature at 250°C and 150°C, respectively. Obtain full scan mass spectra and selected ion monitoring with the *n*-propyl heptafluorobutryl and the *n*-propyl pentafluoropropionyl derivatives of both authentic and isotopically labeled dityrosine in the negative-ion chemical ionization mode with methane as the reagent gas.

The mass spectrum of the n-propyl heptafluorobutryl derivative of dityrosine includes a small molecular ion at mass-to-charge (m/z) 1228 (M^-) and prominent ions at m/z 1208 (M^- -HF) and 1030 (M^- - $\text{CF}_3(\text{CF}_2)\text{CHO}$). Use m/z 1208 to quantify dityrosine.

9. For the analysis of tyrosine, dilute one aliquot of the derivatized amino acid (from step 7) 1:100 (v/v) with ethyl acetate. Inject 1 μl of the sample into the gas chromatograph with a 1:100 split prior to mass analysis. Maintain the initial column temperature of 120°C for 1 min and then increase to 220°C at 10°C/min.

The mass spectrum of the n-propyl heptafluorobutryl derivative of tyrosine reveals prominent ions at m/z 595 (M^- -HF) and 417 [M^- - $\text{CF}_3(\text{CF}_2)\text{CHO}$]. Use m/z 417 to quantify tyrosine.

To ensure that interfering ions are not coeluting with the analyte, monitor the ratio of ion currents of the two most abundant ions of tyrosine and dityrosine in all analyses. Baseline-separate authentic and radiolabeled standards to exhibit retention times identical to those of analytes derived from tissue samples.

SUPPORT PROTOCOL 3

PREPARATION OF *o,o'*-DITYROSINE STANDARD

Dityrosine can be formed by horseradish peroxidase-catalyzed oxidation of tyrosine in the presence of hydrogen peroxide. Dityrosine produced in the mixture can then be purified by chromatographic methods. If radiolabeled dityrosine is to be made, radiolabeled tyrosine should be used as a starting material.

Materials

Horseshradish peroxidase (grade I; Boehringer Mannheim)
0.1 M borate buffer, pH 9.1 (see recipe)
5 mM L-tyrosine (Sigma) or [¹³C₆] L-tyrosine (Cambridge Isotope Laboratories) in
0.1 M borate buffer, pH 9.1
30% (v/v) H₂O₂
2-mercaptoethanol
0.01 M NaOH (APPENDIX 2A)
200 μM borate buffer, pH 8.8: diluted from 0.2 M borate buffer (see recipe) with
H₂O
2.75 × 19.5-cm DEAE cellulose chromatography column (Bio-Rad)
20 μM NaHCO₃, pH 8.8 (see recipe)
Concentrated and 100 mM formic acid
100 mM NH₄HCO₃
Benchtop centrifuge
4 × 34.5-cm BioGel P-2 column (200-4-mesh; Bio-Rad)

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see UNIT 7.1 and APPENDIX 1D).

1. Mix 10 mg horseradish peroxidase with 500 ml of 5 mM tyrosine solution prepared in 0.1 M borate buffer, pH 9.1.

Use [¹³C₆]tyrosine to prepare o,o'-[¹³C₁₂]dityrosine if radiolabeled dityrosine is needed. Follow guidelines on the use and disposal of radioactive materials.

2. Add 142 μl of 30% H₂O₂ and swirl the solution briefly. After incubation at room temperature for 30 min, add 175 μl of 2-mercaptoethanol to the reaction mixture. Immediately freeze the solution in liquid nitrogen and lyophilize to dryness.
3. Dissolve the lyophilized substance in 250 ml of distilled water and adjust the pH to 8.8 with a few drops of 0.01 M NaOH. Load the solution onto a 2.75 × 19.75-cm DEAE column that has been pre-equilibrated with 20 μM NaHCO₃, pH 8.8. Elute the column with 200 μM borate buffer, pH 8.8.

Under these conditions, tyrosine and dityrosine will elute in the breakthrough fractions.

4. Pool and lyophilize the dityrosine-containing solutions. Resuspend the lyophilized material in 20 ml of cold water and centrifuge for 15 min at 1000 × g in a benchtop centrifuge. Extract the pellet with 15 ml of water and combine the two supernatants. Adjust the pH to 7.0 with formic acid and incubate at 0°C overnight.
5. Remove any precipitate by centrifuging for 10 min at 1000 × g in a benchtop centrifuge, 4°C. Load the supernatant on a BioGel P-2 column equilibrated with 100 mM NH₄HCO₃. Elute the column with 100 mM NH₄HCO₃ with a flow rate 40 ml/hr. Monitor the elution of dityrosine at 370 nm.

Under these conditions, dityrosine elutes earlier than tyrosine. Therefore, fractions constituting the first peak should be collected. Dityrosine elution can also be monitored by the use of a fluorometer ($\lambda_{ex} = 280 \text{ nm}$; $\lambda_{em} = 300 \text{ nm}$); however, the collected fractions need to be diluted before measurement.

6. Collect dityrosine fractions and lyophilize. Dissolve the lyophilized dityrosine in 20 ml of 100 mM formic acid. Adjust the pH to 2.5 by adding concentrated formic acid. Remove any precipitate that is formed during the pH adjustment by microcentrifuging

for 10 min at $1000 \times g$, room temperature, in a benchtop centrifuge. Load the solution to a BioGel P-2 column (same as above) equilibrated with 100 mM formic acid. Elute the column with 100 mM formic acid, lyophilize the dityrosine-containing solution, and store at -20°C .

The yield of dityrosine should be around 20%.

SUPPORT PROTOCOL 4

ANALYSIS OF PROTEIN-BOUND NITROTYROSINE BY A COMPETITIVE ELISA METHOD

The availability of anti-nitrotyrosine antibodies provides a very sensitive method for the detection of protein-bound nitrotyrosine in tissue samples, namely enzyme-linked immunosorbant assay (ELISA). In this protocol, nitro-BSA is coated onto ELISA plates, and nitrotyrosines are quantitated by the use of anti-nitrotyrosine antibodies. Competition is accomplished by adding either a potentially nitrated protein sample or a known amount of nitrotyrosine (in the form of nitro-BSA) as a standard. Each competes with the coated nitrated proteins for antibody binding. The amount of antibody that binds to the coated nitro-BSA is inversely proportional to the amount of nitrated protein (sample or standard) present in the solution added to the well of the plate.

Materials

10 $\mu\text{g}/\text{ml}$ nitro-bovine serum albumin (nitro-BSA; Alexis Biochemicals) in plate coating buffer
Nitro-BSA standard (see recipe)
ELISA buffers (see recipe):
Plate coating buffer
1 \times phosphate-buffered saline/Tween 20 (PBST)
Blocking buffer
1 \times diethanolamine (DEA) buffer
Protein sample
Primary antibody: mouse anti-nitrotyrosine antibodies (Upstate Biotechnology)
Secondary antibody: rabbit anti-mouse IgG conjugated with alkaline phosphatase
Tris-buffered saline/Tween-20 (TBST; see recipe)
1 mg/ml *p*-nitrophenyl phosphate (5-mg tablets; Sigma) in DEA buffer (see recipe for ELISA buffer)
96-well ELISA plates
Plastic wrap
Plate reader

Prepare the ELISA plate

1. The day before the assay, coat each of the wells of a 96-well ELISA plate that are to be used to assay protein samples with 100 μl of 10 $\mu\text{g}/\text{ml}$ nitro-BSA in plate coating buffer (1 μg nitro-BSA/well). Leave the wells to be used for blanks empty.

Plate coating may have to be optimized (see Critical Parameters and Troubleshooting).

2. Wrap the plate with plastic wrap and incubate at 4°C overnight.
3. Add 100 μl of each protein sample (1 to 10 $\mu\text{g}/\text{ml}$) to be assayed to 100 μl primary antibody diluted 1:500 with blocking buffer, and incubate at 4°C overnight. Add 100 μl of this solution to the designated well. Prepare nitro-BSA competitor the same way for a standard curve.

The nitrotyrosine in BSA and the protein sample will compete with the coating antigen (nitro-BSA in this case) for antibody binding.

The standard curve range is determined with serial dilutions of nitro-BSA between 10 µg/ml to 1 µg/ml. This will give a curve whose middle part is linear and flanked by a plateau curve on either side. The points that signal the start and the end of the linear part should be taken as the range for the standard curve.

A series of dilutions of the protein sample may need to be tried in order to have readings that will fall within the linear range of nitro-BSA competition curve (see *Critical Parameters and Troubleshooting*).

Perform ELISA

4. The day of the assay, rinse the plate three times with 1× PBST, add 300 µl blocking buffer to each well, including blanks, and incubate for 60 min at room temperature.
5. Remove the blocking buffer from the plate and discard. Add 100 µl of a serial dilution of each of the protein samples prepared in primary antibody solution (step 3) to each sample well. Add 100 µl of a serial dilution of nitro-BSA prepared with primary antibody to the designated wells on the same plate to generate a standard curve. Add 100 µl 1× PBST, without antibody, to blank wells.
6. Incubate at room temperature for 2 hr.
7. Rinse three times with TBST. Add 100 µl of secondary antibody diluted 1:1000 with blocking buffer to each sample and standard well. Add 100 µl PBST to blank wells.
8. Incubate at room temperature for 2 hr.

Detect nitrotyrosine

9. Discard reaction solutions from the wells and wash three times with PBST. Add 100 µl of 1 mg/ml *p*-nitrophenyl phosphate to all wells including the blanks.

Remove any air bubbles with a clean needle.

p-Nitrophenyl phosphate is a substrate for alkaline phosphatase and will provide a colorimetric group upon cleavage.

10. After 30 min, clean the bottom of the plate with a Kimwipe and read at 450 nm on a microtiter plate reader. Estimate nitrotyrosine concentration using the nitro-BSA standard curve. Express nitrotyrosine concentration in the protein sample as an equivalent of nitrotyrosine in nitro-BSA.

ENZYMATIC ANALYSIS OF ISOASPARTATE FORMATION

A novel technique for the determination of isoaspartate formation involves the use of protein-L-isoaspartyl methyltransferase (PIMT). PIMT catalyzes methyl transfer reactions using methyl-S-adenosyl-L-methionine (SAM) as the methyl donor. Because PIMT is highly specific for protein isoaspartate residues, the use of tritiated SAM, where the methyl group is radiolabeled, provides a quantitative method for the determination of isoaspartate residues formed in proteins/peptides.

Materials

- 0.2 M Bis-Tris buffer, pH 6.0 (see recipe)
- 10 µM [³H]methyl-S-adenosyl-L-methionine (5 to 15 Ci/mmol; [³H] SAM; NEN)
- Protein-L-isoaspartyl methyltransferase (PIMT; Promega Corporation or purified from a known source)
- 0.2 M NaOH (*APPENDIX 2A*)
- Safety-Solve II counting fluor (Research Products International)
- Sponge plugs (Jaeco Industries): cut into small pieces
- Scintillation vials with extra caps

**BASIC
PROTOCOL 5**

**Protein Labeling
and Immuno-
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CAUTION: [³H]methanol is volatile at room temperature. Perform all reactions under a hood. When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *UNIT 7.1* and *APPENDIX 1D*).

1. Add [³H]SAM to a final concentration of 10 μM to a protein sample solution in 0.2 M Bis-Tris buffer, pH 6.0, containing 40 U of PIMT in a microcentrifuge tube.

The sample protein amount is usually between 0.5 and 1 mg and the total reaction volume is 50 μl.

Blanks that do not have the protein to be analyzed and standards that have known concentrations of isoaspartate should be carried out under the same conditions at the same time.

The Promega version of the enzyme comes included in the ISOQUANT Isoaspartate Detection kit.

2. Incubate the reactions for 30 min at 30°C.
3. Place the tubes on ice for 15 min to stop the PIMT-catalyzed methyl transfer reaction.
4. Microcentrifuge the tubes at 4°C for 2 min to bring all of the liquid to the bottom of the tube. Place on ice.
5. Add 50 μl of 0.2 M NaOH to hydrolyze the methyl esters so that [³H] methanol can be released from the assayed proteins.

Tubes may be vortexed briefly to collect liquid.

6. Immediately transfer 50 μl of each reaction solution to a sponge insert in a scintillation vial cap and place the cap on a vial containing 4 ml Safety-Solve scintillation cocktail.
7. Incubate the capped vials at 37°C for 60 min.

Diffusion of methanol into the scintillation liquid is time-dependent. To obtain reproducible results, it is critical to incubate all reactions for the same length of time.

8. Remove the caps and replace with new caps that do not contain sponge inserts.
9. Count the samples in a scintillation counter.

SUPPORT PROTOCOL 5

GEL ELECTROPHORETIC ANALYSIS OF ISOASPARTATE FORMATION

To identify which proteins in a tissue sample or a protein mixture contain isoaspartate residues, the protein sample, incubated with [³H]SAM and PIMT (see Basic Protocol 5), can be further analyzed by SDS-PAGE (*UNIT 6.1*) after mixing with acid SDS-PAGE sample buffer (see recipes for acid SDS-PAGE buffers). Protein bands can then be excised from the gel and solubilized in 30% hydrogen peroxide, exactly as described in Support Protocol 2 of this unit; however, for the measurement of isoaspartate formation, acid SDS-PAGE (pH 2.4), rather than the Laemmli gel system (pH 8.3), should be performed (see recipes for acid SDS-PAGE). This is necessary, as high pH running buffer will hydrolyze the base-labile isoaspartate methyl esters, leading to the release of [³H]methanol into the running buffer. Do not use sodium hydroxide to stop the PIMT-catalyzed reaction when the samples are to be analyzed by acid SDS-PAGE.

REAGENTS AND SOLUTIONS

Use deionized distilled water for the preparation of all buffers. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Acid SDS-PAGE buffers (pH 2.4)

2× resolving gel buffer:

0.1 M H₂NaPO₄

2% (w/v) SDS

6 M urea

Adjust pH to 2.4 with HCl

Store up to several months at 4°C

1× running buffer:

0.03 M NaH₂PO₄

0.1% (w/v) SDS

0.2 M acetate

Adjust pH to 2.4 with HCl

Store up to 1 year at room temperature

2× sample buffer:

2% (w/v) SDS

6 M urea

10% (v/v) glycerol

0.01% (w/v) pyronin Y dye

16.5 mM NaCl

Adjust pH to 1.4 with HCl

Store up to 1 year at -20°C

2× stacking gel buffer:

2% (w/v) SDS

6 M urea

0.033 M NaCl

Adjust pH to 1.4 with HCl

Store up to 2 months at 4°C

Bis-Tris buffer, 0.2 M, pH 6.0

41.84 g bis-Tris (Sigma)

Adjust volume to 1 liter with H₂O

Adjust pH with concentrated HCl

Store up to 6 months at 4°C

Borate buffer, 0.1 M, pH 9.1

6.184 g boric acid

Adjust volume to 1 liter with H₂O

Adjust pH with 1 M NaOH

Store up to 2 months at room temperature

Borate buffer, 0.2 M, pH 8.8

12.37 g boric acid

Adjust volume to 1 liter with H₂O

Adjust pH with 1 M NaOH

Store up to 2 months at room temperature

DNP solution

Dissolve 198 mg 2,4-dinitrophenylhydrazine (DNPH; mol. wt. 198.1) in 100 ml 2 M HCl (10 mM DNPH final). Store up to 1 year at room temperature in the dark since DNPH can be destroyed by light.

DNPH may require 1 to 2 hrs of constant stirring for complete solubilization.

ELISA buffers

Blocking buffer: Prepare 0.2% (w/v) gamma globulin (Sigma) in PBST (see below). Filter this buffer through 0.45 μ m filter and store up to 1 week at 4°C.

1 \times diethanolamine (DEA) buffer:

24.25 ml 98% diethanolamine

200 ml H₂O

0.25 ml 20% NaN₃

25 mg MgCl₂·6H₂O

Adjust pH to 9.8 with concentrated HCl

Bring to 250 ml with H₂O

Use Milli-Q purified water or equivalent.

10 \times phosphate-buffered saline/Tween 20 (PBST):

80 g NaCl

2 g KH₂PO₄

11.5 g Na₂HPO₄

2 g KCl

5 ml Tween 20

10 ml 20% (w/v) NaN₃

Dilute to a total volume of 2 liters with H₂O

Store up to 2 months at room temperature

Plate coating buffer:

0.795 g Na₂CO₃

1.465 g NaHCO₃

0.5 ml 20% (w/v) NaN₃

Bring to 500 ml with H₂O

If necessary, adjust the pH with 1 M NaOH to ensure it is ~9.6

Use Milli-Q purified water or equivalent.

[³H]Sodium borohydride ([³H]NaBH₄) working solution

Add 5 ml 1 M nonradiolabeled sodium borohydride dissolved in 0.1 M NaOH to the original bottle in which tritiated sodium borohydride (activity 222.3 mCi/mmol; NEN Life Science Products) was received. Divide the solution into 100- μ l aliquots in microcentrifuge tubes (0.5 ml/tube) and store up to 2 years at -80°C.

NaHCO₃, 20 μ M, pH 8.8

16.8 mg NaHCO₃

Adjust volume to 1 liter with H₂O

Adjust pH with 1 M NaOH

Dilute 1:10 just before use

Store up to 2 months at room temperature

Nitro-BSA standard

Adjust the pH of a 5 mg/ml BSA solution (in water) to 3.5 with acetic acid. Add concentrated sodium nitrite (200 mM) to the BSA solution to obtain a final concentration of 1 mM. Incubate the reaction on a rotator at 37°C for 24 hr. The color change of the solution from colorless to yellow after the incubation usually indicates the presence of nitrotyrosine. Dialyze the nitrated BSA solution overnight against ELISA coating buffer. Determine nitrotyrosine concentration at 430 nm using a spectrophotometer ($\epsilon = 4100 \text{ M}^{-1}\text{cm}^{-1}$, pH > 8.5). Store up to 2 months at 4°C.

SDS-PAGE gels (for Bio-Rad Mini gel system)

10% resolving gel:

6.1 ml 2× resolving buffer (Bio-Rad)
2.5 ml 40% 29:1 acrylamide/bisacrylamide
0.1 ml 0.06%, w/v FeSO_4
0.1 ml 1% (w/v) ascorbic acid
1.1 ml distilled water
0.1 ml 0.3% H_2O_2
Store up to 6 months at 4°C

4% stacking gel:

4.0 ml 2× stacking gel buffer (Bio-Rad)
0.5 ml 40% 29:1 acrylamide/bisacrylamide
0.065 ml 0.06%, w/v FeSO_4
0.065 ml 1%, w/v ascorbic acid
0.305 ml distilled water
0.065 ml 0.3% H_2O_2
Store up to 6 months at 4°C

IMPORTANT NOTE: FeSO_4 , ascorbic acid, and H_2O_2 are used as gel polymerization catalysts; prepare fresh.

Trichloroacetic acid (TCA) solution, 20%, 10%, and 0.1% (v/v)

Prepare TCA stock solution by adding 227 ml water to a 500-g bottle of TCA. Add 10 ml of TCA stock solution to 40 ml of water to prepare 20% (v/v) TCA. Dilute to appropriate percentage with water. Store up to 1 year at room temperature.

Tris-buffered saline with and without Tween-20 (TBST and TBS)

100 mM Tris-Cl, pH 7.5 (APPENDIX 2A)
0.9% (w/v) NaCl
0.1% (v/v) Tween-20 (omit for TBS)
Store up to 2 months at 4°C

COMMENTARY

Background Information

Proteins are acknowledged to be one of the major targets of endogenously generated reactive oxygen species (ROS; Stadtman, 1992). The study of protein oxidative modifications not only contributes to the elucidation of the mechanisms of ROS-mediated damage, but may also aid in understanding how protein oxidation is linked to losses in physiological functions under pathological processes or during aging. Furthermore, the identification of the protein targets of oxidative damage may potentially lead to the development

of strategies that may ameliorate oxidative stress.

Several factors seem to be involved in intracellular accumulation of oxidized proteins. One major factor is the cellular level of oxidative stress, determined by the balance between the rate of ROS generation and the efficiency of antioxidative defenses (Stadtman and Berlett, 1997). The other factor determining the steady-state amount of oxidized proteins is the activity of proteolytic enzymes (Stadtman and Berlett, 1997). It is now firmly established that mild oxidation of proteins

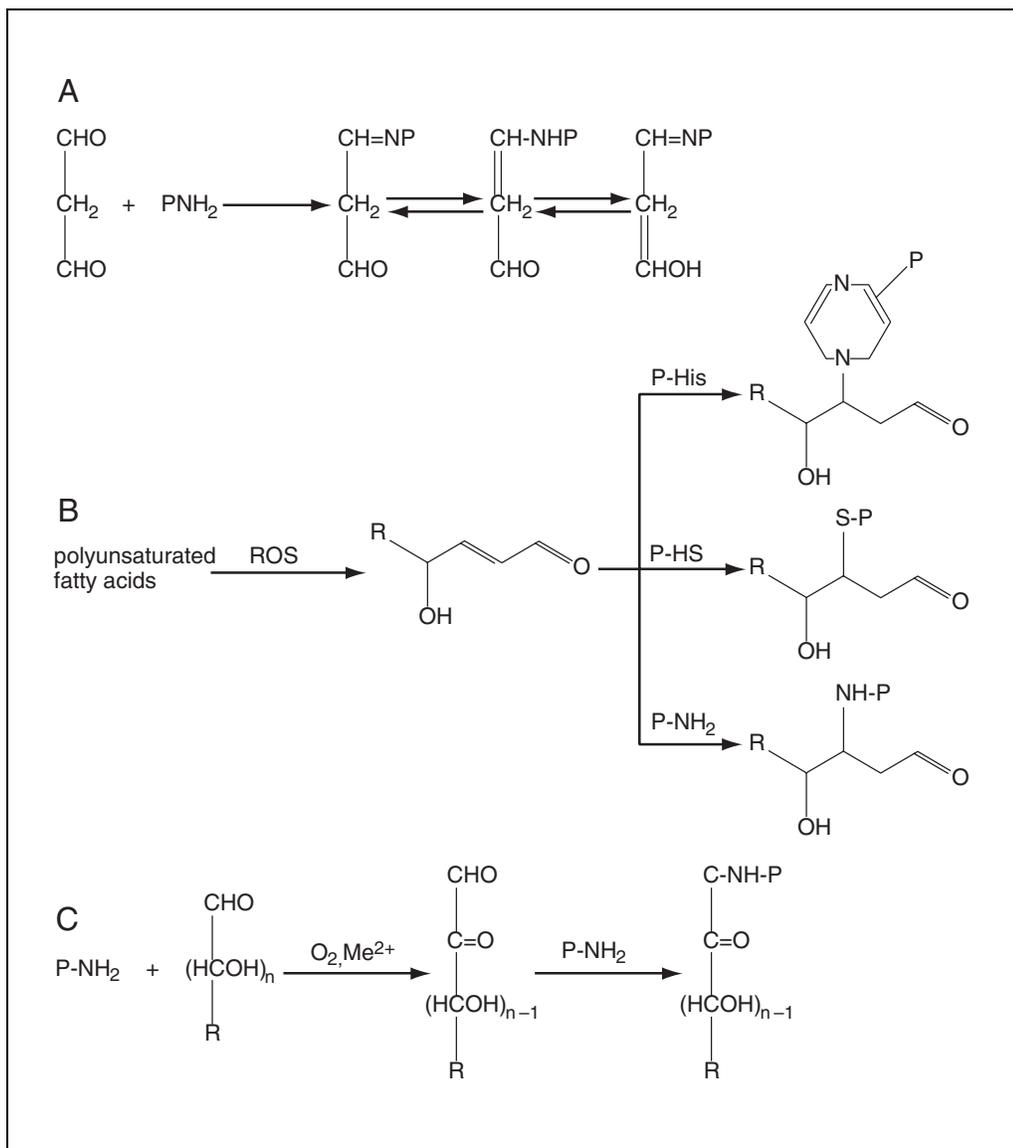


Figure 7.9.1 Generation of protein carbonyls by glycation and glycoxidation and by reactions with lipid peroxidation products of polyunsaturated fatty acids. **(A)** Reactions of protein amino groups (PNH₂) with the lipid peroxidation product, malondialdehyde. **(B)** Michael addition of 4-hydroxy-2-nonenal to protein lysine (P-NH₂), histidine (P-His), or cysteine (PSH) residues. **(C)** Reactions of sugars with protein lysyl amino groups (P-NH₂). “Me” represents “metal ions.” Abbreviation: ROS, reactive oxygen species.

facilitates their eventual degradation by proteases, while heavily oxidized proteins in which cross-links usually occur are resistant to proteolysis (Davies et al., 1987). Oxidative damage to proteases themselves can further attenuate cellular ability to degrade oxidized proteins (Agarwal and Sohal, 1994).

Analysis of protein carbonylation

Some known mechanisms by which protein carbonylation occurs are shown in Figure 7.9.1. The reaction between DNPH and protein carbonyls, shown in Figure 7.9.2, was initially used for the measurement of metal catalyzed

oxidative damage to glutamine synthase (Levine, 1983). The procedure, although very useful for the determination of protein carbonyl content in tissue samples, is not selective and is subject to interference by proteins containing chromophores that have absorbance near 360 nm. In contrast, immunoblotting is highly selective and can detect carbonylation of individual proteins, but the method is not meant to provide quantitative information. The borohydride reduction method, when used in conjunction with gel electrophoresis, is both selective and quantitative (Yan and Sohal, 1998a). Using protein carbonyls as an index, oxidative dam-

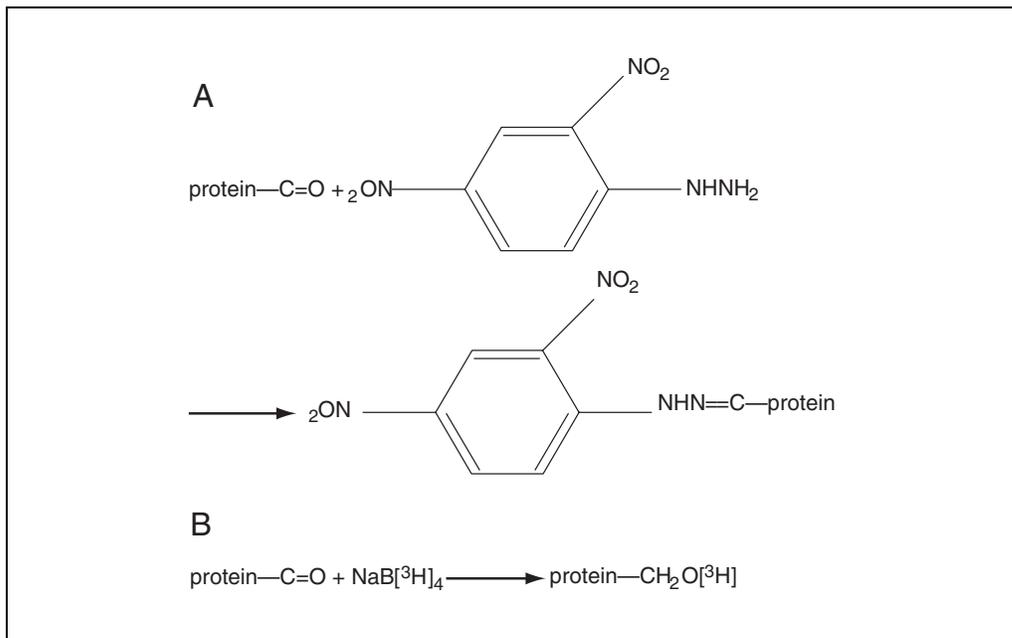


Figure 7.9.2 Methods for labeling protein carbonyls: (1) Derivatization of protein carbonyls with 2,4- dinitrophenylhydrazine (DNPH), forming protein conjugated dinitrophenylhydrazones. (2) Derivatization of protein carbonyls with tritiated sodium borohydride.

age to proteins has been found to be associated with aging and oxidative stress as well as a number of pathologies (Stadtman, 1992). Moreover, protein oxidation during aging has been recently established to be a highly selective phenomenon. Using the housefly as a model system, the authors' laboratory has demonstrated that in flight-muscle mitochondria, only aconitase and adenine nucleotide translocase exhibited a discernible age-dependent increase in protein carbonylation and a corresponding loss in their activities (Yan et al., 1997; Yan and Sohal, 1998b).

The four protocols for the measurement of protein carbonyls described in this unit are relatively simple and easy to perform, without the requirement of elaborate instrumentation. Analysis of protein carbonylation by methods other than those described here may also be used, should the relevant equipment be readily accessible. One such procedure requires HPLC separation, which has the advantage that the removal of free DNPH is unnecessary because the HPLC column can separate proteins from small molecules (Levine et al., 1994). HPLC requires a very small amount of proteins and can monitor both protein and protein-bound DNP absorption at two wavelengths simultaneously. The derivatization of protein carbonyls by DNPH, however, needs to be carried out in solutions that are compatible with HPLC columns. Another method, which also involves the use of anti-DNP antibodies, is the ELISA meas-

urement of protein carbonyls (Buss et al., 1997). This method is quite sensitive and may be used for determination of whole tissue carbonyls, but it is only semiquantitative and lacks selectivity.

Recently, a fairly new method, the MALDI-MS technique, has been applied for the determination of protein carbonyls. The method works very well for small peptides or proteins such as cytochrome c, but for large peptides, the proteins need to be digested with proteases. The resulting small peptides are then separated by HPLC. A potential problem for large proteins with this method is that if a large number of small peptides are generated after protease digestion, HPLC separation of these peptides can become very tedious.

Analysis of protein thiol groups

Protein thiol groups (mainly cysteine residues) not only stabilize and maintain the three-dimensional conformation of many proteins but are also involved in biological redox couplings. Due to their high reactivity, protein thiol groups are very sensitive to oxidative damage induced by a variety of oxidation systems. Loss of protein thiol groups has been demonstrated during aging and in disease (Hughes et al., 1980; McKenzie et al., 1996).

Most of the currently available methods for quantitative analysis of protein thiol groups depend upon the formation of chemical derivatives. Due to the nucleophilic property of the -SH group, alkylation of cysteine thiol groups by



Figure 7.9.3 Radiolabeling of protein thiol groups with [^{14}C]iodoacetamide.

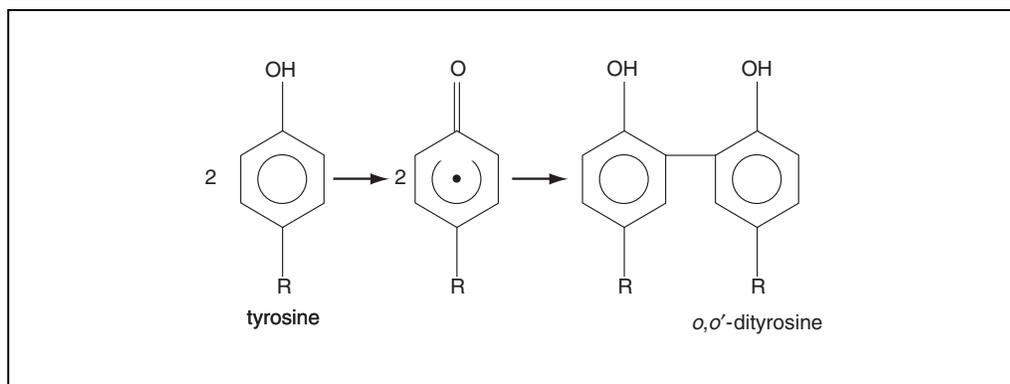


Figure 7.9.4 Formation of one molecule of *o,o'*-dityrosine from two molecules of tyrosine via tyrosyl radical intermediates.

alkylators such as iodoacetamide is widely used for either blocking or quantification of protein-bound thiols. There are two advantages when iodoacetamide is used. First, iodoacetamide does not create a carboxylate functional group; therefore, no new negative charges are introduced into the protein (Fig. 7.9.3). This may be necessary when the alkylated protein is to be analyzed by isoelectric focusing gel electrophoresis. Second, the bond formed from the reaction of iodoacetamide and a thiol group is a stable thioether linkage that is irreversible under normal gel electrophoretic conditions. Furthermore, when radiolabeled iodoacetamide is used, the reaction provides a highly quantitative method for the measurement of protein thiol groups. The use of iodoacetamide, together with gel electrophoresis, has led to a successful identification of glyceraldehyde-3-phosphate dehydrogenase as the enzyme that exhibits the most loss of protein thiol groups during inflammatory bowel disease (McKenzie et al., 1996).

Another classical method for the determination of protein thiol groups involves the use of 5,5'-dithiobis (2-nitrobenzoic acid) also known as DTNB or Ellman's reagent (Ellman, 1959). DTNB undergoes disulfide interchange with the release of 5-thio-2-nitrobenzoic anion (TNB), which has an absorption maximum at 412 nm and can be quantitated spectrophotometrically. The disadvantage of the DTNB method is that TNB is highly subject to reoxidation in an alkaline solution in the presence of oxygen and metal

ions and its molar extinction coefficient changes slightly as a function of the solvent system used. In addition, DTNB itself can undergo hydrolysis of its disulfide bond at a pH higher than 9. Moreover, without prior protein purification, DTNB cannot be used for the quantification of individual proteins that exist in tissue homogenates and may have undergone the most loss of thiol groups caused by oxidative damage during aging or in diseases.

Analysis of tyrosine modification

Dityrosine formation. *o,o'*-Dityrosine is formed either when a hydroxyl radical cross-links tyrosines or by the reaction between protein bound tyrosine and a tyrosyl radical (Fig. 7.9.4). Dityrosine exhibits intense fluorescence at ~400 nm and thus can be measured with an excitation wavelength of ~315 nm in alkaline solutions or ~284 nm in acidic solutions. Based on this property, dityrosine, formed in proteins oxidized in vitro, have been frequently measured by HPLC equipped with a fluorometer as the detection device (Giulivi and Davies, 1993). A disadvantage is that molecules that co-elute, but structurally differ from dityrosine may confound the procedure. Moreover, the HPLC fluorometric method has not yet been successfully used for the detection of dityrosine formation during natural aging, suggesting that the amount of dityrosine in proteins, without challenge by severe oxidative stress, is below HPLC detection limit.

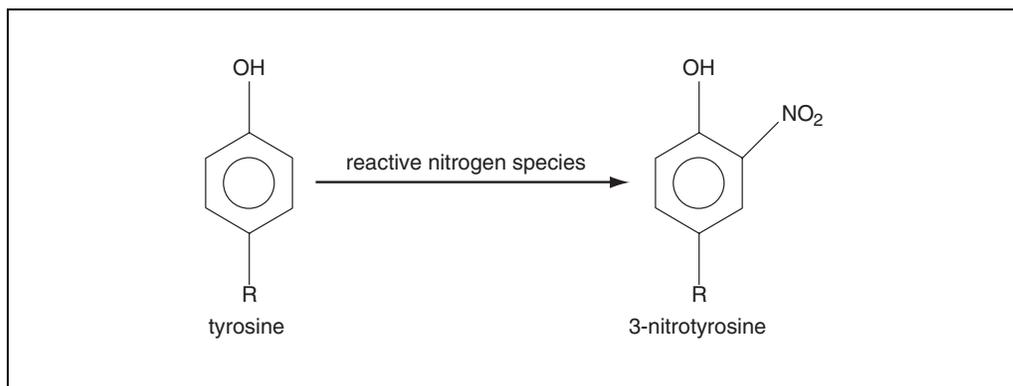


Figure 7.9.5 Formation of nitrotyrosine via reactive nitrogen species-mediated nitration of tyrosine.

In contrast, the GC/MS method described in this unit for dityrosine detection is highly sensitive and can structurally distinguish dityrosine from other tyrosine-modified derivatives, thereby reducing potential confusions caused by compounds that coelute with dityrosine during chromatography. GC/MS analysis also permits the use of a stable, isotopically labeled internal standard which, other than its heavy isotope, is structurally identical to dityrosine, and therefore behaves identically during sample preparation and analysis. Inclusion of such a standard also corrects for the dityrosine loss during processing and thus increases the precision of quantitative measurements. Using the GC/MS method, dityrosine concentrations in various mouse tissues have been found to be elevated during aging and are significantly decreased by caloric restriction (Leeuwenburgh et al., 1997). However, this method lacks selectivity when tissue samples are used as experimental materials, since no distinction can be made in the degree of dityrosine content among different protein species.

Nitrotyrosine formation. Nitrotyrosine is formed by reactions between tyrosine and reactive nitrogen intermediates (Fig. 7.9.5). Its level is elevated, especially during inflammation. Peroxynitrite, produced by the reaction between the nitrogen monoxide radical and superoxide, has been proposed to be the major oxidant that causes nitrotyrosine formation in living systems (Ischiropoulos et al., 1992). Nitrotyrosine can be easily determined spectrophotometrically as its maximum absorbance ranges from 350 nm to 450 nm, shifting from 365 nm (colorless) at pH < 3 to 428 nm (yellow) at pH > 9. However, the amount contained in tissue samples without oxidative challenge is far below the detection limit of a spectrophotometer. The ELISA method described in this unit, although only semiquantitative, is very sensitive for the meas-

urement of nitrotyrosine in tissue samples. This method is especially useful when individual proteins in tissue samples need not be analyzed. For identification of specific proteins that might exhibit selective nitration during aging or under oxidative stress, immunoblot detection using anti-nitrotyrosine antibodies may be preferred.

Analysis of isoaspartate formation

Isoaspartate residues are usually formed from the deamidation of asparaginyl residues or isomerization of aspartyl residues (Fig. 7.9.6). Formation of isoaspartate residues in a protein not only alters the protein's structure, but may also cause the loss of the protein's activity. Although isoaspartate formation was initially found in *in vitro* aging of proteins stored at neutral pH (Graf et al., 1971), the discovery of the enzyme protein-L-isoaspartyl methyltransferase (PIMT) suggests that isoaspartate formation in proteins is physiologically relevant (Clark, 1985). Indeed, it has been found that a variety of tissue samples are able to be tritiated when incubated in the presence of PIMT and [³H]S-adenosyl methionine (SAM; Aswad, 1995), demonstrating the occurrence of isoaspartate formation *in vivo*. Therefore, methods for the detection and quantification of isoaspartate formation in proteins/peptides are of interest to investigators studying post-translational modification of proteins.

The discovery of PIMT also provides a useful tool for the quantification of isoaspartate formation in proteins (Fig. 7.9.7), as described in this unit. The only limitation of the method, however, is that PIMT, as a single enzyme, is not commercially available. Isoaspartate formation can also be measured by isoelectric focusing gel analysis or proteolytic digestion of the protein followed by mass spectral analysis of the peptides resolved by reversed-phase HPLC. The isoelectric focus-

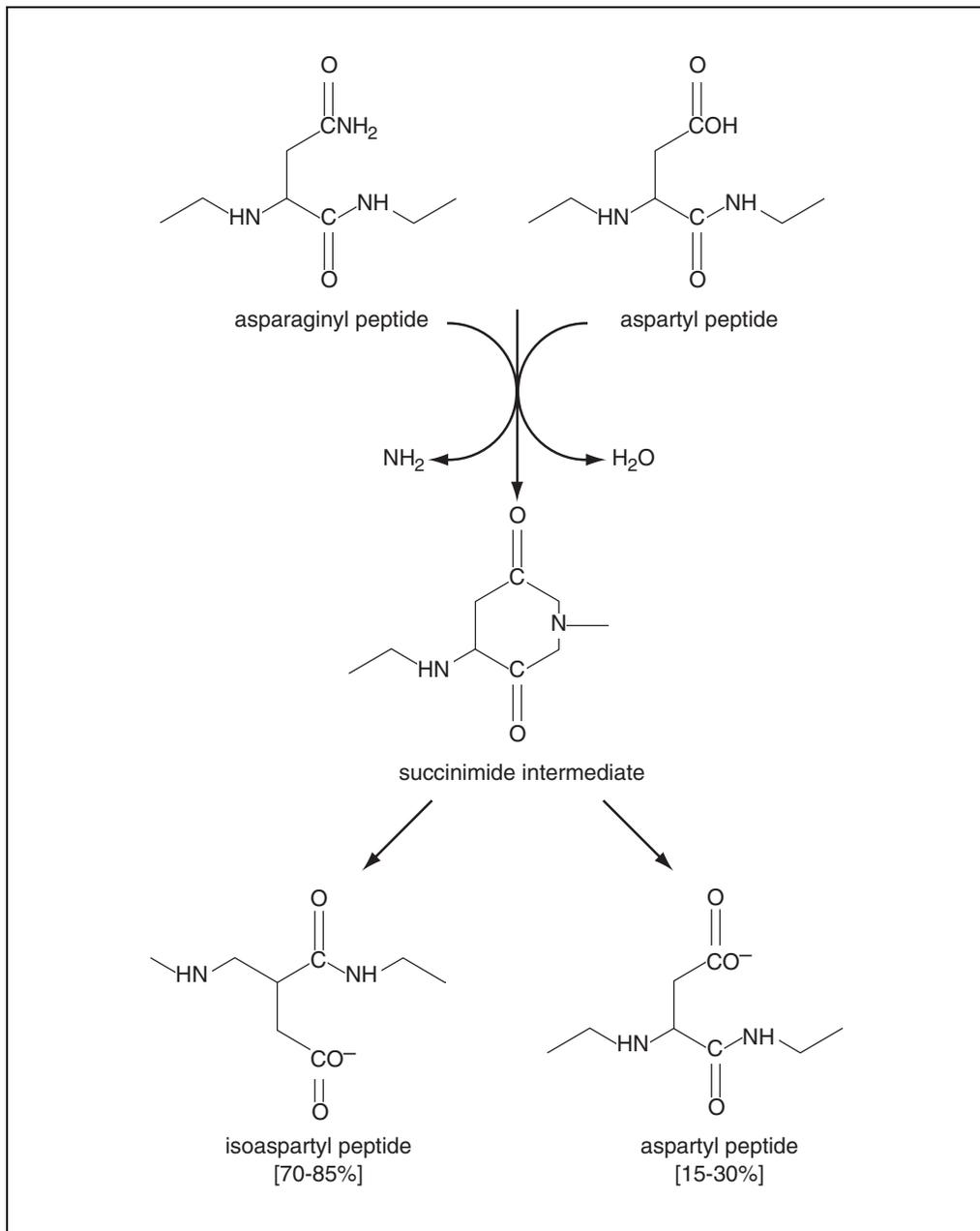


Figure 7.9.6 Formation of isoaspartate by the deamidation of asparagine or the isomerization of aspartate.

ing method is based on the fact that isoaspartate formation in a protein results in a net change of the protein's charge. However, the method cannot detect the formation of isoaspartate residues derived from aspartic acid which do not alter the net charge of the protein (i.e., aspartyl isomerization). In addition, changes in the isoelectric focusing pattern of a protein may also result from the modification of other amino acid residues, such as methionine or lysine oxidation; therefore, the isoelectric focusing method is not specific. Proteolytic digestion of a protein followed by HPLC and mass spectral analysis is capable of detecting the presence of

isoaspartate residues in the protein; however, the HPLC method, based on the retention time of the digested peptides, may not be able to resolve subtle changes in the peptide that arise from the deamidation of a particular peptide. Moreover, many changes may affect the retention time of a particular peptide, which could coelute with the isoaspartate-containing peptide and therefore does not necessarily indicate the presence of isoaspartate residues.

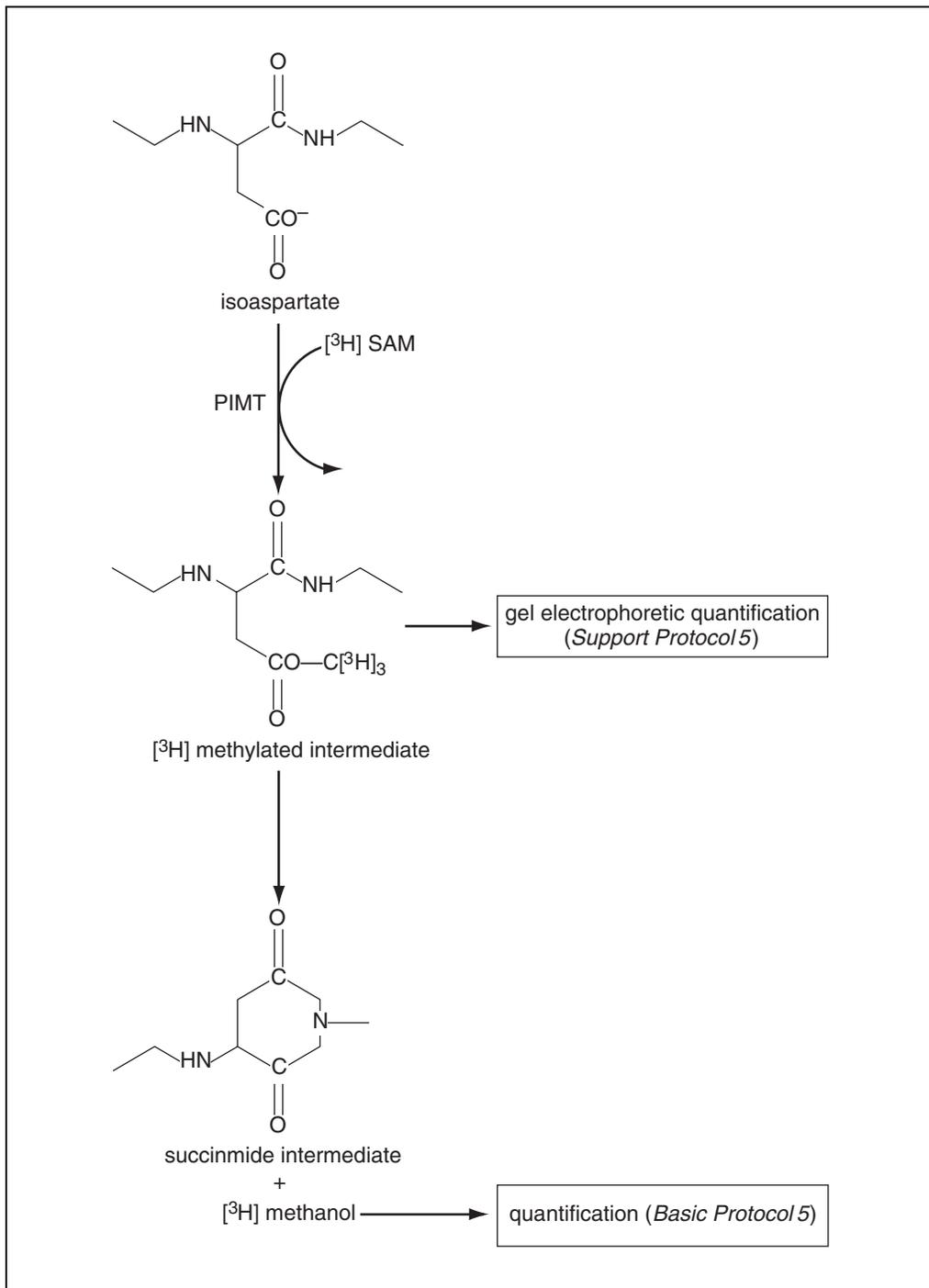


Figure 7.9.7 Pathway for the PIMT-catalyzed methylation of isoaspartate used for quantitation.

Critical Parameters and Troubleshooting

In all studies concerning oxidative damage to proteins, auto-oxidation of the tissues by ambient oxygen, following tissue isolation, should be minimized. Accordingly, tissue collection and preparation must be carried out in buffers supplemented with antioxidants, such as diethylenetriaminepentaacetic acid (DTPA) and butylated hydroxytoluene (BHT). The use of

antioxidant buffers may be especially critical for the detection of trace amounts of oxidized products formed during aging or under oxidative stress. In addition, all buffers should be bubbled with nitrogen before use.

Analysis of protein carbonylation

Depending upon the method used, protein carbonyl content can be expressed as either nmol/mg protein or cpm/mg protein for the

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DNPH (see Basic Protocol 1) and NaBH₄ (see Basic Protocol 2) method respectively. Therefore, the only critical parameter for protein carbonyl quantification is the protein concentration. As a rule, protein concentration after washing with ethanol/ethyl acetate, should always be determined. In addition, it should be noted that a high concentration of protein-conjugated DNP interferes with the BCA protein assay; therefore, for heavily oxidized proteins that have extremely high carbonyl content determined by DNPH, protein concentration should be assayed by UV absorption at 280 nm using BSA as a standard.

Two potential problems may arise when protein carbonyl content is measured spectrophotometrically using DNPH. First, absorption of protein-bound DNP in some experiments may be lower than that of a blank, which is the same protein sample treated with HCl (2 M) rather than DNPH. This problem may be caused by chromophores of certain proteins in the sample. If this is indeed the case, protein carbonyl content may have to be determined by other methods, such as the use of tritiated sodium borohydride (see Basic Protocol 2). The problem can also be caused by the difference in protein concentrations between a blank and a sample due to loss of proteins during washing. Therefore, care should be taken to minimize protein loss during washing steps, and protein concentration should always be determined after the removal of free DNPH (see Basic Protocol 2, step 4). Second, for tissue samples, it may be very difficult to dissolve the final protein pellet after washing. This problem is most likely caused by the use of high concentration of proteins in the initial step where protein samples are treated with DNPH. The authors have found that starting with 1 mg/ml protein will render the final pellet readily soluble. The solubility of a protein pellet will also depend on the nature of the protein; for example, lipoproteins and collagen are always quite difficult to dissolve after treatment with DNPH. In such a case, a denaturing buffer containing 150 mM phosphate and 3% SDS, pH 6.8 should be used.

For immunoblot detection of protein carbonyls, a major concern is the dilution of antibodies. If overdiluted, the immunostaining signal may be very weak; if underdiluted, the background may be too dark. Therefore, it is critical to optimize each antibody's dilution so that the immunostain signal is maximized while background staining is minimized. In addition, the amount of protein loaded onto SDS-PAGE should not be too high. Otherwise, nonspecific immune reactions may occur. Generally, 5 µg of a protein mixture is

enough and less may be used for a purified protein.

When protein carbonyl content is determined by tritiated sodium borohydride (see Basic Protocol 2), protein concentrations should always be determined after the removal of free tritium because protein recovery after washing is rarely 100%. An 80% to 90% recovery for mitochondrial proteins has been routinely obtained in the authors' laboratory. When tritiated proteins are analyzed by SDS-PAGE, solubilization of more than one band of the same protein in one vial may be necessary to obtain enough radioactivity counts.

Analysis of protein thiol oxidation

When protein thiol groups are determined by radiolabeling with [¹⁴C] iodoacetamide in conjunction with gel electrophoresis, it is necessary to include a final concentration of 1% SDS in the reaction mixture so that complete labeling can be ensured. For some proteins, the nucleophilicity of the thiolate (S⁻) anion may be lower than potentially competing alcohol and amino groups in the same proteins; therefore, the iodoacetamide reaction may not be absolutely specific. In such cases, experimental conditions may need to be optimized by varying temperature, pH, and duration of the reactions so that the labeling for cysteine residues will be specific.

It should be noted that iodoacetamide is intrinsically unstable in light, especially in solution; reactions should therefore be carried out in the dark. Adding cysteine, glutathione, or mercaptosuccinic acid to the reaction mixture will quench thiol-reactive species, forming highly water-soluble adducts that are easily separated from proteins during gel electrophoresis.

Analysis of tyrosine oxidative modification

For GC/MS measurement of protein dityrosine residues, dityrosine always needs to be released by acid hydrolysis. Although it has been demonstrated by other investigators that the procedure of acid hydrolysis does not induce more oxidation of tyrosine, which can lead to additional dityrosine formation, care should be taken to ensure no further oxidation indeed takes place for a given sample under given experimental conditions by adding antioxidants to buffers. For such purposes, pure tyrosine should be used as a control during the acid hydrolysis process. In addition, for tissue samples (protein mixtures), the dityrosine level is usually above the detection limit of GC/MS method; however, this may not be the case for an individual protein. Therefore, it may be advisable to determine whether the

protein of interest has a dityrosine level that is above the limit of detection. To achieve this, analyze the protein at two widely different concentrations, 50- to 100-fold. If no dityrosine is detectable in the concentrated sample, it is most likely that the protein does not contain a significant amount of dityrosine residues.

For the measurement of protein nitrotyrosine by competitive ELISA, antibody dilution and the amount of nitro-BSA used for plate coating may have to be optimized. To achieve this, first titrate the amount of nitro-BSA needed to coat the plate versus a constant, high concentration of anti-nitrotyrosine antibody. Plot the absorbance values and select the lowest level that yields a strong signal. Next, coat the plate with this amount of nitro-BSA and vary the concentration of antibodies. Plot the obtained values versus antibody dilution and select a level of the antibody that is within the linear portion of the curve. Optimal dilution for the secondary antibody can also be obtained by similar titration. In addition, in most of the competitive ELISA assays, the first antibody (here anti-nitrotyrosine) may bind preferentially to the coating antigen because the latter on the plate is highly localized. Therefore, to ensure a thorough competition, samples to be analyzed should always be premixed with the first antibody and incubated overnight before the actual ELISA measurement.

Analysis of isoaspartate formation

When protein isoaspartate is measured by PIMT-catalyzed methyl transfer reaction, the protein solution must not contain any detergent such as SDS or chaotropic reagents such as guanidine-HCl, as these will denature the PIMT enzyme. Moreover, for an unknown protein or peptide, as a first step, it is useful to determine whether the protein has any isoaspartate residues. The protein can be diluted to several different concentrations and then analyzed. If the concentration of isoaspartic acid is not protein concentration-dependent, it is most likely that there is little or no isoaspartic acid present in the protein or peptide.

Some proteins, when not denatured, may contain isoaspartate residues in domains that make them poor substrates for PIMT. In such cases, the PIMT methylation reaction may not be complete and isoaspartate concentration may be underestimated. If so, proteins may need to be fragmented by proteolysis before they are used for isoaspartate determination. To determine if proteolysis is necessary, process a sample of undigested protein along with a digested sample. If the amount of isoaspartate measured in the intact protein is similar to that in the digested protein, the protein

need not be fragmented for isoaspartate quantification. If a protein should require digestion by protease, the sample buffer used for blank reactions should contain the same protease and buffer components existing in the digestion, but without the protein sample. This will exclude any isoaspartate residues that may be present in the protease.

Anticipated Results

When performed correctly, protein carbonyl content of native proteins, such as BSA and human plasma, should be around 1 nmol/mg protein (usually 0.5 to 1.5 nmol/mg protein). Literature values for carbonyl content in normal tissues are usually between 1.5 and 2.0 nmol/mg protein (Reanick and Packer, 1994). If immunoblot is performed, immunostaining of protein carbonyls should be differential, which means protein oxidative damage does not occur equally in all proteins. Protein carbonylation analyzed by the use of tritiated borohydride is highly dependent on the source of tritiated borohydride, but for the same batch of the chemical with the same concentration, results are generally reproducible. If protein carbonyls derivatized with tritiated sodium borohydride are to be quantitated by a gel electrophoretic technique (see Support Protocol 2), radioactivity counts for each individual band in a protein mixture (<20 μ g loaded), are usually less than 300 cpm if no more than one band is solubilized in a single vial.

For protein thiol groups measured by gel electrophoresis of iodoacetamide-treated proteins (see Basic Protocol 3), results of autoradiographic intensities should show a highly differential pattern for each individual protein. If a band exhibits the highest intensity among all the proteins resolved by SDS-PAGE, the protein would have the least loss in its thiol groups. In contrast, if a protein band exhibits a very weak intensity following autoradiography, it should have a maximal loss in its thiol groups. Depending on what proteins are being studied, results of liquid scintillation counting of an excised individual band may range from 500 cpm to 2000 cpm.

For mass spectrometric quantification of dityrosine (see Basic Protocol 4), the concentration of protein dityrosine is usually expressed as the ratio of dityrosine to tyrosine. In mouse, dityrosine concentration has been found to be around 0.3 to 0.6 mmol/mol for heart, 0.1 to 0.3 mmol/mol for skeletal muscle and brain, and 0.2 mmol/mol for liver (ter Steege et al., 1998). Nitrotyrosine, when determined by competitive ELISA and expressed as nitro-BSA equivalents, is about 0.12 μ M in human plasma.

Isoaspartate concentration in proteins, usually expressed as pmol/mg protein, is 200 pmol/mg protein in murine brain and heart, and <50 pmol/mg protein in liver with no detectable isoaspartate residues in the plasma (Kim et al., 1997).

Time Considerations

Without the consideration of preparation of protein samples, for a small number of samples (<20), spectrophotometric quantitation (see Basic Protocol 1) can be finished within one day after the DNPH treatment. If a large number of samples are involved, the washing step may take a longer time, but one can always leave the protein pellet in the ethanol/ethyl acetate solution with the tubes capped lightly and stored under a hood.

Immunoblot detection of protein carbonyls (see Support Protocol 1), starting from SDS-PAGE, can be finished within 48 hr. The time can be shortened to 12 hr if the PVDF membrane is incubated for 1 hr each, with both primary and secondary antibodies.

The quantitation of protein carbonyls by electrophoresis (see Support Protocol 2) may require one week. Most of the time is spent on gel solubilization and liquid scintillation counting.

Gel electrophoretic analysis of protein thiol groups labeled with [¹⁴C]iodoacetamide (see Basic Protocol 3) will take at least 2 weeks. Generally, radiolabeling of the protein samples and gel analysis can be finished within 2 days. The rest of the time is used for the exposure of X-ray film to the Whatman 3MM filter paper onto which gels have been dried.

Quantification of protein carbonyls by tritiated borohydride labeling (see Support Protocol 2) typically requires 2 days for ~20 samples. Labeling is generally accomplished in the first day, while the second day is left for scintillation counting.

Quantitation of protein dityrosine by mass spectrometry (see Basic Protocol 4) usually takes up to 1 week. Sample preparation, including tissue hydrolysis, can take 3 days; the actual quantitation by mass spectrometry can be finished within 2 days. Preparation of dityrosine standard (see Support Protocol 3) may take up to 1 week and the time used for lyophilization steps is flexible. In addition, the lyophilized samples can be stored at -80°C for several months before further analysis.

Competition ELISA measurement of nitrotyrosine (see Support Protocol 4) usually takes 2 days. Day one is allotted for sample preparation, plate coating and preincubation of the samples or nitro-BSA with anti-nitrotyrosine antibodies.

Day two is used for antibody incubation, plate washing and data collection.

Enzymatic analysis of isoaspartate formation (see Basic Protocol 5), up to the step of liquid scintillation counting, can be finished in one day. After the addition of scintillation cocktail, the samples can be counted at any time. Gel electrophoretic analysis of isoaspartate formation (see Support Protocol 5) may require a week with most of the time spent on gel solubilization and liquid scintillation counting.

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Radioiodination of Cellular Proteins

UNIT 7.10

Radioiodination with ^{125}I is a useful method for the labeling of cellular proteins. Protocols describing the radioiodination of proteins expressed on the surface of live cells (Basic Protocol 1), proteins in subcellular fractions including membrane-solubilized proteins (Basic Protocol 2), and soluble proteins (Basic Protocol 3 and Alternate Protocol) are described below. Support Protocols 1 and 2 describe methods for preparing solubilized membrane proteins.

SAFETY PRECAUTIONS FOR WORKING WITH ^{125}I -LABELED COMPOUNDS

As with all radioactive isotopes, the experimenter should work only in areas designated for radioactive materials and should follow the guidelines set by the local radiation safety officer. Because ^{125}I is avidly taken up by the thyroid gland, safety guidelines often include a urine test and/or thyroid scan after the procedure.

Because of the risk of exposure to γ radiation, special care should be taken whenever ^{125}I is used. Therefore, a number of extra precautions need to be followed:

1. It is strongly recommended that this procedure be performed by two researchers. This allows one experimenter to do the labeling while the other provides the equipment needed and continually surveys for contamination with the appropriate sodium iodide crystal detector.
2. Vials containing unbound ^{125}I should always be opened in a fume hood designated for use with radioactive materials and equipped with an activated charcoal filter. This should protect the experimenter from the ^{125}I , which is extremely volatile and particularly hazardous to the thyroid gland.
3. All work should be carried out behind a set of lead bricks to block the γ radiation. A double row of standard 4-cm-thick bricks should be sufficient to provide protection from the ^{125}I .
4. When ^{125}I or radioiodinated cells are moved from the protective lead bricks to the fume hood, they should be encased behind lead shields to limit exposure of fellow researchers. Moreover, ^{125}I -labeled cells should be kept within a lead box in a designated incubator.
5. All subsequent processing with radioiodinated cells or proteins should be carried out behind lead shields while wearing protective clothing and gloves, using air monitoring devices, and working with extreme caution.

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *UNIT 7.1* and *APPENDIX 1D*).

CELL SURFACE LABELING WITH ^{125}I USING LACTOPEROXIDASE

In this procedure, cell surface proteins are labeled by adding $\text{Na}[^{125}\text{I}]$ to cells in suspension. The cells are then pulsed with hydrogen peroxide and lactoperoxidase, which catalyzes the oxidation of ^{125}I and converts it to a reactive species. Care should be given to the use of proper concentrations of cells, radioiodine, and lactoperoxidase. The cells must be checked for viability after labeling to ensure that they have tolerated the procedure.

**BASIC
PROTOCOL 1**

**Protein Labeling
and Immuno-
precipitation**

7.10.1

Materials

0.5–1 × 10⁸ cells in suspension
PBS (APPENDIX 2A), azide free and ice cold
Na[¹²⁵I] (0.1 mCi/μl; NEN Life Science Products, Amersham Pharmacia Biotech, or ICN Biomedicals)
Lactoperoxidase enzyme solution (see recipe)
30% (v/v) hydrogen peroxide stock solution
Lactoperoxidase buffer (see recipe), ice cold
10 mM NaI in PBS (APPENDIX 2A), ice cold

Fume hood with double row of 4-cm-thick lead bricks
γ counter and appropriate counting vials

Prepare cells and reagents

1. Centrifuge 0.5–1 × 10⁸ cells in suspension 5 min at 500 × g, 4°C, to pellet cells.
2. Wash cells three times with 25 ml ice-cold PBS by centrifuging as above.
It is critical that there be no sodium azide in the PBS, as this impairs peroxidase activity.
3. While centrifuging, thaw Na[¹²⁵I] and lactoperoxidase enzyme solution. Dilute 30% hydrogen peroxide stock solution 1:1000 in ice-cold lactoperoxidase buffer. Keep all reagents and solutions on ice.
4. Resuspend cells in 1 ml ice-cold lactoperoxidase buffer. Keep suspended cells on ice.

Radiolabel cells

5. While working in a fume hood, add 20 μl Na[¹²⁵I] to the cells (2 mCi/ml cell suspension). Mix gently with pipet tip. Keep cells on ice throughout the entire reaction.
6. Add 100 μl lactoperoxidase enzyme solution to cells. Mix gently with pipet tip.
7. Add 25 μl diluted hydrogen peroxidase solution to the cells. Mix gently with pipet tip. Incubate cells 3 min on ice.
8. Add 50 μl lactoperoxidase enzyme solution to cells and mix gently with pipet tip.
9. Add 25 μl diluted hydrogen peroxidase and mix gently with pipet tip. Incubate cells 3 min on ice.
10. Pulse cells with an additional 50 μl diluted hydrogen peroxidase and incubate 3 min on ice. Repeat this step three times, incubating cells for 3 min on ice after each addition.
11. Wash cells three times with 10 ml ice-cold 10 mM NaI in PBS.

This will terminate the incorporation of ¹²⁵I.

These washes will contain extremely high levels of unincorporated ¹²⁵I, so great care must be taken to avoid contamination.

Determine ¹²⁵I incorporation

12. Suspend cells in 10 ml of 10 mM NaI in PBS and transfer a 10-μl aliquot to a counting vial and count the γ radiation using a γ counter. Pellet the cells.

Whereas this reading is not a measure of specific activity, it does give a preliminary indication of the intensity of the label and can be used for comparisons with subsequent labeling experiments. It is also possible to estimate the general specific activity by first lysing the cells and then precipitating a sample of the protein by trichloroacetate. The

amount of protein and γ radiation can then be measured to attain the radioactivity per milligram protein.

It is also possible to count the number and percentage of live cells after the label with trypan blue staining (UNIT 1.1), in which unstained cells (i.e., those that exclude trypan blue) are counted as live cells. However, extreme caution must be exercised to avoid contamination.

13. Process and analyze pelleted cells.

It is also possible to freeze cell pellets at -70°C . However, the experimenter should bear in mind that the half-life of ^{125}I is ~ 60 days and some radiolysis may occur upon prolonged storage. Iodinated cell surface proteins are useful when there are no available antibodies directed at the extracellular regions of the proteins of interest. They can also be used to determine surface expression and topology of iodinated proteins by SDS-PAGE analysis. Peptide mapping can also be performed on cell surface-iodinated protein.

RADIOIODINATION OF MEMBRANE-SOLUBILIZED PROTEINS

This protocol uses lactoperoxidase beads to catalyze the iodination reaction. The entire radioiodination procedure should be carried out in a fume hood located in a room designated for radioactive work with ^{125}I .

Materials

1 ml membrane-solubilized proteins from 2×10^8 cells (see Support Protocols 1 and 2)

Lactoperoxidase beads in 50% suspension (Worthington Biochemical)

$\text{Na}[^{125}\text{I}]$ (0.1 mCi/ μl ; NEN Life Science Products, Amersham Pharmacia Biotech, or ICN Biomedicals)

3% (w/v) dextrose

Triton X-100 lysis buffer (see recipe) with 0.02% (w/v) BSA

PD-10 columns (10-ml columns prepacked with Sephadex G-25 resin; Amersham Pharmacia Biotech)

γ counter and appropriate counting vials

1. Add the following to 1 ml membrane-solubilized proteins from 2×10^8 cells:

20 μl lactoperoxidase beads in 50% suspension (or follow manufacturer's instructions)

20 μl $\text{Na}[^{125}\text{I}]$ (2 mCi)

150 μl of 3% dextrose.

Using immobilized lactoperoxidase prevents radioiodination of the enzyme and contamination of the desired labeled proteins with labeled lactoperoxidase. Dextrose is added for the generation of hydrogen peroxide in vitro.

To later estimate the specific activity of the iodinated membrane-solubilized proteins, it is advisable to calculate the protein concentration prior to the labeling procedure. This may be done using the Bradford assay or any other method (APPENDIX 3B).

2. Incubate 30 min on ice, mixing well every 10 min.

3. Wash a PD-10 column three times with 10 ml Triton X-100 lysis buffer with 0.02% BSA.

BSA saturates the column so that the small amount of radiolabeled material is not lost nonspecifically on the column.

4. Add the contents of the labeling tube to the column and collect 1-ml fractions until no more fluid drains out.

BASIC PROTOCOL 2

Protein Labeling and Immuno- precipitation

7.10.3

The labeled sample may be spun down prior to its loading on the column to separate the lactoperoxidase-coated beads. Alternatively, the entire sample may be applied to the column, and the lactoperoxidase-coated beads will be stacked at the top of the column, without interfering with the separation.

5. Add 9 ml Triton X-100 lysis buffer with 0.02% BSA and continue collecting 1-ml fractions until eight fractions are collected.

It is important to add the lysis buffer when there is no more sample above the Sephadex resin to avoid dilution of the labeled sample.

6. Transfer a 1- to 10- μ l aliquot from each fraction to a counting vial and measure its radioactivity using a γ counter.

7. Estimate the specific activity (cpm/mg protein) of the peaks.

Usually the main fractions containing the labeled proteins are tubes 3, 4, and 5, and free Na^[125I]-containing fractions are in tubes 7 and 8. Based on the protein concentration obtained for the nonlabeled membrane-solubilized proteins, the specific activity may be estimated. This assumes a similar loss of protein by the end of the time the labeling is carried out, but the method is useful for comparing the equality of the labeling reaction from one experiment to the next.

8. Pool the desired fractions in a 15-ml conical centrifuge tube.

The radiolabeled sample can be used for immunoprecipitation, as a probe for far western analysis (UNIT 17.2), and other applications. It can be stored in 0.05% (w/v) sodium azide at 4°C or in 50% (v/v) glycerol at -20°C for ~2 months, taking into consideration the 60-day half-life of ¹²⁵I.

MEMBRANE PREPARATION

The isolation of membranes serves as an important purification step because it removes cytosolic proteins and nuclei prior to solubilization of membrane proteins. This procedure enables the efficient solubilization of membranes and cytoskeleton-associated proteins without causing high levels of viscosity as a result of chromatin release from the cell nucleus.

NOTE: All steps in these protocols should be carried out at 4°C or on ice.

Membrane Preparation by Homogenization

This protocol utilizes a glass-glass (Dounce) homogenizer, which has proven useful for a number of different cell types. The exact conditions, including the number of strokes required for optimal cell disruption without breakage of nuclei, must be determined for each cell type. This can generally be accomplished by monitoring the procedure with the use of a phase-contrast microscope.

Materials

Cells grown in culture or from dissected organs
PBS (APPENDIX 2A), ice cold
Dounce buffer (see recipe)
10 \times phosphatase inhibitors (see recipe), optional
Tonicity restoration buffer (see recipe), ice cold
0.5 M EDTA, pH 8.0 (APPENDIX 2A)
Triton X-100 lysis buffer (see recipe)
Phase-contrast microscope
Glass-glass (Dounce) homogenizer with pestle B, ice cold

SUPPORT PROTOCOL 1

Radioiodination of Cellular Proteins

7.10.4

10-ml conical centrifuge tube, prechilled
Ultracentrifuge, Beckman TLA100.3 rotor or equivalent, and ultracentrifuge tubes,
4°C

Additional reagents and equipment for cell counting (*UNIT 1.1*)

Prepare cells

1. Harvest the required number of cells.

Cells may be harvested from either tissue culture (UNIT 1.1) or organs by standard procedures. Cells in suspension are centrifuged, whereas it is necessary to collect adherent cells with a cell scraper prior to centrifuging. The number of cells to harvest depends on both the application and the cell type used. Typically, solubilized membranes from 2×10^8 cells are labeled. However, for particularly small cells, such as splenocytes or thymocytes, more cells may be used.

2. Wash cells twice with ice-cold PBS.
3. Count the cells (*UNIT 1.1*), keeping a sample of the intact cells to compare with the ruptured cells using a phase-contrast microscope.
4. Resuspend cells at $1\text{--}5 \times 10^8$ cells/ml in Dounce buffer with $1\times$ phosphatase inhibitors if needed.

Cell concentration is generally $1\text{--}5 \times 10^8$ /ml, depending on the cell size. The Dounce buffer induces cell swelling and facilitates homogenization. The Mg^{2+} concentration in the buffer stabilizes the nuclear envelope, but is also a co-factor for a number of proteases. The lowest Mg^{2+} concentration at which nuclear integrity is maintained during the homogenization process is recommended. Nuclear integrity can be monitored using a phase-contrast microscope.

If phosphorylated proteins are studied, tyrosine phosphatase inhibitors should be included in all solutions containing protease inhibitors.

5. Incubate cells 10 to 15 min on ice.

The cells swell during this incubation.

Homogenize cells

6. Transfer cells to an ice-cold, tight-fitting glass-glass (Dounce) homogenizer.
7. Apply 30 to 50 strokes with pestle B in a straight up and down position.

The number of strokes varies, depending on the lability of the cell membrane. This will have to be determined for each cell type by monitoring membrane disruption using the phase-contrast microscope.

8. Add ice-cold tonicity restoration buffer to a final concentration of 150 mM.

Check to ensure that the cell membranes are ruptured using the phase-contrast microscope and compare them to the sample of intact cells. Tonicity restoration will maintain the integrity of the nuclei.

An additional method for cell disruption is the shearing of cells through a 25-G needle. Whereas this also generally works well, the needle has a tendency to clog, and care must be taken to avoid contamination.

Collect membranes

9. Transfer cells to a prechilled 10-ml conical centrifuge tube and centrifuge 5 min at $500 \times g$, 4°C, to spin out nuclei. Save the supernatant, which contains the membranes, and use the cell pellet to check the results of the Dounce homogenization under the microscope.

The membrane fraction will appear turbid and the nuclear pellet will be white when compared to an intact cell pellet.

10. Add 0.5 M EDTA to supernatant to a final concentration of 5 mM.

EDTA is added to chelate Mg^{2+} and subsequently decrease the activity of magnesium-dependent proteases.

11. Transfer supernatant to an ultracentrifuge tube and ultracentrifuge 1 hr at $100,000 \times g$ (~50,000 rpm), 4°C, in a Beckman TLA100.3 rotor or equivalent.

12. Transfer the pellet, which contains the membranes, to a 1.5-ml microcentrifuge tube.

After ultracentrifugation, a pellet containing the membranes will be seen whereas the supernatant should remain clear. The supernatant obtained after ultracentrifugation contains the cytosol and free lipids. Free lipids will float near the top of the tube.

Solubilize membranes

13. Resuspend membranes at 2×10^8 cell equivalents/ml in Triton X-100 lysis buffer for 20 to 45 min at 4°C with frequent, gentle vortexing.

If a large amount of membrane is prepared (e.g., from 5×10^8 small cells, such as lymphocytes), resuspend the membranes first in lysis buffer without Triton X-100 and then add Triton X-100 to a final concentration of 0.5% (v/v).

14. Centrifuge 15 min at $12,000 \times g$, 4°C, to pellet insoluble material.

15. Save the soluble membrane extract in a small microcentrifuge tube.

Membrane extracts can be frozen at -70°C until needed. However, freezing and thawing extracts may affect protein-protein interactions.

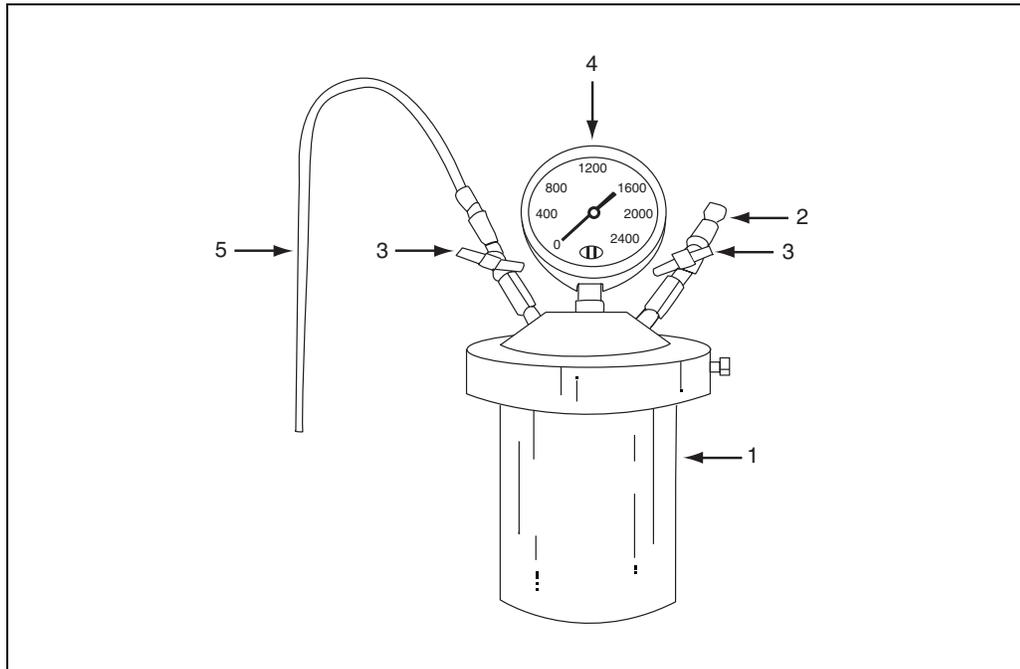


Figure 7.10.1 Nitrogen cavitation device. 1, High-pressure vessel (nitrogen bomb); 2, nitrogen filling tube; 3, valves regulating the flow of nitrogen to and from the vessel; 4, pressure gauge; 5, discharge tube.

Membrane Preparation by Nitrogen Cavitation

This is an alternative approach for membrane preparation. Nitrogen gas is dissolved in a high-pressure vessel (Fig. 7.10.1). Upon decompression, bubbles form and burst the cells. This technique is fast, easy, uniform, and reproducible. The efficiency should be determined by monitoring the samples with a phase-contrast microscope. A nitrogen cavitation device with a nitrogen gas tank is required, however, and this equipment is not inexpensive.

Additional Materials (also see Support Protocol 1)

Nitrogen cavitation device (e.g., Cell Disruption Bomb, Parr Instrument; Fig. 7.10.1)
Nitrogen gas tank

1. Harvest and swell cells as described (see Support Protocol 1, steps 1 to 5).
2. Place cells in a nitrogen cavitation device attached to a nitrogen gas tank and pressurize to 100 psi for 10 min at 4°C.

Before the cavitation device is used, it should be pressurized and checked for leaks.

This level of pressure is good for many cell types; however, the pressure, time of incubation, and rate of release from the nitrogen cavitation device should be optimized for each cell type.

3. Transfer cells to a prechilled 10-ml conical centrifuge tube.
4. Restore tonicity and collect membranes as described (see Support Protocol 1, steps 8 to 15).

RADIOIODINATION OF SOLUBLE PROTEINS

Radioiodinated soluble proteins have been used extensively in cell biology and biochemistry, particularly for receptor binding and internalization assays. The high specificity by which radioiodination targets tyrosine residues (3.6% of the total amino acids in proteins) renders this technique less likely to impair antibody function than nonradioactive methods, such as biotinylation.

Lactoperoxidase-Catalyzed Radioiodination of Soluble Proteins

Soluble proteins can be radioiodinated by the lactoperoxidase-catalyzed or chloramine-T methods. The lactoperoxidase-catalyzed reaction is milder than the chloramine-T method and causes less protein denaturation. However, the specific activity of the radiolabeled proteins obtained by the lactoperoxidase-catalyzed method will be lower than that obtained with the chloramine-T method. Soluble proteins, such as immunoglobulins and protein A, can be radiolabeled using this protocol.

Materials

10 mg/ml BSA in PBS
PBS (APPENDIX 2A), ice cold
30% (v/v) hydrogen peroxide stock solution
0.025 M sodium phosphate buffer, pH 7.4 (APPENDIX 2A), ice cold
1 mg/ml protein sample in PBS
Na^{[125]I} (~0.1 mCi/μl; NEN Life Science Products, Amersham Pharmacia Biotech, or ICN Biomedicals)
1.5 mg/ml lactoperoxidase enzyme (Sigma or Calbiochem) in PBS (APPENDIX 2A), stored in aliquots at -70°C or prepared fresh
15 mM NaI in PBS, prepared fresh and ice cold

PD-10 columns (10-ml columns prepacked with Sephadex G-25 resin; Amersham Pharmacia Biotech)
 γ counter and appropriate counting vials

Set up column

1. Load a PD-10 column with 1 ml of 10 mg/ml BSA in PBS and then wash the column with 20 to 30 ml ice-cold PBS.

The BSA saturates the column so that the small amount of radiolabeled material will not be absorbed nonspecifically to the column, which reduces recovery yields. Other carrier proteins such as fetal calf serum or ovalbumin can be used at the same concentration.

Label proteins

2. Immediately before use, dilute 30% hydrogen peroxide stock solution 1:1000 in ice-cold 0.025 M sodium phosphate buffer to a final concentration of 0.03%.
3. To 10 μ l of 1 mg/ml protein sample, add the following:

0.5 mCi Na^{[125]I}
10 μ l of 1.5 mg/ml lactoperoxidase enzyme
2 μ l of 0.03% hydrogen peroxide.

Incubate 1 min at room temperature with continuous mixing.

Because lactoperoxidase activity is inhibited by azide and sulfhydryl reagents, these should be avoided when the sample is prepared for labeling.

4. Repeat the addition of 2 μ l of 0.03% hydrogen peroxide three more times at 1-min intervals.
5. Add 0.5 ml of 15 mM NaI in PBS.

This serves to compete with the labeled NaI and terminate the reaction.

Isolate labeled proteins

6. Use a disposable pipet to layer the sample onto the PD-10 column (step 1) at room temperature and collect 1-ml fractions until no more fluid drains out.
7. Add 10 ml PBS and continue collecting 1-ml fractions.

It is important to add the PBS when there is no more sample above the Sephadex resin to avoid dilution of the labeled sample.

8. Transfer a 1- to 10- μ l aliquot from each fraction to a counting vial and measure its radioactivity using a γ counter.
9. Determine the specific activity (cpm/mg protein) of the peaks.

The first peak should contain radioiodinated proteins, whereas the second should have unincorporated (free) radioiodide.

10. Pool the desired fractions in a 15-ml conical centrifuge tube.

The radiolabeled sample can be used for immunoprecipitation (UNIT 7.2), as a probe for far western analysis (UNIT 17.2), and other applications. It can be stored in 0.05% (w/v) sodium azide at 4°C or in 50% (v/v) glycerol at -20°C for up to 2 months, taking into consideration the 60-day half-life of ¹²⁵I.

Chloramine-T-Mediated Radioiodination of Soluble Proteins

As described above (see Basic Protocol 3), the chloramine-T method is not as mild as lactoperoxidase-catalyzed radioiodination. It does, however, result in radiolabeled proteins with a higher specific activity.

Additional Materials (also see Basic Protocol 3)

0.5 M sodium phosphate buffer, pH 7.4 (APPENDIX 2A)
Chloramine-T (Sigma)
Sodium metabisulfite (Sigma)

1. Prepare a PD-10 column as described (see Basic Protocol 3, step 1).
2. To 10 μ l of 1 mg/ml protein sample add 10 μ l of 0.5 M sodium phosphate buffer.
The phosphate buffer provides additional buffering capacity required to neutralize the Na[¹²⁵I], which is supplied in 0.1 M NaOH.
3. Immediately before use, prepare 2.25 mg/ml chloramine-T and 7 mg/ml sodium metabisulfite each in 0.5 M sodium phosphate buffer.
The chloramine-T and sodium metabisulfite should be weighed in separate tubes and then dissolved in the proper volume of phosphate buffer immediately before use.
CAUTION: Wear gloves when handling these reagents because they are both toxic.
4. Add 0.5 mCi Na[¹²⁵I] and 10 μ l of 2.25 mg/ml chloramine-T to the protein sample (step 2). Incubate 1 min at room temperature.
Chloramine-T is a potent oxidant that converts iodine into its reactive I⁺ state.
5. Add 10 μ l of 7 mg/ml sodium metabisulfite (step 3) to the mixture to stop the radioiodination reaction. Wait 1 min and then add 50 μ l of 15 mM NaI in PBS.
6. Layer the sample onto the PD-10 column and isolate the labeled proteins as described (see Basic Protocol 3, steps 6 to 10).

REAGENTS AND SOLUTIONS

Use deionized water, preferably filtered, in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers see SUPPLIERS APPENDIX. It is recommended that all solutions be prepared using sterile reagents.

Dounce buffer

10 mM Tris-Cl, pH 7.6 (APPENDIX 2A)
0.5 mM MgCl₂ (APPENDIX 2A)
Store up to 1 year at 4°C
Immediately before use add:
10 mg/ml aprotinin (Boehringer Mannheim–Roche)
10 mg/ml leupeptin (Sigma)
1 mM PMSF (APPENDIX 2A; Sigma)
1.8 mg/ml iodoacetamide (Sigma)

PMSF should be added immediately prior to use, as it undergoes hydrolysis in aqueous solutions.

Lactoperoxidase buffer

Mix 16.8 ml PBS (APPENDIX 2A); 1 ml 0.5 M NaH₂PO₄, pH 7.6 (see recipe); and 2.2 ml water. Store indefinitely at 4°C, but ensure it is not contaminated and check the pH after prolonged storage and adjust as needed.

This buffer is a PBS-based buffer with additional sodium phosphate.

Lactoperoxidase enzyme solution

Dissolve 1.5 mg lactoperoxidase (Sigma or Calbiochem) in 1 ml lactoperoxidase buffer (see recipe). Store in 50- and 250- μ l aliquots at -70°C and thaw only once before use

NaH₂PO₄, 0.5 M, pH 7.6

Add 6.9 g NaH₂PO₄ to 100 ml water. Adjust pH to 7.6 with 0.5 M NaHPO₄. Store indefinitely at 4°C .

Phosphatase inhibitors, 10 \times

4 mM sodium orthovanadate
4 mM EDTA, pH 8.0 (APPENDIX 2A)
100 mM NaF
100 mM sodium pyrophosphate
Adjust pH to 7.6 and freeze aliquots at -20°C

Tonicity restoration buffer

10 mM Tris·Cl, pH 7.6 (APPENDIX 2A)
0.5 mM MgCl₂ (APPENDIX 2A)
0.6 M NaCl (APPENDIX 2A)
Store up to 1 year at 4°C
Immediately before use add:
10 mg/ml aprotinin (Boehringer Mannheim–Roche)
10 mg/ml leupeptin (Sigma)
1 mM PMSF (APPENDIX 2A; Sigma)
1.8 mg/ml iodoacetamide (Sigma)

PMSF should be added immediately prior to use, as it undergoes hydrolysis in aqueous solutions.

Triton X-100 lysis buffer

150 mM NaCl (APPENDIX 2A)
50 mM Tris·Cl, pH 7.6 (APPENDIX 2A)
0.5% (v/v) Triton X-100
Store up to 1 year at 4°C
Immediately before use add:
10 mg/ml aprotinin (Boehringer Mannheim–Roche)
10 mg/ml leupeptin (Sigma)
1 mM PMSF (APPENDIX 2A; Sigma)
1.8 mg/ml iodoacetamide (Sigma)

PMSF should be added immediately prior to use, as it undergoes hydrolysis in aqueous solutions.

COMMENTARY

Background Information

The process of radioiodination occurs when ¹²⁵I (or ¹³¹I) is introduced at the positions *ortho* to the hydroxyl group on tyrosine residues. This reaction may be carried out by either chemical or enzymatic oxidation activity. In most cases, ¹²⁵I is chosen for radioiodination; despite ¹³¹I having about 7-fold greater specific radioactivity, the isotopic abundance of ¹³¹I is usually <20% (due to ¹²⁷I contaminants). Moreover, the

half-life of ¹³¹I is only 8 days as compared to 60 days for ¹²⁵I.

Cell surface radioiodination

Cell surface labeling is an important tool to detect the presence of proteins on the cell surface. This technique is particularly useful for labeling surface membrane proteins for which there are no available antibodies directed against their extracellular domain. Such pro-

teins cannot be detected by simpler methods, such as the sorting of fluorescence-labeled cells or immunostaining. It can also be utilized to determine whether an unidentified protein that co-immunoprecipitates with a known protein is expressed on the cell surface membrane. Another application of cell surface labeling is to follow the kinetics of cell surface-expressed proteins. For example, cells can be stimulated and then surface labeled at various time points. Alternatively, cell surface proteins can be labeled, and then the labeled cells can be cultured without further labeling (i.e., chased) to determine the kinetics of the loss of labeled proteins from the cell surface.

Two commonly used cell surface labeling procedures are radioiodination and biotinylation. Lactoperoxidase-catalyzed radioiodination (Basic Protocol 1) almost exclusively labels tyrosine residues, whereas most methods of protein biotinylation primarily target lysine residues. This is an important consideration in choosing which type of cell surface label to utilize. Whereas the average frequency of tyrosine in proteins is only 3.6% of the total amino acids, lysine occurs with a frequency of 7%. However, one advantage of radioiodination is that it is unlikely to impair the ability of the protein to be recognized by a specific antibody during immunoprecipitation or immunoblot analysis. In contrast, conjugating proteins with biotin has been known to impede antibody binding if the biotinylated lysine residue happens to be within the epitope recognized by the antibody. Moreover, due to the high frequency of lysines in proteins, multiple biotinylation often occurs within the same protein. Each biotin that is conjugated adds an additional 500 Da to the molecular weight of the protein, and as a result, the resolved protein product often displays a range of molecular weights, depending on the number of conjugated biotin moieties. Interpreting these results can be confusing, especially when combined with assays to detect phosphorylated proteins, which often display an upshift in their apparent molecular weight.

Cell surface labeling of proteins by a lactoperoxidase-catalyzed reaction results in the covalent attachment of ^{125}I to accessible tyrosine residues (reviewed by Morrison, 1980). Because of the size of the enzyme, this reaction occurs exclusively on the outside of the plasma membrane. As the reaction and all reagents are kept on ice, limited internalization of radioiodinated surface proteins is expected.

There are various other methods for iodinating cell surface proteins, including the IODO-

Beads reagent (Pierce) and the use of lactoperoxidase-coupled beads (rather than free lactoperoxidase), which can reportedly reduce background for certain applications (Koch and Hausteiner, 1976).

Preparation of membranes

Membrane preparation must precede iodination of membrane-solubilized proteins (Basic Protocol 2). Membrane preparation by homogenization (Support Protocol 1) utilizes one type of homogenizer that consists of a cylindrical glass homogenization vessel, in which either a rod-shaped Teflon-coated pestle or a glass pestle with a ball-shaped tip is inserted. Differences between the use of the two are summarized by Kinne-Saffran and Kinne (1989). The Teflon-glass homogenizer is advantageous for the homogenization of tissue fragments with a lower content of connective tissue, whereas the glass-glass homogenizer is more appropriate for tissue with a higher content of connective tissue and for single cells.

Membrane preparation by nitrogen cavitation (Support Protocol 2) allows quantitative disruption of cells in an inert atmosphere without danger of local heating. This method is mainly used for single cells in suspension or for cells grown in culture.

Radioiodination of soluble proteins

Labeling soluble proteins with radioiodine has been a standard laboratory procedure for many years. The techniques are simple, inexpensive, and reliable. Although different labeling techniques have been developed over the years, such as biotinylation and enzyme- or fluorescein-coupling, the use of radioiodinated proteins has some advantages when compared to these other labeling procedures. For example, samples containing radioiodinated proteins can be exposed to films for a longer time so that the signals emitted accumulate over time on autoradiographic film. (The ^{125}I half-life is 60 days.) Proteins labeled by other methods may provide limited levels of signal. This is particularly important when small amounts of a specific protein are tested. The radioiodination protocols presented here are those that are most commonly used (Morrison 1980). They depend on the availability of tyrosine residues, but in most cases, some histidine residues are also radiolabeled. Other labeling techniques are reviewed in Hubbard and Cohn (1976).

The use of immobilized lactoperoxidase as the catalyst for oxidation allows the easy removal of the enzyme itself, which can often

cause much background due to its own radioiodination. The column allows the removal of free radioiodine.

Critical Parameters

Safety precautions for working with ^{125}I are described at the beginning of this unit.

Cell surface radioiodination

The conditions suggested for this protocol have been optimized for a system in which immunoprecipitation is commonly the method of analysis. The number of cells used in this protocol is appropriate for cells of relatively small size, such as lymphocytes. For larger cells, such as fibroblasts, it may be advisable to radioiodinate $1\text{--}2 \times 10^7$ cells. It is important to test the viability of the cells after the procedure; if the cells have not tolerated the [^{125}I]Na labeling, there is likely to be a higher level of background (i.e., ^{125}I -labeled intracellular proteins). Typically, 95% viability should be anticipated. If lower levels of viability are encountered, the amounts of diluted hydrogen peroxide solution may be lowered.

Preparation of membranes

Success of membrane preparation depends upon the careful optimization of the conditions used for each cell type. For Dounce homogenization, the exact conditions, including swelling time and the number of strokes required for optimal cellular disruption without breakage of nuclei, must be determined. This can generally be accomplished by monitoring the disruption using a phase-contrast microscope. In the case of nitrogen cavitation, shearing and breakage of intracellular organelles is minimal if the pressure and time of equilibration are properly adjusted. It is important that the lysis is relatively rapid (to preserve transient associations of proteins), and that the pressure is high enough to ensure complete disruption of the cells and thereby prevent contamination of the nuclear fraction with intact cells. However, the pressure must be low enough to prevent excessive fragmentation of cell membranes.

Protease inhibitors should be used throughout the procedure and if phosphorylated proteins are of interest, phosphatase inhibitors should be used as well. Iodoacetamide is included with the protease inhibitors but it is added primarily because it blocks free sulfhydryls. This is required because after solubilization, disulfide bonds may be formed between polypeptides that are not normally bound covalently, leading to artifactual results.

Magnesium salts are used during cell breakage to stabilize the nuclear envelope; however, as magnesium is a co-factor for many proteases, these should be removed by chelation with EDTA after removal of nuclei.

Radioiodination of soluble proteins

Radioiodination of proteins may induce protein denaturation and inactivation, and therefore the labeling conditions for a specific protein should be determined. The use of lactoperoxidase beads for labeling membrane proteins, which are solubilized in lysis buffer, enables the researcher to perform the radioiodination by adding a minimal volume of beads and dextrose instead of the addition of lactoperoxidase solution and hydrogen peroxide periodically. Thus lysis buffer is not diluted and ratios between the Triton X-100 and the membrane lipids are maintained throughout the entire labeling procedure. Solubilized membrane proteins can be labeled in a manner similar to soluble proteins, utilizing lactoperoxidase and hydrogen peroxide. For the success of the labeling procedure, care should be taken to use proper concentrations of proteins (soluble or membrane-bound), radioiodine, and catalysts. For sensitive proteins such as IgE for example, a lower concentration of lactoperoxidase or chloramine-T and metabisulfite should be used.

Iodinated proteins should be stored in 0.05% (w/v) sodium azide at 4°C to inhibit bacterial contamination. Alternatively, aliquots may be stored at -20°C with 50% (v/v) glycerol. The presence of a carrier protein such as serum or albumin also helps prevent breakdown of protein due to radiation damage during storage.

Anticipated Results

Cell surface radioiodination

A typical cell surface radioiodination experiment should yield ~95% viable cells. A moderately expressed surface protein (with accessible tyrosines) should be detectable by immunoprecipitation and autoradiographic exposure of dried SDS-PAGE gels or of nitrocellulose membranes in 1 to 4 days. Highly expressed surface proteins, or proteins with multiple tyrosines may be detectable in exposure times of hours. An enhancing screen in the exposure cassette and storage of the cassette at -70°C will expedite results.

For many applications, an important control for surface-labeling experiments is testing the level of leakiness, or the level of intracellular proteins labeled by this procedure. One possi-

bility is to immunoprecipitate an entirely intracellular tyrosine-containing protein, such as actin or tubulin. Although these are highly abundant proteins, little or no label should be expected.

Preparation of membranes

Purification of membranes prior to solubilization results in a 5- to 10-fold purification over total cell extracts, but also in a variable decrease in yield. This will vary depending on the procedure used for cell breakage. In preparing membranes, as the efficiency of cellular disruptions increases, so does the degree of nuclear damage.

Membranes may also be isolated from specific intracellular organelles. Isolation of intracellular organelles is described in Chapter 3 of this book.

Radioiodination of soluble proteins

Radiolabeled soluble proteins can be used for immunoprecipitation (UNIT 7.2), immunoblotting (UNIT 6.2) and far western analysis (UNIT 17.2). Autoradiographic exposure time of dried SDS-PAGE gels or of nitrocellulose membranes can range from 1 to 7 days, depending on the levels of the labeled proteins. An enhancing screen in the exposure cassette and storage of the cassette at -70°C will expedite results.

Time Considerations

Once the cells have been harvested, the procedures described here should take <3 to 4 hr. For radioiodination of solubilized membrane

proteins, membrane preparation takes an additional 2 to 3 hr.

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Key Reference

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Contains background information and standard protocols for radioiodination by chloramine-T- and lactoperoxidase-catalyzed radioiodination.

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CHAPTER 8

Cell Cycle Analysis

INTRODUCTION

The cell cycle is the ordered set of processes through which cells divide, producing two daughter cells possessing the same set of genetic information (Murray and Hunt, 1993). To pass successfully through the cell cycle, all cells must complete three essential tasks: replicate their DNA, faithfully segregate their chromosomes to the daughter cells, and separate the daughter cells from each other. In most cases, cells must also grow during each cell cycle so that the daughters are of sufficient size. One of the most fundamental decisions cells make is whether to progress through the cell cycle. Frequently, this decision is closely linked with choices of cell fate, such as whether to undergo differentiation or programmed cell death.

The last ten years has witnessed a dramatic growth in our understanding of the fundamental mechanisms that regulate the cell cycle. It has become clear that the cell cycles of all eukaryotes are regulated by a family of cyclin-dependent kinases (CDKs). As described in *UNIT 8.1*, these kinases drive both chromosomal DNA replication (S phase) and mitosis (M phase). These kinases are in turn controlled by phosphorylation and by the regulated proteolysis of their cyclin subunits. Orderly progression through the cell cycle is assured by regulatory mechanisms (frequently called checkpoints; Hartwell and Weinert, 1989) that inhibit advancement to the next phase of the cell cycle until necessary functions in the current phase are complete—e.g., by blocking the activation of mitotic CDKs and chromosome condensation until DNA replication is complete.

To address questions related to the cell cycle, it is essential to have clear markers for the cell-cycle state of cells. While mitosis is accompanied by clear morphological changes, biochemical markers are required to determine the state of cells during interphase. Since chromosomal DNA replication occurs during S phase, it is possible to assay directly whether individual cells are in S phase by measuring their capacity to incorporate nucleotide analogs, such as bromodeoxyuridine (BrDU), into DNA (*UNIT 8.2*). DNA content is a general diagnostic marker for whether cells have undergone DNA replication in S phase (thereby doubling their DNA content) or cell division in M phase (thereby reducing their DNA content by half). DNA content can also be monitored by flow cytometric analysis (*UNIT 8.4*). Finally, since different CDKs are active at different phases of the cell cycle, the activities of members of the CDK family also serve as a clear diagnostic marker for the cell-cycle state of cells (*UNIT 8.2*). A combined approach may be used for a more detailed description of the cell-cycle state, monitoring both the abundance of cyclin subunits and the DNA content of cells by flow cytometric analysis (*UNIT 8.4*).

The capacity to obtain populations of cells that are uniform with respect to their cell-cycle state is also essential for cell-cycle studies. Cultured cells can be arrested at various stages of the cell cycle through manipulation of their nutritional status or through treatment with inhibitors that block their progression through S phase or mitosis (*UNIT 8.3*). Alternatively, M phase cells can be obtained from adherent cultures through “mitotic shakeoff”, taking advantage of the decreased attachment of cells to the culture vessel during mitosis (*UNIT 8.3*). Since a relatively small proportion of cells in an exponentially growing culture are in mitosis at any given time, mitotic shakeoff is frequently combined with some

inhibitor treatment or nutrient manipulation. Prolonged arrest in response to nutritional manipulation or drug treatments can frequently perturb cell metabolism. In cases where this is undesirable, it is possible to make use of the fact that cell size is directly correlated with cell cycle stage. Centrifugal elutriation (*UNIT 8.5*), which separates cells on the basis of size, can be employed to obtain synchronous populations of cells from different cell cycle phases without drug treatments.

LITERATURE CITED

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Mary Dasso

Inductive signals from the environment and a variety of intracellular signals determine whether a cell enters the mitotic cell cycle and proliferates or withdraws from the mitotic cycle to meet with a nonproliferative fate. These signals influence the activation of cyclin-dependent kinases (CDKs), which lie at the heart of the intrinsic cell cycle machinery responsible for integrating cell division with developmental and environmental stimuli. A better understanding of these CDKs is expected to provide insight into the diverse processes that involve signal transduction, differentiation, senescence, apoptosis, development, and tumorigenesis.

THE BIOLOGY OF THE CELL CYCLE

A typical cell cycle proceeds with alternating rounds of DNA replication, which takes place during S phase, and nuclear and cytoplasmic division, which take place during M phase. DNA synthesis and mitotic division are separated by the intervening gap phases, G₁ and G₂, during which checkpoint controls are superimposed to preserve the accurate transmission of genetic information. Checkpoints achieve this by monitoring cell cycle events, such as DNA replication and spindle assembly, and generating signals in response to errors in these processes so that cell cycle progression can be halted until repairs are complete (Murray, 1994). These controls ensure that cell cycle events occur in the appropriate sequence and that cell cycle arrest will occur if these events are not carried out in the correct order. Checkpoint controls are responsible, therefore, for ensuring that cells enter S phase only after successful completion of the previous mitosis, attainment of adequate cell size, and repair of any spontaneous or environmentally induced DNA damage. Alternatively, checkpoints induce apoptotic pathways for damaged cells as a way of preventing them from giving rise to mutant progeny. For example, the tumor suppressor protein p53 plays an important role in DNA damage-induced cell death (Lowe et al., 1993) and G₁ phase arrest (Kuerbitz et al., 1992). Signals regulating checkpoints and controlling cell proliferation interact very closely with each other.

After each mitotic cycle, cells traversing G₁ phase receive signals from the extracellular

environment, as well as intracellular cues dictated in part by checkpoints, before continuing in the cell cycle to replicate their DNA and divide once again. Extracellular signals that promote entry into S phase or the beginning of a new mitotic cycle include growth factors, mitogens, nutrients, and cell-substratum interactions. Alternatively, in the absence of mitogenic signals or in the presence of mitogenic antagonists, differentiation inducers, and certain spatial cues, cells withdraw from the cell cycle with unduplicated DNA and meet with alternate, nonproliferative fates, such as quiescence (G₀ phase) or differentiation. Some cell types may transiently undergo cell cycle arrest and then undergo apoptosis because the growth factors required for their proliferation may also be necessary for their continued survival.

A cell's "decision" either to commit to or withdraw from the mitotic cycle occurs at the restriction point late in G₁ phase, after which mitogenic growth factors are no longer required for cells to complete division (Pardee, 1989). In other words, cells that pass the restriction point, which occurs 1 to 3 hr prior to the onset of replication, proceed through the remainder of the cell cycle independently of environmental signals. The restriction point encompasses the critical regulatory mechanism that enables a cell to monitor its external and internal environment as well as the integration of these environmental cues to control the switch between alternative cellular fates, such as proliferation, temporal cell cycle arrest, quiescence, and differentiation. Passage through the restriction point and entry into S phase is determined by the phosphorylation status of the retinoblastoma tumor suppressor protein Rb, which is essentially governed by the activities of the CDKs.

THE CELL CYCLE ENGINE

The CDKs

The division cycle of a eukaryotic cell is currently understood (Morgan, 1995) to be a series of phases or transitions in which temporal order is imposed first by the sequential assembly and activation of the CDKs, and second by their proteolytic inactivation (Fig. 8.1.1). CDKs are enzymes composed of a catalytic subunit (the CDK) and an essential activator subunit (the cyclin). In mammalian cells,

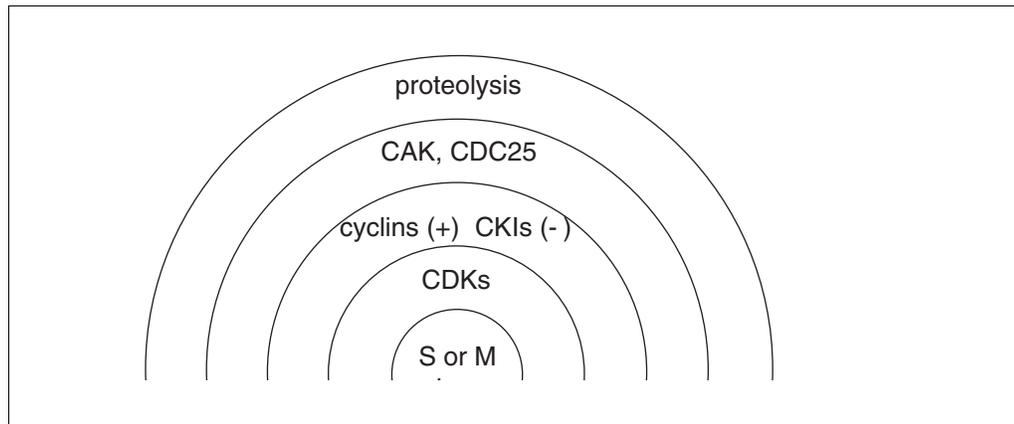


Figure 8.1.1 Peeling back the layers of proliferation control. This “onion” model of proliferation indicates the relationship of each cell cycle component to proliferation or cell division. Cyclin-dependent kinase (CDK) activity, which is absolutely required, depends upon physical interaction with positive regulatory subunits (cyclins) and can be prevented by physical interaction with negative regulatory subunits, called CDK-inhibitory proteins (CKIs). The activation of CDKs also requires phosphorylation by an activating kinase (CAK) and dephosphorylation of phosphates in the ATP-binding motif by members of the Cdc25 family. The entire mechanism can be regulated by proteolysis, because many cyclins and CKIs are targeted by ubiquitin-dependent degradation.

a succession of kinase subunits (CDK4, CDK6, CDK2, and Cdc2) is expressed along with a succession of cyclins (D, E, A, and B) as cells progress from G₁ to M. Cdc2 forms complexes with cyclins A and B to regulate entry into the M phase of the cell cycle.

CDK4/6 and CDK2, whose regulatory partners are the D-type cyclins (D1, D2, and D3) and cyclin E, respectively, represent two distinct classes of G₁ phase-specific CDKs whose activation is required for entry into S phase (Koff et al., 1992; Lew et al., 1991; Xiong et al., 1991). Cyclin D-dependent kinase activity is first detected in mid-G₁ phase as cells approach the G₁/S boundary. Cyclin D-dependent kinases have a distinct substrate preference for the retinoblastoma protein Rb (Matsushime et al., 1992, 1994; Meyerson and Harlow, 1994). Rb binds to and negatively regulates the activities of transcription factors such as E2F-DP1 heterodimers, whose functions are critical for the G₁/S phase transition (reviewed in Nevins, 1992). Under conditions favoring proliferation, the inactivating phosphorylation of Rb by the CDKs in mid- to late-G₁ phase results in liberation of E2F and other Rb-bound transcription factors, which then activate the transcription of S-phase genes. The phosphorylation of Rb is thereby modulated during the cell cycle, in that Rb is present in a hypophosphorylated, active state in G₀ and early G₁ and becomes hyperphosphorylated, and thereby inactive, in late G₁.

Cyclin E-associated kinase activity, although not required for phosphorylation of Rb, is rate limiting for entry into S phase. Cyclin E is expressed periodically at maximum levels near the G₁/S phase transition (Dulic et al., 1992; Koff et al., 1992) and is essential in *Drosophila* for completion of G₁ and progression into S phase (Knoblich et al., 1994). Cyclin E apparently regulates a transition different from that promoted by the D-type cyclins, because cyclin E, but not cyclin D1, is essential for entry into S phase in mammalian cells lacking functional Rb. Inactivation of any of the G₁ CDKs leads to cessation of proliferation and withdrawal from the mitotic cycle.

The activation status of the CDKs is regulated by many mechanisms reflecting both the diversity of the signals they integrate and the central importance of their roles in cell cycle control (Fig. 8.1.2). In general, these regulatory mechanisms act on the synthesis and degradation of the cyclin proteins, formation of cyclin-CDK complexes, positive and negative modification of kinase subunits by phosphorylation, and inhibitory constraints on kinase activity exerted by at least two families of CDK inhibitory proteins (CKIs).

Regulation of CDK activity by cyclins

The primary modulator of CDK activity is the cyclin subunit. Cyclin function is primarily controlled by changes in cyclin levels, which oscillate characteristically throughout the cell

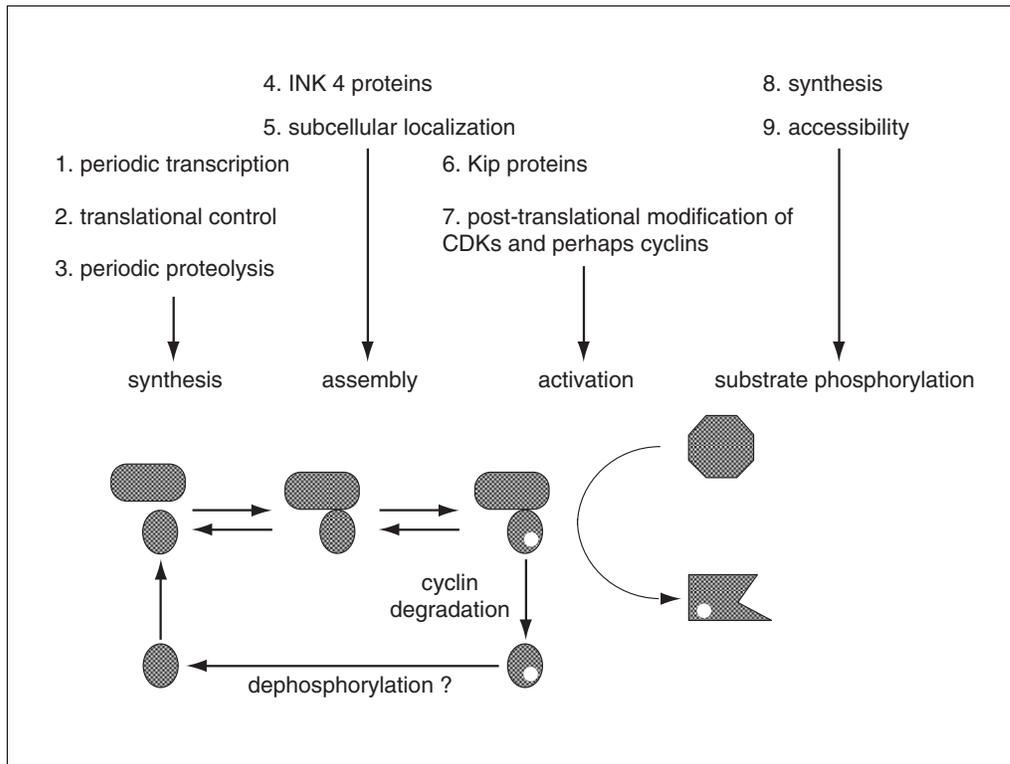


Figure 8.1.2 Activation (and inactivation) of cyclin-dependent kinases (CDKs). This schematic representation shows the five-step pathway (synthesis, assembly, activation, substrate phosphorylation, and cyclin degradation) that leads to CDK activation and proteolytic inactivation. The cyclin is oval, the CDK is round, the substrate is hexagonal, and activating phosphorylation is indicated by the inclusion of a white circle. Phosphorylation of the substrate may affect its structure, as shown here, or may only change the substrate's activity or make it a more attractive binding target for other proteins. Mechanisms that interfere with each step are listed above the step. Abbreviations: INK4, inhibitor of CDK4; KIP, CDK-inhibitory protein.

cycle. In the early *Xenopus* embryo, oscillations in cyclin B levels are mainly controlled by changes in the rate of cyclin degradation. Constant synthesis of cyclin B during the cell cycle provides a roughly linear increase in cyclin concentration until mitosis, at which point cyclin B degradation increases abruptly, resulting in a rapid decline in cyclin levels. Cyclin degradation seems to involve the ubiquitin-dependent proteolytic machinery, which requires the destruction box sequence motif near the N terminus of mitotic cyclins (Glotzer et al., 1991). The trigger for the initiation of cyclin B degradation is not entirely clear, but the proteolytic pathway is well defined.

Regulation of cyclin levels at the transcriptional level occurs in mammalian cells and yeast. In mammalian cells, sequential, periodic oscillations in the levels of the major cyclins largely reflect changes in messenger RNA levels (Pines and Hunter, 1989, 1990), but little is known about the mechanisms that generate these waves of gene expression. These mecha-

nisms have been determined more comprehensively in the yeast *Saccharomyces cerevisiae*, where G₁ cyclins (CLNs) and M cyclins (CLBs) cooperate in overlapping feedback loops to ensure the properly timed execution of their transcription and degradation programs (Amon et al., 1993, 1994). Cyclins may act further to regulate CDK activity by targeting the bound CDK to specific substrates or subcellular locations.

Activation of CDK activity

Because cellular CDK levels tend to remain in constant excess throughout the normal cell cycle, most of the regulatory controls on catalytic activity are post-translational. In addition to cyclin binding, complete activation of the CDK requires phosphorylation at a conserved threonine residue (Thr-160/161). The enzyme responsible for this activating phosphorylation is itself a CDK and has been designated CAK for CDK-activating kinase. CAK is a multi-subunit enzyme composed of a catalytic sub-

unit (CDK7 or MO15) and a regulatory subunit (cyclin H; Fisher and Morgan, 1994). CAK is capable of activating all of the major CDK-cyclin substrates involved in vertebrate cell cycle control. During a normal cell cycle, activation of phosphorylation at the threonine residue by CAK tends to oscillate in parallel with cyclin binding.

Changes in phosphorylation are probably not caused by changes in CAK activity, which remains relatively constant throughout the cell cycle. It is possible, therefore, that cyclin binding may stimulate CDK activation, perhaps by inducing conformational changes that permit CAK-mediated phosphorylation. CDK7, like its substrates, harbors a potential site of phosphorylation at a conserved threonine residue. Mutation of this residue greatly reduces kinase activity, suggesting that activation of CAK itself may require phosphorylation at this site (Fisher and Morgan, 1994). Therefore, although CAK activity is not rate limiting during normal cell proliferation, its regulation by other kinases or additional subunits may be critical under different environmental growth conditions.

Inactivation of CDK activity

Negative regulation of cell cycle progression by inactivation of CDK activity occurs during development, differentiation, senescence, and cell death. These negative controls most likely play a crucial role in preventing tumorigenesis, and an understanding of the balance between negatively and positively acting signals on CDK activities will be of utmost importance in future studies of the cell cycle.

A cyclin-CDK complex can be inactivated by a decrease in the synthesis or an increase in the degradation of cyclins, and through inhibitory phosphorylation at two sites near the N terminus (Thr-14 and Tyr-15 in human Cdc2 and CDK2). Phosphorylation of these two residues is particularly important in the control of Cdc2 activation during mitosis. Phosphorylation of Thr-14 and Tyr-15 parallels the rise in cyclin B levels that occurs as cells approach mitosis. The Cdc2–cyclin B complex is maintained in an inactive state until dephosphorylation of Thr-14 and Tyr-15 at the G₂/M border activates Cdc2 (Krek and Nigg, 1991).

The kinase responsible for phosphorylating Tyr-15 is Wee1, originally identified in *Schizosaccharomyces pombe* (Parker et al., 1991, 1992). Thr-14 and Tyr-15 are both dephosphorylated by the dual-specificity phosphatase Cdc25. Near the onset of mitosis, the Cdc25 protein undergoes a marked elevation in phos-

phatase activity that coincides with extensive phosphorylation of the protein in its N-terminal region. During mitosis, the kinase responsible for this phosphorylation is activated and the corresponding phosphatase is inhibited. The Cdc25-stimulatory kinase may be Cdc2 itself, creating an elegant positive-feedback loop to induce the mitotic dephosphorylation and activation of Cdc2.

Negative regulation of CDK activity occurs also through the actions of CKIs. Two families of these proteins exist that antagonize CDKs by binding to and inhibiting their kinase activity (reviewed in Sherr, 1994; Sherr and Roberts, 1995). The INK4 group (inhibitor of CDK4), whose members include p15, p16, p18, and p19, selectively compete with the D-type cyclins for binding to CDK4/6 to inhibit their kinase activity (Hannon and Beach, 1994; Serrano et al., 1993). The KIP family of inhibitors, which includes p21 (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993), p27 (Koff et al., 1993; Polyak et al., 1994a,b; Toyoshima and Hunter, 1994), and p57 (Lee et al., 1995; Matsuoka et al., 1995), is defined by a conserved domain that is sufficient for stable binding to cyclin-CDK complexes and inhibition of their kinase activity. The KIPs have been described as promiscuous inhibitors because they can bind to and inhibit each of the G₁ CDKs essential for G₁ progression and S-phase entry.

The CKIs seem to function as stoichiometric inhibitors by setting an adjustable threshold for cyclin-dependent activation of the CDKs. In the presence of growth inhibitory stimuli or antimitogenic signals the levels of CKIs in the cell increase. High levels of these proteins prevent proliferation by establishing an inhibitory threshold that cannot be suppressed by the steady-state levels of cyclin-CDK complexes present in the cell. Conversely, mitogenic signals lower the inhibitory threshold to below the level of cyclin-CDK complexes, permitting CDK activation and cell cycle progression. The decision to proliferate or not to proliferate relies, therefore, on a balance of growth-inhibitory and growth-stimulatory signals transduced from the extracellular environment and modulated by CDK activity and the antagonizing effects of the CKIs.

Proteolysis

CKIs have been shown to prevent cell proliferation by negatively regulating cyclin-CDK complexes. Recent findings suggest that the converse may be true as well, i.e., that cyclin-

CDK complexes may inhibit CKI function (Sheaff et al., 1997). Cyclin-CDK complexes in yeast and in mammalian cells seem to promote cell cycle progression through degradation of CKIs by the ubiquitin-proteasome pathway. This pathway also regulates the turnover of certain cyclins, thereby determining their periodic expression as well as their rapid proteolytic inactivation in response to growth factor deprivation.

The ubiquitin-proteasome pathway is involved in protein degradation in mammalian cells and is responsible for the tightly regulated selective turnover of intracellular proteins. Experimental evidence has implicated the ubiquitin system in the degradation of mitotic cyclins, oncoproteins, the tumor suppressor protein p53, several cell surface receptors, transcriptional regulators, and mutated and damaged proteins. Some of these proteolytic events occur throughout the cell cycle, whereas others occur following cell cycle-dependent post-translational modifications (such as site-specific phosphorylation) of the target proteins. Although the signals that designate proteins for degradation are still being elucidated, the enzymes of the ubiquitin-proteasome proteolytic pathway have been well characterized.

Degradation of a protein by the ubiquitin pathway involves two distinct steps: targeting of the protein by covalent attachment of multiple ubiquitin molecules and degradation of the targeted protein. Conjugation of ubiquitin to proteins destined for degradation generally occurs in a three-step process involving three specific classes of enzymes called E1, E2, and E3. E1 is the ubiquitin-activating enzyme that catalyzes the ATP-dependent activation of ubiquitin to produce a high-energy thiol ester intermediate. Following activation, the E2 ubiquitin-carrier (or ubiquitin-conjugating) proteins transfer ubiquitin from E1 proteins to the E3 ubiquitin-protein ligase. Finally, the E3 ligase catalyzes the formation of an isopeptide bond between the ubiquitin polypeptide and the protein substrate. While E1 is a general factor that does not seem to be cell cycle dependent, many E2 and E3 proteins, whose expression might be regulated in a cell cycle-dependent manner, are yet to be identified.

Although the signals responsible for directing the ubiquitin-proteasome machinery to specific target proteins are still being elucidated, recent findings on the turnover of cyclins D1 and E have revealed exciting evidence for phosphorylation-dependent promotion of proteolysis. Unlike cyclins B1 (Brandeis and Hunt,

1996) and A, which are targeted for degradation by the ubiquitin-mediated pathway through a proteolysis targeting signal (known as the destruction box) located within their sequence, cyclins D1 and E are targeted for degradation by an alternative mechanism. Cyclin E degradation by the ubiquitin-proteasome system is regulated by both CDK2 binding and CDK2 catalytic activity (Clurman et al., 1996). The binding of cyclin E to CDK2 protects it from ubiquitination, while CDK2-activated pathways abrogate this protective effect.

Site-specific phosphorylation as a mechanism for substrate targeting in ubiquitin-mediated degradation is further supported by recent reports on the degradation of cyclin D1. It has recently been reported that cyclin D1 turnover is governed by ubiquitination and proteasomal degradation, which are positively regulated by cyclin D1 phosphorylation on Thr-286 (Diehl et al., 1997). Furthermore, this site-specific phosphorylation does not appear to be dependent on CDK4 catalytic activity, suggesting the existence of another kinase that can phosphorylate cyclin D1 to accelerate its destruction by the ubiquitin-proteasome machinery.

Significant findings on the ubiquitin-dependent degradation of CKIs in yeast as well as in mammalian cells (Pagano et al., 1995) have recently been reported. CDK-dependent phosphorylation seems to play a critical role in the targeting of cyclins and certain CKIs for degradation by the ubiquitin-proteasome degradation machinery. The two CKIs that have been identified in the budding yeast *S. cerevisiae* (p40^{Sic1}, which regulates entry into S phase by inhibiting the Cdc28p-Clnp kinases, and Far1p, which is specifically required to arrest the cell cycle in G₁ in response to pheromones) are degraded by a common ubiquitination system. The components of this G₁-S ubiquitination system in *S. cerevisiae* are Cdc34p, Cdc4p, Cdc53p, and Skp1p.

Henchoz et al. (1997) have shown that Far1p is regulated by this ubiquitination system and that an important determinant of the recognition signal for ubiquitination is specific phosphorylation of Far1p by the Cdc28p-Clnp kinase. A similar pathway has been proposed for controlling degradation of the CKI p40^{Sic1} (Feldman et al., 1997). Analogously to Far1p, an essential requirement for ubiquitination and degradation of p40^{Sic1} appears to be phosphorylation by the Cdc28p-Clnp kinase, thereby ensuring that S phase cannot be initiated before the G₁-specific Cdc28p-Clnp kinase has been activated.

The two components of the yeast ubiquitin machinery responsible for recruitment of phosphorylated substrates (in particular p40^{Sic1}) for degradation are Cdc4p and Skp1p. The interaction between Cdc4p and Skp1p is mediated by the F-box, a newly recognized structural motif that seems to be required for Skp1p association (Bai et al., 1996). Skp1p connects many cell cycle regulators to the ubiquitin proteolysis machinery through the F-box motif. It seems likely that Skp1p association may designate active CDKs as targets of the proteolysis machinery.

Ubiquitin- and phosphorylation-dependent degradation may serve as a general mechanism to regulate CKIs. Evidence for the operation of this mechanism in mammalian cells has been provided by reports on the phosphorylation-dependent degradation of the CKI p27 by the ubiquitin-proteasome machinery. Mutations that affect putative sites of phosphorylation by associated cyclin E-CDK2 kinase stabilize p27 in vivo (Clurman et al., 1996; Sheaff et al., 1997; Vlach et al., 1997), demonstrating that p27 is an inhibitor as well as a biologically relevant substrate of cyclin E-CDK2 kinase activity. Ubiquitin-dependent degradation triggered by phosphorylation is a conserved mechanism to regulate the activity of CKIs and cyclins throughout the cell cycle and in response to extracellular signals.

TARGETS OF THE CELL CYCLE MACHINERY

The molecular networking of the CDKs and CKIs seems to affect the fundamental cell cycle regulator Rb. The intricate nature of multiple regulatory systems designed to modulate the activation status of CDK proteins reflects the importance of the substrate protein Rb as a functional unit that controls G₁ phase and as an obligatory oncogenic target (Bartek et al., 1996). Rb is unique as a prototypic member of the “pocket-protein” family, whose members include p130 and p107. All three proteins share structural homology, bind to oncoproteins of small DNA tumor viruses, interact with the E2F transcription factors, and suppress proliferation when overexpressed in cell culture.

Rb is functionally different from p130 and p107 in several important ways, however. Rb-knockout mouse embryos die at embryonic day E14.5 (Jacks et al., 1992; Lee et al., 1992), while certain strains of mice lacking p130 or p107 are viable and fertile, and display no obvious developmental abnormalities (Cobrinik et al., 1996; Lee et al., 1996). Further-

more, heterozygous Rb mice all develop and eventually succumb to specific tumors, while neither heterozygous nor homozygous p130- or p107-mutant mice exhibit increased tumor incidence.

Rb is, therefore, a bona fide tumor suppressor protein whose upstream regulators and downstream effectors comprise a multicomponent biochemical pathway that serves as the molecular mechanism underlying cell cycle control. A better understanding of the functional relationship between Rb and its negative and positive regulators will provide greater insight into the control of cell proliferation and tumorigenesis.

Some targets for CDK activity reside in the replicative machinery, and a brief discussion of the role of CDKs in replication follows. This aspect of control is not yet clearly understood, however, and any discussion at this time will be incomplete.

Activation of a replication origin is controlled at two levels. First, a protein-DNA complex termed the prereplicative complex (pre-RC) forms at the origin; this complex forms only in G₁ phase. Second, the pre-RC is converted into a replicative complex; this occurs only during S phase (reviewed in Stillman, 1996). This control must ensure that origins fire only once per cell cycle; therefore, origin activation must be coordinated with cell cycle progression, presumably by CDKs. Proteins that participate in origin activation have been identified in yeast and *Xenopus*, and, more recently, mammalian homologs (pre-RCs) have been described. Briefly, pre-RCs form only in cells in G₁ phase and are activated at the G₁/S transition. An origin of replication complex (ORC) binds to the origin of replication and acts as a foundation upon which the pre-RC is assembled in G₁ cells. Assembly of the pre-RC occurs with production of Cdc6 and recruitment of MCM (minichromosome maintenance mutants) proteins. After it is assembled, the pre-RC is activated first at the G₁/S boundary, and later throughout S phase for late-firing origins. This event requires the activation of the Cdc7-dbf4 kinase complex. Phosphorylation by this complex stimulates the dissociation of Cdc6 and MCM from the ORC, and replication ensues.

Mitogens and their downstream effectors, the CDKs, can affect this process by regulating the expression of Cdc6 at both the transcriptional and post-transcriptional levels. Oscillations of CDK activities also prevent the formation of the pre-RC. For example, G₂-phase cells cannot support pre-RC formation even when

provided Cdc6 (Piatti et al., 1996). Thus, one active research area is to determine how a cell coordinates the activation of CDKs, the conversion of a pre-RC to a replicative complex, and ultimately does this once, per cell cycle.

A PARADIGM OF CELL CYCLE ANALYSIS

Researchers with little experience in cell cycle studies may benefit from use of the following analytical paradigm, distilled from the literature, which addresses the mechanism of

cell cycle arrest following drug/cytokine treatment or arrest-inducing conditions (also see Table 8.1.1). This analysis should take approximately 3 months to complete. Subsequent units in this chapter will describe in detail some of the techniques that can be used.

First, it is necessary to establish that the cell is arrested in a particular stage of the cell cycle under arrest-inducing conditions. Second, the amount of CDK activity in treated and untreated cells is measured. This can be accomplished in a G₁ cell by determining the extent

Table 8.1.1 A Beginner's Guide to the Cell Cycle Engine^a

Protein	Activity
<i>Positive regulators of CDKs participating directly in the cell cycle</i>	
Cyclin A	Required for G ₁ /S transition and progression through S-phase; a role in G ₂ /M transition
Cyclin B	The mitotic cyclin; essential for G ₂ /M transition
Cyclins D1, D2, D3	Not required in the absence of Rb; cell type-specific patterns of expression
Cyclin E	Required for G ₁ /S transition
<i>Essential CDKs participating directly in the cell cycle</i>	
Cdc2	Binds to cyclin B and cyclin A; essential for mitosis
CDK2	Binds to cyclin E and cyclin A; may associate with cyclin D1; essential for entry into S phase
CDK3	Unknown partner; essential for entry into S phase
CDK4	Binds to D-type cyclins; cell type-specific pattern of expression
CDK6	Binds to D-type cyclins; cell type-specific pattern of expression
<i>Regulators</i>	
CAK	Complex composed of cyclin H and CDK7 with or without a novel protein MAT1
Cdc25	Three isoforms with patterns of expression that suggest one works at G ₁ /S and the others in G ₂ and M
CIP/KIP family (p21, p27, p57)	Inhibit activity of all G ₁ CDK complexes by forming a ternary complex and either blocking activation by CAK or by inhibiting the active complex
INK4 family (p15, p16, p18, p19)	Inhibit activity of cyclin D-CDK4 and cyclin D-CDK6 complexes either by forming a ternary complex or by competing for the CDK subunit
<i>Most frequently used targets of the engine</i>	
Cdc6	Accumulation may be regulated by phosphorylation
E2F (1-5), DP (1-2)	Heterodimeric family of transcription factors that interact with Rb family members
Lamins	Phosphorylation by B-type cyclins correlates with dissolution of the lamin structure at mitosis
Rb, p107, and p130	Phosphorylation by CDKs generally correlates with inactivation
RFA, human SSB	One subunit of the trimeric single-stranded binding protein is phosphorylated by CDKs
Histone H1	One of the first-identified substrates of the CDKs and a convenient in vitro substrate

^aAbbreviations: CDK, cyclin-dependent kinase; CAK, CDK-activating kinase; CIP, CDK-inhibitory protein; KIP, CDK-inhibitory protein; INK4, inhibitor of CDK4; Rb, retinoblastoma; RFA, replication factor A; SSB, single-stranded DNA binding protein.

of phosphorylation of histone H1 by CDK2 and of glutathione-S-transferase-linked Rb by CDK4 and CDK6. These results can be correlated with the phosphorylation and expression of the various CDK substrates (i.e., Rb). Third, it is important to develop a steady-state expression profile of the cyclins, CDKs, and CKIs by immunoblotting as well as by northern blotting (if desired) to measure mRNA levels. Fourth, the formation of active CDK-cyclin complexes is investigated. If no kinase activity is detected, it may be interesting to determine whether this absence is due to CKI binding, post-translational modification of the ATP-binding cleft of the kinase, aberrant localization of one of the cyclins or CDKs, or other events. Finally, it may be relevant to determine whether cyclin-CDK complexes form at all. Are the components in the right place? Are the INK proteins sequestering CDK4 in the cytosol? Is cyclin D1 in the nucleus? These questions may provide insights into the mechanism of cell cycle arrest and may be a source of ideas for future research. If all the results indicate that the cell should not be, but is, in an arrested state, congratulations! You may be on your way to elucidating a novel mechanism. Good luck!

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Assays for CDK Activity and DNA Replication in the Cell Cycle

The somatic cell cycle is normally divided into four phases: S phase, during which DNA is replicated, M phase (mitosis), when chromosomes and cells divide, and the periods in between—G₁ phase (after M and before S) and G₂ phase (after S and before M). Progress through the cell cycle is regulated by a complex formed by a cyclin subunit and a cyclin-dependent kinase (CDK). The catalytic subunit (the CDK) is activated by binding to the cyclin subunit.

In mammalian cells, three different cyclins—denoted cyclins A, B, and E—are primarily involved in regulating the cell cycle. These bind to one of two different CDKs, CDK1 (often called *cdc2*) and CDK2, such that specific cyclin-CDK complexes are present and active at particular phases of the cell cycle (see below). The amount of protein kinase activity of these cyclin-CDK complexes indicates, therefore, where a cell is in the cell cycle. This is useful for analyzing effects on cell proliferation and on cell cycle arrest. Whether a cell is in S phase can be inferred by determining the activation status of specific CDK complexes, but it can also be assayed directly by the incorporation of nucleotide analogs into DNA, which can also show how many cells entered S phase in the course of the experiment. As well as marking particular cell cycle stages in normal cells, the behavior of some cyclin-CDK complexes is perturbed in some transformed cells; therefore, assays for cyclin-CDK activity are often informative in studies on cellular transformation. This unit outlines how to assay cyclin-CDK activity (see Basic Protocol 1) and how to detect DNA replication by incorporation of 5-bromodeoxyuridine (BrdU) into DNA (see Basic Protocol 2).

Particular stages in interphase can be identified according to which cyclin-CDK complexes are present and active as follows:

Cyclin E-CDK2 activity appears in late G₁ stage and disappears in early S phase.

Cyclin A-CDK2 activity appears in early S phase and disappears in early M. In some cell types, cyclin A-CDK1 activity appears in G₂ phase and also disappears in early M.

Cyclin B-CDK1 activity marks the end of G₂ phase and disappears at mid-M.

MEASURING CDK ACTIVITY

Cyclin-CDK activity is assayed by a protein kinase assay using either a peptide substrate or, more usually, histone H1. Histone H1 can also be phosphorylated by a number of other protein kinases, notably cAMP-dependent protein kinase (PKA). For this reason cyclin-CDK assays using histone H1 should be carried out in the presence of a PKA inhibitor. A sharp increase in the histone H1 kinase activity in a whole cell lysate can be measured during mitosis, which is a gross indication of cyclin B-CDK1 activity, the major mitotic kinase. To measure the activity of other cyclin-CDK complexes, however, or to assay specific B-type cyclin-CDK complexes, one must first isolate a specific cyclin-CDK complex by immunoprecipitation.

Alternatively, proteins of the cyclin-dependent kinase subunit (Cks) family can be used as an affinity matrix. These proteins, which include the p13^{suc1} protein from the fission yeast *Schizosaccharomyces pombe* and the p9^{Cks} proteins from *Xenopus* and from humans, bind a number of cyclin-CDK complexes in cell lysates—notably cyclin B-CDK1—with high affinity. Cks proteins covalently linked to Sepharose beads are commercially

BASIC PROTOCOL 1

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available from a number of companies and are often used as a quick and easy way to measure the increase in cyclin B–CDK1 activity at mitosis. They should not be considered specific for cyclin B–CDK1, however, because they bind other cyclin-CDK complexes as well as related kinases, such as MAP kinase.

In choosing an antibody before embarking on the immunoprecipitation and the protein kinase assay, there are two considerations that must be taken into account.

1. Because many CDKs are able to bind more than one cyclin, using an antibody directed against the cyclin will give a more precise indication of the stage of the cell cycle than using one that recognizes the CDK. Unfortunately, most anti-cyclin antibodies are fairly species specific, probably because the primary structure of the cyclins is much less well conserved than that of the CDKs.
2. Some domains of the cyclin and of the CDK are masked in the active complex (Jeffrey et al., 1995). Antibodies raised against the PSTAIRE motif for the conserved part of the CDK of a CDK will recognize only the unbound, inactive kinase and, therefore, are useful only for immunoblotting, not for the kinase assay. Similarly, antibodies raised against the C terminus of the cyclin should not be used because these often only recognize the monomeric protein.

Basic Protocol 1 will give a good indication of cell cycle stage because it works well for cyclins A, B, and E, with their partner kinases CDK1 and CDK2. Complexes between cyclin D and CDK4 or CDK6 cannot be assayed in this manner, however, because they do not phosphorylate histone H1 and may be sensitive to some detergents. (See Background Information for references to more appropriate procedures.) Depending on the equipment available, the results can be assayed using a scintillation counter to measure the incorporation of radiolabeled phosphate into a substrate, or by SDS–polyacrylamide gel electrophoresis (SDS-PAGE; *UNIT 6.1*) followed by autoradiography (*UNIT 6.3*) or phosphorimaging.

Materials

- Cells grown in 6-cm tissue culture dishes
- 1× PBS (*APPENDIX 2A*), ice cold
- Lysis buffer (see recipe), ice cold
- Protein A– or protein G–Sepharose conjugate (Amersham Pharmacia Biotech) or formaldehyde-treated *Staphylococcus aureus* cells (Pansorbin, Calbiochem)
- Anti-cyclin antibody, anti-CDK antibody, or Cks–Sepharose bead conjugate (Upstate Biotechnology)
- Kinase buffer (see recipe), ice cold
- Histone H1 solution (see recipe) or consensus cdc2 peptide (New England Biolabs)
- 1 mM ATP (diluted from 100 mM ATP [see recipe] in distilled H₂O)
- 2000 Ci/mmol [γ -³³P]ATP or 3000 Ci/mmol [γ -³²P]ATP
- 100 mM EDTA (*APPENDIX 2A*)
- 2× SDS sample buffer (*UNIT 6.1*)
- PKA-inhibitory peptide: 1 mM of peptide (Sigma) in 10 mM sodium phosphate, pH 7.2 (see *APPENDIX 2A* for buffer recipe; store peptide solution up to 1 year at –20°C)
- 50 mM roscovitine *or* 100 mM olomoucine (both from Calbiochem) in DMSO (store both solutions in aliquots up to 1 year at –20°C, and thaw only once; store olomoucine in the dark)
- Negative control: immunoprecipitated preimmune serum or anti-IgG antibody
- Positive control: purified cyclin B–CDK1
- 75 mM phosphoric acid (7.5 ml of 1 M phosphoric acid + 92.5 ml distilled water; store up to 2 years at room temperature)
- 96% ethanol

15% to 20% SDS-polyacrylamide gel (UNIT 6.1)
Coomassie blue G-250 staining solution (see UNIT 6.1)
Destaining solution: 10% (v/v) acetic acid

1-ml syringes and 21-G needles, prechilled to 4°C
1.5-ml microcentrifuge tubes, prechilled to 4°C
1-ml pipet tips, prechilled to 4°C
Phosphocellulose units (Pierce) or 1.5-cm squares of phosphocellulose P81 filter paper (Whatman)
Microcentrifuge, 4°C
End-over-end rotator or rotating wheel
Whatman 3MM filter paper
Phosphorimager (optional)

Additional reagents and equipment for SDS-PAGE and Coomassie brilliant blue staining (UNIT 6.1) and for autoradiography and densitometry (optional; UNIT 6.3)

Immunoprecipitate

1. Place 6-cm tissue culture dish containing cells on a glass plate on ice, preferably in a cold room. Remove culture medium with a Pasteur pipet, add 3 ml ice-cold PBS, and let stand for 1 min on ice. Tip the dish and remove PBS with a Pasteur pipet. Repeat wash with ice-cold PBS and carefully remove as much PBS as possible by draining the dish.

Adjust the volumes appropriately for other size dishes. Ideally samples should be assayed in duplicate.

2. Add 1 ml lysis buffer and leave dish on ice for 20 min. Tip the dish and scrape the lysate to one edge with a rubber policeman or cell scraper.

The lysis buffer, also known as RadioImmunoPrecipitation Assay (RIPA) buffer, is a fairly stringent buffer that removes some of the more weakly associated proteins from the cyclin-CDK complex. It is an appropriate buffer to measure the basic level of cyclin-CDK activity, although any modulation by weakly associated proteins will be lost. A variety of other lysis buffers have also been used to assay cyclin-CDK complexes, using the same basic procedure.

3. Take up the lysate through a prechilled 21-G needle attached to a prechilled 1-ml syringe, and transfer to a prechilled 1.5-ml microcentrifuge tube. Draw up the lysate through the needle three times to shear the DNA. Alternatively, sonicate the tube, keeping the lysate cold at all times.
4. Add 10 μ l protein A- or protein G-Sepharose, or 100 μ l formaldehyde-treated *S. aureus* cells, to the lysate. Cap the tube and microcentrifuge 20 min at 10,000 \times g, 4°C, to clear lysate. Remove lysate (~900 μ l) with a prechilled 1-ml pipet tip, being careful not to disturb the pellet.

At this point, the lysate can be frozen in liquid nitrogen and stored at -80°C. Samples should be thawed only once and not refrozen.

5. Add lysate (~900 μ l) to anti-cyclin antibody, anti-CDK antibody, or Cks-Sepharose beads in a prechilled 1.5-ml microcentrifuge tube on ice. Incubate 1 hr to overnight at 4°C, with continuous mixing on an end-over-end rotator or rotating wheel for Cks-Sepharose beads.

The amount of antibody will vary depending on the titer. Most available anti-cyclin or anti-CDK polyclonal antibodies are used at 1:1000 or less. Most monoclonal antibodies are used at 0.1 to 1 μ g/ml, which will be ~1 μ l monoclonal or polyclonal antibody per 6-cm dish of cells.

The tube does not need to be continuously mixed if antibodies are being used.

6. Microcentrifuge the tube 5 min at $10,000 \times g$, 4°C . Transfer lysate to a fresh prechilled 1.5-ml microcentrifuge tube containing 30 to 50 μl of a 50% mixture of protein A- or protein G-Sepharose in ice-cold lysis buffer. Mix on an end-over-end rotator or rotating wheel for 30 to 45 min at 4°C .

When using protein A-Sepharose and a mouse monoclonal antibody, adding 1 μg of a rabbit anti-mouse antibody often increases the efficiency of immunoprecipitation, because some subtypes of mouse IgG do not bind well to protein A. In general, mouse monoclonal antibodies bind better to protein G-Sepharose than to protein A-Sepharose. See Harlow and Lane (1988) for a more detailed discussion and Table 7.2.1.

7. Microcentrifuge 5 sec at maximum speed, 4°C . Remove lysate with a 1-ml pipet tip or 21-G needle attached to an aspirator. Add 700 μl ice-cold lysis buffer to pellet and microcentrifuge 5 min at $10,000 \times g$, 4°C . Repeat microcentrifugation three times with 700 μl ice-cold lysis buffer each time.

Perform kinase reaction

8. Remove supernatant, add 1 ml ice-cold kinase buffer to pellet, and microcentrifuge 5 min at $10,000 \times g$, 4°C .

For SDS-PAGE analysis, transfer the Sepharose beads in the last wash solution to a screw-cap microcentrifuge tube.

9. Prepare kinase assay cocktail:

80 μl kinase buffer
4 μl 1 mM ATP (40 μM final)
4 μl 10 $\mu\text{Ci}/\mu\text{l}$ [γ - ^{33}P]ATP (2000 Ci/mmol) or [γ - ^{32}P]ATP (3000 Ci/mmol)
0.5 μl 10 mg/ml histone H1 (50 $\mu\text{g}/\text{ml}$ final)
2 μl 1 mM PKA-inhibitory peptide (20 μM final)
9.5 μl double-distilled water.

Either ^{33}P - or ^{32}P -labeled ATP can be used. ^{33}P may be preferable, because it emits β particles of a lower energy, and is therefore less harmful to the researcher and requires less shielding to use.

10. Remove as much of the last wash from the pellet as possible without leaving it completely dry. Add 20 μl kinase assay cocktail to the immunoprecipitate or Cks-Sepharose beads on ice and incubate 30 min at 30°C . Add 20 μl kinase assay cocktail to negative control immunoprecipitate and positive control as well.

The amount of protein kinase in an immunoprecipitate should always be compared with that of a negative control. Purified cyclin B-CDK1 to be used as a positive control can be purchased from a number of different suppliers.

As a further test for the specificity of the assay, one can inhibit a parallel set of immunoprecipitates with chemical inhibitors of the cyclin-CDKS. The cyclin-CDK complexes can be inhibited by several different chemicals. Thus far the most specific inhibitors have proved to be roscovitine (use at a final concentration of 5 μM) and olomoucine (use at a final concentration of 10 μM). Either of these inhibitors can be added to a separate reaction to indicate whether the bulk of the protein-kinase activity is due to a cyclin-CDK complex.

Assay for kinase activity

Assay by scintillation counting

- 11a. Add 100 μl of 100 mM EDTA to the samples to stop the reaction. Set up phosphocellulose units in a rack and apply samples to phosphocellulose units using a pipet, being careful not to touch the membrane with the pipet tip. Alternatively, spot samples on 1.5-cm squares of phosphocellulose P81 paper in a glass dish.

Phosphocellulose units and P81 paper are negatively charged; therefore, the substrate must have some positively charged residues. This makes them appropriate to assay either histone H1 or a consensus cdc2 peptide.

- 12a. Microcentrifuge phosphocellulose units 30 sec at $10,000 \times g$, room temperature. Add 500 μ l of 75 mM phosphoric acid and spin again. Transfer phosphocellulose units to fresh microcentrifuge tubes, add 500 μ l 75 mM phosphoric acid, and spin again. Alternatively, rinse P81 squares three times with 5 ml of 75 μ M phosphoric acid, and one time with 5 ml 96% ethanol.
- 13a. Transfer phosphocellulose units or P81 filter papers to scintillation vials, add 2 to 10 ml scintillation fluid, and count on the ^{32}P channel if using ^{32}P , or on the ^{35}S channel if using ^{33}P .

Assay by SDS-PAGE

- 11b. Add 10 μ l of 2 \times SDS-PAGE sample buffer to the samples to stop the reaction. Boil samples 3 min in a screw-cap microcentrifuge tube to reduce the risk of vaporizing radioisotope.
- 12b. Run the samples on a 15% to 20% SDS-polyacrylamide gel (UNIT 6.1).
- 13b. Place the gel in a flat container, add 500 ml staining solution, and let stand 15 min at room temperature. Remove staining solution and add 500 ml destaining solution for 20 min. Repeat destaining three times with 250 ml destaining solution for 15 min each time.
- 14b. Place the gel on Whatman 3MM filter paper, cover with plastic wrap, and dry the gel in a gel dryer for 1 hr at 80°C. Expose the gel in a phosphorimager or to X-ray film.

Phosphorylated histone H1 can usually be detected on the dried gel with a hand-held β counter. If using ^{32}P , place a lead screen with a hole punched in it over the gel to assess the amount of radioisotope in individual lanes. If using ^{33}P , use another piece of X-ray film to block the signal from the other lanes.

- 15b. Measure the amount of incorporated label using a phosphorimager or by densitometry of the exposed film.

The labeled histone H1 will appear as a doublet of bands running at ~30 kDa.

Given the limited linear response of X-ray film, densitometry is an inaccurate way to measure the incorporation of label. It will provide only a rough guide to the relative amount of kinase activity in each sample.

MEASURING DNA REPLICATION USING INCORPORATION OF BrdU

This protocol describes how to use the nucleotide analog BrdU to label and detect cells in the process of DNA replication. BrdU freely diffuses into cells and therefore can simply be added to the culture medium. Cells are grown on poly-L-lysine-coated coverslips, which allows them to be visualized using high-resolution optics on an epifluorescence microscope. The cells are then fixed on the coverslips and the DNA is denatured to expose the BrdU epitope to an anti-BrdU antibody. If BrdU is added for a short time, this method can be used to identify only those cells undergoing DNA replication. Alternatively, a continuous pulse of BrdU will identify all the cells whose DNA was replicated during the experiment. Anti-BrdU antibodies can be either unconjugated, in which case a secondary antibody labeled with a fluorophore must also be used, or conjugated to a fluorophore, in which case the antibodies can be visualized directly by fluorescence microscopy. Conjugated antibodies are generally less sensitive but are sufficient for most purposes, except for the very short pulses of BrdU that are used to visualize individual DNA replication foci.

BASIC PROTOCOL 2

Cell Cycle Analysis

8.2.5

Materials

- 1 mg/ml poly-L-lysine: 20 mg poly-L-lysine (average mol. wt. 400,000)/20 ml dH₂O; filter sterilize through 0.45 µm filter and store at -20°C for 3 months
- Cell culture medium
- Cell suspension: 10⁵ cells/ml in PBS (*UNIT 1.1*; see *APPENDIX 2A* for PBS recipe)
- 10 mg/ml BrdU (see recipe)
- 1 mg/ml 5-fluorodeoxyuridine (FrdU; see recipe)
- PBS (*APPENDIX 2A*)
- Blocking solution: 3% (w/v) BSA in PBS
- 50% methanol/50% acetone (fixative; prepared fresh); *or* formaldehyde solution (cross-linking agent; see recipe) and Tris·Cl/MgCl₂/Triton X-100 (TSM; see recipe)
- Eco*R1 exonuclease and exonuclease III, *or* HCl/Triton X-100 (see recipe)
- Anti-BrdU antibody, conjugated; *or* unconjugated anti-BrdU antibody and fluorophore-labeled secondary antibody (Amersham)
- Slide-mounting solution containing antifade agent
- Grade 1 (0.15-mm) glass coverslips, cleaned and sterilized with dry heat
- Cytocentrifuge (optional)
- Humidified chamber: e.g., inverted petri dish with dampened filter paper in lid

Prepare and seed cells onto coverslips

1. Incubate clean, dry heat-sterilized coverslips for 5 to 15 min at 37°C in poly-L-lysine solution in a 10-cm tissue culture dish. Wash the coverslips with water and air dry at room temperature.

To handle the coverslips, use fine watchmakers forceps with one of the tips bent to ~20° angle.

2. Place coverslips in 6- or 10-cm tissue culture dishes and add enough cell culture medium to cover the surface of the dish (3 to 5 ml). Pipet in the cell suspension. Spin down suspension cells onto coverslips in a cytocentrifuge, or simply incubate them as a 10⁵ cell/ml suspension in PBS with poly-L-lysine coverslips for 10 min at 37°C.

The amount of cells added will depend on the experimental design, but cells should not be confluent after they have reattached to the dish. Note that cells often adhere better to coverslips than to plastic, so they will tend to be a little denser on the coverslip than in the rest of the dish.

Seed cells at least 3 to 4 hr before staining to allow them to adhere properly. Some cell lines may require a different incubation temperature.

Label S-phase cells with BrdU

3. Add BrdU to the cell suspension at a final concentration of 25 to 100 µM. Incubate at 37°C for 15 min to several hours.

Use higher concentrations for shorter times. For labelings >2 hr, FrdU can be added to a final concentration of 0.4 µM. This inhibits thymidylate synthetase, reducing endogenous pools of thymidine and increasing the effective concentration of BrdU in the cell.

4. Remove BrdU and wash coverslips three times with 5 ml PBS each time. Transfer coverslips to a glass petri dish containing 5 to 10 ml PBS. Proceed to either fixation or permeabilization of cells.
- 5a. *Fix/permeabilize cells with methanol/acetone:* Rinse coverslips once with 5 ml PBS and drain off the PBS. Carefully add enough fixative to cover the coverslips (3 ml) and incubate 2 min at room temperature. Remove fixative and rinse coverslips with 5 to 10 ml PBS.

Other fixatives include 100% methanol or 100% acetone. Fixation can also be done at -20°C. If methanol or acetone is used, the permeabilization step is not necessary (go to step 6).

- 5b. *Fix cells with formaldehyde, then permeabilize with TSM:* Rinse the coverslips once with 5 ml PBS and drain off PBS. Carefully add enough formaldehyde solution to cover the coverslips (3 ml) and let stand for 2 to 5 min at room temperature. Carefully rinse the cells twice with 5 ml PBS. Drain off PBS, add enough TSM to submerge the coverslips (3 to 5 ml), and let stand for 5 to 10 min at room temperature. Drain off TSM and wash three times with 5 to 10 ml PBS for 2 min each.

An optional step is to wash once in 50 mM glycine in PBS after removing the TSM to quench the cross-linking reagent. This is not usually necessary unless there is a very high autofluorescent background at the end of the procedure.

6. Place a piece of Parafilm on top of the dampened filter paper in the humidified chamber and pipet ~25 μ l blocking solution onto the paper. Drain the excess PBS from the coverslip by touching the edge to a Kimwipe. Lay the coverslip cell side down on top of the drop of blocking solution and leave for 15 to 30 min. Proceed to denaturation of DNA by either acid or nuclease treatment.

Nuclease treatment is easier to combine with other antibodies in double-immunofluorescence experiments; acid denaturation is sometimes more sensitive.

- 7a. *Denature DNA with acid:* Place coverslips in a glass petri dish, add 5 to 10 ml HCl/Triton X-100, and let stand 10 min at room temperature. Remove coverslips to a fresh petri dish and wash twice with 10 ml PBS for 5 min each to neutralize the acid.

In dual-labeling experiments, the sample should be incubated with the second primary and the secondary antibodies and then fixed again before acid treatment. The first secondary antibody should be labeled with Texas red or TRITC, not FITC, because FITC is sensitive to acid pH.

- 7b. *Denature DNA with nuclease:* Dilute *Eco*RI and exonuclease III in their recommended buffers according to the manufacturers' instructions. In the humidified chamber, place the coverslip cell side down on a drop of ~150 U/ml of *Eco*RI and incubate for 30 min at 37°C. Then place the coverslip on a drop of 300 U/ml exonuclease III and incubate 30 min at 37°C.

*Some commercially available kits contain a cocktail of *Eco*RI, exonuclease III, and anti-BrdU antibody, or DNase I and anti-BrdU antibody, which will denature the DNA and stain with the antibody in a single step.*

Incubate coverslips with antibodies

8. Dilute anti-BrdU antibody in blocking solution according to manufacturer's instructions. Place coverslips cell side down on a drop of anti-BrdU antibody. Incubate coverslips with anti-BrdU antibody for 30 to 60 min at room temperature.

Usually 2 to 5 μ g/ml is a good dilution for monoclonal antibodies.

9. Lift the coverslip off the Parafilm with forceps and place cell side up in a petri dish with 5 ml PBS. Wash the coverslips four times in 5 ml PBS for 5 min each time.

Coverslips stained with anti-BrdU antibody conjugated to a fluorophore can now be mounted on slides (step 12).

10. Dilute fluorophore-labeled secondary antibody in blocking solution according to manufacturer's instructions. Remove coverslips from PBS with forceps and place cell side down on a drop of fluorophore-labeled secondary antibody. Wash the coverslips four times in 5 ml PBS for 5 min each time.

Antibodies and fluorophores vary in their affinity and brightness, so there is no universally appropriate dilution. Most suppliers give a recommended dilution; otherwise it will have to be determined empirically—1:200 to 1:5000 is usually appropriate. Higher dilutions will result in less background staining.

Mount coverslips on slides

11. Dip coverslips in a beaker of water to remove any salts and then drain the water off by holding the edge of the coverslip to piece of Kimwipe.
12. Place each coverslip cell side down onto a small drop of mounting solution containing antifade agent on a good-quality, clean microscope slide. Remove any excess mounting solution from around the edge of the coverslip with a Kimwipe, being careful not to dislodge the coverslip. Seal the coverslip around the edge with a small amount of nail polish.

Commercial mounting solutions are readily available. All mounting solutions should be adjusted to pH 8.5 or greater, because FITC fluorescence is pH sensitive. Mounting solutions containing Gelvatol or Mowiol harden overnight, after which they do not need to be sealed.

Slides can be stored in the dark up to 3 months at 4°C or –20°C.

13. View the slides under epifluorescence using the appropriate filter set for the fluorophore on the primary or secondary antibody.

It is helpful to first locate the optical plane with the cells using transmitted light and then visualize the anti-BrdU staining by epifluorescence.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

ATP, 100 mM

Dissolve ATP lithium salt (Boehringer Mannheim) in water. Adjust pH to 7.0 with 1 M NaOH. Store in aliquots at –20°C for 1 year.

Bromodeoxyuridine (BrdU), 10 mg/ml (27 mM)

Dissolve 100 mg BrdU in 10 ml water. Filter sterilize through 0.45- μ m filter. Divide into aliquots and store up to 6 months at –20°C.

5-Fluorodeoxyuridine (FrdU), 1 mg/ml (4 mM)

Dissolve 10 mg FrdU in 10 ml water. Filter sterilize through 0.45- μ m filter. Divide into aliquots and store up to 6 months at –20°C.

Formaldehyde solution

3.7% (v/v) formaldehyde

2% (w/v) sucrose in PBS (see APPENDIX 2A for PBS recipe)

Prepare fresh

HCl/Triton X-100

17.4 ml of 11.5 N HCl (2 N final)

0.5 g Triton X-100 (0.5% final)

82 ml double-distilled H₂O

Prepare fresh

Histone H1 solution

10 mg/ml histone H1 from calf thymus (Boehringer Mannheim)

10 mM NaPO₄, pH 7.2

Store in aliquots up to 1 year at –20°C

Kinase buffer

250 μ l of 1 M Tris-Cl, pH 7.5 (*APPENDIX 2A*; 25 mM final)
300 μ l of 5 M NaCl (*APPENDIX 2A*; 150 mM final)
100 μ l of 1 M MgCl₂ (*APPENDIX 2A*; 10 mM final)
100 μ l of 0.1 M dithiothreitol (DTT; *APPENDIX 2A*; 1 mM final)
Store in aliquots up to 6 months at -20°C

Lysis buffer

30 ml of 5 M NaCl (0.15 M final)
12 ml of 0.5 M Na₂HPO₄
8 ml of NaH₂PO₄
10 ml of 0.5 M EDTA (5.0 mM final)
10 ml Nonidet P-40 (1.0% v/v final)
10 g sodium deoxycholate (1.0 % w/v final)
1 g SDS (0.1% w/v final)
2.1 g NaF (50.0 mM final)
1.8 g NaVO₄ (1.0 mM final)
10 ml aprotinin (1.0% v/v final)
928 ml double-distilled H₂O
Filter through a 0.45- μ m membrane
Store up to 1 month at 4°C

Final sodium phosphate concentration is 10.0 mM and final pH is 7.2. This buffer is also known as RIPA buffer.

Tris/sodium/magnesium detergent (TSM)

0.5 g Triton X-100 (0.5% v/v final)
2 ml of 1 M Tris-Cl, pH 7.4 (*APPENDIX 2A*; 20 mM final)
1 ml of 5 M NaCl (*APPENDIX 2A*; 50 mM final)
0.3 ml of 1 M MgCl₂ (*APPENDIX 2A*; 3 mM final)
96.2 ml double-distilled H₂O
Filter sterilize through 0.45- μ m filter
Store up to 6 months at room temperature

COMMENTARY

Background Information

A number of markers can be used to assess the progress of a cell through the cell cycle. Cells in mitosis (M phase) are easily recognized under a phase contrast or differential interference contrast (DIC) microscope by their condensed chromosomes, and DNA replication (S phase) was first recognized many years ago by the incorporation of radiolabeled nucleotides into DNA. The long periods between mitosis and DNA replication were defined simply by the absence of any assayable event (except growth), as is signified by the name given to these periods, G₁ and G₂ (the gap phases). With the advent of flow cytometry, all four cell cycle phases can be easily recognized by the replication state of the DNA. G₁-phase cells have a diploid (2*n*) content of DNA, G₂- and M-phase cells have completed DNA replication and therefore have a tetraploid (4*n*) DNA content,

and S-phase cells have an intermediate content.

Much more is understood now about the molecular events underlying progress through the cell cycle than when the phases were named. Progress is controlled by sequential waves of cyclin-dependent kinase (CDK) activity (Fig. 8.2.1). Precisely where a cell is in the cell cycle can be determined by which cyclin-CDK complex is present and active. Cyclin E-CDK2, cyclin A-CDK2, and cyclin B-CDK1 are particularly useful in this respect. The D-type cyclins, with their associated kinase subunits CDK4 and CDK6, are affected by a number of parameters in addition to the stage of the cell cycle. For example, D-type cyclins rapidly disappear when growth factors are removed (Matsushime et al., 1991). The D-type cyclin-CDK complexes must be assayed under different conditions from A-, B-, and E-type cyclin-CDKs because they recognize only a very limited set

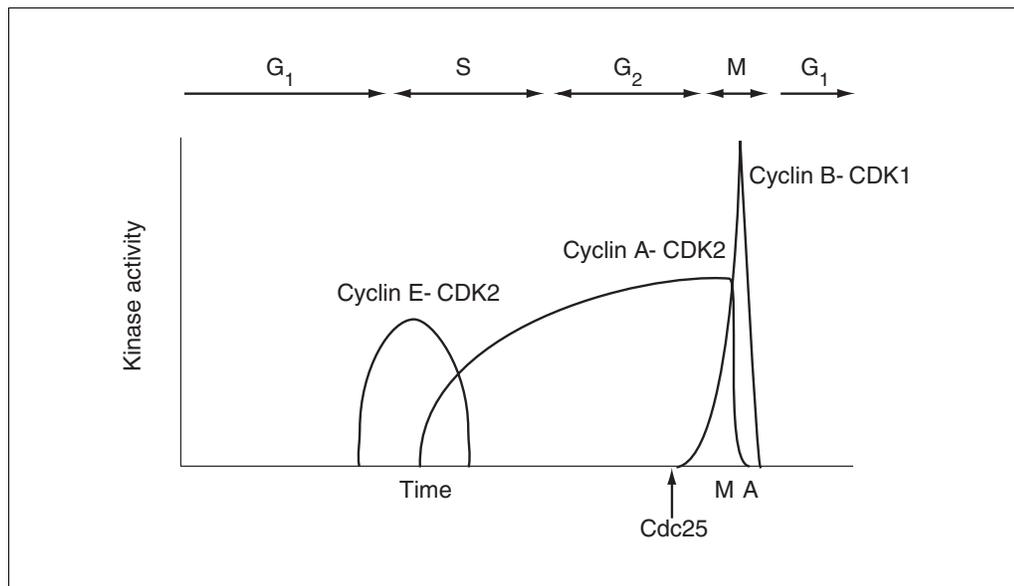


Figure 8.2.1 Appearance and disappearance of specific cyclin-cell-dependent kinase (CDK) activities through the cell cycle. The arrow marks the end of G₂ phase, at which point cyclin B–CDK1 is activated by the Cdc25 phosphatase. The relative kinase activities are not to scale. Abbreviations: M, metaphase; A, anaphase.

of substrates, which does not include histone H1. Of these, the retinoblastoma protein (Rb) is the best characterized (see Phelps and Xiong, 1997, for a more detailed discussion).

The amounts of CDK1 and CDK2 do not vary through the cell cycle, and therefore their activity is regulated by the levels of their cyclin partners. Cyclin levels are regulated both by their synthesis (effectively controlled at the level of transcription) and by their destruction. This is just the first level of regulation, however. Once the CDK has bound its partner cyclin, the complex must be phosphorylated on the T-loop threonine to generate a fully active and stable protein kinase complex. This step is performed by a specific enzyme, CDK-activating kinase (CAK), which in animal cells is itself another cyclin-CDK complex, cyclin H–CDK7, and is sometimes associated with a third protein, MAT1 (reviewed in Morgan, 1995). The phosphorylated cyclin-CDK complex can in turn be regulated by binding a member of a family of specific inhibitor proteins, and by phosphorylation of a threonine and/or tyrosine residue in the ATP-binding region of the kinase (reviewed in Lew and Kornbluth, 1996). These phosphorylation events alter the mobility of the CDK on SDS-PAGE. The final activation of a cyclin-CDK complex is performed by a specific family of phosphatases that dephosphorylate Tyr-14 and Thr-15. This event is responsible for the rapid activation of a preformed pool of cyclin B–CDK1 that drives cells into mitosis. The

cyclin B–CDK1 complex is the most active histone H1 kinase in the cell, and its rapid activation is responsible for the peak of histone H1 kinase activity found in whole cell lysates when cells enter mitosis.

Critical Parameters and Troubleshooting

With the exception of the D-type cyclin–CDK4/6 kinases, the cyclin-CDKs are fairly robust kinases. Active cyclin-CDKs can be isolated from cell lysates that have been frozen immediately after isolation, although the kinases are not stable to repeated rounds of freeze/thaw. Cyclin-CDKs can be inactivated if the phosphate is removed from the T-loop threonine, however, and phosphatase inhibitors must be included in the lysis buffer. Okadaic acid is a poor choice as a phosphatase inhibitor, because it can prematurely activate cyclin B–CDK1 (Yamashita et al., 1991). Another phosphatase inhibitor, β -glycerophosphate, seems to have a stabilizing effect on cyclin-CDK complexes.

Negative results in CDK assays are more likely to be caused by failure to immunoprecipitate the kinase than by denaturation of the protein, which underlines the importance of choosing the correct antibody. If there is no detectable activity in samples that should contain a particular cyclin-CDK, the immunoprecipitates should be immunoblotted with anti-CDK and anti-cyclin antibodies. The anti-

PSTAIRES antibody, which recognizes a conserved alpha helix in CDKS with the sequence PSTAIRES in the center, is useful in this respect because it recognizes both CDK1 and CDK2 on immunoblots. Because the CDKs migrate at between 30 and 34 kDa, a normal reducing SDS-polyacrylamide gel can be used for immunoblots. Cyclins A and E migrate close to the heavy chain of the antibody in an immunoprecipitate, however, and can be masked if the secondary antibody binds to the heavy chain. This problem can be ameliorated by using antibodies from different species for the immunoprecipitation and the immunoblot; by cross-linking the antibody to the Sepharose beads; or by using *N*-ethyl maleimide or iodoacetamide in place of β -mercaptoethanol in the SDS-PAGE sample buffer.

A lack of signal in BrdU labeling is generally caused either by loss of cells from the coverslips during too-vigorous washing (especially before fixation) or by addition of the solutions to the wrong side of the coverslip. Check that there are cells on the slide at the end of the protocol by phase contrast or DIC microscopy. If the coverslips are dropped, it is very difficult to tell which side the cells are on. If the cells are dense enough, try scraping a small area with a needle and looking under a microscope to see if cells have been scraped away. Other problems are usually caused by defective antibodies (e.g., the fluorophore of the secondary antibody) or by defective enzymes in the nuclease method.

Anticipated Results

The cyclin-CDK complexes differ in their activity against histone H1 in the order: cyclin B-CDK1 > cyclin A-CDK1 = cyclin A-CDK2 >> cyclin E-CDK2. The amount of histone H1 kinase activity in any individual immunoprecipitate will vary, therefore, according to which cyclin-CDK is being measured and the stage of the cell cycle. Moreover, some transformed cells have altered levels of different cyclin-CDKs (e.g., cyclin E-CDK2 is overexpressed in a variety of cancer cell lines); therefore, results may differ between cell types.

The length of S phase will depend on the cell type being studied; S phase is ~6 to 8 hr long in many common human and mouse cell lines. When detecting DNA replication using BrdU, the number of positive cells will depend on the length of time for which BrdU is added to the medium and the proliferation state of the cells. With short pulses of BrdU, the sites of DNA replication will appear as distinct dots in

the nucleus of the cell. They are easily seen with a $\geq 40\times$ magnification lens. The number and size of the dots will depend on whether the cell is in early S phase (many small dots) or late S phase (fewer, bigger dots, more around the edge of the nucleus).

Time Considerations

Assuming a 1-hr incubation with the primary antibody, it should take ~3 hr to perform the steps from cell lysis through the kinase assay, and 90 min more to load the samples in the scintillation counter. Analysis of the samples by SDS-PAGE will take another 3 hr before the gel is exposed to X ray film.

For Basic Protocol 2, it should take ~3.5 hr from the time the coverslips are removed from the labeling medium until it is possible to view the slides under the microscope.

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Key Reference

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Presents a wide variety of protocols for analyzing the cell cycle.

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Methods for Synchronizing Cells at Specific Stages of the Cell Cycle

The protocols presented here describe procedures used to synchronize cells in various stages of the cell cycle (Fig. 8.3.1). Synchronization is particularly useful for investigating a particular cell cycle–regulated event or preparing cells for extraction of transient factors whose expression is dependent on cell cycle stage. Exponentially growing cultures are generally asynchronous; i.e., each cell progresses through the cell cycle independently of the cell cycle stage of its neighboring cells. Cells that are synchronized are artificially induced to cycle in a homogeneous manner. The ability to continue cycling is an important distinction between a homogeneous population of cells created by synchronization and one created by blocking cells from cycling. Blocking cells from cycling may also result in a homogeneous population of cells at a particular stage of the cell cycle, but often results in death of the cell. In contrast, the purpose of synchronization is to create an enriched population of cells at a single stage of the cell cycle; these cells will then be able to continue through the cell cycle with as little disruption of normal events as possible. For a comprehensive review of events and explanation of the salient features of each of these stages see Pines (1995), Hartwell and Kastan (1994), *UNIT 8.1*, and the chapter introduction.

Techniques will be presented for synchronizing cells in the G₁, S, and M phases of the cell cycle. These techniques include a selection of methodologies that capitalize on the biology and biochemistry of eukaryotic cells, such as selective nutrient depletion (e.g., isoleucine deprivation, see Alternate Protocol 3; and serum withdrawal, see Basic Protocol 2), feedback control through addition of excess nutrients (e.g., thymidine, see Basic Protocol 4), morphological differences (e.g., mitotic shake-off, see Basic Protocol 1), or the use of chemical agents to reversibly arrest cells at a particular cell cycle stage (e.g., lovastatin, see Basic Protocol 3; mimosine, see Alternate Protocol 4; and nocodazole, see Alternate Protocol 2). The protocols can be modified to enhance for mitotic cells (see Alternate Protocol 1) or to provide sequential G₁/S blocks (see Alternate Protocol 5). Methods are provided for determining the mitotic index (see Support Protocol 1) and for measuring DNA synthesis by trichloroacetic acid (TCA) precipitation of [³H]thymidine-labeled DNA (see Support Protocol 2).

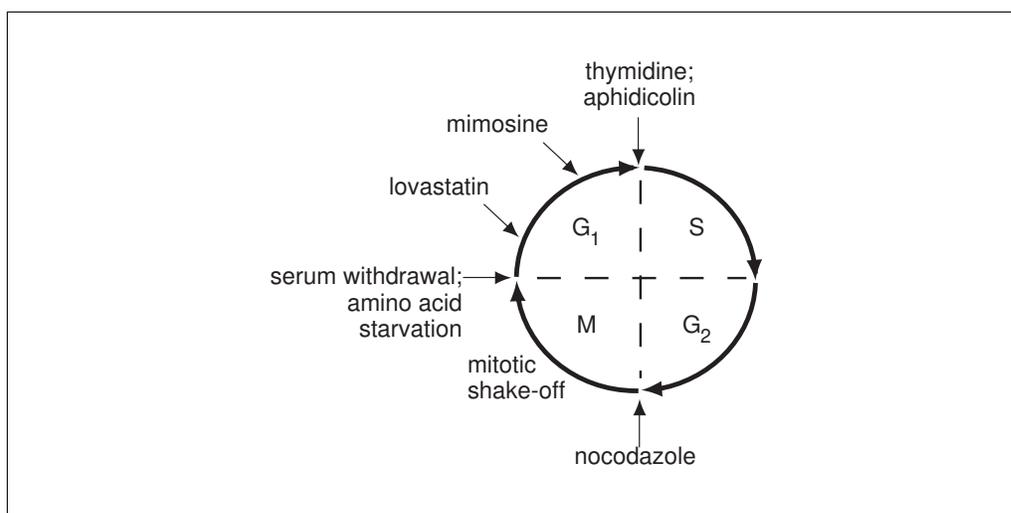


Figure 8.3.1 Relationship between synchronization methods and cell cycle. Arrows indicate the point in the cell cycle at which cells are enriched by each method.

STRATEGIC PLANNING

Prior to selecting a method of synchrony there are several factors to consider.

Stage of Analysis Versus Stage of Synchrony

In general, it is best to synchronize cells as close to the start of the cell cycle phase to be studied (Fig. 8.3.2). In most cases, cells will not maintain a high degree of synchrony through several rounds of cell division. In order to follow the expression of factors as a function of progression through a cell cycle phase, it is usually best to synchronize cells in the phase prior to the actual phase to be investigated. For example, to investigate the S phase-specific expression of a G₁/S phase-cyclin protein (Gong et al., 1995), it may be necessary to synchronize cells in G₁ or at the G₁/S boundary using isoleucine deprivation or isoleucine deprivation coupled with aphidicolin treatment, respectively. The most difficult phase in which to investigate events related to both entry into and exit from is G₁, mainly because of the high degree of variability in G₁-phase progression.

Degree of Uniformity

Inherent in the strictness of this requirement is the specificity of the event to be analyzed. Ideally, cell cycle events that exhibit a broad window of action or expression would not require a high degree of synchrony, and measurements could be made over a longer period of time. In contrast, investigations of rare events with a short window of detection are often the most difficult, requiring a high degree of synchrony in the cell population in order to detect differences.

Number of Cells Required

This is determined in part by the sensitivity of the detection method selected. Cost and time are typically inversely related to the number of cells and degree of synchrony required. In cases where a large number of synchronized cells are required for biochemical

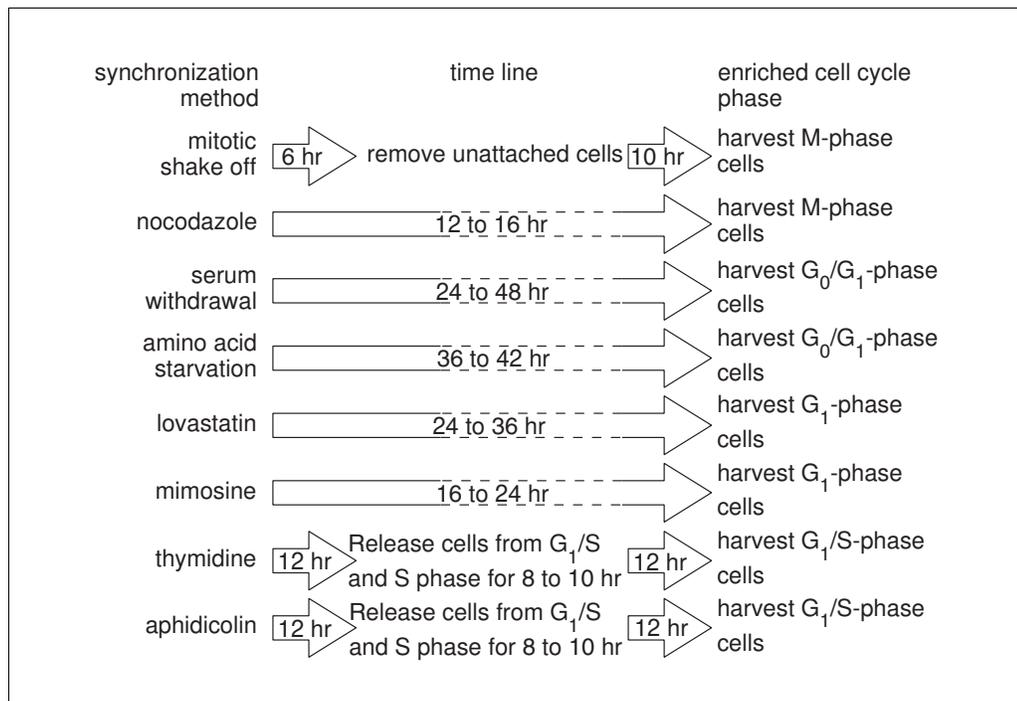


Figure 8.3.2 Quick reference guide to synchronization methods. Indicated times are for cultures with 24 hr doubling time.

characterization of a rare cell cycle event, some procedures, such as double aphidicolin exposure (see Alternate Protocol 5), can be expensive, while the same procedure may be an inexpensive and time-efficient means of obtaining a few hundred highly synchronized cells for analysis by immunofluorescent microscopy.

Doubling Time

All of the protocols discussed here are recommended for cells with doubling times <30 hr. Some may be used for cells with longer doubling times, but the efficacy of the protocol will depend on the specifics of the cells used. While all synchrony methods result in a population of cells enriched in a particular cell cycle phase, cell synchronization is relatively transient, producing synchronous populations for a single cycle. Synchrony generally diminishes each time cells pass through G₁ phase, since this is the most temporally variable stage of the cell cycle. Within a population of cells, G₁ phase averages between 8 and 16 hr for most rapidly dividing cell lines. Progression through S phase requires ~8 hr, through G₂ ~2 additional hours, and completion of M phase occurs 0.5 to 1 hr later.

Chemical Inhibitors

Chemicals that inhibit cell cycle progression are advantageous for synchronization because they are effective in a large variety of cell types, require no special equipment, and lend themselves to applications that require large numbers of cells. Indeed, chemical inhibitors are often the primary method of synchronizing cells at a particular stage of the cell cycle. There are two main disadvantages to chemical synchronization: (1) chemical treatment has been linked to the possible disruption of normal cell cycle regulatory processes (Schimke et al., 1991; Gong et al., 1995), and unbalanced cell growth induces an apoptotic phenotype in some cell types; and (2) chemicals may have more than one target in cells, some of which may not be fully known. With any experiment, however, careful design of controls is of greatest importance when using chemical synchronization.

Assessment of Synchronization

Assessment of the quality of synchronization is dependent on the equipment available. The best method for following overall changes in the distribution of cycling cells is flow cytometry. The most up-to-date benchtop versions of flow cytometers, such as the Becton Dickson FACScan/FACSCaliber and the Coulter Epics, are relatively simple to use, and the preparation of material for analysis is both rapid and inexpensive. If no flow cytometer is readily available, there are biochemical ways to determine cell synchrony. The first method measures the progression of cells through S phase. Short pulses of [³H]thymidine in control and synchronized cultures are compared over time to monitor for peaks in [³H]thymidine incorporation as an indicator of active replication and the maximum number of cells in S phase. This method can also be adapted to the fluorescent microscope using bromodeoxyuridine (BrdU)-labeled cells rather than [³H]thymidine (UNIT 8.2). All cells that were in S phase during the pulse of BrdU can be followed through the cell cycle by labeling them with FITC. The second method measures the expression or sequential activation of cyclin-dependent kinases as an indicator of cell cycle stage. As demonstrated by Hunter, Pines and others (Minshull et al., 1989; Pines, 1995; Hunter and Pines, 1994; Brandeis and Hunt, 1996), cyclins are sequentially expressed and/or stabilized as cells move from G₁ into M phase (Table 8.3.1).

Immunoblotting (UNIT 6.2) for the various cyclins or in vitro kinase assays following cyclin-specific immunoprecipitation (UNIT 8.2) can be used to assess cyclin expression or activity. One caveat to this methodology is the fact that a large number of transformed cell lines inappropriately or constitutively express one or more cyclins (Buckley et al.,

Table 8.3.1 Characteristic Cyclin Expression Profiles and Activation as a Function of Cell Cycle Stage^a

Cell cycle stage	Cyclin	Kinase activated
Early G ₁	D-type	CDK4, CDK6
Mid-late G ₁	E	CDK2
gc S phase	A	CDK2
G ₂ /M	A, B	cdc2

^aAbbreviations: CDK, cell-dependent kinase; cdc, cyclin-dependent kinase.

1993; Keyomarsi and Pardee, 1993; Leach et al., 1993). This should be evaluated prior to using this method of assessment of cell cycle progression and synchronization. Finally, the mitotic index (a morphological evaluation of cell synchrony) can be used to compare asynchronous controls and synchronized cells for the appearance of rounded cells with condensed chromatin (O'Connor et al., 1993). This method has the advantage that no special equipment is required, although the evaluation of ≥ 500 events per sample is somewhat labor intensive and does not lend itself well to evaluating early G₁ synchronization.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

**BASIC
PROTOCOL 1**

ENRICHMENT OF MITOTIC CELLS BY MITOTIC SHAKE-OFF

Mitotic shake-off is one of the oldest and perhaps easiest methods for obtaining mitotically enriched populations of cells (Morla et al., 1989; Pines and Hunter, 1989; Pagano et al., 1992). This method is based on the observation that as cells progress into metaphase during mitosis they become rounded and have fewer points of attachment with the culture vessel (Elvin and Evans, 1983; Zwanenburg, 1983).

This procedure is applicable to most monolayer cells. Collection of pure populations of mitotic cells allows the measurement of cell cycle-related events during the M/G₁/S phase transitions. Each cell type requires some characterization to determine the most suitable times at which cells should be harvested following release.

This procedure has several advantages. This technique is based on normal cellular processes rather than extrinsic control by experimental manipulation and does not require special equipment or reagents other than those commonly available in the average cell culture facility. However, this procedure is not applicable to suspension cultures or weakly adherent cultures, such as some neuronal cell types. Only a small proportion of the cells, 1% to 5% of the typical exponential asynchronous culture, may be in mitosis at any one time; therefore, a limitation of the procedure is the low yield of mitotic cells obtained from exponentially growing cultures. To overcome this drawback and pre-enrich for mitotic cells, one can carry out short incubations with mitotic inhibitors, and/or release cells from G₁/S-phase synchronization using the procedures described below, and capture cells by shake-off as they progress into M. Although this procedure is used for following the progression of cells into G₁, the same degree of synchrony is not maintained as cells progress through subsequent S and G₂ phases.

The following protocol describes mitotic shake-off using the adherent HT-29 colon cell line. It has also been used with CHO cells, HeLa cells, and a number of other colon and breast cell lines.

Materials

Human colon carcinoma cell line (HT-29 cells), grown in 162-cm² tissue culture flasks

RPMI-15: RPMI 1640 supplemented with 15% (v/v) heat-inactivated fetal bovine serum (FBS; *APPENDIX 2A*), prewarmed to 37°C

RPMI 1640 medium (*APPENDIX 2B*), prewarmed to 37°C

0.5× trypsin solution (see recipe)

Additional reagents and equipment for trypsinizing and counting cells (*UNIT 1.1*)

1. Trypsinize (*UNIT 1.1*) subconfluent cultures of HT-29 cells and replat in prewarmed RPMI-15 at one-half to one-third maximum density. Incubate at 37°C until 70% to 80% confluent.

This ensures that the maximum number of cells will be replicating in the culture.

2. Remove the growth medium and wash cells once with prewarmed RPMI 1640. Add 3 ml of 0.5× trypsin solution and incubate cells 5 min at 37°C.
3. Tap the flasks on the bench to shake off any loosely attached rounded cells and add 20 ml prewarmed RPMI-15. Transfer cell suspension to 15-ml centrifuge tubes and centrifuge 5 min at 500 × g, room temperature.
4. Discard supernatant, resuspend pellet in 20 ml prewarmed RPMI-15, and centrifuge 5 min at 500 × g, room temperature. Discard supernatant and resuspend pellet in 20 ml prewarmed RPMI-15.
5. Count cells (*UNIT 1.1*) and adjust concentration to 2.5 × 10⁵ cells/ml in prewarmed RPMI-15.
6. Transfer 20 to 40 ml of cell suspension to 162-cm² flasks and incubate 6 hr at 37°C. Observe the cultures to see if cells have reattached.
7. Shake flasks, swirl medium over the flask surface, and pipet off the medium. Repeat wash twice with 20 ml prewarmed RPMI-15 each time. Add 20 ml fresh, prewarmed RPMI-15 and incubate 10 hr at 37°C.

The washing step removes any unattached cells.

8. Observe the cultures for evidence of cell rounding. Gently shake or tap the flasks on the bench and swirl the medium around the flask. Remove the medium, which contains mitotic cells.
9. Pool the medium from several flasks and centrifuge 5 min at 500 × g, room temperature, to pellet the mitotic cells.
10. Resuspend cells in RPMI-15, count cells (*UNIT 1.1*), and adjust cell density to 5 × 10⁵ cells/ml in RPMI-15. Analyze cells by flow cytometry, Giemsa staining for mitotic chromatin condensation, or phase-contrast microscopy for rounded cells.

For Giemsa staining, submerge an air-dried slide in 0.1 to 0.2 mg/liter Giemsa (Sigma, Life Technologies) for 10 min. Gently rinse stain away by dipping in 1× PBS and air dry the slide.

PRE-ENRICHMENT OF EXPONENTIAL CULTURES FOR MITOTIC CELLS

Short incubations with S phase–synchronizing agents, such as excess thymidine (see Basic Protocol 4), will increase the proportion of cells in M phase. Release cells from this block by thoroughly washing and collecting them ~10 hr later, when significant numbers of rounded cells are observable in the culture. Alternatively, add 0.4 µg/ml nocodazole 5 to 6 hr before shake-off to arrest cells as they progress through M phase.

ENRICHMENT OF MITOTIC CELLS BY NOCODAZOLE ARREST

Nocodazole chemically interferes with the organization of microtubules in cells. Microtubule formation is an important structural feature of cells as they enter mitosis. Several drugs, including vincristine and colcemid, are similar to nocodazole in that they interfere with microtubules and cause arrest in G₂/M. Unlike nocodazole, however, the effects of these agents may not be completely or readily reversed.

Nocodazole arrests both suspension and adherent cells in a prophase to pseudo-metaphase state. Interpretation of results and discernment of mitotic figures may be more difficult when nocodazole is used since nocodazole disrupts microtubules. Microtubules are required for the condensation of chromatin and alignment on the metaphase plate. This procedure has been used to obtain large amounts of activated cyclin B-cdc2-dependent kinases.

Nocodazole is inexpensive and specific in acting on cells in the post-replicative period. It can be added to actively growing cultures without residual effects on other cell cycle stages. Nocodazole is useful for obtaining large numbers of highly synchronized cells. Some cell lines are defective in sensing disruption of the microtubular network and will not arrest in G₂/M, but will instead continue to cycle and undergo abnormal mitosis. Nocodazole can exhibit toxicity at concentrations close to its effective concentration for some cell lines, so the optimal concentration should be determined prior to preparing large amounts of material. The concentration used in this protocol works well with CA46 cells, but lower concentrations may be used with other cell lines. Effective concentrations range from 10 to 400 ng/ml. High concentrations of nocodazole notably diminish cell synchrony following reentry into the cell cycle.

The use of the suspension cell line, CA46, is described in this protocol. Other lymphoma cell lines, breast cell lines, colon cell lines, and HeLa cells have also been used.

Materials

CA46 cells, grown in 75-cm² tissue culture flasks
0.4 mg/ml nocodazole (diluted from 4 mg/ml nocodazole; see recipe)
RPMI-15: RPMI 1640 supplemented with 15% heat-inactivated FBS (*APPENDIX 2A*),
prewarmed to 37°C

Additional reagents and equipment for counting cells (*UNIT 1.1*)

1. Grow CA46 cells to subconfluency (0.5 to 1.0 × 10⁶ cells/ml). Count cells (*UNIT 1.1*) and adjust concentration to 5 × 10⁵ cells/ml in 20 ml prewarmed RPMI-15.

At this dilution, the maximal number of cells should be replicating within the culture.

2. Add 0.4 mg/ml nocodazole to cultures at a final concentration of 400 ng/ml.
3. Incubate cells 12 to 16 hr at 37°C.

4. Centrifuge cells 5 min at $500 \times g$, room temperature, discard supernatant, and resuspend pellet in 20 ml fresh, prewarmed RPMI-15. Repeat centrifugation and addition of 20 ml prewarmed RPMI-15.
5. Transfer cell suspension to fresh 75-cm² tissue culture flasks and incubate 4 hr at 37°C. Monitor cultures during this next 4-hr period for an increase in cell number.

An increase in cell number indicates progression into G₁ phase. Mitotic index, due to the effect of nocodazole on microtubules, may be more difficult to measure if nocodazole is used. The mitotic figures seen following release from nocodazole are sometimes aberrant and less distinct (these probably result from cells trapped in mitosis at the time of application). In addition it is easier and faster to merely count cells than to perform mitotic index measurements if progression through M phase is being measured.

ENRICHMENT OF CELLS AT G₀/G₁ BY SERUM STARVATION

This protocol describes enrichment of cells at G₀/G₁ by serum starvation (Campisi et al., 1984). Although some fibroblasts begin entering G₀ within 1 hr (Pardee, 1989), it may take 24 to 48 hr for an entire population to respond to serum withdrawal. Before using this technique, it is important to determine the confluent/maximum cell density the cells will achieve (cells/ml or cells/cm²).

The method described below is for monolayer cultures, although the procedure can be adapted to suspension cultures. This procedure has been used in many cell cycle-related studies, including analysis of the cell cycle-dependent reversible tyrosine phosphorylation of cdc2 (Morla et al., 1989) and regulation of cyclin A (Girard et al., 1991; Carbonaro-Hall et al., 1993). This simple method for obtaining G₀ cells requires no special equipment or reagents and is effective on cell types that do not respond to confluence arrest. Serum withdrawal may require extensive optimization of both the amount of serum withdrawn and the length of withdrawal. Withdrawal of serum for extended periods will actually reduce the synchronicity of the restimulated population. For some cell types, serum starvation is not a viable method of synchronization, because cells permanently arrest in G₀, do not arrest, or undergo apoptosis.

The use of 3T3 cells, a fibroblastic cell line, is described in this protocol. This procedure has also been shown to be effective in producing a G₀-like state in myoblasts (Puri et al., 1997), CHO fibroblasts (Tesfaigzi and Carlson, 1996), and MOLT-4 leukemia cells (Dbaibo et al., 1995).

Materials

- NIH-3T3 cells, grown in 100-mm tissue culture plates
- 1× trypsin (Life Technologies)
- DMEM (APPENDIX 2A), prewarmed to 37°C
- DMEM-0.5: DMEM supplemented with 0.5% (v/v) heat-inactivated FBS (APPENDIX 2A), prewarmed to 37°C
- DMEM-10: DMEM supplemented with 10% (v/v) heat-inactivated FBS, prewarmed to 37°C

Additional reagents and equipment for trypsinizing and counting cells (UNIT 1.1)

1. Trypsinize (UNIT 1.1) exponentially growing NIH-3T3 cells grown in 100-mm tissue culture plates, using 1× trypsin.
2. Transfer detached cells to 15-ml conical centrifuge tubes and centrifuge cells 5 min at $500 \times g$, room temperature. Discard supernatant, resuspend pellet in 10 ml prewarmed DMEM, and repeat centrifugation. Repeat wash.

3. Plate cells at 30% to 40% of their confluent density in 15 ml DMEM-0.5 and incubate 24 to 48 hr at 37°C.

A 24- to 48-hr period of serum starvation allows cells time to sequester into a G₀-like state. This time period gives cells that have passed the restriction point (Pardee, 1974, 1989) the opportunity to proceed through the remainder of the cell cycle and arrest upon reentry into G₁ of the next cell cycle.

4. Remove DMEM-0.5 and add 15 ml DMEM-10. Incubate at 37°C.

The addition of medium containing 15% FBS stimulates the cells to reenter the cell cycle. NIH-3T3 cells progress into S phase ~12 hr after stimulation; this time will vary depending on the cell type.

ALTERNATE PROTOCOL 3

ENRICHMENT OF CELLS AT G₀/G₁ BY AMINO ACID STARVATION

A large number of synchronous G₀ cells can be obtained by amino acid starvation (Tobey and Crissman, 1972). Omission of isoleucine, an essential amino acid, from the medium causes cells to sequester in a G₀/G₁-like state. Methionine depletion has also been successfully used to synchronize cells (Sallot et al., 1996). In some tumor cell lines, however, this treatment may produce a late S/G₂-phase arrest (Hoshiya et al., 1996). In the author's experience, depleting both isoleucine and methionine does not result in greater synchronization, but rather increases the likelihood of irreversible G₁ arrest and cell death. Before using this technique, it is important to determine the doubling time of the exponentially growing culture.

This procedure can be used with suspension or adherent cell lines to follow the progression of early G₁ cells into the cell cycle. Cells cycle synchronously upon release (addition of complete medium) for an entire cell cycle through cytokinesis. This method does not require exogenous chemicals to cause cell cycle arrest but makes use of the intrinsic biology of the cell. Notably, not all cell types will become synchronous with this procedure. Some transformed cells lines are defective in nutrient checkpoint control and will continue through the cell cycle irregularly. In other cell types, nutrient deprivation induces cell death or partial differentiation rather than transient arrest.

Materials

CA46 cells, grown in 162-cm² tissue culture flasks
Isoleucine-free minimal essential medium (e.g., Select-Amine Minimal Medium, Life Technologies), prewarmed to 37°C
Dialyzed FBS (Life Technologies)
RPMI-15: RPMI 1640 supplemented with 15% (v/v) heat-inactivated FBS
(APPENDIX 2A)

1. Centrifuge exponentially growing CA46 cells 5 min at 500 × g, room temperature. Discard supernatant, resuspend pellet in 50 ml prewarmed isoleucine-free minimal essential medium without serum, and centrifuge again. Repeat wash.
2. Resuspend cells at 35% to 40% of their confluent density in (4 × 10⁶ cells/ml) isoleucine-free minimal essential medium/10% dialyzed FBS, transfer to appropriate size tissue culture flasks, and incubate 36 to 42 hr at 37°C.

This time period is equivalent to one and one-half to two cell cycles for CA46 cells. This time should be appropriately adjusted for each cell type.

Prolonged culture under these conditions will lead to increased toxicity, genetic aberrations, and, in some cases, irreversibility of the arrest.

3. Transfer cells to 50-ml centrifuge tubes and centrifuge 5 min at $500 \times g$, room temperature. Discard supernatant and resuspend pellet in RPMI-15 at a final cell density of 0.5×10^6 cells/ml.

Avoid rough or unnecessary handling of the cells following the starvation procedure because the cells may be more fragile than exponentially growing cells.

Adherent cells should be treated cautiously as they may not be as tightly attached to the plate and may be dislodged by the removal of the isoleucine-free medium or addition of the fresh complete medium.

ENRICHMENT OF G₁-PHASE CELLS USING LOVASTATIN

Lovastatin inhibits HMG-CoA reductase (3-hydroxy-3-methylglutaryl-coenzyme A reductase), resulting in the depletion of mevalonate, which is an essential precursor of cholesterol synthesis and a requirement for isoprenylation of substrate molecules, including p21 ras (Wejde et al., 1993).

Lovastatin has been demonstrated to block a variety of cell types, including adherent and suspension cells, in very early G₁ phase rather than in G₀, as defined by the presence of the proliferation-specific antigen Ki-67 (Keyomarsi et al., 1991). Lovastatin has been effectively used to synchronize breast cancer cells (Bonapace et al., 1996; Wilcken et al., 1997) and Jurkat cells (Martel et al., 1997). Lovastatin is a convenient and rapid inhibitor of early G₁ cells, requiring a single cell cycle to induce a G₁ block. Lovastatin is not effective with all cell types, and in some cell types it also induces a partial arrest of cells in G₂/M (Jakobisiak et al., 1991). Lovastatin is more expensive than some other methods described for generating G₁ populations, and it requires careful optimization of concentration and timing in order to obtain maximal synchronization.

This protocol describes a method by which lovastatin can be used to synchronize breast cancer cells in G₁ phase.

Materials

MCF-7 cells, grown in 100-mm tissue culture plates

1× trypsin (Life Technologies)

DMEM-10: DMEM supplemented with 10% (v/v) heat-inactivated FBS (APPENDIX 2A), prewarmed to 37°C

10 mM activated lovastatin (see recipe)

DMEM-10 containing 5 mM mevalonate (Sigma)

Additional reagents and equipment for trypsinizing and counting cells (UNIT 1.1)

1. Trypsinize (UNIT 1.1) exponentially growing MCF-7 cells using 1× trypsin and replate in 15 ml DMEM-10 at a cell density equivalent to 30% to 35% of maximum.

This prevents growth arrest due to confluence.

2. Add 10 mM activated lovastatin to the culture medium at a final concentration of 40 μM and incubate 24 to 36 hr at 37°C.

This incubation should last for at least one doubling time; times may vary according to the cell type.

Effective concentrations of lovastatin vary according to the cell type and should be optimized; concentrations ranging between 10 and 60 μM have been reported (Keyomarsi et al., 1991).

3. Trypsinize cells in 3 to 5 ml 1× trypsin. Add 5 ml prewarmed DMEM-10 to cells and centrifuge 5 min at $500 \times g$, room temperature. Discard supernatant, resuspend pellet in 10 ml prewarmed DMEM-10 and repeat centrifugation. Repeat wash.

**ALTERNATE
PROTOCOL 4**

4. Resuspend cells at 35% to 40% of their confluent density in 15 ml DMEM-10 containing 5 mM mevalonate.

The mevalonate concentration should be ~100-fold the concentration of lovastatin used. Key regulatory proteins that require isoprenylation for activity will be modified following release of the cells from the lovastatin block.

5. Monitor cells for progression through G₁ and S phases by flow cytometry, activation of cyclin E- or cyclin A-dependent kinases, or incorporation of labeled nucleotide precursors into DNA (see Support Protocol 2).

ENRICHMENT OF G₁-PHASE CELLS BY MIMOSINE ARREST

Mimosine, a plant-derived amino acid whose cellular target is still unclear, blocks cells either in late G₁ phase (Lalande, 1990) or at the G₁/S boundary (Watson et al., 1991; Mosca et al., 1992), depending on the relevance of the recovery period to the timing of initial [³H]thymidine incorporation. When used alone or in combination with prior isoleucine synchronization, mimosine arrests cells with a diploid DNA content (Mosca et al., 1992, Dijkwel and Hamlin, 1992). Mimosine is an effective inhibitor of S-phase entry in mammalian fibroblastic cell lines and human leukemic cell lines, and it inhibits DNA replication in *Xenopus* extracts (Gilbert et al., 1995). Mimosine is an inexpensive and stable inhibitor of cell cycle progression and is relatively non-toxic compared with other chemical inhibitors, such as aphidicolin. Unlike aphidicolin, however, mimosine requires several hours to inhibit cell cycle progression. Studies monitoring cell cycle progression indicate that replication is initiated 1 to 2 hr after release from mimosine-induced blockade.

There is some debate as to the target and position in the cell cycle at which mimosine arrests cells. It has been reported that mimosine inhibits enzymes involved with thymidine biosynthesis (Kalejta and Hamlin, 1997) and other enzymes involved with nucleotide biosynthesis (Gilbert et al., 1995). The lack of clarity as to the exact target and site of action of mimosine could complicate the use of mimosine to study defined events in nucleotide regulation or incorporation.

Materials

CA46 cells, grown in 75-cm² tissue culture flasks

RPMI-15/mimosine: RPMI supplemented with 15% (v/v) heat-inactivated FBS (APPENDIX 2A) and 400 μM mimosine (diluted from 100 mM mimosine; see recipe), prewarmed to 37°C

RPMI-15: RPMI supplemented with 15% (v/v) heat-inactivated FBS, prewarmed to 37°C

1. Centrifuge 5–25 × 10⁶ exponentially growing CA46 cells or CA46 cells synchronized by prior isoleucine deprivation (see Alternate Protocol 3) 5 min at 500 × g, room temperature. Discard supernatant and resuspend pellet in prewarmed RPMI-15/mimosine to give a final cell concentration of 0.4 × 10⁶ cells/ml.

Effective concentrations of mimosine range from 100 to 400 μM.

2. Incubate cells 6 to 24 hr (exponentially growing cells) or 14 hr (isoleucine-deprived cells) at 37°C.
3. Centrifuge cells 5 min at 500 × g, room temperature. Discard supernatant, resuspend pellet in 10 ml prewarmed RPMI-15, and repeat centrifugation. Repeat wash and resuspend cells in prewarmed RPMI-15 at a cell density equivalent to 35% to 40% of their confluent density (0.4 × 10⁶ cells/ml).
4. Monitor progression of cells into S phase by incorporation of labeled nucleotide precursors into DNA (see Support Protocol 2) or flow cytometry.

SYNCHRONIZING CELLS AT THE ONSET OF S PHASE BY DOUBLE-THYMIDINE BLOCK

Synchronization of cells at the G_1/S phase border is generally achieved by inhibition of DNA synthesis using chemical inhibitors, including aphidicolin, hydroxyurea, or excess thymidine (Tobey and Crissman, 1972; Thomas and Lingwood, 1975; Heintz et al., 1983).

Synchronization of cells with excess thymidine was the first reliable method to be widely used (Bootsma et al., 1964, Stein and Borun, 1972). The method involves two sequential exposures to high levels of thymidine, with each exposure separated by an interval determined from knowledge of the normal cell cycle distribution of the exponentially growing cells and the recovery time from the thymidine block. The method is most useful to measure S- and G_2/M -phase events. The procedure is good for one cell cycle passage; the cells lose synchrony rapidly thereafter.

Thymidine is an inexpensive and readily available inhibitor of the cell cycle. Unlike many inhibitors, the mechanism by which thymidine blocks the cell cycle is well understood and attributable to feedback inhibition of the nucleotide synthesis (Thomas and Lingwood, 1975). It may be difficult to accurately measure DNA synthesis by measuring [3H]thymidine incorporation, because of the large pools of thymidine in cells, especially early in S phase. Prolonged disruption of the cell cycle with thymidine results in genetic aberrations presumably caused by misreplication events.

Before using this technique, it is important to determine the doubling time of the exponentially growing culture. The procedure described below is for the synchronization of HeLa cells based on a doubling time of 24 hr.

This protocol can be used for both adherent and suspension cells. The only requirement is that cells display a robust doubling time of less than 24 to 30 hr. Doubling times outside this range may require alterations in the timing of thymidine application and release (see Critical Parameters and Troubleshooting).

Materials

HeLa cells, grown in 162-cm² tissue culture flasks

DMEM-10/thymidine: DMEM supplemented with 10% (v/v) heat-inactivated FBS (APPENDIX 2A) and 2 mM thymidine (diluted from 100 mM thymidine; see recipe), prewarmed to 37°C

DMEM, prewarmed to 37°C

DMEM-10: DMEM supplemented with 10% (v/v) heat-inactivated FBS (APPENDIX 2A), prewarmed to 37°C

1× trypsin (Life Technologies)

1× PBS, pH 7.4 (APPENDIX 2A)

Additional reagents and equipment for trypsinizing and coating cells (UNIT 1.1)

1. Plate exponentially growing HeLa cells at 30% to 50% confluence in 15 ml DMEM-10/thymidine and incubate 12 hr at 37°C.

This time period is equivalent to the progression through G_2/M and G_1 phases. During this period, the G_2/M cells will progress into G_1 and then with the original G_1 cells will achieve a biochemical state equivalent to G_1/S -phase border cells. Any cells in S phase when thymidine is added will become blocked in S phase.

2. Remove the the thymidine-containing medium with a pipet and rinse the dishes twice with 10 ml prewarmed DMEM each time.

Thoroughly rinse all surfaces of the dish to prevent carryover of thymidine.

3. Trypsinize cells using 1× trypsin, resuspend in 10 ml DMEM-10, and count the number of cells in suspension (UNIT 1.1). Replate the cells at 30% to 35% of their confluent density in 15 ml prewarmed DMEM-10 and incubate 16 hr at 37°C.

During this period, the cells will recover from the thymidine block (~1 to 2 hr), progress through the cell cycle, divide, and enter G₁ of the next cell cycle. If the cells are expected to progress through two more cycles, they should be plated at ~25% of their confluent density.

Progression of cells through the first thymidine block can be monitored by determining cell number. Approximately 5 to 6 hr after removal of the thymidine, cells that were blocked at the end of S phase (the leading-edge cells) will enter G₁ of the next cell cycle and an increase in cell number will be detectable. Incubation in medium without added thymidine should occur ~8 to 10 hr later in order to give lagging-edge cells (those arrested at G₁/S in the first block) sufficient time to progress through S phase.

4. Remove medium with a pipet and replat with 15 ml prewarmed DMEM-10/thymidine and incubate 12 to 14 hr at 37°C.

Cells in G₂/M or G₁ will progress and arrest at the G₁/S-phase border.

5. Remove the thymidine-containing medium with a pipet and rinse the dishes twice with 15 ml DMEM-10 each time.

Thoroughly rinse all surfaces of the dish to prevent carryover of thymidine.

6. Monitor the progression of cells into S phase by flow cytometry, into G₂/M by assembly and activation of cyclin B-cdc2-dependent kinase (UNIT 8.2), or through M phase by cell rounding, mitotic index (see Support Protocol 1), or increases in cell number.

ALTERNATE PROTOCOL 5

PERFORMING SEQUENTIAL G₁/S BLOCKS

Variations on the double thymidine–block strategy have evolved with time. A commonly used variant technique involves a first block with thymidine and a second block with aphidicolin (Heintz et al., 1983) or alternatively a double-aphidicolin block (O'Connor et al., 1993). Essentially the same basic principles as described in Basic Protocol 4 for the double-thymidine block apply to the use of aphidicolin. The use of thymidine and aphidicolin procedures for synchronizing cells at the G₁/S-phase border proved useful in determining the regulation of various cell cycle–related processes, including the regulation of histone gene expression (Heintz et al., 1983), regulation of cyclin B (Pines and Hunter, 1989), and effect of DNA damage on the formation and activation of cyclin A– and cyclin B–dependent kinases (O'Connor et al., 1993). Starvation methods paired with sequential aphidicolin treatment are particularly useful for obtaining highly synchronous populations of G₁/S cells (Heintz and Hamlin, 1982, Burhans et al., 1986).

Aphidicolin is a specific inhibitor of eukaryotic DNA polymerase (Heintz and Hamlin, 1982). Treatment of cells with aphidicolin prohibits S-phase cells from progressing through the remainder of S phase, while G₁ and G₂/M cells continue to cycle to the G₁/S boundary (Burhans et al., 1986). For this reason, a single aphidicolin block is not usually sufficient to obtain a highly synchronous culture. Aphidicolin can be substituted for thymidine during both blocks in Basic Protocol 4, by adding aphidicolin (see recipe) to exponentially growing cells at a final concentration of 0.1 µg/ml aphidicolin.

The point at which aphidicolin blocks the entry of cells into S phase is much better characterized and closer to the start of S phase than the point at which excess thymidine blocks. Incorporation of nucleotide precursors into DNA begins <15 min after release of cells from aphidicolin (Burhans et al., 1986); therefore, aphidicolin may be more appropriate for some studies examining S-phase progression. It is important to note that

aphidicolin blocks the replicative DNA polymerase but not other events related to replication such as unwinding of replication origins and assembly of replication complexes. For this reason, there is some controversy about the exact phase in which aphidicolin arrests cells from cycling. It is clear that aphidicolin blocks cells at a cell cycle phase before the incorporation of deoxynucleotides into long polymers of DNA at the very beginning of S phase.

Aphidicolin can be used in suspension cultures or adherent cell lines. While aphidicolin is rapid and dependable and produces a highly synchronous population of cells immediately poised to enter S phase, it is also more expensive to use than excess thymidine. In addition, prolonged treatment of cells with aphidicolin results in DNA fragmentation (Schimke et al., 1991).

DETERMINING MITOTIC INDEX

The mitotic index is used to monitor the progression of cells through M phase, when chromatin condensation occurs. This procedure is labor intensive, but it correlates well with the progression of cells through M phase and cytokinesis (O'Connor et al., 1993). This method is useful to monitor cell cycle progression into M phase in the presence of inhibitors of cell division, such as colcemid or vincristine, when observation of the progression of cells through a single cell cycle is desired.

This protocol is adaptable to attached cells that have been trypsinized or to suspension cells. When working with adherent cells it is important to guarantee that you are observing the entire population of cells and not a subset of easily detached cells. Adherent cells staged at certain phases in the cell cycle attach more tightly to their substratum (see previous protocol) and may detach more poorly than other cells in the same vessel. Therefore, to avoid an experimental artifact and overestimation of mitotic cells due to uneven detachment of cells, culture vessels should be carefully viewed after trypsinization and removal of cells to ensure that no or few cells are left behind. Mitotic index should be determined at several timepoints (see below). Elevation of the percentage of cells exhibiting mitotic figures relative to the asynchronous control is evidence that the cells are actively cycling and are synchronized.

Materials

Burkitt's lymphoma cell line (CA46 cells), grown in RPMI-15, synchronized and unsynchronized

RPMI-15: RPMI supplemented with 15% (v/v) heat-inactivated FBS

1× PBS, pH 7.4 (APPENDIX 2A), ice cold

0.5× PBS, pH 7.4 (APPENDIX 2A), or 75 mM KCl, ice cold

3:1 (v/v) ethanol/glacial acetic acid, ice cold

Giemsa stain (Sigma, Life Technologies; optional)

1. Stimulate $>1 \times 10^3$ CA46 cells for each timepoint to enter the cell cycle by the method indicated in the synchronization protocol or by changing to fresh RPMI-15.
2. At the appropriate timepoints, transfer cells to a 15-ml centrifuge tube and centrifuge 5 min at $500 \times g$, room temperature. Discard supernatant, resuspend cells in 2 vol ice-cold 1× PBS, and repeat centrifugation. Discard supernatant and resuspend cells in 0.5 ml ice-cold 0.5× PBS for 10 min or ice-cold 0.75 mM KCl for ≥ 20 min, on ice to permit swelling of the cells without rupture.

This hypotonic swelling makes mitotic figures easier to distinguish. Alternatively, add 6 ml of 2% (v/v) 3:1 ethanol/glacial acetic acid and store overnight at 4°C.

The number and frequency of timepoints depends on the experiment. For G_1 - or S-synchronized cells, mitotic index can be monitored every 4 hr; for late S- or M-synchronized cells,

it should be monitored every 30 min. Unsynchronized cells should be analyzed at the same timepoints to assess the success of the synchronization protocol.

3. Centrifuge cells 5 min at $500 \times g$, room temperature. Discard supernatant and resuspend pellet in 0.5 ml of 3:1 ethanol/glacial acetic acid. Finger vortex gently while resuspending to avoid cell clumps.

The final cell concentration should be $1-5 \times 10^3$ cells/ml.

4. Drop $\sim 100 \mu\text{l}$ cells onto glass slide, two to three slides per timepoint. Allow slides to air dry for 0.5 to 2 hr.

Cells should not overlap, therefore, the optimal concentrations of cells to be placed on each slide can be determined empirically by dropping cells onto one or two slides and observing them for the distribution on the slide. To help cells spread evenly, the slide can be tilted at a slight angle to improve the spread of cells.

5. Stain cells with 0.1 to 0.2 mg/liter Giemsa stain for 10 min. Gently rinse Giemsa stain from slides by dipping in $1 \times$ PBS and air dry slides.
6. Observe slides under the microscope for the presence of cells with mitotic figures with condensed nuclear material and a lack of nuclear membrane. Represent the data as a percentage of mitotic cells in the total number of cells observed.

Generally, 200 to 500 cells are scored for each timepoint.

SUPPORT PROTOCOL 2

MONITORING [^3H]THYMIDINE INCORPORATION INTO DNA BY TCA PRECIPITATION

Pulse-labeling of cells with [^3H]thymidine (Thomas and Lingwood, 1975) followed by TCA precipitation onto filters (Jackman et al., 1994) has been used to monitor synchronous cells for peak DNA synthesis (>3 - to 4-fold increase over baseline incorporation of labeled nucleotides). Peak DNA synthesis reflects the point in time when the maximum number of cells are in S phase.

Materials

CA46 cells, released from synchronization
RPMI-15: RPMI supplemented with 15% (v/v) heat-inactivated FBS (APPENDIX 2A),
prewarmed to 37°C
[^3H]thymidine (20 to 60 Ci/mmol)
 $1 \times$ PBS (APPENDIX 2A)
 $0.5 \times$ PBS (see recipe)
20% (v/v) TCA, ice cold
5% (v/v) TCA, ice cold
95% ethanol

Dry ice/methanol bath
Wet ice in bucket
Glass fiber filters
Vacuum manifold
Vacuum drying oven, 80°C

1. Following release of cells into the cell cycle by replacement of amino acids or growth factors (Basic Protocol 2, Alternate Protocol 3) or by removal of inhibitors (Basic Protocol 3, Basic Protocol 4, Alternate Protocol 4), transfer several aliquots of 1×10^5 CA46 cells to 15-ml plastic centrifuge tubes.

The number of cells can be reduced 10-fold, if necessary

2. Centrifuge the cells 5 min at $500 \times g$, room temperature. Discard supernatant and resuspend pellet in an equivalent volume of prewarmed RPMI-15 supplemented with $5 \mu\text{Ci/ml}$ [^3H]thymidine. Incubate 30 min at 37°C .
3. Centrifuge cells 5 min at $500 \times g$, room temperature, discard supernatant, and resuspend pellet in 5 ml ice-cold $1\times$ PBS. Repeat PBS wash and resuspend pellet in 0.5 ml $0.5\times$ PBS.
4. Place cells in tube rack in dry ice/methanol bath and let stand for 1 to 3 min. Allow cells to thaw for 3 to 5 min at 37°C . Repeat twice.

The freeze-thaw process lyses the cells.

5. Place tubes containing cell lysates in bucket containing wet ice. Add an equal volume of ice-cold 20% TCA to the lysates and let stand 30 min on ice.
6. Moisten glass fiber filters with 5% TCA, then place in vacuum manifold and seal in place according to the manufacturer's directions.

Label filters with pencil prior to wetting or, alternatively, label the caps of scintillation vials into which filters will be placed.

7. Transfer precipitates to glass fiber filters under vacuum. Wash each tube twice with 2 vol ice-cold 5% TCA and transfer this material also to the appropriate filter.
8. Wash filters three times under vacuum with 5 ml ice-cold 5% TCA and once with 5 ml ice-cold 95% ethanol.
9. Transfer filters to scintillation vials and set caps loosely in place. Place vials in 80°C vacuum oven for ~ 45 min.

Include blank filters as a negative control for background.

10. Add 5 to 10 ml scintillation fluid to each vial. Place vials in scintillation counter and count on the ^3H channel for 5 min each. Average the cpms from multiple aliquots and plot against time from release.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Aphidicolin, 10 μM

10 μM aphidicolin (Sigma) in dimethylsulfoxide (DMSO)
Store <3 months at -80°C

Lovastatin, activated, 10 mM

Dissolve 52 mg lovastatin (Merck, Sharp, and Dohme Research Pharmaceuticals) in 1.04 ml of 95% ethanol. Add 813 μl 1 N NaOH. Adjust to pH 7.2 with 1 M HCl and dilute to 13 ml (4 mg/ml; 10 mM) with sterile-filtered, deionized H_2O or medium. Store <1 month at -20°C .

This preparation is "activated" lovastatin, which is ready for use.

Mimosine, 100 mM

100 mM L-mimosine (Sigma) in PBS, pH 7.4 (APPENDIX 2A)
Store <2 months at 4°C

Nocodazole, 4 mg/ml

DMSO in 4 mg/ml nocodazole (Aldrich)
Store <6 months at -20°C or <1 month at 4°C

Thymidine, 100 mM

100 mM thymidine (Sigma) in PBS, pH 7.4 (APPENDIX 2A)
Sterile filter or autoclave and store <3 months at -20°C or -80°C

Trypsin, 0.5×

Dilute 1× trypsin (Life Technologies) 1:1 with 50 ml 1× HBSS (APPENDIX 2A) supplemented with 1.25 ml of 0.5 M disodium EDTA and 1 ml of 0.5 M HEPES, pH 7.2. Store <3 months at -20°C or <1 week at 4°C .

COMMENTARY

Background Information

An essential task in the study of cell cycle-related processes is synchronization of cells into specific cell cycle phases. Synchronization improves the conditions by which an actual process under scrutiny can be studied and helps link the process to a particular cell cycle-phase transition. This unit provides methods that have proven effective in synchronizing mammalian cells into defined cell cycle phases. The advantages and disadvantages of each procedure are discussed and some key situations where such procedures have been employed successfully in the biochemical dissection of cell cycle-control processes are described. This unit focuses on the use of chemicals or growth-restriction conditions to synchronize exponentially growing cells.

An additional method for obtaining enriched populations of phase-specific cells, using centrifugal elutriation to obtain pure populations of cells, has been omitted from this unit. Centrifugal elutriation uses mechanical separation to isolate cells in different cell cycle phases. Centrifugal elutriation is often a superior method of synchronization: it allows one to obtain pure populations of cells without introducing potential artifacts of imbalanced growth, since no chemicals are employed and no transient arrests are induced. Its expense and specific requirements for optimization of the elutriator preclude the use of this methodology in most laboratories performing occasional synchronizations or just getting started in methods of cell cycle analysis. This method will, however, be described in a future unit.

Critical Parameters and Troubleshooting

The goal of cell synchronization is to create a completely synchronized population of cells that progress uniformly through the cell cycle. Achieving this task requires that (1) the cells be completely arrested at one defined point in the cell cycle, (2) the arrest procedure be com-

pletely reversible, and (3) the released cells recover and progress uniformly through the cell cycle. In the practical sense, the procedures currently available for cell synchronization aim towards, but do not entirely meet, these requirements. Problems arise in the degree of arrest obtainable and the uniformity with which the released population progresses through the cell cycle. For a cell line not yet characterized for synchronization, one may have to experiment with several different procedures to find the most suitable technique.

Mitotic shake-off: If cell lines are poorly adherent, synchrony obtained by this shake-off will be poor as well. Therefore, the time of trypsinization or the concentration of trypsin should be reduced. Care should be taken with more fragile cell lines not to damage their membranes during manipulation of cells, which might prevent their reattachment or reentry into the cell cycle.

Poorly growing cultures or cultures that are not in logarithmic growth will produce lower yields of cells than fresh, rapidly dividing cultures. If too few cells are obtained by this method despite optimal cell growth, removal of unattached cells to enhance synchrony should be omitted in favor of obtaining larger numbers of cells. Alternatively, this technique can be combined with nocodazole to enhance the number of cells and purity of the synchronized population.

Nocodazole: If an increase in cell number is not observed or a decrease in cell number is observed following release from nocodazole, the concentration of the drug is most likely too high, resulting in an irreversible block in G_2/M phase or causing cells to die. Reducing the concentration should alleviate this problem.

Increasing the concentration of nocodazole should prevent any leakiness of the G_2/M -phase block. In cell lines with doubling times >24 hr, 16 hr may not be sufficient time to accumulate the bulk of the cells in G_2/M . Caution should be used when extending the time of nocodazole

exposure, as longer exposure times may permit all cells to exit G₁ and S phases, but may be toxic to cells held for prolonged periods of time in G₂ and M phases. Finally, a number of tumor cell lines are defective in their ability to arrest at G₂/M. Nocodazole will not be an effective synchronizing agent for these checkpoint-defective cell lines.

Serum starvation: A range of reduced serum concentrations are effective (0.1% works well for most cell lines). Serum withdrawal can also be accomplished by complete withdrawal (0% serum) or performed with 1% serum, depending on the cell line. The optimal concentration must be determined for each cell line. In addition, plating cells too densely interferes with the ability of cells to fully arrest. Densely plated cells arrest as a result of confluence rather than nutritional depletion. If cells are overgrown following removal of serum, cells should be plated at a lower density prior to initiating serum withdrawal.

Amino acid depletion: Insufficient washing of the cells early in amino acid starvation will reduce the ability of the cells to become uniformly synchronized. It is very important to use dialyzed (not charcoal-treated) FBS rather than reduced serum concentrations of standard serum. Keeping cells below confluence is also important during amino acid depletion, as excess cell numbers can prevent maximum growth of synchronized cultures.

Low numbers of synchronized cells can result from toxicity of amino acid withdrawal. The unfortunate side effect of toxicity is that dying cells can provide a source of isoleucine, which will further reduce the quality of the synchrony. Toxicity can result if cells are exposed to amino acid-deficient medium for two or three cell cycles. Extended withdrawal times may also cause cells to remain in G₁ as statically arrested cells rather than transiently blocked cells.

Lovastatin: The results of synchrony obtained with lovastatin have shown some variation among cell types. Several fibroblastic cell lines have demonstrated strong G₁-arrest characteristics in the presence of lovastatin, while other tumor cell lines have demonstrated a persistent arrest in both G₁ and G₂. In those cases where cells show a significant G₂ rather than G₁ population of cells in the presence of lovastatin, it would be best to select another method of synchronization.

Mimosine: As mentioned previously, the site and mode of action of mimosine arrest has not been clearly defined. Therefore, it is not clear that mimosine inhibits cells from pro-

gressing into S phase or inhibits the progression of cells through S phase (Kalejta and Hamlin, 1997). If mimosine does, in fact, have two sites of action in the cell cycle, then a single mimosine treatment may produce populations contaminated with cells trapped in S phase. For this reason, it may be prudent to use mimosine as an adjunct to G₀/G₁-synchronizing protocols to obtain pure, late G₁/S-synchronized cultures.

Double-thymidine block: Timing is of the essence with this technique! With the procedure outlined in Basic Protocol 4, the leading-edge cells will be at the G₁/S phase border of the next cell cycle at the end of the sixteenth hour, while the lagging edge of cells will be in G₂/M at the end of the sixteenth hour. If thymidine is added too quickly, cells at the end of S phase will become trapped until released following the second thymidine block. If thymidine is added too late, cells already in S phase will become trapped in S phase during the block and may interfere with detection of early S-phase events.

Mitotic index: This is a relatively straightforward technique with few steps that require optimization. Gentle handling of the cells following resuspension and fixation with 3:1 ethanol/glacial acetic acid will improve cell yields and cell morphology.

[³H]thymidine incorporation into DNA: Insufficient cell numbers, poorly growing cultures, and use of medium with a large pool of cold thymidine can all lead to poor labeling of DNA. Also insufficient washing of cultures synchronized by double-thymidine exposure can lead to a large pool of excess cold thymidine, which reduces the relative amount of [³H]thymidine. If cell number is the problem, precipitating materials from increasing numbers of cells should demonstrate an optimum cell concentration for detection of labeled DNA. Alternatively, if cell numbers are limiting, the addition of 1 μg of cold carrier DNA or RNA to cell lysates may improve the recovery of labeled DNA. Poorly growing cultures are detectable by their reduced cell cycle time and failure to increase logarithmically in cell number with time. If the required medium for cell growth (e.g., some hematopoietic cell lines require the addition of hypoxanthine/thymidine to the medium), the medium used for labeling cells can be substituted for another medium with lower thymidine concentration (e.g., DMEM) during the 30-min labeling period with little or no effect on cell replication.

The lack of increase in DNA synthesis above background can be the result of insufficient washing of TCA precipitates, poor or ineffec-

tive synchronization of cells, or failure of cells to re-enter the cell cycle.

Failure of cells to re-enter the cell cycle can be determined by monitoring cell numbers. In cases where synchrony is poor, the cell number will increase uniformly rather than punctately, as occurs with synchronized cultures. Thorough and complete washing of cell precipitates is critical to the success and reproducibility of this protocol.

Anticipated Results

Mitotic shake-off: Cells obtained by mitotic shake-off should be enriched in G₂/M phase by at least 75%. Contamination of cells in M with cells in other stages is most often related to the stringency of trypsin treatment or rigor of shaking or tapping of the cultures. Pure populations of G₁ cells can be gained using the mitotic shake-off technique by placing the mitotic cells in a flask and incubating them for 2 hr at 37°C. The mitotic cells will re-attach to the flasks and flatten out as they enter G₁ phase. Mitotic lag-guards can be removed from the flask by gently swirling the medium over the attached cells and then decanting the medium. The remaining attached cells will consist of primarily G₁ cells, which can be confirmed by flow cytometry.

Nocodazole: It is possible to obtain nearly pure populations of G₂/M-phase cells (up to 95% with CA46 cells) by using nocodazole. It is necessary to confirm that the cell cycle block has been reversed by monitoring cell number. Nocodazole is generally used at 10 to 400 ng/ml (final concentration). Excessive concentrations or prolonged incubation times can be toxic. The authors have found that nocodazole-arrested lymphoma cells do not display the characteristic proportional increase in cell volume that is observed with other types of G₂-arrest agents, when evaluated using a Coulter counter and channelzyer. Therefore, methods that monitors changes in cell volume should be not be used to evaluate the effectiveness of nocodazole arrest.

Serum starvation: Over 90% of the population should be in G₀/G₁ following serum starvation. By 12 to 16 hr after addition of serum-containing medium, 40% to 60% of the population should enter S phase in a parasynchronous wave. Peak synthesis of DNA, as measured by [³H]thymidine incorporation, should occur by 16 hr after restimulation.

Amino acid depletion: With this technique, >90% of CA46 cells will arrest in G₁ after two cell cycles. Following release into the cycle, >80% of these cells will re-enter the cell cycle and progress into S phase. The quality of the

synchronization depends on obtaining maximal replication in cells prior to depletion of amino acids and thorough washing of cells prior to resuspension in isoleucine-free medium. Conditions that lead to extensive cell death during the procedure may interfere with this procedure, since lysed cells can act as a significant source of free isoleucine. Depending on the cell type, cells will enter S phase as a synchronous or parasynchronous wave within 8 to 12 hr. Starvation methods are useful when combined with other types of synchronizing agents, in particular, release from starvation in the presence of aphidicolin or mimosine to enrich for G₁/S cells (Heintz and Hamlin, 1982, Burhans et al., 1990, Dijkwel and Hamlin, 1992).

Lovastatin: Up to 80% reversible sequestration of cells in G₁ phase has been seen with the use of lovastatin (Wilcken et al., 1997). It is important to note that recovery from lovastatin does prolong the G₁ phase of many cell types (Keyomarsi et al., 1991).

Mimosine: Synchronization with mimosine produces late G₁/S populations of >70% purity. These cells initiate DNA synthesis <30 min after release from mimosine arrest.

Double-thymidine block: Cell populations treated in this manner can often be enriched to contain >65%, and sometimes as much as 80%, of the cells in S phase. The real trick to this procedure is to prevent the leading-edge cells from entering S phase of the next cell cycle while ensuring the lagging-edge cells have left S phase of the previous cell cycle. Most cells will progress into S phase within 1 to 2 hr following release from excess thymidine.

Mitotic index: As cells enter M phase, the number and percentage of mitotic figures observed in the cultures will increase.

[³H]Thymidine incorporation: Peak DNA synthesis as reflected by an increase of at least 3-fold over background represents the point in time when the maximum number of cells are in S phase. The increase in peak DNA synthesis can be substantially >5-fold over background, depending on the culture conditions and the degree of synchrony obtained.

Time Considerations

Because these methods involve manipulations of cultures of cells, they required one or more days, although the hands-on time for any one day may be limited. For an estimate of the total time required for each synchrony protocol see Figure 8.3.2.

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Determining Cell Cycle Stages by Flow Cytometry

The most common approach to determining the cell cycle stage is based on measurement of cellular DNA content. This allows one to discriminate between cells in the $G_{0/1}$ versus the S versus the G_2/M phases of the cell cycle (Fig. 8.4.1). DNA is generally stained with a fluorescent dye and cellular fluorescence is measured by flow, image, or laser scanning cytometry (Crissman and Steinkamp, 1990). A variety of fluorochromes can be used for DNA staining. The spectral properties of the dyes most frequently used for this purpose are presented in Figure 8.4.2. Following cell staining with one of these dyes, the intensity of fluorescence integrated over the analyzed cell is expected to be in stoichiometric relationship to DNA content, and thereby can be used to determine the cell cycle stage. Deconvolution of the DNA content frequency histograms, usually done using special computer software (Rabinovitch, 1994), reveals the percentage of cells residing in the respective phases of the cell cycle.

A plethora of techniques for DNA content measurement utilizing the fluorochromes depicted in Figure 8.4.2 have been published during the past two decades. Description of many methods, which are applicable to different cell systems including eukaryotes and prokaryotes, as well as to clinical samples, can be found in a separate monograph (Darzynkiewicz et al., 1994). The techniques differ primarily according to the mode of cell permeabilization (detergent versus fixation with alcohols) and composition of the stain solution. Four types of procedures are described in this unit. Relatively simple and rather universally applicable methods for staining ethanol-fixed cells are presented in Basic Protocol 1 and Alternate Protocol 1. Because cells may be stored in the fixative for extended periods of time and/or transported while in the fixative, these methods allow one to prepare and collect the cells without regard to the timing of their analysis. The methods presented utilize the two most commonly used DNA fluorochromes, propidium iodide (PI; see Basic Protocol 1) and 4',6-diamidino-2-phenylindole (DAPI; see Alternate Protocol 1). The DAPI staining procedure, which does not require incubation with RNase A, is simpler and more rapid as compared to the one using PI. However, it requires a flow cytometer equipped with a UV excitation source.

The methods utilizing detergent to permeabilize cells are presented in Basic Protocol 2 and Alternate Protocol 2. These methods provide more accurate estimates of the DNA content, and therefore better discrimination of the cell-cycle phases as compared to measurement of fixed cells. This is due to the fact that exposure of live cells to detergents results in rupture of the plasma membrane and elimination of the cytoplasmic constituents, which contain components that are autofluorescent or that nonspecifically interact with DNA fluorochromes. Isolated nuclei are then stained rather than whole cells. It should be stressed that analysis of the detergent-treated cells may lead to an underestimation of M cells. Lacking a nuclear envelope, these cells may totally disintegrate into chromosomes or chromosome aggregates. In Basic Protocol 2 cells are stained with PI; in Alternate Protocol 2 they are stained with DAPI. As in the case of fixed cells, staining with of detergent-permeabilized cells with DAPI does not require incubations with RNase A. It should be stressed that fluorochromes other than PI or DAPI (Fig. 8.4.2) can be used in these protocols, provided that their fluorescence is excited and measured at appropriate wavelengths, as indicated in the figure.

A different approach is used for cell cycle analysis of live cells (see Basic Protocol 3). The main application of this method is for cell sorting, where cells that have been selected on the basis of their DNA content (cell cycle phase) can be cultured for analysis of their

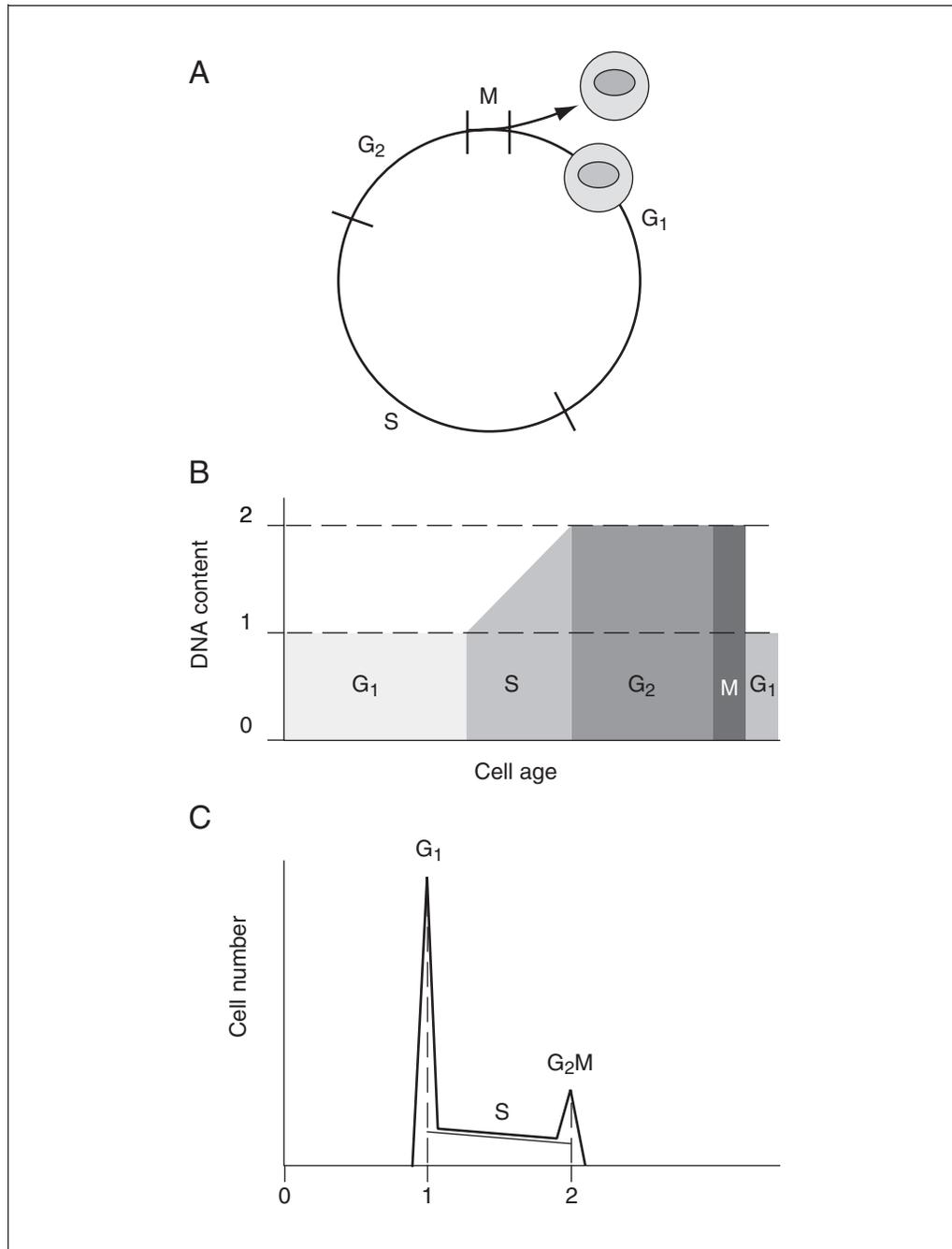


Figure 8.4.1 Relationship between DNA content and the cell cycle. **(A)** Stages of the cell cycle. **(B)** Estimation of cell position in the cell cycle based on DNA content measurement. Content of cellular DNA doubles during S, and therefore the cell age during S can be estimated based on the amount of replicated DNA (increase in DNA content). On the other hand, cells in G₁ and G₂/M are uniform with respect to DNA content, which is equivalent to the DNA ploidy index (DI) 1.0 (for G₁) and 2.0 (for G₂/M). **(C)** If DNA content could be measured with absolute accuracy, based on DNA-specific fluorescence, the G₁ and G₂/M cells would have uniform fluorescence values and be represented on the frequency histograms as the bars of a single channel width (dashed lines). Due to inaccuracy in DNA-content measurement, the actual data are in the form of G₁ and G₂/M peaks. Percentage of cells represented by these peaks, and in the S phase, are estimated by deconvolution of the histograms, using a variety of mathematical techniques (Bagwell, 1993; Rabinovitch, 1994). The interactive software based on these techniques deconvolutes the histograms and is available from several sources (see Background Information).

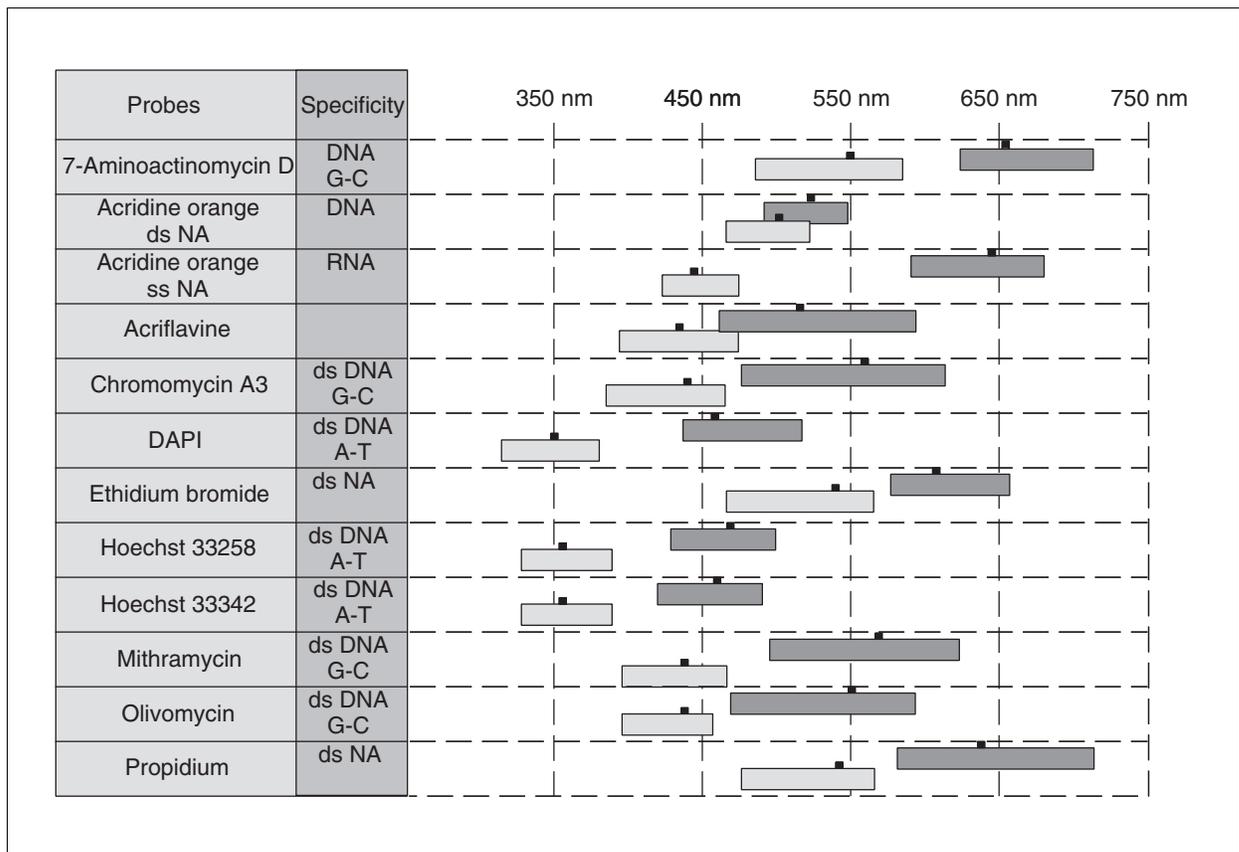


Figure 8.4.2 Fluorescence excitation (shaded bars) and emission (solid black bars) wavelengths of the most commonly used DNA fluorochromes. Abbreviations: ds, double-stranded; ss, single-stranded; NA, nucleic acid.

growth characteristics or sensitivity to drugs or for purposes of cloning or expansion. The method presented in Basic Protocol 3 is simple, based on cell staining with Hoechst 33242 fluorochrome, which, unlike PI or DAPI, is able to penetrate through the plasma membrane and stain DNA in live cells.

The final procedure combines analysis of cellular DNA content with expression of cyclins D, E, A, or B1. Cyclins are the key components of the cell cycle progression machinery and are expressed discontinuously during the cell cycle (see Background Information). Thus, the presence of a particular cyclin detected immunocytochemically within the cell, provides an additional marker of the cell cycle position. Simultaneous bivariate analysis of DNA content and expression of cyclins (Basic Protocol 4) makes it possible to distinguish additional stages of the cell cycle, which cannot be identified by analysis of DNA content alone (Darzynkiewicz et al., 1996).

CELL CYCLE ANALYSIS OF FIXED CELLS STAINED WITH PROPIDIUM IODIDE

In this protocol, ethanol is used to fix and permeabilize cells to make them accessible to propidium iodide (PI). As mentioned above, the fixation step makes this protocol applicable in instances when samples have to be stored or transported before analysis. Following fixation, the cells are rinsed with PBS and stained with PI in a solution containing Triton X-100 and RNase A. Triton X-100 additionally permeabilizes the cells, and to some extent decreases cell loss resulting from electrostatic cell attachment to tubes. Because double-stranded sections of RNA also stain with PI, RNase A is included to digest these sections and thereby to increase specificity of DNA staining.

BASIC PROTOCOL 1

Cell Cycle Analysis

8.4.3

Materials

Cells to be stained
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Fixative: 70% ethanol
Propidium iodide staining solution I (see recipe)

Low-speed centrifuge
12 × 75-mm centrifuge tubes, preferably polypropylene or silanized
Flow cytometer with 488-nm argon-ion laser fluorescence excitation source
Software to deconvolute cellular DNA content frequency histograms (e.g.,
Multicycle from Phoenix Flow Systems)

Additional reagents and equipments for counting and trypsinizing cells (*UNIT 1.1*)

Prepare cell suspension for fixation

- 1a. *For cells growing in suspension or hematologic samples:* Rinse cells once by centrifuging 6 min at $200 \times g$, room temperature, with PBS. Count cells (*UNIT 1.1*) and thoroughly resuspend 1×10^6 to 1×10^7 cells in 0.5 ml of PBS.
- 1b. *For cells growing attached to tissue culture dishes:* Collect cells from flasks or petri dishes by trypsinization (*UNIT 1.1*) and pool the trypsinized cells with the cells floating in the medium (the latter consist of detached mitotic, apoptotic, and dead cells). Centrifuge the cell suspension 6 min at $200 \times g$, room temperature. Remove the supernatant and resuspend the pellet in medium containing serum (to inactivate trypsin), then centrifuge again and remove the supernatant. Count cells (*UNIT 1.1*) and thoroughly resuspend 1×10^6 to 1×10^7 cells in 0.5 ml of PBS.

Other means of trypsin inactivation, such as addition of protease inhibitors, may also be used.

- 1c. *For cells isolated from tissues (e.g., tumors):* Rinse free of any enzyme used for cell dissociation using the centrifugation technique described in step 1a, above, and thoroughly resuspend (well dispersed, not in aggregates) 1×10^6 to 1×10^7 cells in 0.5 ml of PBS.

Fix cells in ethanol

2. Prepare for fixation by adding 4.5 ml of 70% ethanol fixative to each of an appropriate number of 12 × 75-mm centrifuge tubes. Keep tubes on ice.
3. Using a Pasteur pipet, transfer 0.5-ml aliquots of cell suspensions prepared as in step 1a, 1b, or 1c into the appropriate tubes containing the cold 70% ethanol fixative and keep cells in fixative ≥ 2 hr on ice.

It is important to achieve a single-cell suspension. Fixation of cells that are in aggregates while suspended in PBS stabilizes the aggregates, which then become impossible to disperse. It is essential, therefore, to have a monodisperse cell suspension at the time that cells are mixed with ethanol.

Cells suspended in 70% ethanol can be stored at 0° to 4°C for several months if not years.

Stain cells with PI

4. Centrifuge the ethanol-suspended cells 5 min at $200 \times g$. Decant ethanol thoroughly.
5. Suspend the cell pellet in 5 ml PBS, wait 60 sec, then centrifuge again as in step 4. Decant supernatant
6. Resuspend cell pellet in 1 ml of propidium iodide staining solution I. Incubate either 15 min at 37°C or 30 min at room temperature.

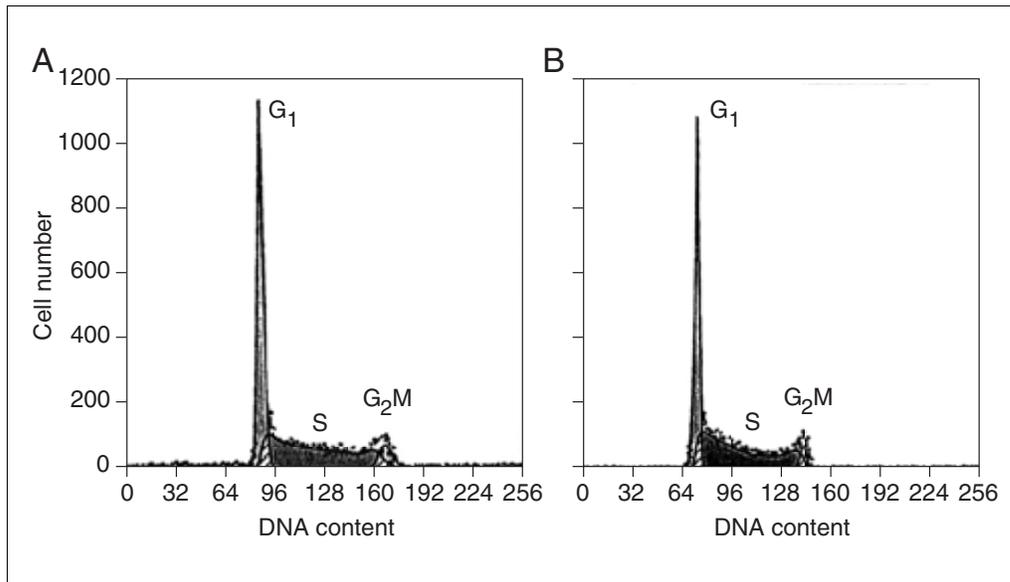


Figure 8.4.3 (A) DNA content frequency histograms of fixed HL-60 cells stained with DAPI (Alternate Protocol 1) and (B) detergent-permeabilized HL-60 cells stained with DAPI (Alternate Protocol 2). The histograms were deconvoluted using Multicycle software (Phoenix Flow Systems).

Measure cell fluorescence by flow cytometry

- Set up and adjust flow cytometer for excitation with blue light and detection of PI emission at red wavelengths.

For excitation, the 488-nm argon-ion laser line may be used. Alternatively use a BG 12 optical filter when the source of illumination is a mercury-arc or xenon lamp. A long-pass (>620-nm) emission filter is recommended.

- Measure cell fluorescence by flow cytometry. Use pulse-width/pulse-area signal to discriminate between G₂/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using DNA content histogram deconvolution software (e.g., Multicycle from Phoenix Flow Systems).

CELL CYCLE ANALYSIS OF FIXED CELLS STAINED WITH DAPI

This protocol is similar to Basic Protocol 1, except the cells are stained with DAPI rather than PI. Because DAPI does not stain RNA, there is no need to treat the cells with RNase A. Excitation of DAPI, however, requires UV light source, which is not universally available. Emission of DAPI is measured at blue wavelengths.

ALTERNATE PROTOCOL 1

Additional Materials (also see Basic Protocol 1)

DAPI staining solution I (see recipe)

Flow cytometer with UV illumination source (e.g., mercury-arc lamp or laser tuned to UV at 340 to 380 nm)

- Collect cells, fix in 70% ethanol, and wash (see Basic Protocol 1, steps 1 to 5).
- Resuspend cell pellet in 1 ml DAPI staining solution. Incubate 30 min in the dark at room temperature.
- Set up and adjust flow cytometer for UV excitation at 340 to 380 nm and detection of DAPI emission at blue wavelengths.

For excitation, an UG-1 optical filter (short-pass, 390 nm) may be used when the source of excitation is a mercury-arc or xenon lamp. For detection of DAPI emission a band-pass filter at 470 ± 20 nm is recommended.

Cell Cycle Analysis

8.4.5

4. Measure cell fluorescence by flow cytometry. Use the pulse-width/pulse-area signal to discriminate between G₂/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using software that deconvolutes DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems).

**BASIC
PROTOCOL 2**

**CELL CYCLE ANALYSIS OF UNFIXED, DETERGENT-PERMEABILIZED
CELLS STAINED WITH PI**

In this protocol the cells are lysed with detergent to aid in the staining of DNA for flow cytometric analysis. The cells in suspension are mixed with staining solution that contains Triton X-100, PI, and RNase A. DNA content of the stained nuclei is then measured by flow cytometry, using excitation with blue light. Because there is no fixation step, the procedure is simpler and more rapid, and because there are fewer centrifugations, there is less cell loss as compared to Basic Protocol 1

Materials

Cells to be stained: 1×10^6 to 5×10^6 cells/ml suspended in PBS (*APPENDIX 2A*) or culture medium

Propidium iodide staining solution II (see recipe)

Flow cytometer with 488-nm argon-ion laser fluorescence excitation source

Software to deconvolute cellular DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems)

1. Mix 0.2 ml of cell suspension with 2 ml of propidium iodide staining solution II. Incubate 20 min at room temperature.
2. Set up and adjust flow cytometer for excitation with blue light and detection of PI emission at red wavelengths.

For excitation, the 488-nm argon-ion laser line may be used. Alternatively use a BG 12 optical filter when the source of illumination is a mercury-arc or xenon lamp. A long-pass (>620-nm) emission filter is recommended.

3. Measure cell fluorescence by flow cytometry. Use pulse-width/pulse-area signal to discriminate between G₂/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using DNA content histogram deconvolution software (e.g., Multicycle from Phoenix Flow Systems).

**ALTERNATE
PROTOCOL 2**

**CELL CYCLE ANALYSIS OF UNFIXED, DETERGENT-PERMEABILIZED
CELLS STAINED WITH DAPI**

This protocol is essentially identical to Basic Protocol 2 except that the cells are stained with DAPI. As mentioned previously, there is no need for incubation with RNase A, which simplifies the protocol, but the procedure requires a flow cytometer equipped with UV light excitation source.

Additional Materials (also see *Basic Protocol 2*)

DAPI staining solution II (see recipe)

Flow cytometer with UV-illumination source (e.g., mercury-arc lamp or laser tuned to UV at 340 to 380 nm)

1. Mix 0.2 ml of cell suspension (1×10^5 to 1×10^6 cells suspended in PBS or culture medium) with 2 ml of DAPI staining solution II. Incubate 10 min at room temperature.
2. Set up and adjust flow cytometer for UV excitation at 340 to 380 nm and detection of DAPI emission at blue wavelengths

For excitation, an UG-1 optical filter (short-pass, 390 nm) may be used when the source of excitation is a mercury-arc or xenon lamp. For detection of DAPI emission a band-pass filter at 470 ± 20 nm is recommended.

3. Measure cell fluorescence by flow cytometry. Use the pulse-width/pulse-area signal to discriminate between G₂/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using software that deconvolutes DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems).

STAINING OF LIVE CELLS WITH HOECHST 33342

Supravital staining of DNA offers the possibility of sorting live cells at different phases of the cell cycle, based on differences in their DNA content. The protocol uses Hoechst 33342 fluorochrome, which stains cellular DNA without a need for cell fixation or permeabilization with detergent. The actual procedure for cell staining is simple. Cells are suspended in culture medium or PBS and incubated in the presence of 2.0 to 5.0 $\mu\text{g/ml}$ of Hoechst 33342 for 20 to 90 min. Cell fluorescence is then measured directly, without any additional treatments or centrifugations. Because Hoechst 33342 is excited at UV wavelengths, the procedure requires a flow cytometer with a UV light illumination source. It should be stressed, however, that accuracy of DNA content measurement and therefore the ability to discriminate cells at different phases of the cycle, is much lower with supravital cell staining as compared to staining of ethanol-fixed or detergent-permeabilized cells.

Materials

1 mg/ml Hoechst 33342 in H₂O (store up to several weeks at 4°C in dark or foil-wrapped bottles)

Cells to be stained: 1×10^6 cells/ml suspended in PBS (APPENDIX 2A) or culture medium

Flow cytometer with UV light illumination source (e.g., mercury-arc lamp or laser tuned to UV at 340 to 380 nm)

1. Add sufficient 1 mg/ml Hoechst 33342 to cells suspended in PBS or culture medium (1×10^6 cells/ml) to obtain a final fluorophore concentration of 2.0 $\mu\text{g/ml}$. Incubate 20 min at 37°C.
2. Set up and adjust flow cytometer for UV excitation at 340 to 380 nm, and detection of Hoechst 33342 fluorescence at blue wavelengths.
3. Measure cell fluorescence by flow cytometry. Use the pulse-width/pulse-area signal to discriminate between G₂/M cells and the cell doublets, and gate out the latter. Analyze the data (Fig. 8.4.3) using software that deconvolutes DNA content frequency histograms (e.g., Multicycle from Phoenix Flow Systems).

When intensity of cell fluorescence or resolution of cells in the cell-cycle phases is inadequate, prolong the staining time (up to 90 min) and/or increase Hoechst 33342 concentration in the medium (up to 5 $\mu\text{g/ml}$). The same sample may be reanalyzed after prolonged incubation and/addition of more staining solution.

This protocol is predominantly used for sorting live cells. However, because sensitivity of cells to Hoechst 33342 varies depending on the cell type (the dye also sensitizes cells to UV light), it is possible that viability and cell cycle progression of the sorted cells may be affected by the staining procedure.

**BIVARIATE ANALYSIS OF DNA CONTENT AND EXPRESSION OF
CYCLINS D, E, A, OR B1**

A special category of methods for cell-cycle analysis combines measurement of DNA content and expression of the proliferation-associated proteins. The latter are detected immunocytochemically, using specific antibodies that are labeled with fluorochrome either directly, or indirectly via a secondary antibody. This protocol is devoted to cyclins, whose analysis provides an insight into the actual components of the cell-cycle progression machinery (see Background Information and *UNIT 8.2*). Because some cyclins are expressed transiently, at very specific time intervals in the cell cycle, their presence in the cell can be considered as a marker of this particular portion of the cycle. The protocol combines measurement of DNA content with expression either of one of the D-type cyclins, or cyclins E, A, or B1. Cells are fixed and labeled with anti-cyclin antibody followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody, and finally stained with PI for DNA measurement. If directly conjugated FITC-anti-cyclin antibody is available, secondary-antibody labeling (steps 8 and 9) can be omitted. This analysis is adapted to the most commonly used flow cytometers, such as those equipped with a single-laser (488-nm) illumination source.

Materials

Cells to be analyzed

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Fixative: 80% ethanol or absolute methanol, -20°C .

0.25% (v/v) Triton X-100 in PBS, pH 7.4 (store at 4°C)

Rinsing buffer: 1% (w/v) bovine serum albumin (BSA) in PBS, pH 7.4 (store at 4°C)

Anti-cyclin IgG1 antibodies: e.g., mouse monoclonal antibodies to cyclin B1 (clone GNS-1), cyclin A (clone BF-683), cyclin D1 (clone G124-326), cyclin D3 (clone G107-565), and cyclin E (clone HE12); all provided by PharMingen; antibodies to cyclin D1 may also be obtained from Immunotech/Coulter

Mouse IgG1 (isotypic control)

FITC-conjugated goat anti-mouse IgG

Propidium iodide staining solution III (see recipe)

15-ml conical tubes, polypropylene or silanized

Low-speed centrifuge

Flow cytometer equipped with 488-nm argon laser fluorescence excitation source

Additional reagents and equipment for collecting and preparing cells for fixation (see Basic Protocol 1, step 1a, b, or c)

Prepare and fix cell suspension

1. Collect cells and resuspend in PBS (see Basic Protocol 1, step 1a, b, or c).
2. Prepare for fixation by adding 10 ml of 80% ethanol or absolute methanol fixative to each of an appropriate number of 15-ml tubes. Keep tubes on ice (0° to 4°C).

In addition to preparing a tube for each aliquot of cells to be tested with an anti-cyclin antibody, prepare an appropriate number of tubes for isotypic controls.

3. With a Pasteur pipet, transfer 1 ml of the each cell suspension into the appropriate tubes containing cold fixative. Incubate on ice.

Time of fixation (storage) at 4°C may vary from 4 hr to several days.

To minimize cell loss, all the subsequent steps should be done in the same tube.

Label cells with anti-cyclin primary antibody

4. Centrifuge fixed cells 5 min at $300 \times g$, room temperature. Remove alcohol, resuspend cells in 5 ml PBS, and centrifuge as before.
5. Remove supernatant and resuspend cell pellet ($\leq 1 \times 10^6$ cells) in 1 ml of 0.25% Triton in PBS. Keep on ice for 5 min, then add 5 ml of PBS and centrifuge at $300 \times g$ for 5 min at room temperature. Remove supernatant.
6. Dissolve each of the anti-cyclin antibodies of interest (primary antibodies) in rinsing buffer at 5 $\mu\text{g}/\text{ml}$. For each sample to be analyzed, take 100 μl of the 5 $\mu\text{g}/\text{ml}$ anti-cyclin antibody solution. Also, prepare 100 μl of 5 $\mu\text{g}/\text{ml}$ mouse IgG₁ (isotypic control) for each tube of control cells prepared.
7. Resuspend each cell pellet in 100 μl of rinsing buffer containing the appropriate primary antibody or isotypic control. Incubate 60 min at room temperature with gentle agitation or at 4°C overnight.

Label cells with FITC-conjugated secondary antibody

8. Add 5 ml of rinsing buffer to each tube and centrifuge 5 min at $300 \times g$, room temperature. Remove the supernatants.
9. Make a 1:30 dilution of the secondary antibody (FITC-conjugated goat anti-mouse IgG) in rinsing buffer. Resuspend each of the cell pellets in 100 μl of the diluted FITC-conjugated secondary antibody and incubate 30 min in the dark at room temperature with gentle agitation.

If cyclin antibody that is directly conjugated to FITC is available, this protocol can be simplified by omitting steps 8 and 9.

Stain cells with PI

10. Add 5 ml of rinsing buffer to each tube and centrifuge 5 min at $300 \times g$, room temperature. Remove the supernatants
11. Resuspend each cell pellet in PI staining solution III. Incubate 20 min at room temperature in the dark before measurement.

Measure cell fluorescence by flow cytometry

12. Set up and adjust the flow cytometer for excitation with blue light (488-nm laser line). Use a 530 ± 20 nm band-pass filter for detection of FITC emission and 620-nm long-pass filter for PI emission.
13. Measure the cyclin-associated green fluorescence of FITC and DNA-associated red fluorescence of PI.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

DAPI staining solution I

To 100 ml of 0.1% (v/v) Triton X-100 in PBS (APPENDIX 2A) add 0.1 mg 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes). Store up to 1 month at 4°C in the dark.

DAPI staining solution II

To 100 ml of PIPES buffer (see recipe) add 0.1 mg 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes). Store up to several weeks at 0° to 4°C in dark or foil-wrapped bottles.

PIPES buffer

3.02 g piperazine-*N,N'*-bis(2-ethanesulfonic acid (PIPES; Calbiochem; 10 mM final)
5.84 NaCl (0.1 M final)
406 mg MgCl₂ (2 mM final)
1 ml Triton X-100 (0.1% final)
H₂O to 1000 ml
Adjust to pH 6.8
Store up to 6 months at 0° to 4°C

Dissolve the dry ingredients and Triton X-100 in ~800 ml water; adjust pH with NaOH or HCl, and add water to 1000 ml.

Propidium iodide (PI) staining solution I

To 100 ml of 0.1% (v/v) Triton X-100 in PBS add 20 mg DNase-free RNase A and 2 mg of propidium iodide (PI; Molecular Probes). Store up to 2 weeks at 4°C in the dark.

If RNase is not DNase-free, boil a stock solution of RNase A (2 mg in 1 ml water) for 5 min, then use it to prepare the PI staining solution.

Propidium iodide (PI) staining solution II

Add 2 mg of propidium iodide (PI; Molecular Probes) to 100 ml of PIPES buffer (see recipe). Store up to several weeks in dark or foil-wrapped bottles at 0° to 4°C. Prior to use add DNase-free RNase A to 200 µg/ml final concentration.

Propidium iodide staining solution III

Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing:
5 µg/ml propidium iodide (PI)
200 µg/ml DNase-free RNase A
Prepare fresh

COMMENTARY

Background Information

Univariate cellular DNA content analysis

Choice of protocol: Choosing a particular protocol from among those presented in this unit depends on the sample type (i.e., determining whether fixed or unfixed cells should be used), availability of UV light illumination in the flow cytometer (which will dictate preference for DAPI over PI), or the need to identify and quantify apoptotic cells in addition to discrimination of the cell cycle phases. In the discussion that follows, characteristics and applicability of each of the methods is presented. Because a variety of different fluorochromes can be used to stain cellular DNA, and occasionally some of them may be preferred over DAPI, PI, or Hoechst 33342, the spectral properties of the most commonly used dyes are presented in Figure 8.4.2.

In Basic Protocol 1 and Alternate Protocol 1, DNA content is measured in prefixed cell samples. The preference for analysis of fixed cells often is dictated by the need to store or

transport samples. Extended storage of unfixed cells, unless done at low temperatures following suspension of the cells in cryopreservative medium, leads to cell deterioration and DNA degradation. Fixed cells, on the other hand, often can be stored for months if not years without much deterioration. The fixative essentially has two functions: (1) it preserves the cells by preventing lysis and autolytic degradation, and (2) it makes the cells permeable and hence makes DNA accessible to the fluorochrome. For DNA-content analysis, the precipitating fixatives (ethanol, methanol, and acetone) are preferred over the cross-linking agents (formaldehyde, glutaraldehyde). It should be stressed, however, that damaged DNA, especially DNA having a large number of double-strand breaks (e.g., as are present in apoptotic cells) leaks out from the ethanol-prefixed cells during hydration and subsequent staining. This allows one to identify apoptotic cells as those with fractional DNA content (“sub-G₁ cell population”). Methods for detecting apoptotic cells are reviewed in Darzynkiewicz et al. (1997).

As mentioned, a variety of DNA fluorochromes can be used to stain DNA in the prefixed cells. Staining with dyes that react with DNA and RNA, such as PI, requires incubation of cells with RNase A. The enzyme is included in the staining solution; a 30-min incubation at room temperature in that solution is adequate to remove RNA from the subsequently measured cells. Since most flow cytometers use blue light (488-nm line of the argon ion laser) as the fluorescence excitation source, PI is a useful label.

Alternate Protocols 1 and 2 employ DAPI instead of PI. One advantage of DAPI is its greater specificity towards DNA, which often is reflected in lower coefficient of variation (CV) values of the mean DNA content of G_1 cell populations. Another advantage is that it does not require incubation with RNase A. However, not all flow cytometers are equipped with a UV-light illumination source, which is needed for DAPI excitation.

The major advantage of detergent-based methods (see Basic Protocol 2 and Alternate Protocol 2) is greater accuracy in DNA-content estimates. However, because the cells are lysed by detergents, mitotic cells, which lack a nuclear envelope, may disintegrate to such an extent that individual chromosomes or chromosome clusters are measured. This usually happens when the samples are vigorously pipetted or vortexed. Therefore, one has to be cautious in interpreting the data, because mitotic cells may not be detected by methods utilizing detergents or hypotonic staining solutions. This is of special importance in instances when a large proportion of cells are in mitosis—e.g., during incubations with mitotic blockers. Furthermore, the presence of chromosomes or chromosome aggregates in the sample may contribute to an increased frequency of detection of objects with low fluorescence values, generally classified as debris or apoptotic cells (“sub- G_1 ” cell population, see Darzynkiewicz et al., 1997). Likewise, the lysis of apoptotic cells, which have fragmented nuclei, releases several fragments from a single cell. Each such fragment may erroneously be identified as an individual apoptotic cell.

DNA staining in live cells (see Basic Protocol 3) is generally performed in combination with cell sorting to obtain cells synchronized at particular cell cycle phases. The fluorochrome for supravital cell staining is expected to be nontoxic and not alter the cell metabolism. Such a probe has yet to be developed. Most DNA fluorochromes are charged molecules

that do not adequately penetrate the plasma membrane. Some uncharged Hoechst dyes that can pass through the membrane, though at a limited rate, are exceptions. The most frequently used supravital DNA fluorochrome is Hoechst 33342. The procedure of staining with Hoechst 33342 followed by sorting appears not to induce immediate cytotoxicity (Loken, 1980). Delayed toxicity attributed to Hoechst 33342 has been observed, however, especially when the cells were treated with some antitumor drugs or radiation subsequent to staining. Hoechst dyes photosensitize cells that have BrdU incorporated into their DNA, in particular to UV light at ~300-nm wavelengths. Viability of the sorted BrdU-labeled cells, counterstained with Hoechst dyes and illuminated with UV light laser, is expected to be impaired.

The intensity of supravital cell staining with Hoechst 33342 and the resolution of DNA content (i.e., possibility of discrimination of cells in different cell-cycle phases) varies among different cell types. This variability, to a large degree, is due to a rapid efflux of the dye from the cell generated by the P glycoprotein transport pump. Cells characterized by rapid efflux mechanisms (e.g., multidrug-resistant tumor cells or stem cells) stain poorly with Hoechst 33342. It has been observed, however, that agents that may impair the efflux function (e.g., calcium channel-blocking drugs such as verapamil), at least in some cell types, improve stainability with Hoechst 33342 (Krishan, 1987).

Cell cycle and kinetic parameters: All cells in $G_{0/1}$ have a uniform DNA content, as do cells in G_2/M . Under ideal conditions of DNA staining and measurement, the fluorescence intensities of all $G_{0/1}$ and G_2/M cells are expected to be uniform, and, after digitization of the electronic signal from the photomultiplier, to have uniform numerical values with G_2/M cells having twice the fluorescence of cells in $G_{0/1}$. This, however, is never the case, and on frequency histograms the $G_{0/1}$ and G_2/M populations are represented by peaks of various width because of inaccuracy of the measurement. The coefficient of variation (CV) of the mean value of DNA-associated fluorescence of the $G_{0/1}$ population is a measure of the width. The CV value, therefore, is a reflection of the inaccuracy of the DNA estimate. Because of the inaccuracy there is an overlap between early S and $G_{0/1}$ as well as between late S and G_2/M cells on the histograms. Several mathematical methods of deconvolution of the DNA content frequency histograms have been developed to estimate the

percentage of cells in the respective phases of the cell cycle (Bagwell, 1993; Rabinovitch, 1994). The goal of these techniques is to evaluate the extent of the overlap and thereby to correct for the inaccuracy of the measurement. These methods provided the basis for development of software that allows one to estimate percentage of cells in particular phases of the cycle from the histograms, through simple interactions with the computer. Such software is generally provided upon the purchase of instruments or can be obtained separately from other vendors. The most common software programs used to deconvolute DNA content frequency histograms are provided by Phoenix Flow and Verity Software House.

It is often assumed that the cells in S and G₂/M represent the “proliferative” cell fraction and that their frequency reflects the proliferative potential of the cell population. While this indeed may be the case in some cell systems, the DNA content frequency histograms alone do not provide any direct information on cell kinetics. For example, the histogram cannot reveal whether the cell progression through the cycle is slowed down (e.g., compared to control) if the slowdown affects all phases of the cycle proportionally. Likewise, the histogram cannot show whether the cells are “frozen” in the cycle, which may happen during treatment with high doses of certain drugs, hyperthermia, or radiation. However, when the cell-proliferation rate (e.g., the cell-doubling time in culture) is measured in parallel with DNA content, it is possible, with certain assumptions and approximations, to estimate cell kinetics, including the duration of individual phases of the cycle. One of the assumptions is that the cells are in exponential phase of growth. In such a situation there are always twice as many daughter cells as mother cells, and the cell age distribution across the cycle is represented by the diagram shown in Figure 8.4.4B. Duration of a particular phase of the cycle (e.g., G₁) can then be estimated from the equation:

$$\frac{T_{G_1}}{T_C} = \frac{\ln(F_{G_1} + 1)}{\ln 2}$$

where T_{G_1} is duration of G₁ phase, T_C is duration of the cell cycle, and F_{G_1} is a fraction of cells residing in G₁. T_C is estimated from the cell growth curves—i.e., when the number of live cells is plotted as a function of time (cell number on exponential scale versus time on linear scale of the x and y coordinates), the exponential growth is reflected by a straight

slope. The slope allows one to calculate the cell doubling time in culture. The latter, with generally acceptable approximation, equals T_C . A similar formula, of course, applies for estimating duration of phases of the cell cycle other than G₁. Thus, for example, when the duration of the cell cycle is 24 hr and the fraction of cells in G₁ is 0.5 (50%), the duration of G₁ (T_{G_1}) is $\ln(1 + 0.5) \times 24 / \ln 2 = (0.405 \times 24) / 0.693 = 14.0$ hr. The graphical approach for measuring duration of the cell-cycle phases from the DNA content frequency histograms is illustrated in Figure 8.4.4.

Bivariate analysis of DNA content and expression of cyclins D, E, A, or B1

Cyclins are the key elements of the cell cycle progression machinery. They combine with particular cyclin-dependent protein kinases (CDKs) forming the holoenzymes that phosphorylate different sets of proteins at consecutive stages of the cell cycle, thereby driving the cell through the cycle (Pines and Hunter, 1991; Draetta, 1994; Hartwell and Kastan, 1994; Sherr, 1994; Cardon-Cardo, 1995; Morgan, 1995; Bartek et al., 1997). The function of cyclins in these holoenzymes is to activate their partner CDKs and to target them to specific protein substrates whose phosphorylation is essential for traversing a particular section of the cell cycle.

Several cyclins, notably D-type cyclins as well as cyclins E, A, and B, are expressed transiently during the cell cycle. Cyclins D and E belong to the family of G₁ cyclins, whereas cyclins A and B are G₂ cyclins (also see Fig. 8.2.1). During unperturbed growth of normal, nontumor cells, the scheduled timing of synthesis and degradation of cyclins takes place at very specific points of the cycle. The periods of expression of these cyclins by the cell, therefore, can be considered landmarks of the cell cycle. These landmarks complement the traditional milestones of the cycle detected by DNA content measurement, namely mitosis and DNA replication.

The development of antibodies to cyclins made it possible to detect them immunocytochemically and to investigate their expression in individual cells by cytometry (Gong et al., 1994; Lukas et al., 1995; Urbani et al., 1995; Juan et al., 1997). Bivariate analysis of DNA content and cyclin expression provides a framework for subdividing the cell cycle into several subcompartments and for defining, with a greater precision than before, the point at which the cell cycle is arrested by some antitumor

drugs (Darzynkiewicz et. al., 1996). Furthermore, the differences in cyclin expression make it possible to discriminate between cells having the same DNA content but residing at different

phases, such as in G_2 versus M or G_2/M cells of a lower DNA ploidy versus G_1 cells of a higher DNA ploidy.

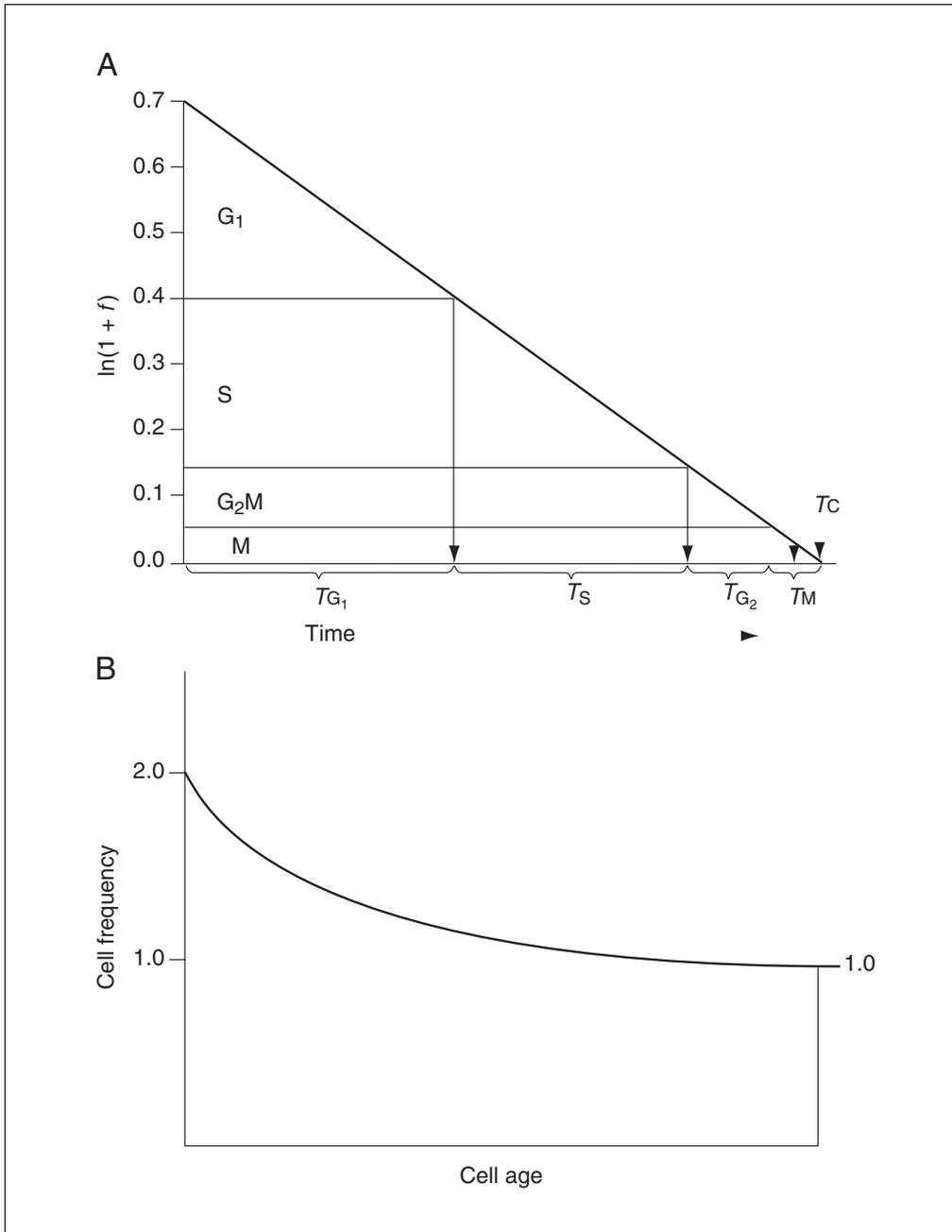


Figure 8.4.4 Graphical method for estimating duration of particular cell cycle phases (**A**) The method is based on the assumption that all cells are in the exponential phase of proliferation—i.e., that there are twice as many daughter as mother cells. (**B**) The proportions of cells in different phase of the cycle are obtained from DNA content frequency histograms (Fig. 8.4.3), and the value of T_C (cell generation, or cell cycle time), which is in turn calculated from the growth curves as the cell-doubling time in culture. T_C equals the cell-doubling time when all cells are in the proliferative cell pool—i.e., when the growth fraction equals 1.0. Fractions of cells in particular phases of the cycle (f) are plotted exponentially as $\ln(1+f)$. T_C is then connected with $\ln 2$ (0.693). The points of intersection of the line connecting $\ln 2$ and T_C with the levels representing frequency of cells in particular phases of the cycle, projected on the time coordinate, represent the duration times of these phases, as shown.

The bivariate analysis of cyclin expression versus DNA content, unlike any other approach, detects the inappropriate (“unscheduled”) expression of cyclins—i.e., the presentation of G₁ cyclins by cells in G₂/M and of G₂/M cyclins by G₁ cells—without the need for cell synchronization. Such unscheduled expression of cyclins B1 and A was seen when cell cycle progression was halted, e.g., after synchronization at the G₁/S boundary by inhibitors of DNA replication (Gong et al., 1995; Urbani et al., 1995). The unscheduled expression of cyclins B1 or E, representing a characteristic feature of a particular tumor phenotype, was also observed in some tumor cell lines when their growth was unperturbed (Gong et al., 1994). Likewise, while the expression of cyclins D1 or D3 in nontumor cells was restricted to an early section of G₁, the presentation of these proteins in many tumor cell lines was also seen during S and G₂/M (Juan et al., 1996). As specific markers of cell proliferation, cyclins are expected to reflect proliferative potential of tumors and therefore to be the key prognostic markers in neoplasia.

Critical Parameters

Univariate cellular DNA content analysis

The most critical issue in DNA content analysis is the accuracy of DNA content measurement. The accuracy, as mentioned (see Background Information), is reflected by the extent of variation in cellular fluorescence between the cells with identical DNA content, such as G_{0/1} cells. The CV of the DNA-associated mean fluorescence of G₁ cells, therefore, is considered as an index of accuracy of DNA content measurement. High accuracy is required, in particular, in studies of DNA ploidy, to distinguish between DNA diploid and aneuploid cells, which may differ minimally in DNA content. High accuracy of DNA content measurement is also critical in analysis of the cell cycle distribution. Regardless of the type of the software used to deconvolute DNA frequency histograms, the accuracy in estimation of cell proportions in respective phases of the cell cycle directly correlates with accuracy of DNA content measurement. There is no formal consensus regarding the acceptable maximal CV value of the mean DNA content of G_{0/1} cell population—i.e., maximal error in cellular DNA content estimate. Most researchers, however, would consider the accuracy to be poor and results unacceptable if CV values of G_{0/1}

populations of normal, nontumor cells exceed 6%.

A variety of factors can contribute to poor accuracy in DNA content analysis. The most common is inappropriate optical alignment (spatial position of laser beam with respect to sample flow and fluorescent light-collecting lenses) of the flow cytometer. When the instrument is optimally aligned, the measured cell passes precisely through the center of the laser beam, and at the same time, is in the focus of the fluorescence emission collection lenses. A minor adjustment of the sample flow or any of the optical parts leads to a loss in accuracy of DNA content measurement. Proper maintenance of the instrument, and careful adjustment with standard fluorescent beads of known uniformity prior to analysis of the experimental samples, is essential to achieve accurate DNA content measurements. Problems in sample preparation, either resulting in mechanical damage to the cells or involving incorrect composition of buffers and staining solutions, represent another common cause of poor resolution in DNA analysis.

It should be stressed that there may be situations when, in spite of good accuracy in DNA content measurement (in terms of proper instrument adjustments and sample staining), the CV of the mean DNA content of G₁ cell populations is relatively large. This may happen when significant numbers of dead or dying cells are present in the sample or when the cells were treated with drugs interacting with DNA. Many antitumor drugs are known to impair stainability of DNA with the fluorochromes used for flow cytometry. Furthermore, because of the nature of the tumor, which may either be multiclonal or have developed drug resistance by gene amplification mechanisms (e.g., as reflected by the presence of minute chromosomes), the tumor cell populations may have variable DNA content, and therefore intrinsically high CV values for the G_{0/1} cell populations.

Bivariate analysis of DNA content and expression of cyclins D, E, A, or B1

The critical steps for immunocytochemical detection of intracellular proteins are cell fixation and permeabilization. These steps often have to be customized for particular antigens. The fixative is expected to stabilize the antigen in situ and preserve its epitope in a state where it continues to remain reactive with the available antibody. The cell has to be permeable to allow access of the antibody to the epitope.

Most studies on cyclins have employed precipitating fixatives such as 70% to 80% ethanol, absolute methanol, or a 1:1 mixture of methanol and acetone cooled to -20° to -40°C . Brief (15- to 30-min) treatment with 1% paraformaldehyde followed by 70% cold ethanol has been used for fixation of D-type cyclins although this cyclin can also be detected following fixation with cold methanol. The choice of fixative, thus, appears not to be a critical factor for cyclin detection and, although the absolute level of the immunofluorescence may vary, various fixation protocols yield essentially similar cyclin distributions with respect to the cell cycle position. Each fixative has some undesirable effects (e.g., increased cell clumping in the case of the ethanol/acetone mixture or cell autofluorescence and poor DNA stainability when formaldehyde is used), and one often has to compromise between these effects and the optimal detection of a particular cyclin. Fixation in 80% cold ethanol, as presented in this protocol, offers such a compromise.

Much more critical for the detection of cyclins is the choice of a proper antibody. Often an antibody that is applicable to immunoblotting fails in immunocytochemical applications, and vice versa. Likewise, the antibody may show nonspecific reaction with denatured proteins (i.e., reveal several bands on immunoblots), and yet be acceptable in immunocytochemical assays. These discrepancies may be due to differences in accessibility of the epitope or differences in the degree of denaturation of the antigen on the immunoblots as compared to the situation in its *in situ* location. Some epitopes may not be accessible *in situ* at all. This especially pertains to epitopes that are the cyclin segments involved in formation of complexes with the partner CDKs, CDK inhibitors, or other molecules such as proliferating cell nuclear antigen (PCNA). Since there is strong homology between different cyclin types, cross-reactivity may also be a problem. Because commercially available monoclonal antibodies may differ, e.g., in specificity and degree of cross-reactivity, it is important to use reagents that have been already tested and referenced in published papers. It is required that the authors provide information (the vendor and hybridoma clone number) on the reagent used in their study.

A very important control is examination of the stained cells by UV light microscopy, or preferably confocal microscopy. All cyclins, with the notable exception of cyclin B1, have nuclear localization. Cyclin B1 has cytoplas-

mic localization during late S and most of G_2 , and is localized in the chromatin only at mitosis. Cyclin B2 is localized to Golgi (Jackman et al., 1995). Inappropriate localization of the fluorescence of the cells stained with the presumed anti-cyclin antibody may be an indication of its nonspecific reactivity with other cell constituents.

The relative cellular content of a particular cyclin plays a role in its detection. The signal-to-noise ratio (ratio of fluorescence intensity of the cyclin-positive cells to the control cells, stained with the isotype immunoglobulin), for example, is higher in the case of cyclin B1 than in the case of cyclins E or A, most likely due to the fact that the absolute level of cyclin B1 is higher compared to cyclins E or A. The level of expression of D-type cyclins varies markedly depending on the cell type and the phase of cell growth. High sensitivity of the instrument and low level of cell autofluorescence, therefore, are of greater importance for the detection of cyclins E or A than of cyclin B1 or D-type cyclins.

While isotypic immunoglobulin or irrelevant isotypic antibody are generally accepted as appropriate controls, they are not perfect for this purpose, as their fluorescence may also vary depending on the source (vendor) and may not be representative the actual background. Ideal controls represent the cells of the same type and of the same species, but with the gene that codes for the detected protein deleted. Such control cells should be subjected to identical immunocytochemical procedure as the studied cells. Unfortunately, few cell lines with deleted cyclin genes are currently available.

Anticipated Results

Univariate cellular DNA content analysis

Figure 8.4.3 presents DNA frequency histograms of HL-60 cells stained with DAPI according to Alternate Protocol 1 and Alternate Protocol 2, respectively. As is evident based on differences in DNA content, one can identify a population of $G_{0/1}$ cells with uniform low DNA content values, G_2/M cells with DNA content twice that of $G_{0/1}$ cells, and S phase cells with intermediate DNA content. To reveal the percentage of cells in $G_{0/1}$, S, and G_2/M , the DNA frequency histograms in the figure were deconvoluted using the Multicycle software (Phoenix Flow Systems). Similar results are expected when using Basic Protocols 1 and 2. Supravital cell staining with Hoechst 33342, however, yields less accurate DNA measurements, which

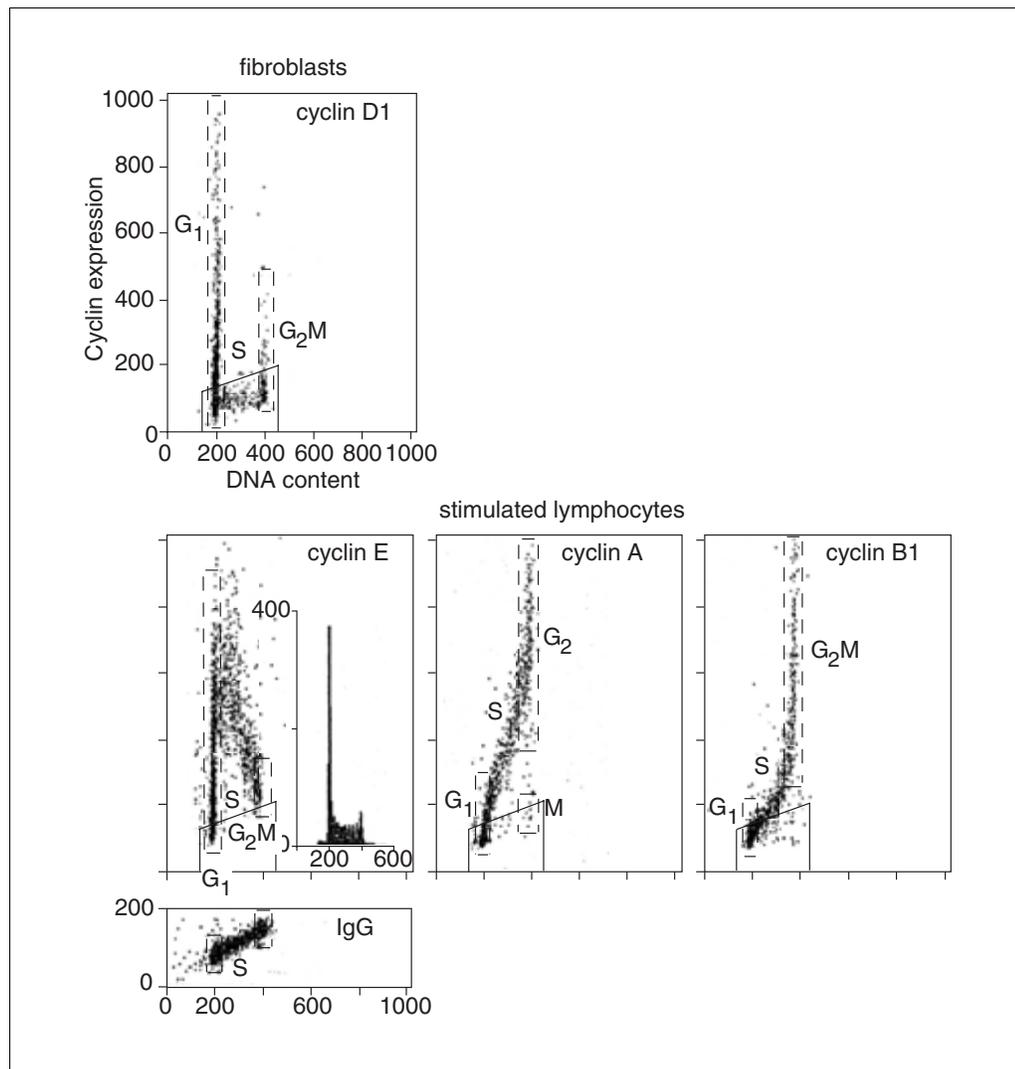


Figure 8.4.5 Bivariate cyclin versus DNA content distributions (scatter plots) showing expression of cyclin D1 in normal human fibroblasts and cyclins E, A, and B1 in mitogen-stimulated human lymphocytes. The trapezoidal windows represent level of fluorescence of the respective control cells stained with the isotype IgG, rather than the respective cyclin-anti-cyclin antibody. The $G_{0/1}$ and G_2/M populations gated based on differences in DNA content are marked by dashed lines.

are reflected by the increased width of the $G_{0/1}$ and G_2/M peaks (i.e., increased CV of the mean fluorescence of $G_{0/1}$ and G_2/M cell populations).

Bivariate analysis of DNA content and cyclin expression

The scheduled timing of expression of cyclins B1, A, E, and D1 in relation to the major phases of the cell cycle is reflected by a very characteristic pattern of the bivariate cyclin-versus-cellular DNA content distributions. These distributions are shown in Figure 8.4.5 for normal human proliferating lymphocytes (cyclins B1, A, and E) and fibroblasts (cyclin D1). As is evident from the cytograms, the

expression of cyclin B1 is limited to late S phase cells and the cells with a G_2/M DNA content, while early- and mid-S phase cells show a very low level of this protein. Cells in G_1 phase are essentially cyclin B1-negative.

Similar to cyclin B1, expression of cyclin A is minimal in G_1 cells. It becomes pronounced, however, during S phase where its level progressively increases as the cells advance towards G_2 . Maximal expression of cyclin A is seen in cells having a G_2/M DNA content (Fig. 8.4.5). It should be mentioned, however, that because cyclin A is abruptly degraded during prometaphase (Pines and Hunter, 1991), mitotic cells that have advanced past prometa-

phase are essentially cyclin A–negative (not shown).

Expression of cyclin E can be summarized as follows: (1) the maximal level of this protein is detected in the cells undergoing transition from G₁ to S; (2) its level continuously decreases during cell progression through S, with the result that most G₂/M cells are cyclin E–negative; and (3) a distinct threshold in cyclin E expression is apparent at the G₁/S transition. As it is evident from the continuity of the cell clusters on scatter plots (Fig. 8.4.5) the cells have to accumulate cyclin E above the threshold level to enter S phase.

The presence of cyclin D1 in exponentially growing normal fibroblasts is limited to cells in G_{0/1} (Fig. 8.4.5). Most cells in S and G₂/M are cyclin D1–negative, with the exception of a few cells with a G₂/M DNA content. The latter may be G₁ cell doublets, since not all doublets can be identified by analysis of the shape (pulse width) of the electronic signal.

It should be stressed that, as mentioned before (see Background Information), the cyclin distributions as shown in Figure 8.4.5 characterize only those cells that are growing normally, exponentially, and asynchronously. The distributions are very different when the cell-cycle progression is perturbed, or in the case of some tumor cell lines that display unscheduled expression of these cyclins.

Time Considerations

For Basic Protocol 1 (PI staining), cell fixation takes ~10 min, but cells have to be kept in fixative 2 hr; the cell staining procedure takes ~45 min. Alternate Protocol 1 (DAPI staining) requires similar times for cell fixation. However, because there is no need to incubate cells with RNase, the time of staining is shorter. The detergent-based cell-staining procedure of Basic Protocol 2 takes ~60 min. For Alternate Protocol 2, which is simpler, cell staining takes ~15 min. Supravital staining of cells (Basic Protocol 3) requires ~20 min for staining, although extended staining times (up to 90 min) may be needed for some cell types.

For Basic Protocol 4 (DNA and cyclins) cell fixation takes ~10 min, but the cells have to be kept in fixative for ≥2 hr. The cell-staining procedure takes ~1 hr if the antibodies used are directly conjugated with a fluorochrome. The staining may take an additional 40 min if the primary antibody is not fluorochrome-tagged and therefore indirect labeling has to be used. It is sometimes convenient, however, to leave

the cells for incubation with the primary antibody overnight, at 4°C.

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Centrifugal Elutriation to Obtain Synchronous Populations of Cells

Counterflow centrifugal elutriation (CCE) is a generally noninvasive method for separating large numbers of mixed particles (bacteria, yeast, mammalian cells) into populations on the basis of their size and mass. For mammalian cells the method is particularly useful for separating mixed cell populations (e.g., T cells or monocytes from peripheral blood mononuclear cells or, as described here, for obtaining proliferating cell populations partitioned at progressive stages of the cell division cycle. CCE does not perturb cell metabolism, a fundamental problem encountered with metabolic synchronizing agents (see UNIT 8.3). Thus, the characteristics of populations separated by CCE more closely reflect normal cell cycle regulation. The standard protocol permits separation of $\sim 2 \times 10^8$ cells into six to ten progressive populations using a Beckman JE-6B rotor (see Basic Protocol). For separation of a larger number of cells (~ 10 -fold more), parameters are modified for use with a large JE-5.0 rotor (see Alternate Protocol). Because the task of harvesting cells from large volumes of culture medium by repetitive centrifugation is time consuming and can adversely effect cell viability, this protocol also presents a convenient method for concentrating cells from culture media by continuous flow using the large elutriation rotor. To accurately assess elutriated cell fractions for purity and to verify cell cycle position, short protocols are provided to quantify DNA content by flow cytometry with propidium iodide (see Support Protocol 1), to quantify nascent DNA synthesis by incorporation of [^3H]thymidine deoxyribose (see Support Protocol 2), and to quantify DNA synthesis and content by flow cytometry using PI and incorporated 5-bromo-2'-deoxyuridine (BrdU; see Support Protocol 3).

SEPARATION OF CELLS INTO PROGRESSIVE STAGES OF THE CELL DIVISION CYCLE BY COUNTERFLOW CENTRIFUGAL ELUTRIATION

**BASIC
PROTOCOL**

This section describes the setup and equilibration of the Beckman J-6B elutriator system for sterile separation of mammalian cells into progressive populations through the cell division cycle based on cell size and mass.

NOTE: Methodical tissue culture technique (Chapter 1) is the single most important parameter to ensuring reproducible results and good yields at all cell cycle phases. Healthy cells are less prone to lysis and clumping from released DNA and eliminate the need for DNase treatment during the run. Large clumps in the elutriation chamber will adversely effect fluid flow and separation. Sustained log-phase growth is required for high viability, low debris, and sufficient levels of S- and G₂/M-phase cells.

Materials

70% (v/v) ethanol

Elutriation medium (see recipe) equilibrated ≥ 2 hr at ambient temperature

Nonadherent cells in log-phase growth: suspension cultures at $2\text{--}5 \times 10^5$ cells/ml with fresh medium added at each doubling

Beckman J-6B or J25I centrifuge with view port door and strobe assembly

Beckman JE-6B rotor with standard elutriation and bypass chambers

Elutriation liquid system assembly (Fig. 8.5.1), consisting of:

Medium reservoir: 2-liter roller bottle and 2-ml pipet

Loading syringe: 10-ml syringe barrel and three-way valve

High-quality peristaltic pump with 10-turn potentiometer or digital control (e.g., Masterflex 7520 or 7550), capable of 100 rpm, 0 to 150 ml/min in 0.1 ml/min increments

continued

**Cell Cycle
Analysis**

Contributed by Alan F. Wahl and Karen L. Donaldson

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8.5.1

Supplement 2

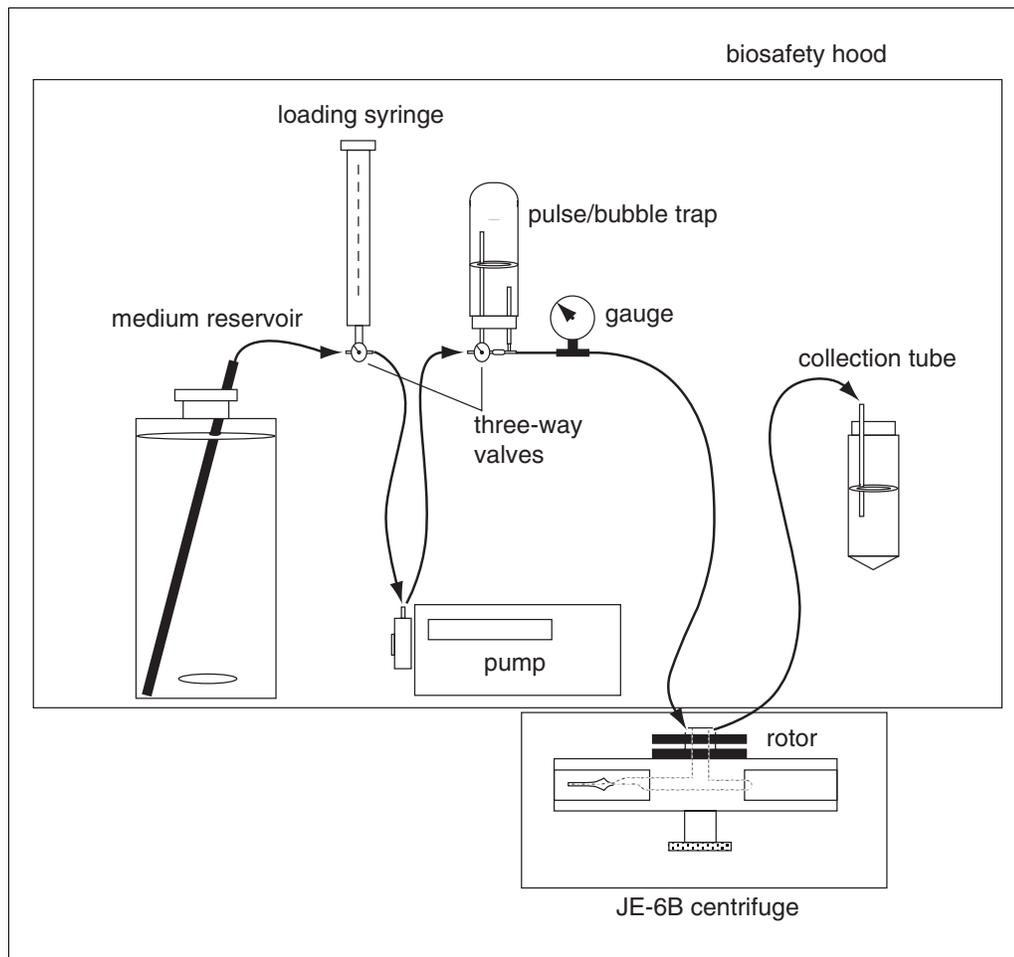


Figure 8.5.1 Schematic of the counterflow centrifugal elutriation system. Fluid is drawn from a reservoir past a valved loading syringe and through a peristaltic pump. It proceeds through a pulse/bubble trap compensator and pressure gauge to the rotor, and returns from the chamber to a 2-ml pipet into a collection vessel. The entire setup (with the exception of the centrifuge itself) is assembled in a hood.

Pulse/bubble trap and three-way valve (Beckman)

Pressure gauge (Beckman)

Collection tube: 250-ml conical centrifuge bottle (Corning)

¼-in.-o.d. (0.64-cm) Silastic tubing (Masterflex 6411-14)

Ring stand and clamps

18-G needle

10-ml syringe

250-ml conical centrifuge bottles

Beckman GPR centrifuge (or equivalent) with swinging bucket rotor

Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*)

NOTE: Elutriation is performed with the centrifuge, media, and cells at ambient temperature. Cell fractions are processed immediately after collection or stored on ice in multiple runs.

Set up elutriation system

1. Set up a JE-6B elutriation rotor, strobe assembly, and J-6B or J25I centrifuge according to manufacturer's specifications (Beckman Instruments, 1981, 1989).

Ensure that the internal rotating seal is free from wear and is correctly seated against the head assembly.

Failure of the rotating seal will either result in leakage into the centrifuge chamber or allow input and output fluid to mix and bypass the elutriation chamber.

2. Ensure that the elutriation and bypass chambers are in the correct positions and that the fluid flowing into the rotor enters the bottom (outermost) region of the elutriation chamber.
3. Set up the elutriation liquid system assembly as shown in Figure 8.5.1. Secure the loading syringe, pulse/bubble trap, and pressure gauge using clamps and a ring stand. Use ¼-in.-o.d. Silastic tubing throughout the system. Bevel the input and output pipet ends to prevent flow obstruction during the run.

This design differs from that described by the manufacturer.

The pulse/bubble trap is held inverted (as shown) with a three-way valve connected to the first input tee. This allows the cells to bypass this chamber. After loading, the chamber is switched in-line to compensate for any flow pulsation caused by the pump.

4. To ensure sterile sorting, place the entire assembly (step 3) in a laminar flow hood and place the centrifuge directly adjacent to the hood (Fig. 8.5.1). Minimize the length of tubing used in the system.

Sterilize elutriation system

5. With the centrifuge off, sterilize the entire system by pumping 70% ethanol through at ~30 to 40 ml/min, being careful to fill the loading reservoir, bypass chamber, pressure gauge, bypass tubing, and all valves. Allow to sit for 10 min.
6. Remove ethanol, being careful to drain all components by starting at the loading chamber and finally drain the rotor by lifting and tilting.
7. Rinse any residual ethanol by pumping sterile water through the system as described for ethanol.

Residual ethanol will cause precipitate in the elutriation medium.

8. Allow to recirculate 5 to 10 min by putting both input and output lines into a sterile water vessel, then remove lines and drain the system.

Equilibrate elutriation system

9. Equilibrate the system with elutriation medium that is well equilibrated at the operating (ambient) temperature. Circulate the medium as described in step 5, placing both input and output lines into a 2-liter vessel containing elutriation medium.

Cold medium placed in a warm system will degas and create bubbles in the system.

10. Begin removing trapped air from the system by pinching off tubing to create ~20 lb back pressure and quickly releasing. Start at the loading chamber and progress toward the elutriation chamber, switching valves as necessary to ensure that all bubbles are removed. Remove air in the elutriation chamber by lifting the rotor and tilting 90 degrees to allow medium to fill the chamber.
11. Turn on the centrifuge, bring the rotor to running speed, and repeat step 10 to remove trapped air.
12. Stop rotor and lift to check for leakage.

This step also helps to dislodge additional trapped air.

13. Restart rotor and recheck the entire system for trapped air bubbles.

Air that becomes trapped in the elutriation input will impede flow into the chamber and ruin the run.

An air cushion of ~1 in. (2.5 cm) should remain in the pulse/bubble chamber to dampen any pump pulsation during the run.

Calibrate pump

14. Set rotor speed to 1800 rpm and loading flow rate at 12 ml/min.

As an example, this protocol loads cells at 12 ml/min at a rotor speed of 1800 rpm, with ten samples taken at 3 ml/min increments up to 42 ml/min. Elutriation of most mammalian cells is done at rotor speeds between 1800 and 2500 rpm, with corresponding loading flow rates ranging between 12 and 20 ml/min. See Table 8.5.1 for examples of flow rates used to elutriate Jurkat cells using the JE-6B rotor. See Beckman Instruments (1981, 1989) for a detailed discussion of the selection of rotor speed and flow rate based on particle size and mass.

Although separation of populations is achieved by varying either speed or flow rate, the authors have found that varying flow rate with a digitally controlled pump provides the most reproducible results.

15. Calibrate the pump to the loading flow rate with the complete system in place and the rotor running at the desired speed. To calibrate, collect medium from output line into a graduated cylinder for 1 min and measure volume. If necessary, readjust pump calibration setting and collect again for 1 min. Repeat until pump speed is accurate. Also check maximum flow rate to ensure that this is obtainable with minimum back pressure (~5 psi).

Obstruction of input and output tubes or a change in their respective heights will affect pressure, flow rates, and separation. A high-quality digital pump will retain linearity through the range of flow rates. For pumps utilizing a 10-turn potentiometer, settings should be determined to deliver each incremental flow rate.

Harvest cells

16. Transfer 2×10^8 nonadherent log-phase cells (~0.5 to 1 liter) to 250-ml conical centrifuge bottles and centrifuge for 10 min at $200 \times g$ (e.g., in a Beckman GPR centrifuge). Gently resuspend pellet in 10 ml elutriation medium and verify cell density (final 2×10^7 /ml).

Table 8.5.1 Jurkat Elutriation Parameters^a and Cell Cycle Phase Content of the Separated Populations

Fraction	Flow rate (ml/min)	Cell number	% of load	% G ₁ ^b	% S ^b	% G ₂ ^b
Load	13	2.0×10^8	100 ^c	55.6	27.3	17.1
1	18	3.3×10^6	1.6	96.2 ^d	3.6	0.2
2	21	2.1×10^7	10.5	91.0	5.2	3.8
3	24	3.7×10^7	18.5	86.8	11.0	2.2
4	27	3.2×10^7	16.0	78.0	19.4	2.6
5	30	2.3×10^7	11.5	59.7	35.4	4.9
6	33	1.9×10^7	10.6	29.9	55.0	15.1
7	36	1.5×10^7	7.5	13.0	50.7	36.3
8	39	1.2×10^7	6.0	4.8	40.3	54.9
9	42	7.7×10^6	3.8	4.1	32.3	63.6
10	45	5.7×10^6	2.8	2.9	19.8	77.3
11	48	4.3×10^6	2.2	2.7	10.4	86.9

^aJE-6B rotor maintained at constant speed of 1800 RPM.

^bPercentage distribution calculated from histograms of total DNA content (Fig. 8.5.3).

^cThe percentages from fractions 1 to 11 add up to <100% because small amounts are lost to the system during loading (load fraction) or aggregate in the chamber and are unrecoverable (dump fraction).

^dApproximately 3% of these cells contain apoptotic DNA with sub-G₁ content.

For most efficient use of time and to maintain cells in optimal condition, cells can be centrifuged during equilibration of the elutriator.

17. Load concentrated cells into a 10-ml syringe and pass three times *slowly* through an 18-G needle just prior to loading. Check cells under a microscope to ensure that the culture is monomeric with high viability.

Load elutriator and collect fractions

18. Check that the pump and rotor are at speed (12 ml/min, 1800 rpm). Transfer the 10-ml cell suspension directly from the syringe used for dispersion (step 17) into the loading syringe. Place elutriator output line into a 250-ml conical centrifuge bottle (collection vessel). Set the valve at the pulse/bubble trap to bypass the trap.

Loading can be done through the pulse/bubble trap, but this dilutes the cells and increases the time needed for loading and equilibration.

19. Open the valve to the loading syringe to allow cells to be drawn into the medium stream, being careful to prevent air from being drawn into the system. Do not allow the loading syringe to empty completely (shut the valve once all but ~250 μ l of the cell suspension has been drawn into the system).
20. Add ~2 to 4 ml elutriation medium to the loading syringe and load the additional volume to ensure complete loading of the cells. Again, leave ~250 μ l remaining in the loading syringe.

If air has entered the elutriator line during loading, the rotor must be stopped and purged of air and the cells must be reloaded.

21. Once cells have bypassed the pulse/bubble trap, open this valve to trap bubbles and to compensate for pump pulsation. Carefully monitor cells through the view port of the centrifuge door.

Cells appear as a broad translucent band that should fill the chamber to just below the elutriation boundary (Fig. 8.5.2), below the top of the chamber.

22. Watch the cells carefully. Do not allow the cells to pack (increase flow rate if necessary) or to flow over the top of the elutriation boundary (decrease flow rate). Allow cells to equilibrate while collecting 125 ml medium (load fraction).

For pilot runs, examine the load fraction to ensure that it contains only debris. If a significant number of viable cells are present, the load should be repeated at a lower-flow rate.

Elutriation is a balance of g force and counterflow. If cells do not appear to be filling the chamber, gradually increase pump speed. If cells are reaching the elutriation boundary, reduce the pump speed to prevent loss of cells and then gradually increase until they are at the appropriate level. Do not change rotor speed.

23. Once cells have equilibrated, transfer the output line to a fresh 250-ml collection vessel to collect fraction 1. Gradually increase pump speed by 0.1 ml/min increments from 12 ml/min to 15 ml/min and collect another 125 ml medium. Place cells on ice.

Cold shock will cause sustained growth arrest of some cell types. Placing these populations on ice is not recommended when kinetic cell growth studies are to follow elutriation.

24. Collect remaining fractions in 125 ml medium each at 3 ml/min increments in pump speed. Collect ten samples up to 42 ml/min and place on ice.

25. Once the last fraction has been collected, remove any remaining cells from the rotor by stopping the rotor and continuing to pump 125 ml medium through the system.

For multiple elutriation runs of the same cell line, fractions may be overlaid into the same 250-ml bottle; however, all run conditions must be reproduced. The same number of cells must be loaded and rotor/pump speeds and fractions must be kept the same in order to

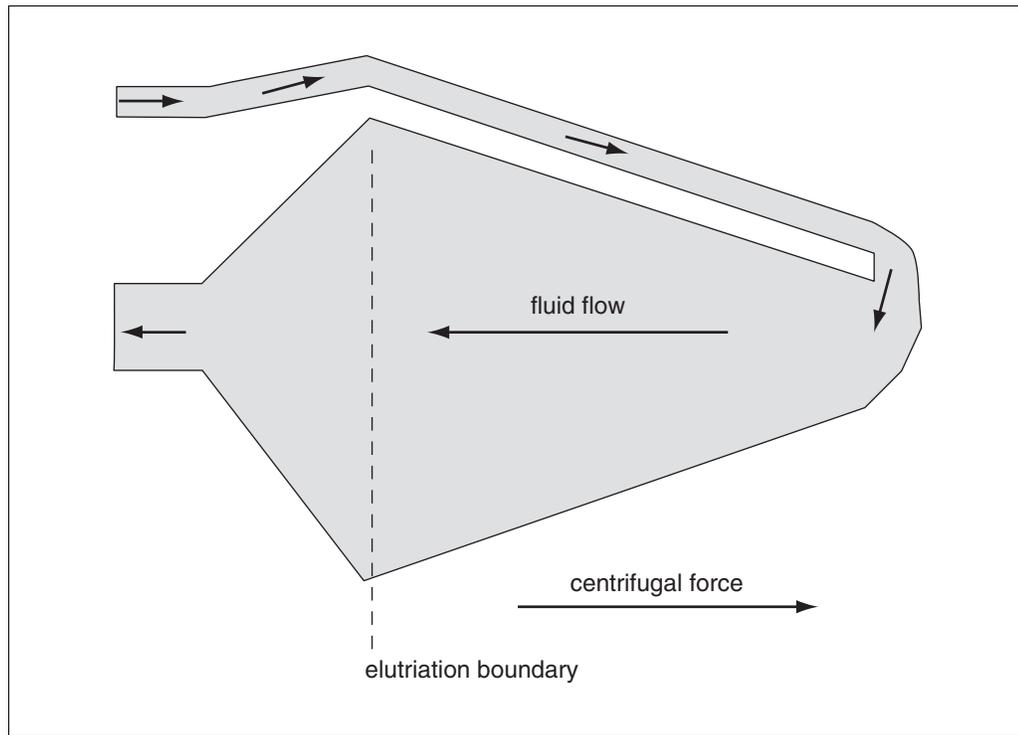


Figure 8.5.2 Schematic of the standard elutriation chamber.

duplicate each fraction. Store fractions on ice to slow cell cycle progression during subsequent runs.

26. Carefully and thoroughly resuspend cells in centrifuge bottles for each fraction. Count cells using a hemacytometer (UNIT 1.1) and concentrate the desired number of cells by centrifugation for use in experiments.

Very early and late fractions contain fewer cells. To obtain a more accurate cell count, cells from these fractions must first be concentrated by centrifugation.

ALTERNATE PROTOCOL

LARGE-SCALE ELUTRIATION WITH THE JE-5.0 ROTOR

Setup and equilibration of the larger JE-5.0 elutriator for concentration and large-scale elutriation is as described above (see Basic Protocol) with minor modifications (e.g., the size of the system tubing is increased to accommodate increased flow rate). If large and small rotors are used interchangeably, it is convenient to keep a separate, preassembled plumbing system (loading syringe, pulse/bubble trap chamber, gauges, and so on) for each rotor. The careful handling of healthy cells and the loading conditions are as described in the Basic Protocol.

To accommodate the volume of cells needed to fuel the large rotor, cells are concentrated from one large vessel (such as a 10-liter spinner flask) using the JE-5.0 rotor. With careful monitoring to prevent cells from pelleting or flowing through the chamber, a large volume of cells can be conveniently concentrated by gradually increasing rotor speed while maintaining a constant pump speed. The flowthrough medium is collected in a large waste flask and the concentrated cells are collected by transferring the output line to a 250-ml bottle and stopping the rotor. This concentration procedure is less time consuming and labor intensive than traditional centrifugation procedures and also preserves better viability. Thus, it is very useful on its own to concentrate cells for any purpose.

Additional Materials (also see *Basic Protocol*)

Beckman JE-5.0 rotor with 40-ml chamber
3/8-in.-o.d. (1.0-cm) Silastic tubing (Masterflex 6411-16)
30-ml syringes for loading chamber and for dispersing cells
4-liter vessel
Large sterile vessel
Large waste flask

Concentrate cells

1. Set up elutriator (see *Basic Protocol*, steps 1 to 4), but use a Beckman JE-5.0 rotor, 3/8-in.-o.d. Silastic tubing throughout the assembly, a 30-ml syringe for the loading chamber, and a 4-liter vessel for the medium reservoir.
2. Sterilize, equilibrate, and calibrate elutriator (see *Basic Protocol*, steps 5 to 15), calibrating at a pump speed of 70 ml/min and a rotor speed of 2000 rpm.
3. Pool nonadherent log-phase cultured cells into a large sterile vessel.
At 2×10^5 cells per ml, this procedure will require ~10 liters of cells to load the chamber.
4. Place both input and output lines in the loading vessel and load cells into the chamber at a pump speed of 70 ml/min and rotor speed of 2000 rpm. Continue to recirculate for 8 to 10 min to ensure that cells are visible in the chamber.
5. Gradually increase rotor speed to 2400 rpm over a 5-min interval, and then transfer the output line into waste flask.
6. Continue to gradually increase the rotor speed to 2900 rpm, keeping cells well below the elutriation boundary near the top of the chamber (Fig. 8.5.2). Be careful not to allow cells to pack into a pellet (increase flow rate if necessary).
7. Once the desired number of cells are collected based on cells/ml medium, transfer the output line into a 250-ml centrifuge bottle. Stop the rotor and collect 250 ml medium in each of two centrifuge bottles (500 ml total).

Prepare cells and equipment for elutriation

8. Centrifuge cells for 10 min at $200 \times g$ (e.g., in a Beckman GPR centrifuge) and gently resuspend in 30 ml elutriation medium.
9. While the cells are centrifuging, reequilibrate the system with elutriation medium and recalibrate at a pump speed of 50 ml/min and a rotor speed of 1800 rpm.

Preparing the system for elutriation while the cells are centrifuging provides for the most efficient use of time and helps maintain cells in optimal condition.

See Table 8.5.2 for example of flow rates used for WEHI-231 cells elutriated using the JE-5.0 rotor.

10. Load concentrated cells into a 30-ml syringe and pass three times slowly through an 18-G needle just prior to loading. Check cells under a microscope to ensure mono-dispersion.

Load elutriator and collect fractions

11. Load elutriator (see *Basic Protocol*, steps 18 to 22), making sure pump and rotor speed are set at 50 ml/min and 1800 rpm. Follow cells with ~10 ml elutriation buffer to ensure complete cell loading (step 20), and leave 1 ml to prevent complete emptying of the syringe (steps 19 and 20). Collect 250 ml for the load fraction (step 22).

Table 8.5.2 WEHI-231 Elutriation Parameters^a and Cell Cycle Phase Content of Separated Populations

Fraction	Flow rate (ml/min)	Cell number	% of load	% G ₁ ^b	% S ^b	% G ₂ ^b
Load	50	2.0 × 10 ⁹	100 ^c	24.3	58.8	16.9
1	60	7.8 × 10 ⁶	0.4	98.7	1.2	0.1
2	70	2.6 × 10 ⁸	13.0	67.7	32.2	0.1
3	80	3.5 × 10 ⁸	17.5	39.0	60.4	0.6
4	90	3.3 × 10 ⁸	16.5	18.4	79.2	2.4
5	100	2.0 × 10 ⁸	10.0	7.5	82.2	10.3
6	110	2.1 × 10 ⁸	10.5	4.0	69.4	26.6
7	120	2.1 × 10 ⁸	10.5	3.8	50.5	45.7
8	130	1.5 × 10 ⁸	7.5	5.2	36.3	58.5
9	140	4.2 × 10 ⁷	2.1	6.2	29.5	64.3

^aJE-5.0 rotor maintained at constant speed of 1800 RPM

^bPercentage distribution calculated from dot plots of BrdU and total DNA (Fig. 8.5.5).

^cThe percentages from fractions 1 to 11 add up to <100% because small amounts are lost to the system during loading (load fraction) or aggregate in the chamber and are unrecoverable (dump fraction).

12. To collect first fraction, transfer output line to a fresh 250-ml collection vessel. Gradually increase pump speed by 1.0 ml/min increments to 60 ml/min and collect the next 250-ml fraction. Place cells on ice.

Cold shock will cause sustained growth arrest of some cell types. Placing these populations on ice is not recommended when kinetic cell growth studies are to follow elutriation.

13. Collect remaining fractions in 250 ml medium each at 10 ml/min increments in pump speed. Collect ten samples up to 150 ml/min and place cells on ice.
14. Remove any remaining cells from the rotor using 250 ml medium, and count and concentrate cells as desired (see Basic Protocol, steps 25 and 26).

ANALYSIS OF CELL FRACTIONS TO DETERMINE CELL CYCLE POSITION

Three protocols are presented here for accurately assessing elutriated cell fractions for purity and for verifying cell cycle position. To determine DNA content, elutriated fractions may be examined by flow cytometry using the DNA-binding fluorophore propidium iodide (PI; see Support Protocol 1; also see *UNIT 8.4*). To determine cell cycle stage, nascent DNA synthesis can be assessed by [³H]TdR incorporation into cells (see Support Protocol 2). This is one way of characterizing the proportion of cells that are in S phase. DNA content and active DNA synthesis can be assessed simultaneously by a dual staining method using both PI and 5-bromo-2'-deoxyuridine (BrdU) incorporation (see Support Protocol 3; also see *UNIT 8.2*). The PI staining procedures, both alone and in combination with BrdU incorporation, generally follow the protocols from Becton Dickinson Immunocytometry. Cell cycle stage can also be determined by characterizing the cyclin-dependent kinase (CDK) activity of the cells (see *UNIT 8.2*).

SUPPORT PROTOCOL 1

Standard PI Staining and Flow Cytometric Analysis

Materials

Elutriated cell populations (see Basic Protocol or Alternate Protocol)
PBS (*APPENDIX 2A*), ice cold
Methanol, ice cold

continued

Buffered PI solution (see recipe)

200 U/ml RNase in PBS

12 × 75-mm tubes

Nylon cloth, 41- μ m mesh

Flow cytometer (Becton Dickinson Immunocytometry FACScan or equivalent)

Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*)

1. Set up 12 × 75-mm tubes with 1×10^6 cells from each elutriated population in 1 ml ice-cold PBS.

Maintain equal cell density and PI concentration for each sample for comparison of DNA in different populations (also see step 7).

2. While gently vortexing cells, gradually add 1 ml ice-cold methanol in a dropwise fashion. Incubate on ice for 30 min.

The methanol must be ice cold. Be sure to begin vortexing cells before adding methanol to prevent clumping. Cells are now fixed and may be stored at 4°C for several days prior to analysis.

3. Centrifuge cells at $300 \times g$, room temperature, for 10 min. Carefully aspirate liquid from cell pellet and then loosen pellet by flicking tube or vortexing.

4. Combine equal volumes of buffered PI solution and 200 U/ml RNase solution, and add 500 μ l to each tube.

5. Incubate for 30 min at room temperature, protected from light. Transfer tubes to ice and keep on ice until analysis.

6. Immediately before analysis, carefully vortex samples and then filter through a 41- μ m nylon mesh to remove any cell clumps.

7. Analyze on a flow cytometer using an FL2 linear detector.

A histogram of FL2 area versus number of events will show DNA content and cell cycle stage (see Fig. 8.5.3).

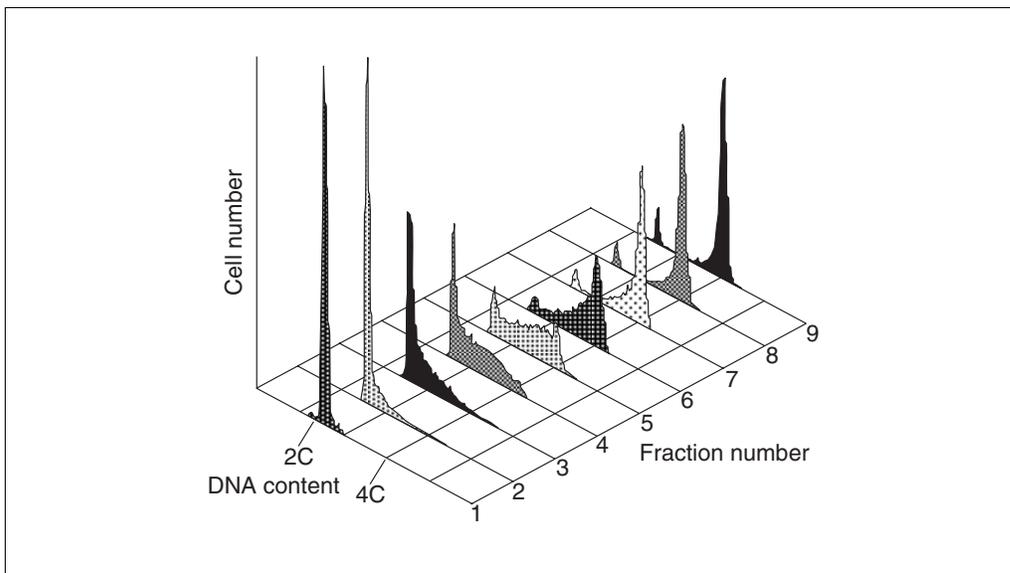


Figure 8.5.3 Three-dimensional histogram of DNA content by flow cytometry versus elutriation fractions of WEHI-231 cells, a murine lymphocytic B cell line. An aliquot of each fraction was fixed in methanol and stained with propidium iodide. The relative DNA content of each fraction was determined by flow cytometry, collecting 10,000 events (x axis, propidium iodide fluorescence intensity for DNA content; 2C, diploid; 4C, tetraploid; y axis, relative cell number).

**SUPPORT
PROTOCOL 2**

Equal cell density between samples is important for equilibration of PI incorporation. Detection of fluorescence intensity will be decreased if greater than equal numbers are used (resulting in a leftward shift in the DNA histogram), or increased if using less than equal cell numbers (resulting in a rightward shift). If cell numbers between samples are fairly close, compensation for this shift may be made by setting a marker at 200 and adjusting FL2 detectors for each sample so that the G_1 peak appears at 200. This should only be done when the position of the G_1 peak is easily recognized.

[^3H]Thymidine Deoxyribose Incorporation

Materials

- Elutriated cell populations (see Basic Protocol or Alternate Protocol)
- Culture medium prewarmed to 37°C
- [Methyl- ^3H]thymidine deoxyribose ([^3H]TdR; 6.7 Ci/mmol)
- Aqueous liquid scintillation fluid
- 96-well microtiter plate
- Glass fiber filters
- Cell harvester

1. Prepare an aliquot of each elutriated cell population at a density of 4×10^5 cells/ml in an appropriate culture medium. Plate 150 μl of each aliquot into triplicate wells of a 96-well microtiter plate and set up control wells with 150 μl culture medium. Warm the plate at 37°C for 10 min.

Maintain equal cell numbers between samples for comparison of thymidine incorporation between cell populations.

2. Prepare an aliquot of 37°C culture medium containing [^3H]TdR at 10 $\mu\text{Ci/ml}$. Add 50 μl to each well containing cells and each control well.
3. Incubate for 30 min at 37°C.

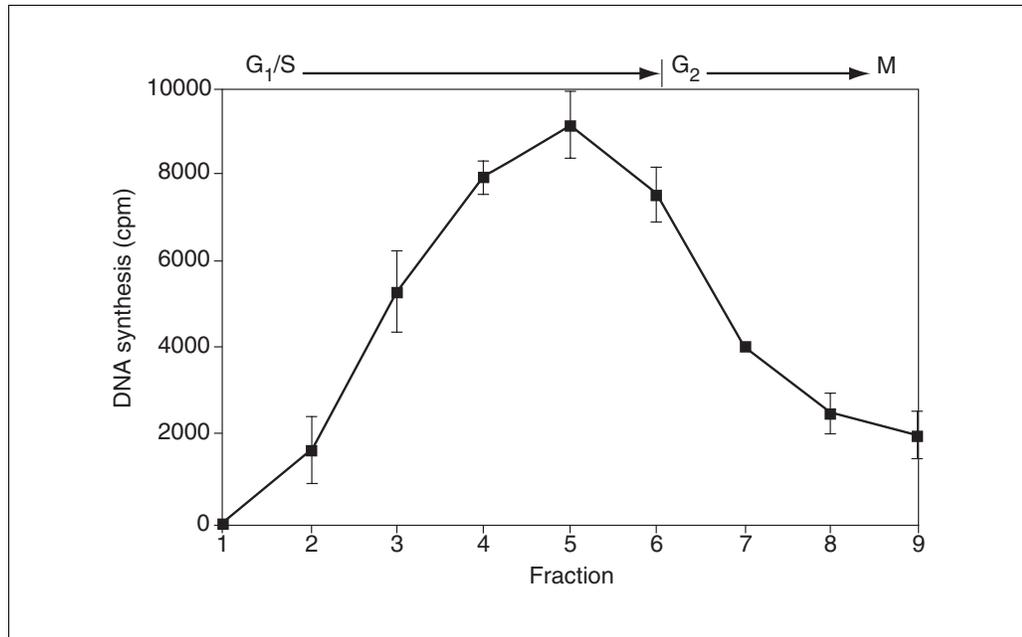


Figure 8.5.4 DNA synthesis profiles of elutriated WEHI-231 murine B cells by [^3H]TdR incorporation. The level of cellular DNA synthesis was determined by incubating equal numbers of cells from each elutriated fraction with [^3H]TdR for 30 min prior to cell harvest and scintillation counting (x axis, elutriation fraction number; y axis, DNA synthesis in cpm). On the basis of flow cytometry and DNA synthesis data, relative positions within the cell cycle are shown at the top of each plot. Each data point is the mean of triplicate determinations; bars are SD.

4. Transfer medium and cells onto glass fiber filters using a cell harvester and wash with water.
5. Air dry filters and seal each in a bag with aqueous liquid scintillation fluid.
6. Quantify radioactivity in a β scintillation counter.

A representative graph of DNA synthesis as a function of elutriation fraction number is shown in Figure 8.5.4.

Combined BrdU Incorporation and PI Staining

**SUPPORT
PROTOCOL 3**

Materials

Elutriated cell populations (see Basic Protocol or Alternate Protocol)

Culture medium

1 mM BrdU solution (see recipe)

PTB buffer (see recipe)

PBS (*APPENDIX 2A*), ice cold

100% ethanol, ice cold

2 N HCl/0.5% (v/v) Triton X-100 (store at 4°C)

0.1 M sodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7$), pH 8.5 (store at 4°C)

Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody

5 $\mu\text{g}/\text{ml}$ PI solution (see recipe)

12 \times 75-mm tubes

Flow cytometer (Becton Dickinson Immunocytometry FACScan or equivalent)

Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*)

1. Set up 12 \times 75-mm tubes with 1×10^6 cells from each elutriated population in 1 ml culture medium.

Maintain equal cell density and PI and BrdU concentration for each sample for comparison of DNA in different populations (also see step 12).

2. Add 10 μl of 1 mM BrdU solution (final 10 μM) and incubate for 15 min at 37°C.

For cell lines with shorter cell division times, incubation time may be reduced to 2 min; slower cycling cells may require a 30-min incubation.

3. Wash cells twice in 3 ml PTB buffer and centrifuge at $300 \times g$ for 10 min at room temperature. Aspirate medium, resuspend cell pellet in 500 μl ice-cold PBS, and place cells on ice.

Be certain that PBS is cold and cell pellets are placed on ice both before and after cold ethanol is added.

4. While gently vortexing cells, gradually add 1 ml ice-cold ethanol in a dropwise fashion. Incubate on ice for 30 min.

It is critical that ethanol be ice cold. Be sure to begin vortexing cells before adding ethanol and maintain vortexing to prevent clumping. Cells are now fixed and may be stored several days at 4°C.

5. Centrifuge cells at $300 \times g$, 10°C, for 10 min. Carefully aspirate liquid from cell pellet and then loosen pellet by flicking tube or vortexing.

6. Add 1 ml of 2 N HCl/0.5% Triton X-100 and incubate for 30 min at room temperature.

This step denatures the DNA, producing single-stranded molecules that allow the subsequent binding of anti-BrdU antibody.

**Cell Cycle
Analysis**

8.5.11

7. Centrifuge at $300 \times g$, room temperature, for 10 min. Resuspend pellet in 1 ml of 0.1 M $\text{Na}_2\text{B}_4\text{O}_7$, pH 8.5, to neutralize. Incubate ~10 to 15 min at room temperature.
8. Centrifuge at $300 \times g$, room temperature, for 10 min. Resuspend pellet in 80 μl FITC-conjugated anti-BrdU antibody diluted 1:4 in PTB buffer. Incubate for 30 min at room temperature.
9. Add 3 ml PTB buffer to each tube, centrifuge cells, and resuspend pellet in 500 μl of 5 $\mu\text{g}/\text{ml}$ PI solution.
10. Incubate for 15 min at room temperature, protected from light, and then place on ice.
11. Analyze on a flow cytometer using FL1 log (FITC) and FL2 linear (PI) detectors. Show dot plot as FL2 area versus FL1 height.

A histogram of FL2 area will also show DNA content and cell cycle stage (see Fig. 8.5.5).

Equal cell density between samples is important for equilibration of PI incorporation. Detection of fluorescence intensity will be decreased if greater than equal numbers are used (resulting in a leftward shift in the DNA histogram), or increased if using less than equal cell numbers (resulting in a rightward shift). If cell numbers are fairly close, compensation for this shift may be made by setting a marker at 200 and adjusting FL2 detectors for each sample so that the G_1 peak appears at 200. This should only be done when the position of the G_1 peak is easily recognized.

12. To calculate percentage of cells in each stage of the cell cycle, draw regions in dot plot and then calculate with Region Stats (standard with FACScan).

Calculations can also be done using the PI histogram and CELLFIT (Becton Dickinson) formulas; however, the relatively pure cell cycle populations in transition between cell cycle phases obtained through elutriation cells cannot be analyzed, as the Becton Dickinson algorithm requires profiles with G_1 , S, and G_2 content. Manually marking peak areas is also inaccurate as S phase overlaps into G_1 and G_2 .

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

BrdU solution, 1 mM

30.7 mg 5-bromo-2'-deoxyuridine (BrdU)
 100 ml PBS (APPENDIX 2A)
 Divide into 1-ml aliquots
 Store up to 6 months at -20°C (do not refreeze)

Buffered PI (propidium iodide) solution

10 mg propidium iodide
 0.1 ml Triton X-100
 3.7 mg EDTA
 90 ml PBS (APPENDIX 2A)

Combine, stirring gently to dissolve completely (protect from light). Adjust volume to 100 ml with PBS. Store up to 1 month at 4°C protected from light.

CAUTION: Use gloves. Propidium iodide is a mutagen and suspected carcinogen.

Elutriation medium

1.9 liters RPMI culture medium
 100 ml heat-inactivated FBS (APPENDIX 2A)
 Make fresh and use at room temperature

The composition of elutriation medium can vary. Typically, it is either culture medium or PBS without Ca^{2+} or Mg^{2+} , and is supplemented with 5% (v/v) FBS or 5% (w/v) BSA.

PI (propidium iodide) solution, 5 µg/ml

Prepare a 100 µg/ml stock solution by mixing 10 ml propidium iodide in 100 ml PBS (*APPENDIX 2A*) and stirring gently to dissolve completely (protect from light). Store up to 6 months at 4°C protected from light. Immediately before use, dilute 1/20 in PBS (final 5 µg/ml).

CAUTION: Use gloves. Propidium iodide is a mutagen and suspected carcinogen.

PTB (PBS/Tween/BSA) buffer

2.5 ml Tween 20 (20% final)
5 g BSA (1%)
Adjust to 500 ml with PBS (*APPENDIX 2A*)
Mix slowly to dissolve
Pass through 0.2-µm filter
Store up to 6 months at 4°C

COMMENTARY

Background Information

A detailed characterization of the physical separation properties of the elutriation system can be found in Keng et al. (1981) and the respective JE-6B and JE-5.0 instruction manuals (Beckman Instruments, 1981, 1989). Additional information on cell cycle separation is found in Kaufman et al. (1990) and Donaldson et al. (1997). B cell separation is described in Thompson et al. (1983), separation of T cells in Marjanovic et al. (1991), and separation of mixed populations in Meistrich (1983). CCE is relatively simple to perform and has the advantage of providing large quantities of cells with minimal impact on cell metabolism.

The combined separation and analysis protocols presented here are generally applicable to a wide variety of human and mouse nonadherent cell lines. Specific parameters are given for Jurkat cells using the standard rotor chamber (Table 8.5.1) and for WEHI-231 cells with the large chamber (Table 8.5.2). Although published protocols and the rotor speed and flow rate Nomogram contained in the Beckman elutriator manual will provide approximate starting conditions, a pilot run is likely to be required for each cell line. Cycling cells will separate differently depending on a wide variety of parameters including culture and medium conditions and ploidy. Some primary cells such as stimulated human peripheral T cells are much smaller than their transformed (Jurkat) counterparts, allowing twice as many cells to be loaded (Donaldson et al., 1997). As the majority of stimulated proliferating cells are in the G₁ phase of the cell cycle, with few cells in G₂/M, it may be difficult even in the best logarithmic cultures to obtain highly enriched G₂ fractions. Although elutriation can be carried out with a refrigerated rotor

and cold media, the authors have found little advantage to cell viability, and degassing becomes more problematic.

The JE-5.0 rotor with the 40-ml chamber can be used to elutriate ten times as many cells as the JE-6B rotor, and the JE-5.0 is useful for harvesting large volumes of cells from culture medium prior to elutriation. The general conversion from the low-volume rotor used for analysis and methods development to the high-volume rotor for preparative work requires an ~4-fold increase in flow rate. As concentrating cells from large volumes of culture media by repetitive centrifugation can be time consuming and diminish cell viability, harvesting by continuous flow using the JE-5.0 rotor is a convenient alternative. Although it is possible to proceed from harvest in the JE-5.0 directly to sorting the cells without emptying the chamber, in practice, most cells types must first be removed and passed through an 18-G needle to ensure monodispersion prior to sorting.

Cell cycle position and distribution of the elutriated cell populations can be confirmed by several methods, the simplest being pulse labeling of each population with [³H]TdR (Fig. 8.5.4). DNA content can be determined by PI staining and flow cytometry. Fluorescence intensity is directly proportional to DNA content (Fig. 8.5.3). Three-dimensional histograms show total DNA content of each elutriated fraction of WEHI-231 cells determined by flow cytometry of PI-stained nuclear DNA. The method of combined labeling of cell populations with PI for total DNA content and pulse BrdU for nascent DNA synthesis allows for the simultaneous and accurate determination of total DNA and active synthesis (Fig. 8.5.5). Dot plots of DNA content detected by PI staining (*x*

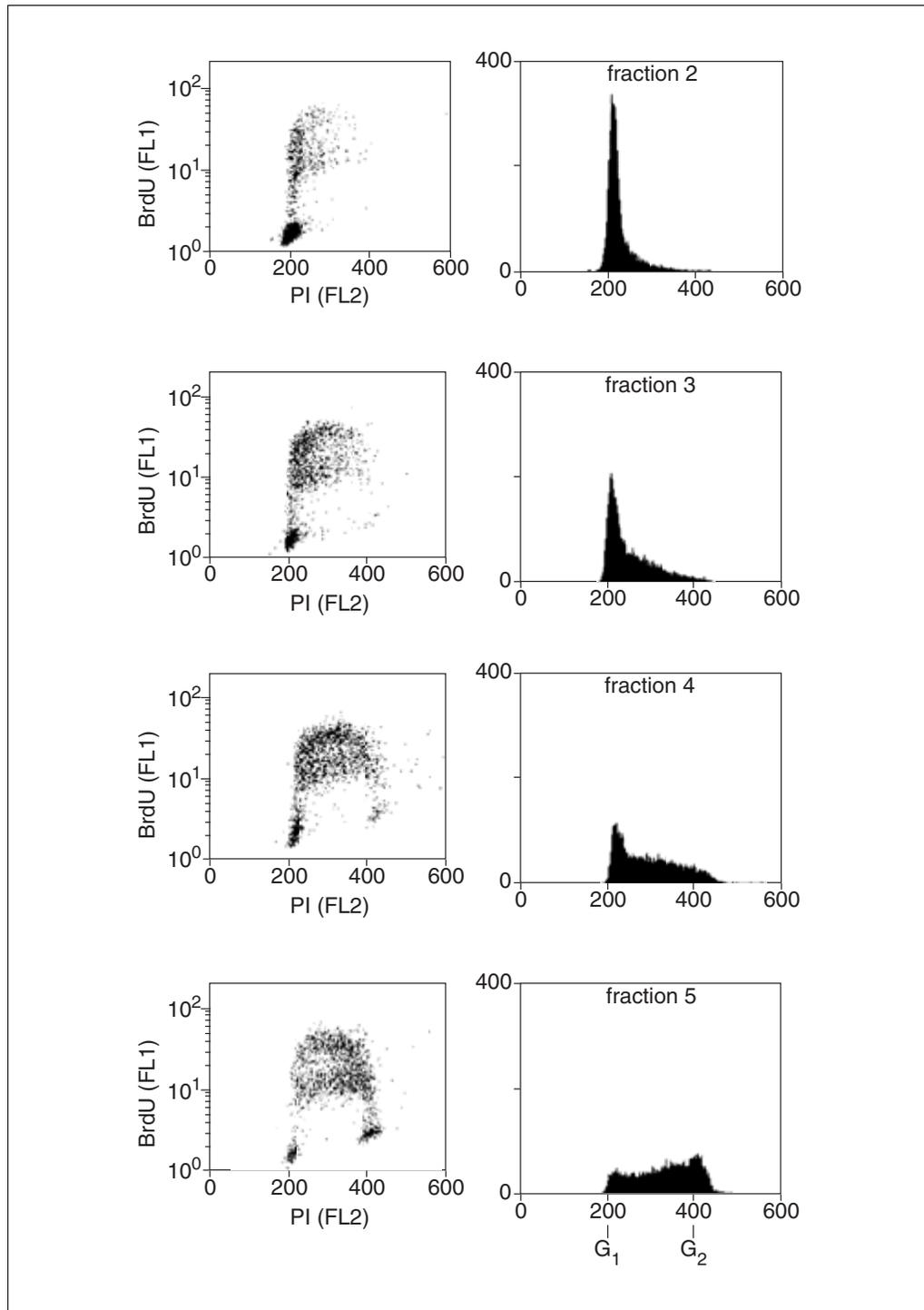
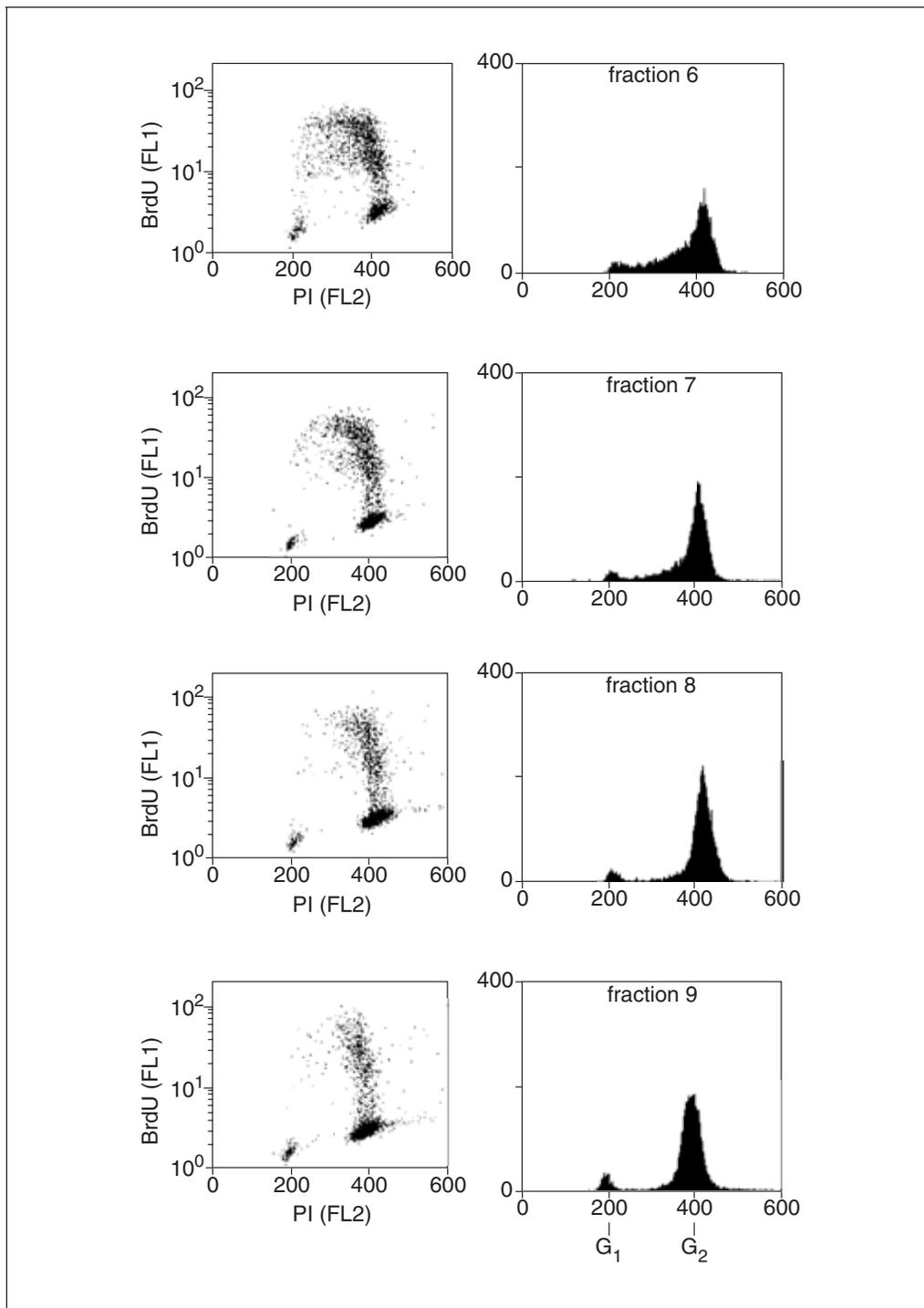


Figure 8.5.5 (*above and at right*) Simultaneous analysis of DNA synthesis and DNA content of elutriated WEHI-231 cells by BrdU incorporation, PI staining, and flow cytometry. Aliquots of cells from each elutriated fraction were pulsed with BrdU for 15 min, fixed in ethanol, and stained for DNA synthesis with FITC-conjugated anti-BrdU antibody and for DNA content with propidium iodide. Dot plots (left) were generated by analysis of 10,000 events and show DNA content by FL2 area (linear; x axis) and DNA synthesis by FL1 height (log; y axis). The corresponding histogram for DNA content is also shown immediately to the right (x axis, PI fluorescence intensity; y axis, relative cell number).



axis) with DNA synthesis by FITC-labeled anti-BrdU (y axis) are shown for each elutriated fraction. Cells synthesizing DNA shift upward on the y axis (FL1 height) and appear as an arc above G_1 and G_2 populations of cells. DNA content by PI staining on the x axis (FL2 area) is also shown as a histogram immediately to the right of each dot plot. Accurate percentages of cell cycle populations can be determined by

gating separate regions around these G_1 , S, and G_2 populations.

Critical Parameters

Experiments should be fully planned and all equipment, reagents, and tubes should be prepared and ready well in advance. Set up, sterilization, and equilibration are time consuming and it is helpful to coordinate these tasks with cell concentration. Even the best biophysical

predictions will not eliminate the need for a pilot elutriation run to verify speed and flow rates needed for optimal separation.

Cells in poor condition are not worth elutriating. Maintaining cells at $2\text{--}5 \times 10^5$ cells/ml with systematic 1:2 dilution with fresh medium for the week prior to elutriation will ensure healthy, log-phase cells. For cell cycle studies, this also ensures good yields of S- and G₂/M-phase populations. Cells must be monomeric. Doublets of G₁ cells can approximate the mass of a G₂ cell and contaminate later fractions. Similarly, highly aneuploid cells will not sort well due to overlaps in mass with near-diploid cells at different stages of their division cycle. Highly aneuploid cells can be eliminated from a mixed population by elutriating the mixed cells and harvesting the smallest population. Upon reculture, cells with high ploidy are greatly diminished.

Although the authors do not recommend it for these protocols, some protocols include EDTA or DNase treatment to reduce aggregation. The release of chromosomal DNA from damaged cells is the major cause of clumping within the chamber. Careful handling during concentration, resuspension, and syringe dispersion will greatly reduce lysis. Resuspend the cells in elutriation medium and load the same number of cells each time. Varying the medium or cell number can shift the buoyant density and eluting population.

Anticipated Results

A combination of CCE and analysis of elutriated fractions should provide ten fractions enriched for cells at specific stages of the cell cycle. The viability of the cells is maintained and the elutriated cells are suitable for culture and/or biochemical analysis.

Time Considerations

Effective CCE requires 90 min to set up, sterilize, equilibrate, and calibrate the equip-

ment; 90 min to harvest the cells; and 30 min to elute the fractions. PI staining takes 2 hr, [³H]TdR labeling takes 90 min, and PI staining plus detection of BrdU incorporation takes 4 hr.

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CHAPTER 9

Cell Adhesion

INTRODUCTION

Cell-adhesive interactions determine the organization of tissues and mediate and guide precise cell migrations during embryonic development, inflammation, the immune response, and wound repair. They also help to regulate gene expression, growth, differentiation, and apoptosis. Research into cell adhesion has undergone dramatic evolution over the past two to three decades, from phenomenological studies of the biophysical and morphological mechanisms used by cells to adhere to other cells and the extracellular matrix, to the discovery and in-depth mechanistic analyses of a variety of specific cell adhesion systems, and then to the recent paradigm shift whereby adhesion molecules are now viewed as activators or regulators of a remarkably wide range of signal transduction pathways.

FUNCTIONS OF ADHESION MOLECULES

Cell adhesion molecules function by forming specific protein-protein or protein-carbohydrate bonds at the cell surface to mediate cell interactions. In addition, cell adhesion molecules or cell adhesion receptors often form direct links to multimolecular protein complexes on the cytoplasmic face of the plasma membrane. These cytoplasmic adhesion and signaling complexes interact with the cytoskeleton and signal transduction pathways. As a result, cell adhesion not only links cells with other cells and the extracellular matrix, but also helps to integrate extracellular physical information with the major signal transduction pathways within cells.

Cell adhesion is often categorized into cell-to-cell adhesion and cell-to-substrate adhesion. In each case, the physical structures that mediate cell adhesion can be either specialized adhesive structures or broad expanses of plasma membrane. For example, cells can initially adhere to other cells along large areas of plasma membrane using general-purpose adhesive molecules such as cadherins. But they can also form attachments to each other by using specialized adhesive structures, such as desmosomes, adherens junctions, and tight junctions. Each type of junctional complex involves specific adhesion-molecule components, such as cadherins and associated cytoplasmic components involved in linkage to the actin-containing cytoskeleton. Fibroblasts can also form adhesions to other fibroblasts, but they most characteristically form adhesive interactions with extracellular matrix molecules. Such cell-to-matrix adhesion sites can also be either broad zones or specialized structures. For example, rapidly migrating cells typically interact with tissue culture substrates through broad areas of contact termed “close contacts,” and the entire basal surface of epithelial cells adheres via integrins to their underlying basement membranes. Specialized adhesive structures include the hemidesmosomes of certain epithelial cells and the focal adhesions of fibroblastic and endothelial cells. The protein composition of the complexes involved in cell-to-matrix adhesions can sometimes involve a few of the same proteins as in cell-to-cell adhesions, such as vinculin and actin, but they mainly consist of distinct types of adhesive, cytoskeletal, and signal transduction molecules.

GENERAL CHARACTERISTICS OF CELL ADHESION PROTEINS

Cell adhesiveness generally involves specific binding of a cell adhesion protein or receptor to a target molecule. Adhesion mediated by the binding of an adhesion molecule to the same type of protein on a neighboring cell is termed a “homophilic” interaction. Members of the calcium-dependent cadherin family are major mediators of such interactions, and some calcium-independent adhesion proteins, such as N-CAM, can also provide homophilic interactions. Cadherin adhesion molecules characteristically form tightly packed dimeric and multimeric complexes that mediate adhesion with high avidity via cooperativity, even though the affinity of a single cadherin protein-protein interaction alone is not high.

A particularly common type of adhesive interaction involves the binding of a receptor to a specific ligand. In the process of cell-to-cell adhesion, the target protein of an adhesion receptor can be either a “counter-receptor” or a complex carbohydrate linked to a protein anchor in the plasma membrane. In cell-to-matrix interactions, a plasma membrane adhesion receptor such as an integrin binds to an adhesive extracellular matrix protein. For example, a number of types of integrin receptors can bind to fibronectin, laminin, or collagen. Integrin receptors directly mediate cell adhesion, migration, and anchorage to these structural components of the extracellular matrix.

Molecules that mediate cell-cell or cell-matrix adhesion fall into two broad structural classes. One type is anchored to the plasma membrane, often as a transmembrane protein. These membrane-anchored molecules are generally receptors, homophilic adhesion molecules, or counter-receptors. They often consist of an extracellular domain containing one or more specific cell interaction domains or sites, as well as a stalk region, a hydrophobic transmembrane domain, and a cytoplasmic domain or tail. This type of adhesion molecule is often likely to be involved in the transmembrane transmission of signals after binding to their target molecule.

The second broad class of adhesion molecules consists of cell surface or extracellular matrix proteins (see Chapter 10) that contain domains involved in cellular adhesion. Nearly all matrix proteins contain such sites. For example, this class includes fibronectin, laminins, vitronectin, collagens, and many other extracellular proteins. These proteins contain one or more cell-binding domains, which are comprised of a primary recognition motif consisting of a short peptide sequence (e.g., Arg-Gly-Asp or Leu-Asp-Val) and often a synergy site or other structural feature that substantially enhances receptor-binding specificity and affinity.

Adhesion molecules frequently have many of the following characteristics (for reviews, see Alberts et al., 1994; Chothia and Jones, 1997; Edelman and Thiery, 1985; Gumbiner, 1996; Hay, 1991; Richardson and Steiner, 1995).

1. The backbone structure of adhesion proteins is often based on multiple repeats of protein motifs, such as the immunoglobulin (Ig) motif, EGF repeat, or fibronectin motif. The basic immunoglobulin repeat is quite common, as is the structurally related fibronectin type III repeat.
2. They have specialized functional domains, including one or more domains for binding molecules on other cells or in extracellular matrix. Often a domain for forming dimers or higher polymers is also present, as well as another for binding to complex carbohydrates.
3. They often bind with only moderate affinity, e.g., in the range of $K_d = 10^{-6}$ to 10^{-7} M for fibronectin, and as low as 10^{-4} M for leukocyte adhesion molecules involved in rolling adhesion. This modest affinity appears to be important for allowing dynamic changes in cell adhesions and to permit cell migration involving cyclic attachment and detachment of cell adhesion sites to a substrate or other cells.

4. They often function by clustering to generate complexes of high avidity: adhesion molecules such as integrins and cadherins often organize into large local clusters or aggregates in the plane of the plasma membrane, which can produce strong total avidity as a result of the summation of the otherwise weak binding of many individual molecules.
5. Their binding functions can be regulated by activation, e.g., “inside-out” signaling that changes their ability to bind ligands effectively; examples are integrin activation during leukocyte adhesion and platelet activation, and the suppression of the cadherin system by tyrosine phosphorylation.

MAJOR FAMILIES OF CELL ADHESION MOLECULES

Adhesion molecules that are anchored in the plasma membrane include several large groups of proteins that share common structural motifs, such as the immunoglobulin repeat. **Cadherins** comprise a large family of proteins particularly centrally involved in cell-to-cell adhesive interactions (Takeichi, 1990; Yap et al., 1997; see *UNIT 9.3*). Cadherins on one cell bind in homophilic fashion to the same type of cadherin on other cells by means of specific cell interaction domains, which can include the short peptide recognition sequence His-Ala-Val. The cytoplasmic domain of cadherin molecules characteristically binds to catenins, which provide direct linkages to the actin cytoskeleton (e.g., via α - and β -catenins).

The “classical” cadherins such as E-cadherin and N-cadherin mediate adhesion over broad expanses of cell-cell contact, or they can become further organized into adherens-type junctions that are linked to cytoplasmic molecules such as vinculin and actin. A number of other types of cadherins have been discovered recently, whose functions are probably also adhesive, but which remain to be characterized. Finally, certain highly specialized cadherins found exclusively in desmosomes, termed desmocollins and desmogleins, link cells together at particularly strong attachment sites connected to intermediate filaments such as keratins or vimentin.

Cadherin activity is quite sensitive to calcium ion concentration, which accounts for the ability of calcium chelators such as EDTA to dissociate tissues into their individual component cells. Chelation of divalent cations appears to disrupt conformation, so that the cadherin molecules become quite sensitive to general proteolytic attack; this latter property has been used to classify cell-cell adhesion molecules (see *UNIT 9.3*). Although desmosomal and other cadherins can be found widely in epithelia of organisms at all ages, the cadherins are particularly important during embryonic development, when they appear to organize groups of cells and tissues by specific cell-cell adhesions. They help to define tissues by binding primarily to cadherins of the same type, rather than to other cadherins on unrelated cell types; this activity can lead to “sorting out” of different types of cells from others.

Integrins are nearly ubiquitous cell surface receptors for a wide variety of extracellular matrix proteins, as well as for “counter-receptor” ligands on other cells. There are more than 20 distinct integrin subunits, which are combined to form heterodimers that always consist of one α and one β subunit. Genetic loss of almost any integrin subunit leads to disease or death, often during embryonic development or near the time of birth. Integrin molecules have a head domain containing a ligand-binding site, two spindly legs, and usually rather short cytoplasmic domains. Integrin binding is often inhibited by depletion of divalent cations.

CAMs (cell adhesion molecules) and integrin counter-receptors are structurally related by their use of the immunoglobulin repeat, and they often have the term “-CAM” in their names, yet they differ functionally. Molecules such as NCAM are calcium-independent,

homophilic adhesive molecules that bind to the same type of molecule on an adjacent cell surface, often a cell of the same tissue type (Edelman and Crossin, 1991). In contrast, counter-receptors such as the ICAMs and VCAM have specialized peptide recognition sites that are bound specifically by integrins such as LFA-1 (CD11a/CD18 or $\alpha_L\beta_2$) or VLA-4 ($\alpha_4\beta_1$), which are often present on other types of cells (e.g., endothelial cells and lymphocytes). The functions and sites of expression of these molecules can consequently differ widely. For example, molecules such as NCAM are implicated in embryonic developmental events such as axonal guidance and bundling, whereas ICAM counter-receptors are present as targets for binding by cells circulating in blood. The levels of these counter-receptors on the cell surface can often also be regulated rapidly in response to cytokines, for example during inflammatory responses.

A family of transmembrane proteins termed **ADAMs** (membrane proteins with a disintegrin and metalloprotease domain; Wolfsberg and White, 1996) contain a protease-like domain plus an integrin recognition site in the disintegrin domain. They appear to play roles in cell adhesion; for example, one member of this family is present on sperm and has been implicated in sperm-egg adhesion. **Syndecans** are cell-surface heparan sulfate proteoglycans with a protein core that crosses the plasma membrane and terminates in a cytoplasmic tail. Syndecans appear to function as “co-receptors,” mediating signaling in association with a primary adhesion molecule. For example, syndecans synergize with fibronectin during formation of focal adhesion sites and form linkages to protein kinase C (Woods and Couchman, 1998).

Adhesive extracellular matrix molecules are described in Chapter 10. One interesting aspect of their function involves the striking effects of adsorption or attachment to a substrate. Molecules such as fibronectin and vitronectin circulate in blood at relatively high concentrations (e.g., 100 to 300 $\mu\text{g/ml}$), yet binding to cell surface receptors is relatively limited. On the other hand, even very low concentrations of the same proteins (1 to 10 $\mu\text{g/ml}$) bind to substrates and then mediate strong cell adhesion. This functional enhancement of binding of cells to molecules attached to a substrate has been variously ascribed to activation of molecules such as fibronectin and vitronectin by conformational changes resulting from binding to a substrate, to enhanced ability to interact with cells due to multivalency, or to a physical chemical enhancement in free energy (overall avidity) due to immobilization of a ligand. Whatever the mechanisms, it is clear that the binding of extracellular adhesion molecules to substrates can enhance functional activity, which appears to be important biologically to generate strong adhesion only when a circulating ligand is immobilized. For example, in wounds, immobilized fibronectin forms a crucially important provisional matrix that permits adhesion and migration of cells to close the wound.

FUNCTIONS OF CELL ADHESION MOLECULES IN SIGNAL TRANSDUCTION AND CYTOSKELETON

The functions of cell adhesion molecules and their receptors can be understood intuitively as physical mechanisms by which cells attach to other cells, adhere to extracellular matrix molecules, and provide traction during cell migration. This concept can be extended to intracellular structural effects—i.e., the organizing of the cytoskeleton. For example, transmembrane adhesion proteins provide membrane anchors for a host of cytoskeletal proteins such as keratin or vimentin in desmosomes and hemidesmosomes, actin microfilaments and other proteins in epithelial adherens junctions, and a number of adhesion plaque proteins and actin in cell-substrate adhesion plaques (e.g., see Jockusch et al., 1995; Yamada and Geiger, 1997).

It is now clear, however, that adhesion molecules also play crucial roles in helping to trigger or modulate many major signal transduction pathways. In fact, their signaling

functions are probably at least as important in cell biology as their attachment and cytoskeletal activities. A notable characteristic of the intracellular complexes of proteins induced by binding of adhesion molecules is that their formation also induces accumulation of a variety of signal transduction molecules that can trigger downstream signaling pathways (Clark and Brugge, 1995; Schwartz et al., 1995; Lafrenie and Yamada, 1996). As a consequence, adhesion proteins are actually cell-interaction proteins that have multiple functions in the bidirectional transfer of information at the cell surface. They can mediate outside-in transfers of signaling information, such as cellular responses to binding of specific extracellular matrix proteins involving the activation of intracellular signaling, but they can also mediate inside-out information in which intracellular signals modulate the activity of integrins and assembly of extracellular matrix. Even classical adhesive proteins such as fibronectin can trigger a bewildering range of activities after binding by integrins; these can include activation or modulation of many well-known mammalian signal transduction pathways (such as tyrosine phosphorylation, MAP kinases, protein kinase C, Ca^{2+} and H^+ fluxes, and phosphoinositide pathways) which can activate specific gene transcription, mediate anchorage-dependent growth stimulation, and prevent apoptosis.

Two structural regions in transmembrane adhesion molecules that allow them to function as signal transduction receptors are their extracellular ligand-binding domains and their cytoplasmic domains. The ligand-binding domains are obviously essential for binding to extracellular molecules, but in at least some cases, they have additional, intriguing roles. For integrin adhesion receptors, there appear to be separable functions for ligand occupancy (filling the binding site with a ligand), as opposed to receptor clustering (which can be induced by multivalent ligands such as fibrils of fibronectin or collagen). These two inputs can synergize to promote the accumulation of specific cytoskeletal proteins such as α -actinin and actin to form strong adhesions (Yamada and Miyamoto, 1995).

Even though they lack intrinsic enzymatic activities, integrins appear to function as signaling receptors and regulators of actin cytoskeletal organization by recruiting other molecules to bind to their cytoplasmic domains. Integrins appear to bind directly to certain cytoplasmic proteins, such as talin, α -actinin, and tensin, in a process that is sometimes regulated by ligand occupancy on the outside of the cell. Also important, however, are docking proteins such as focal adhesion kinase, which can bind to integrins as well as binding to at least eight cytoplasmic molecules to form molecular complexes. Other docking proteins such as p130^{Cas} probably increase the repertoire of cross-linked and complexed proteins. Integrin clustering appears to play a central role in forming large complexes that can consist of over 30 different types of molecules. These integrin-induced multimolecular complexes can serve as signaling centers, e.g., for tyrosine phosphorylation and MAP kinase activation (reviewed by Yamada and Miyamoto, 1995; see *UNIT 14.2* and *UNIT 14.3* for protocols measuring these post-translational modifications).

Cell-to-cell adhesion molecules also appear to undergo a very similar process of local clustering, complex formation, binding of cytoskeletal proteins such as actin, and accumulation of signaling molecules (Kirkpatrick and Peifer, 1995; Yap et al., 1997; Yamada and Geiger, 1997). It will become increasingly important to understand how these processes are controlled. The cadherin system can be suppressed by tyrosine phosphorylation mediated by v-Src kinase, and certain integrin functions can be down-modulated by the phosphatase PTEN (Takeda et al., 1995; Tamura et al., 1998). There are likely to be large numbers of other regulatory pathways that affect the widespread intracellular effects of cell-cell and cell-substrate adhesion molecules on cytoplasmic processes. It is clear that adhesion molecules are crucial, integral components of the basic signaling and regulatory mechanisms of cells, providing dynamic links to the external environment.

CELL ADHESION ASSAYS

A crucial first step for effective analysis of a cell adhesive process is to characterize it in a quantitative *in vitro* assay. Cell-to-substrate adhesion assays are used to examine the ability of cells to attach to matrix molecules, such as fibronectin or laminin, and to determine which specific adhesion receptors are involved using antibodies or synthetic peptide inhibitors. Roles of cytoplasmic molecules such as cytoskeletal proteins and signaling molecules can also be evaluated with these assays using pharmacological inhibitors of intracellular processes—for instance inhibitors of kinases and other molecules.

There are several types of cell-substrate adhesion assays, which can quantitate (1) cell attachment, (2) cell spreading, or (3) detachment of previously adherent cells. Each assay measures different parameters of the adhesion process: cell attachment assays generally determine the numbers of cells that can attach to a substrate in a specific time period after washing off nonattached cells (*UNIT 9.1*); cell spreading assays determine the percentage of cells that show spread morphologies, or measure the surface area of spread cells, after certain times of incubation (*UNIT 9.1*); and cell detachment assays measure the ease with which cells can be detached after a particular period of time, for example by determining the numbers of cells detached from a dish using each of a series of increasing centrifugal force levels (*UNIT 9.2*). Although conceptually distinct, these three types of assays can in practice have features that overlap significantly.

In order to compare these assays, it is useful to consider the steps in cell-substrate adhesion and spreading. The first step in cellular adhesion to a substrate is the attachment of plasma membrane receptors or other cell interaction molecules to substrate molecules such as extracellular matrix (ECM) proteins (Chapter 10). For example, if cells are cultured on tissue culture dishes or glass in serum-containing medium, they often adhere to serum proteins such as vitronectin and fibronectin that are nonspecifically adsorbed to the substrate. Integrins frequently mediate such adhesions (e.g., attachment of cells to an extracellular matrix protein), although various other cell attachment proteins, such as the selectins, also exist.

The binding of integrins to ligands can occur even at 4°C, because it is a direct protein-protein interaction. However, this mechanism of cell adhesion is initially quite weak and can often require centrifugation of the cell against the substrate to produce close enough apposition of the plasma membrane to substrate proteins for effective binding. In contrast, normal adhesion appears to involve active cellular processes involving the plasma membrane and the cytoskeleton. The latter, poorly understood process of cytoskeletal strengthening of adhesions can produce enormous increases in attachment strength (see *UNIT 9.2* for a conceptual discussion). This strengthening phenomenon may be related to the formation of multimolecular complexes in adhesion structures containing a variety of cytoskeletal and signal transduction proteins (Burridge et al., 1997; Yamada and Geiger, 1997). Cell attachment assays are generally performed shortly after plating cells before much cell spreading can occur (e.g., within 10 to 20 min for fibroblasts).

Attachment is rapidly followed by spreading of the cell on the substrate in a dynamic adherence response involving cell movements; spreading can become maximal by 30 to 60 min. Even in simple attachment assays, it appears likely that there are some contributions from cell spreading, as the area of attachment of the cell to the substrate expands, accompanied by increasing organization of the cytoskeleton. As spreading and firmer adhesion ensue, cells often form strong focal adhesions to the substrate that are sites of termination of actin microfilament bundles (also known as stress fibers, since the bundles appear to follow lines of linear stress between focal adhesions). As cells attach and spread, they rapidly acquire resistance to detachment, for example by centrifugal force.

Although assays for cell attachment, spreading, or detachment each focus on one particu-

lar facet of cell-substrate adhesion, each assay has strengths, weaknesses, and partial overlaps with the others. Attachment assays are conceptually the simplest, but nonetheless usually unavoidably involve an element of measuring resistance to detachment of the cells by shear forces. In order to rinse unattached cells out of 96-well plates, medium must be poured in and then aspirated or flicked out, thereby generating substantial shear forces as the meniscus passes over attached cells. Cell spreading assays can provide rapid semiquantitative data and evaluate the cytoskeletal facet of cell-adhesive interactions. As a consequence, however, they do not measure only the simple interaction of an adhesion receptor with its ligand, but instead the more complete adhesion response. Spreading assays also involve an element of judgment in choosing to record whether a cell is spread or not spread, which can sometimes perturb a researcher unaccustomed to microscopy. The advantage of centrifugal detachment assays is that they can provide direct physical measurements of adhesive strength as measured in force units ($\times g$). However, this sort of assay is particularly sensitive to timing, and waiting slightly too long before an assay can permit rapid cytoskeletal organization and strengthening responses that anchor cells so tightly to the substrate that they cannot be detached without being destroyed. Nevertheless, centrifugal detachment assays arguably provide the most rigorous quantitative data.

Methods for quantification of cell-to-cell adhesion generally depend on determining the rates of reaggregation of dissociated cells (see *UNIT 9.3*). Although it is also possible to estimate strengths of adhesion by determining the force needed to separate two cells using microneedles, aggregation assays are the norm. These assays are conceptually related to the platelet aggregometer assays used routinely in clinical laboratories to measure platelet adhesion (aggregation). An added feature with nucleated cells, however, is that multiple types of adhesion molecules can often be involved in any particular cell-cell adhesive event. Three commonly observed types of adhesion molecule are the calcium-dependent cadherins, calcium-independent molecules such as N-CAM and other immunoglobulin-repeat molecules, and integrins, though other adhesion molecules may also be involved.

CHARACTERIZATION OF CELL ADHESION MECHANISMS

Once the adhesive characteristics of a cell toward a particular cellular or substrate adhesive system are established, a variety of inhibitors or modulators of adhesion can be tested in order to better define the adhesion system. Examples include function-blocking antibodies against the many dozens of known adhesion molecules to determine which are required for an adhesive event, competitive peptide inhibitors that target the active sites of adhesion receptors, and pharmacological and ionic activators and inhibitors. The latter categories include phorbol esters or Mn^{2+} to activate certain integrins, and chelators for depleting Ca^{2+} to inhibit cadherins and integrins. For routine analyses of integrins, the pertinent receptor(s) involved in a particular adhesive function can be identified by targeting a broad class of integrins first—for instance using an antibody that inhibits all β_1 or all α_v integrins—and then narrowing down the possible candidate receptors involved in a particular function by using more specific antibodies within that class (e.g., anti- α_5 within the β_1 integrin class). Monoclonal antibodies against integrins are widely available. For cadherins, specific antibodies against classical but not novel cadherins are also available, as are various cell biological approaches as described in this chapter. Reagents for analyzing other types of adhesion molecules should become increasingly available from commercial sources.

PROCEDURES DESCRIBED IN THIS CHAPTER

Two semiquantitative assays for measuring cell adhesion to a substrate are provided in *UNIT 9.1*. The first is a cell spreading assay in which adhesion is evaluated by determining

the percentage of cells that spread on a substrate, using microscopy to count spread cells. The second assay quantitates cell attachment after washing out nonattached cells, using a colorimetric protocol for quantification. The relative advantages of each of these widely used assays are compared.

The McClay centrifugation assay for directly quantitating the strength of adhesion of cells to a substrate is described in *UNIT 9.2*. This assay determines the proportion of cells detached by the graded application of centrifugal force. A new variation of the original method provides a simple method for quantitation by counting numbers of stained cells under a light microscope. This simplification of the assay should further enhance the applicability and popularity of this assay. *UNIT 9.2* also provides a helpful in-depth conceptual discussion of the issues involved in quantitating cell adhesiveness.

In *UNIT 9.3*, the original discoverer of the important cadherin cell-cell adhesion system provides an overview of cadherins, as well as detailed laboratory protocols for assaying cell-cell adhesion. Both quantitative and qualitative methods for identifying cadherin function are presented. Besides providing complete protocols for assaying cell-cell adhesion of a single cell type in short- and long-term cultures, this unit describes a method for assaying the sorting-out behavior of different types of cells in a mixed-cell aggregation culture. In addition, a variety of approaches for the detection and characterization of cadherin and associated catenin systems are discussed, along with dominant-negative inhibitor approaches to characterizing this centrally important cell-cell adhesion system.

UNIT 9.4 describes current experimental approaches used to analyze integrin functions in mediating adhesive and other interactions of cells with specific substrates. This unit also provides detailed procedures for in vitro analyses of binding interactions between purified integrins and their ligands.

UNIT 9.5 provides a variety of protocols for studying the major group of calcium-independent cell-cell adhesion proteins termed the immunoglobulin superfamily of cell adhesion molecules (IgSF-CAMs). This superfamily contains over 100 adhesion molecules. After describing how to purify IgSF-CAMs from tissues or culture supernatants, this unit describes a number of approaches to characterizing their biological functions in vitro and in living cells. Assays with fluorescent beads provide ways to mimic and to analyze IgSF-CAM adhesive functions in isolation or in interactions with cells. Complementary approaches using various transfection, adhesive-substrate, inter-molecular interaction, and functional disruption analyses provide a powerful collection of tools to understand the roles and mechanisms of this major class of adhesion proteins.

UNIT 9.6 turns to dynamic analyses of cell adhesion. Cell adhesion often consists of sequential steps in dynamic processes that can be difficult to visualize and experimentally analyze in terms of mechanisms using simple cell attachment protocols. For example, leukocytes in the blood stream do not simply settle by gravity onto surfaces such as endothelial cells or exposed matrix in vessel walls, but they instead interact dynamically in the presence of flow and shear stress. In addition, some adhesive events that occur rapidly are difficult to study in static assays but can be analyzed effectively using dynamic flow methods. *UNIT 9.6* describes methods for such analyses using commercially available flow chambers in which cells being carried in suspension attach in the presence of laminar flow. Flow assays can measure cell adhesion either to a monolayer of endothelial cells or to purified extracellular matrix proteins attached to a substrate. The resulting data obtained in the form of video microscopy recordings can then be analyzed using approaches described in the second basic protocol.

Further supplements will provide protocols for analyzing other types of cell adhesion systems.

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Cell-Substrate Adhesion Assays

UNIT 9.1

This unit describes two standard assays for quantitating the adhesion of cells to an immobilized substrate. The first is an assay employing phase-contrast microscopy to measure the spreading of adherent cells (see Basic Protocol 1); the second is a colorimetric assay measuring attachment of cells (see Basic Protocol 2). The relative usefulness of spreading versus attachment assays is discussed later in this unit (see Background Information). Both assays employ microtiter plates and are performed in a humidified 5% to 10% (v/v) CO₂ atmosphere at 37°C. These protocols can be used to determine whether there is adhesion between a given cell type and a given adhesion molecule. Additionally, they can be used to test the regulatory effects of exogenous agents on cell-substrate adhesion.

When the adhesive substrate is a peptide, it should be conjugated to an inert protein to improve immobilization. An additional protocol is provided for preparation of such peptide-protein conjugates (see Support Protocol).

CAUTION: Glutaraldehyde and crystal violet are hazardous chemicals; follow appropriate precautions for handling, storage, and disposal.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be performed in a humidified 37°C, 5% to 10% (v/v) CO₂ incubator unless otherwise specified. CO₂ levels should be adjusted to maintain pH 7.4.

SPREADING ASSAY

In this protocol, cells are added to a microtiter plate coated with an adhesive molecule and are incubated to allow spreading. After the incubation period, all cells are fixed with glutaraldehyde, and the wells are examined for the percent spread cells using morphological criteria and phase-contrast microscopy.

Materials

- Adhesion molecule of interest
- Dulbecco's PBS (DPBS; Life Technologies; *APPENDIX 2A*)
- 10 mg/ml heat-denatured BSA solution (see recipe)
- Cells of interest
- Dulbecco's modified Eagle medium with 25 mM HEPES (DMEM/HEPES; Life Technologies; *APPENDIX 2B*), prewarmed to 37°C and gassed with 5% to 10% (v/v) CO₂
- 2× exogenous agent (e.g., antibody, peptides) in DPBS
- 5% (w/v) glutaraldehyde (dilute 50% stock solution with water)
- CMF-DPBS/NaN₃: divalent cation-free Dulbecco's PBS (CMF-DPBS; Life Technologies; *APPENDIX 2A*) containing 0.05% (w/v) sodium azide (NaN₃)
- 96-well tissue culture microtiter plates (Costar)
- Aspirator
- Multichannel pipettor
- 15-ml polypropylene tubes
- Glass coverslips
- Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*)

**BASIC
PROTOCOL 1**

Cell Adhesion

9.1.1

Immobilize substrate

1. Dilute adhesion molecule with DPBS.

For adhesion to extracellular matrices or purified extracellular matrix molecules (see Chapter 10), 1 to 20 $\mu\text{g/ml}$ is usually adequate. Purified matrix proteins and other molecules can be purchased from a variety of commercial suppliers, including Sigma, Life Technologies, Becton Dickinson Labware, and Calbiochem. If a nonmatrix molecule or a complex mixture is to be tested, a higher concentration should be used (see Critical Parameters and Troubleshooting).

The handling of adhesion molecules prior to dilution varies; some molecules (e.g., fibronectin) are best thawed quickly at 37°C, while others (e.g., laminin) are best thawed slowly on ice.

Occasionally, it may be necessary to measure adhesion of cells to peptides rather than proteins. In this case, peptides should be conjugated to inert protein carriers as this permits efficient immobilization (see Support Protocol for details).

2. Add 100 μl diluted adhesion molecule to the appropriate wells of a 96-well, tissue culture microtiter plate. Leave a blank well or wells for measuring background spreading on blocked plastic.

There is generally no need to carry out spreading assays with replicate wells, as quantification is performed by counting multiple fields from within the same well.

3. Incubate 60 min at room temperature or overnight at 4°C.

Time-course studies have shown that there is substantial coating of proteins onto plastic within an hour at room temperature, which allows the assay to be performed quickly. However, if the adhesion molecule binds weakly to plastic, or if it is more convenient to carry out the experiment the next day, the wells can be coated overnight, usually without detrimental effects.

Block nonspecific adhesion

4. Aspirate fluid, add 200 μl of 10 mg/ml heat-denatured BSA solution to each well with a multichannel pipettor, and incubate 30 min at room temperature.

Although simple BSA solutions, or other inert proteins, can be used for blocking plastic, heat-denatured BSA is the most effective agent in the author's hands. Nevertheless, its preparation is not straightforward and the procedure must be followed closely. To ensure blocking of the sides of the wells, 200 μl is used rather than 100 μl .

The time can be varied at this step; 30 min is probably the minimum time for efficient blocking of plastic, but longer times are not detrimental. Even overnight blocking at 4°C produces good results.

Prepare cells

5. During blocking, prepare a suspension of the cells to be examined (e.g., UNIT 1.1) in a 15-ml polypropylene tube.

Trypsin, EDTA, or trypsin/EDTA solutions are commonly used to detach adherent cells. The action of these reagents must be terminated—e.g., by resuspending the cells in DMEM with 10% (v/v) FBS—prior to using the cells in spreading assays. However, the use of these agents usually has no deleterious effect on adhesive activity provided that the cells are not overtrypsinized. It is important to guard against clumping or aggregation of cells; therefore, gentle conditions should be used when centrifuging and resuspending cells. All solutions used during the preparation of cell suspensions should be warmed to 37°C.

Freshly isolated cells from tissue can also be used, but results are much more variable.

- Count cells using a hemacytometer (*UNIT 1.1*), and resuspend the cells at 2×10^5 /ml in warm DMEM/HEPES gassed with 5% to 10% (v/v) CO₂. Incubate 10 min at 37°C with the lid off in a CO₂ incubator.

For most cells, this density prevents cells added to the microtiter plate from interfering with each other during the spreading process. If cells contact each other, substrate-dependent adhesive effects can be altered; on the other hand, if cells are too sparse, quantification becomes difficult.

Cells are left in a polypropylene tube with the lid off to allow them to recover from the process of detachment. Alternatively, the tube can be capped and left on its side in an incubator to prevent the cells from settling and aggregating into a large clump at the bottom of the tube. However, they should not be left too long (to prevent nonspecific adhesion to the sides of the tube), and they should be pipetted gently prior to use (to ensure dispersion).

Gassing the cell suspension with CO₂ can sometimes give enhanced spreading, although this is not always needed.

Molecules that mediate spreading are not always resistant to trypsin treatment. However, this 10-minute recovery period is generally sufficient for reexpression.

Perform adhesion assay

- Aspirate the BSA solution and wash the wells with 100 µl DPBS.

The heat-denatured BSA is toxic for some cells.

- To test spreading of specific cell types on given adhesion molecules, add 50 µl DPBS followed by 50 µl cells to the appropriate wells. To examine the effects of exogenous agents (e.g., antibodies, peptides) on spreading, add 50 µl of 2× exogenous agent followed by 50 µl cells. In this case, also add 50 µl DPBS followed by 50 µl cells to control wells.

Spreading can sometimes be increased by incubating the plate containing exogenous agents several minutes at 37°C, allowing them to warm up prior to addition of cells.

To ensure good spacing of cells, guard against swirling, tapping, or shaking the wells once cells have been added. In the author's experience, a single pipetting of cells down the side of the well and into the DPBS solution produces good dispersion.

A number of adhesion studies have focused on divalent cation-dependence, particularly when integrins are involved. Calcium, magnesium, and manganese ions are the most frequently studied. It is worth noting that manganese ions will precipitate phosphate from PBS. Therefore, in the presence of manganese, HEPES-buffered saline should be used in place of DPBS.

- Incubate 60 to 90 min at 37°C in a 5% to 10% (v/v) CO₂ incubator with the microtiter plate lid off.

*It is important that the DMEM/DPBS mixture has the opportunity to equilibrate as rapidly as possible with gaseous CO₂ in order to reestablish the buffer (see *Critical Parameters and Troubleshooting*).*

Ensure that the shelves holding the microtiter plates are level, as uneven shelves lead to uneven settling of cells.

Fix and analyze cells

- Aspirate medium and fix cells by directly adding 100 µl of 5% (w/v) glutaraldehyde down the side of the well and incubating 30 min at room temperature.
- Aspirate fixative and store cells in 200 µl CMF-DPBS/NaN₃.

Fixed assay plates can be stored at 4°C for several weeks protected from evaporation.

12. Add sufficient CMF-DPBS/ NaN_3 to form an inverted meniscus at the top of the well and carefully place an appropriately sized glass coverslip over the plate.

Understandably, the optical quality of the plastic that is used to make microtiter plates is not ideal for phase-contrast microscopy. However, adding a coverslip to an overly full well can greatly improve the observation of adherent cells in microtiter plates.

13. Using an inverted phase-contrast microscope, quantify percent spreading in each well by counting three separate, randomly selected fields of 100 cells each. Use an eyepiece graticle to aid the selection of cells and to minimize double counting. Use specific morphological criteria, such as a phase-dark cell body and cytoplasm that is visible around the entire circumference of the nucleus, to identify cells with spread morphology.

Alternatively, image analysis software can be used to measure average cell area (see Critical Parameters and Troubleshooting).

ATTACHMENT ASSAY

This protocol measures the attachment of cells to microtiter plates coated with an adhesion molecule. After incubation, nonattached cells are removed and the remaining attached cells are fixed with glutaraldehyde, washed, and stained with crystal violet. The plates are read by a microtiter plate reader, and the percent attached cells is calculated.

Materials

- Adhesion molecule of interest
- Dulbecco's PBS (DPBS; Life Technologies; *APPENDIX 2A*)
- 10 mg/ml heat-denatured BSA solution (see recipe)
- Cells of interest
- Dulbecco's modified Eagle medium with 25 mM HEPES (DMEM/HEPES; Life Technologies; *APPENDIX 2B*), prewarmed to 37°C and gassed with 5% to 10% (v/v) CO_2
- 2× exogenous agent (e.g., antibody, peptides) in DPBS
- 5% (w/v) glutaraldehyde (dilute 50% stock solution with water)
- 0.1% (w/v) crystal violet solution (see recipe)
- 10% (v/v) acetic acid
- 96-well tissue culture microtiter plates (Costar)
- Aspirator
- 15-ml polypropylene tubes
- Microtiter plate reader
- Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*)

Immobilize substrate

1. Dilute adhesion molecule with DPBS.

For adhesion to extracellular matrices or purified extracellular matrix molecules (see Chapter 10), 1 to 20 $\mu\text{g}/\text{ml}$ is usually adequate. Purified matrix proteins and other molecules can be purchased from a variety of commercial suppliers, including Sigma, Life Technologies, Becton Dickinson Labware, and Calbiochem. If a nonmatrix molecule or a complex mixture is to be tested, a higher concentration should be used (see Critical Parameters and Troubleshooting).

The handling of adhesion molecules prior to dilution varies; some molecules (e.g., fibronectin) are best thawed quickly at 37°C, while others (e.g., laminin) are best thawed slowly on ice.

Occasionally, it may be necessary to measure adhesion of cells to peptides rather than proteins. In this case, peptides should be conjugated to inert protein carriers as this permits efficient immobilization (see Support Protocol for details).

2. Add 100 μ l diluted adhesion molecule to the appropriate wells of a 96-well, tissue culture microtiter plate using a P200 pipettor. Prepare sufficient wells to perform assays in triplicate or quadruplicate. Leave sufficient blank wells to determine 100% attachment at three cell concentrations and to determine background binding of crystal violet to plastic.

A standard pipettor should be used rather than a multichannel pipettor, as attachment assays rely much more heavily on the accuracy of pipetting than do spreading assays.

The blank wells for 100% attachment are not blocked with BSA. The background wells are treated as shams, and therefore are blocked with BSA.

3. Incubate 60 min at room temperature or overnight at 4°C.

Time-course studies have shown that there is substantial coating of proteins onto plastic within an hour at room temperature, which allows the assay to be performed quickly. However, if the adhesion molecule binds weakly to plastic, or if it is more convenient to carry out the experiment the next day, the wells can be coated overnight, usually without detrimental effects.

Block nonspecific adhesion

4. Aspirate fluid, add 200 μ l of 10 mg/ml heat-denatured BSA solution to each sample well, and incubate 30 min at room temperature. Block wells for background crystal violet binding, but not those for 100% attachment.

Although simple BSA solutions, or other inert proteins, can be used for blocking plastic, heat-denatured BSA is the most effective agent in the author's hands. Nevertheless, its preparation is not straightforward and the procedure must be followed closely. To ensure blocking of the sides of the wells, 200 μ l is used rather than 100 μ l.

The time can be varied at this step; 30 min is probably the minimum time for efficient blocking of plastic, but longer times are not detrimental. Even overnight blocking at 4°C produces good results.

Prepare cells

5. During blocking, prepare a suspension of the cells to be examined in a 15-ml polypropylene tube. Prepare a sufficient number of cells to perform assays in triplicate or quadruplicate.

Trypsin, EDTA, or trypsin/EDTA solutions are commonly used to detach adherent cells. The action of these reagents must be terminated—e.g., by resuspending the cells in DMEM with 10% (v/v) FBS—prior to using the cells in spreading assays. However, the use of these agents usually has no deleterious effect on adhesive activity provided that the cells are not overtrypsinized. It is important to guard against clumping or aggregation of cells; therefore, gentle conditions should be used when centrifuging and resuspending cells. All solutions used during the preparation of cell suspensions should be warmed to 37°C.

Freshly isolated cells from tissue can also be used, but results are much more variable.

6. Count cell density on a hemacytometer (UNIT 1.1), and prepare working cell suspensions at 5×10^5 /ml for fibroblasts and similarly sized cells, and at 1×10^7 /ml for leukocytic cells in warm DMEM/HEPES gassed with 5% to 10% (v/v) CO₂. Incubate 10 min at 37°C with the lid off in a CO₂ incubator.

It is advisable to use pipet tips that have their ends cut off for attachment assays. This is to prevent coated proteins and/or cells from being washed off directly by a fine stream of liquid.

These densities prevent cells from interfering with each other during the attachment process. If cells contact each other, substrate-dependent adhesive effects can be altered; on the other hand, if cells are too sparse, quantification becomes difficult.

Cells are left in a polypropylene tube with the lid off in a CO₂ incubator to allow them to recover from the process of detachment. Alternatively, the tube can be capped and left on its side in an incubator to prevent the cells from settling and aggregating into a large clump at the bottom of the tube. However, they should not be left too long (to prevent nonspecific adhesion to the sides of the tube), and they should be pipetted gently prior to use (to ensure dispersion).

It is more important to gas the medium for an attachment assay than it is for the spreading assay, particularly when the level of adhesion is predicted to be low. Gassing the cell suspension with CO₂ can sometimes give enhanced attachment, although this is not always needed.

Perform adhesion assay

7. Aspirate the BSA solution and wash the wells with 100 μ l DPBS.

The heat-denatured BSA is toxic for some cells.

8. To estimate 100% attachment, dilute cells to 20%, 50%, and 100% of the working cell suspension using warm DMEM/HEPES gassed with 5% to 10% (v/v) CO₂. Add 50 μ l DPBS followed by 50 μ l cells to uncoated wells.

Cells can also be plated on poly-L-lysine-coated plastic (APPENDIX 2A) to determine 100% attachment.

Using cell dilutions and extrapolating the resulting graph is the most accurate way of determining 100% attachment, as the absorbance value for 100% attachment with undiluted cells may be off the linear range of the plate reader.

To ensure good spacing of cells, guard against swirling, tapping, or shaking the wells once cells have been added. In the author's experience, a single pipetting of cells down the side of the well and into the DPBS solution produces good dispersion.

9. To test attachment of specific cell types to given adhesion molecules, add 50 μ l DPBS followed by 50 μ l cells to the appropriate wells. To examine the effects of exogenous agents (e.g., antibodies, peptides) on attachment, add 50 μ l of 2 \times exogenous agent followed by 50 μ l cells. In this case, also add 50 μ l DPBS followed by 50 μ l cells to control wells.

Attachment can sometimes be increased by incubating the plate containing exogenous agents several minutes at 37°C, allowing them to warm up prior to addition of cells.

A number of adhesion studies have focused on divalent cation-dependence, particularly when integrins are involved. Calcium, magnesium, and manganese ions are the most frequently studied. It is worth noting that manganese ions will precipitate phosphate from PBS. Therefore, in the presence of manganese, HEPES-buffered saline should be used in place of DPBS.

10. Incubate 15 to 20 min at 37°C in a 5% to 10% (v/v) CO₂ incubator with the microtiter plate lid off.

The incubation time for attachment assays may need to be adjusted depending on the cell type, as some cells adhere more quickly than others.

It is important that the DMEM/DPBS mixture has the opportunity to equilibrate as rapidly as possible with gaseous CO₂ in order to reestablish the buffer (see Critical Parameters and Troubleshooting).

Ensure that the shelves holding the microtiter plates are level, as uneven shelves lead to uneven settling of cells.

Remove loose cells and fix adherent cells

11. Fix cells only in the wells to be used for determining 100% attachment by adding 100 μ l of 5% (w/v) glutaraldehyde.
12. Remove nonadherent and loosely attached cells from remaining wells by either tapping the plate or gently washing the wells 1 to 3 times with 100 μ l DPBS and cut-off pipet tips.

This is the most critical stage in an attachment assay, and needs to be optimized for each cell type used (see Critical Parameters and Troubleshooting).

13. Aspirate the final wash and fix attached cells by adding 100 μ l of 5% (w/v) glutaraldehyde and incubating 20 min at room temperature (or at 4°C overnight, if necessary).
14. Wash wells three times with 100 μ l water.

Stain and analyze adherent cells

15. Add 100 μ l of 0.1% (w/v) crystal violet solution to each well and incubate 60 min at room temperature.

Avoid getting crystal violet solution on the rims of the wells, as this dries during incubation and can be difficult to remove by washing.

Staining can also be performed overnight without detriment to the final results.

16. Wash wells three times with 400 μ l water.

A larger wash volume is used to remove all traces of stain.

17. Solubilize dye in 100 μ l of 10% (v/v) acetic acid and incubate 5 min on an orbital shaker at 150 rpm, room temperature.
18. Measure absorbance at 570 nm using a microtiter plate reader. Subtract background crystal violet staining from all experimental and 100% attachment results. Plot data from 20%, 50%, and 100% inocula (A_{570} versus cell density) and determine the value for 100% attachment by extrapolation. Use this value to express experimental data as percent attachment.

PREPARATION OF PEPTIDE-PROTEIN CONJUGATES

The discovery that many large adhesion proteins contain short peptide sequences that are recognized by cellular receptors has enabled these receptor sites to be probed with the use of synthetic peptide reagents. By definition, these peptides are small and usually bind poorly to plastic. In addition, immobilization of peptides may reduce their cellular adhesivity by masking key residues that endow adhesive activity. For these reasons, it is necessary to use an alternative method to simple coating from a peptide solution. The author prefers to prepare covalent conjugates of peptides to protein carriers that are inert in terms of adhesive activity. Many different cross-linkers and carriers are available for such syntheses, but consistent success can be obtained with *N*-succinimidyl 3-(2-pyridyl-dithio)propionate (SPDP), a heterobifunctional cysteine-lysine cross-linker, and rabbit IgG as carrier. *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) also works well as a cross-linker, and BSA and keyhole limpet hemocyanin are good carriers. A detailed description of the use of these agents can be found in Humphries et al. (1994). The first step in the conjugation reaction is the conjugation of the cross-linker to the carrier. The second step is the conjugation of the cross-linker/carrier to the peptide, which requires disulfide bond formation between the cross-linker and peptide, and is slow. Thus, peptides are usually designed with a terminal cysteine residue.

SUPPORT PROTOCOL

Cell Adhesion

9.1.7

Materials

6 mg/ml normal rabbit IgG (Sigma) in CMF-DPBS
3 mg/ml *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Pierce) in ethanol
Divalent cation-free Dulbecco's PBS (CMF-DPBS; Life Technologies;
APPENDIX 2A)
Cysteine-containing peptide, solid
Dulbecco's PBS (DPBS; Life Technologies; *APPENDIX 2A*)
10-ml Sephadex G25 columns (PD-10, Amersham Pharmacia Biotech)
Additional reagents and equipment for dialysis (*APPENDIX 3*)

1. Pipet 1 ml of 6 mg/ml normal rabbit IgG into a microcentrifuge tube. Add 0.2 ml of 3 mg/ml SPDP by rapid injection and mix thoroughly. Incubate 30 min at room temperature on a rotator.

SPDP is not very soluble in aqueous solution. It should be added to and mixed with IgG rapidly to avoid precipitation.

2. Equilibrate a 10-ml Sephadex G25 column with 10 to 20 vol CMF-DPBS.
3. Add the rabbit IgG/SPDP mixture to the column followed by CMF-DPBS. Discard the first 2.5 ml eluate and collect the next 3.5 ml (containing cross-linker-derivitized IgG) in a single tube.
4. Aliquot the collected fraction as necessary (depending on the number of conjugates to be made) and add to 1 to 2 mg solid cysteine-containing peptide. Vortex briefly and incubate overnight at room temperature on a rotator.

Radiolabeled peptide can be used to quantify coupling if necessary, although, in the author's experience, coupling is quite consistent from peptide to peptide.

5. Dialyze the mixture (*APPENDIX 3*) against several changes of DPBS (with divalent cations) to remove uncoupled peptide. Dialyze using standard dialysis tubing in ≥ 1000 -fold DPBS at 4°C with gentle stirring. Dialyze >3 hr for each change of DPBS.
6. Divide into 100- μ l aliquots and store indefinitely at -80°C.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

Crystal violet solution, 0.1%

Dissolve 0.1% (w/v) crystal violet in 200 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6. Filter through a 0.22- μ m filter. Store at room temperature.

CAUTION: *Crystal violet is hazardous; handle with caution and dispose of properly.*

It is imperative to filter this crystal violet solution. It can be difficult to determine whether it has dissolved properly due its intense color. If the solution is not filtered, specks of solid crystal violet can sometimes be added to the experimental wells, resulting in spuriously high absorbance readings.

Heat-denatured BSA solution, 10 mg/ml

Dissolve BSA at 10 mg/ml in divalent cation-free Dulbecco's PBS (CMF-DPBS; Life Technologies; *APPENDIX 2A*) in a 15-ml polystyrene tube. Filter through a 0.22- μ m filter to remove undissolved protein, and incubate 10 to 12 min in an 85°C water bath. Cool to room temperature and use the same day.

The major aim is to prepare a solution that contains microparticulate BSA, as this will coat plastic very efficiently. The solution should go slightly hazy. A clear solution indicates insufficiently aggregated BSA, while a white solution indicates that aggregates are too large. After cooling, the solution is ready for use.

COMMENTARY

Background Information

As the name suggests, adhesion assays measure cell adhesion. However, because adhesion is a complex process involving receptor-ligand binding, modulation of intracellular signaling, and cytoskeletal modulation, these assays can also be a source of information about other cellular events and a means of probing the contribution of these processes to adhesion. In general, adhesion assays are used to test whether a certain cell type can adhere to a certain adhesive substrate, and to test the sensitivity of a particular cell-substrate interaction to inhibitors. This unit describes two standard assays for quantifying the adhesion of cells to an immobilized substrate. The spreading assay is based on an original publication by Yamada and Kennedy (1984), and the attachment assay is based on Kueng et al. (1989). As presented here, the attachment assay employs colorimetric detection of bound cells. However, this is actually one of many different readouts that can be applied to the basic assay. Common alternatives include the use of radiolabeled or fluorescently labeled cells (Dustin and Springer, 1989; Chan et al., 1992), or the measurement of endogenous gene activity (e.g., phosphatase; Prater et al., 1991). In general these are more time-consuming to perform.

A number of considerations will affect the decision whether to use a cell spreading assay or a cell attachment assay. Spreading assays take longer to perform, but are less prone to nonspecificity. For example, many molecules can mediate attachment of cells that is not physiologically relevant, but very few of these molecules can mediate morphological spreading. Importantly, by observing cells in a spreading assay, a lot of information can be gained about the biological response of the cells to the substrate. The morphology of cells can differ even if the percent spreading is the same. Additionally, now that signaling mechanisms that control cell morphology are better understood, spreading assays can give indirect indications of the intracellular events that are triggered by certain substrates. Spreading assays are more sensitive when used to measure the inhibitory activity of an exogenous agent, because the readout from the assay is more reliant on multiple adhesive interactions, and partial disruption by an inhibitor is sufficient to see a biological effect. A greater degree of receptor blockade is probably needed to observe an inhibitory effect in an attachment assay. Finally,

as spreading assays do not need replicate wells, it is easier to construct detailed dose-response curves.

Nonetheless, use of attachment assays is sometimes obligatory, because not all cells are able to spread and because some cells only spread on certain substrates. It is misleading to think that attachment assays measure single adhesive contacts: multiple contacts are needed for a cell to withstand the washing steps in an attachment assay. Nevertheless, fewer contacts are needed for attachment than for a cell to spread.

Critical Parameters and Troubleshooting

General considerations for adhesion

The major problem likely to be encountered in both assays is that cells fail to adhere. Many factors can lead to this, including coating plates with insufficient amounts of adhesive substrate, using bad batches of adhesive substrate, using poor protein-binding microtiter plates or badly constructed plates with uneven wells, squirting liquids too vigorously onto the bottom of the wells, using cultures that are growing poorly or have a mycoplasma infection, having variations in pH during adhesion, and (for attachment assays) overwashing. Several of these factors are discussed in further detail below.

Substrate concentration. The concentration of adhesion molecule required for coating depends on a number of factors, including the size of the molecule, the efficiency with which it coats plastic, and the apparent affinity with which it is bound by cellular receptors. In most cases, spreading assays are used to measure the adhesion of cells to extracellular matrices or purified extracellular matrix molecules (also see Chapter 10). The key components of such matrices are usually large macromolecules that coat plastic relatively well; they are also bound with at least moderate affinity by cells. For these reasons, a concentration range between 1 and 20 $\mu\text{g/ml}$ is usually adequate, although it is advisable to carry out a range-finding dose-response experiment before focusing on a narrow range. If a nonmatrix molecule or a complex mixture is to be tested, a higher concentration should be used.

Plates. Most tissue culture microtiter plates are adequate for spreading and attachment experiments, although the author finds Costar to be excellent. Immulon 4HB plates from Dynex

Technologies (formerly Dynatech Labs) have higher protein-binding capacity and are particularly good for assays involving small proteins.

Cultures. For cell spreading assays, an important parameter is the health of the cells. Cultures should be actively growing, but should have been passaged >24 hr previously. Relatively poor spreading responses have been observed in cells that were passaged the day before a spreading assay.

pH. When cells and DPBS (with or without exogenous agents) are combined in the coated well, it is important that the DMEM/DPBS mixture has the opportunity to equilibrate as rapidly as possible with gaseous CO₂ in order to reestablish the buffer. This process can be aided by leaving the lid off the microtiter plate in the incubator. It is also the author's experience that the adhesion of some cell types is improved by raising the concentration of gaseous CO₂ from 5% to 10% (v/v). This can be particularly effective at increasing binding to poorly adhesive substrates. It may also be advisable to use a particular incubator and/or time of day when the door to the incubator will not be opened, as this helps prevent alkalinization of the medium.

Washing. For attachment assays, the key parameter is the washing protocol, as this is the major determinant of the signal-to-noise ratio. When washing unattached cells (see Basic Protocol 2, step 12), different cells respond differently to tapping and washing, and it is recommended to vary the number of tapping or washing cycles to obtain the best signal-to-noise ratio (i.e., attachment to an adhesive substrate compared to attachment to BSA-blocked plastic). Sometimes this can even be judged by eye in a pilot experiment. Cut-off pipet tips are particularly important at this stage to avoid removal of attached cells.

More specific problems include cell death in the assay (which can be caused by exposure of sensitive cells to heat-denatured BSA) and clumping of cells either in the center of a well or around the perimeter (which is caused by swirling the plate). In addition, large errors in attachment assays can result from inaccurate pipetting, which can come from use of multichannel pipettors, or suboptimal washing of wells (note that the volume of BSA blocking solution is higher than the volume of adhesive substrate, and that the wash step after crystal violet staining is larger still).

Assessing cell spreading

Different methods can be used to determine whether a cell is spread or not. Perhaps the most quantitatively accurate method is to use image analysis software to measure average cell area; however, this tends to produce small differences that may be hard to interpret without the application of other criteria relating to cell shape. Instead, the author prefers to assign specific criteria to a definition of spreading and apply these to each cell individually. The usual criteria are that the cell body should be phase-dark and that cytoplasm should be visible around the entire circumference of the nucleus. Different cells adopt different morphologies and therefore these criteria might need to be slightly modified on a case-by-case basis.

Anticipated Results

Both the spreading and attachment assays measure percent adhesion, and the level of adhesion obtained depends upon the cell type and adhesive substrate under study. In spreading assays, a level of 80% should be anticipated, and often higher levels can be obtained. It is critical that the background level of spreading on BSA-coated plastic be as low as possible. Frequently, this is actually zero, but certainly this should not rise above 2% to 3%. The level of attachment observed by the crystal violet staining method is usually not as high as for spreading, but 60% to 70% should be attainable. The background level of attachment (i.e., on BSA-coated plastic) should also be as low as possible; however, for attachment assays, this level is usually higher (5% to 10%).

Time Considerations

Both spreading and attachment are relatively quick to perform. Coating and blocking the wells requires <2 hr. The actual assays can be carried out easily in half a day, and the subsequent quantification of spreading assays can take a similar length of time. Quantification of attachment assays is much quicker (i.e., <2 hr).

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Quantitative Measurement of Cell Adhesion Using Centrifugal Force

The following protocol was developed in order to study the biophysical sequence of events in cell-substrate adhesion, and it can also be adapted to study cell-cell adhesion. The method allows quantification of the weak association between cells and their substratum at 4°C, thereby giving a measure of the receptor-ligand affinity only. The approach also allows measurement of the ATP-dependent events that strengthen adhesion and that involve the cytoskeleton following initial binding. This is done by incubating cells in contact with substrate at 37°C for increasing periods of time. Centrifugal force is the only shear force involved in the assay; thus the strength of any adhesion that resists this applied force can be accurately measured. The original centrifugal adhesion assay developed by McClay et al. (1981) used radioactively labeled cells in a rather cumbersome procedure to quantify adhesion. The protocol given here is much easier than any previously published version. At the same time, the assay remains highly quantitative and has simple innovations that can accommodate many kinds of adhesion studies.

The procedure is as follows: 96-well polyvinyl chloride (PVC) plates with flat bottoms are coated with the substrate target for the cells. The wells are then treated with BSA or another nonadherent protein that blocks nonspecific binding sites. Cells are added to the PVC plates on ice, the wells are filled to the brim, and then sealed with clear packing tape. The idea is to enclose each well as a sealed, fluid-filled compartment without any air bubbles. The cells are then gently centrifuged into contact with the substrate. To measure cell-substrate affinity alone, the wells are kept at 4°C, flipped upside down, and centrifuged to provide a defined dislodgement force for the cells (Fig. 9.2.1). Using replicates challenged with different centrifugal forces one can determine the relative avidity of cells for a substrate. If cells are incubated at 37°C after being centrifuged onto the substrate, one can follow the progress of adhesive strengthening due to cytoskeletal engagement. This process generally occurs rapidly, and progress can be monitored by this assay.

There are many ways to quantify cells adhering to the substrate. Currently, the simplest is to image the well bottom and count cells bound relative to controls and to the total

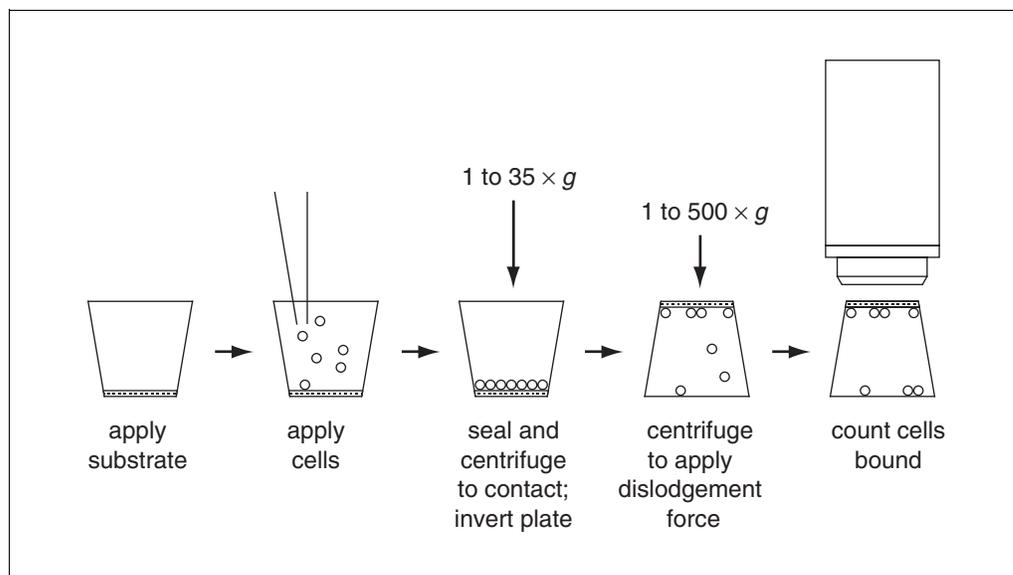


Figure 9.2.1 Centrifugal force adhesion assay with light microscopic readout.

number of cells originally added. Other approaches can easily be adapted; however, this method provides a simple visual measure of adhesion that is quite easy to learn, is easily adapted by any laboratory at low cost, and allows for a number of variations to be applied, including morphometrics (see Background Information).

CENTRIFUGATION CELL ADHESION ASSAY

Materials

- 96-well flat-bottom polyvinyl chloride (PVC) plates (Falcon)
- Substrate molecule of interest in PBS
- 40 mg/ml BSA (fraction V; Sigma) in PBS
- PBS (*APPENDIX 2A*)
- Tissue culture medium without FBS
- Dissociated cells of interest, suspended at $5\text{--}10 \times 10^5/\text{ml}$ in calcium-free physiological solution (see Critical Parameters, discussion of suitable media)
- Clear packing tape (3M Scotch 375, 4.8 cm wide, although any clear packing tape will probably work)
- Microtiter plate support template
- Low-speed refrigerated cell centrifuge with microtiter plate carrier

1. Cut away the sides of a 96-well flat-bottom PVC plate with scissors, and then cut the plate into a 3×8 -well rectangle.

Only the six wells in the middle are used. Since the assay involves filling the wells over the brim to eliminate air bubbles, some of the medium will spill into the surrounding empty wells. Plates can be cut into other configurations depending upon the centrifuge carrier, the microscope, or other variables that are particular to the lab.

2. Add 50 μl substrate per well, leaving blank wells for 100% attachment (no substrate or blocking) and for background binding (no substrate). Use at least three replicates for each parameter tested. Incubate ~ 30 min at room temperature.

Polyvinyl chloride has a natural avidity for many proteins.

As a control, determine how much of the substrate protein is attached to the plate. In the first set of experiments with a substrate protein or peptide, determine the threshold concentration for binding (relative to the BSA control wells and at a low g force), and then operate just above that threshold in subsequent experiments (see Critical Parameters).

3. Wash three times with 100 μl PBS. Then block substrate-coated and background binding wells (but not 100% attachment control wells) ~ 30 min with 50 μl of 40 mg/ml BSA at room temperature. Wash three times with 100 μl PBS, flicking the contents of the wells into the sink to eliminate the fluid after each wash.

It is important that the background control well does not bind cells. BSA treatment should block all spaces not covered by the substrate molecule. Typically 100% of cells bind to the untreated wells, and $<1\%$ bind to the background wells. Although this varies according to the cell type used and the dislodgement force applied, in most cases the conditions can be adjusted so that $<1\%$ of cells bind to the BSA-blocked wells. This leaves the test substrate with a wide range in which to examine adhesion avidity.

If desired, poly-L-lysine can be added to the 100% attachment control well (no substrate, no blocking) to bind cells even more tightly.

4. Place the plate on ice. Add 100 μl tissue culture medium to all wells, add 100 μl cell suspension ($5\text{--}10 \times 10^4$ cells), and then fill all wells with another 100 μl tissue culture medium.

Fewer cells can be added, especially if fluorescent cells are used for later quantification. For fluorescent cells, good results can be obtained with as few as 1000 cells per well. Also,

it is possible to employ two different fluorescent colors and unstained cells in the same well, so that two experimental cell types can be compared to a control cell population under exactly the same conditions.

Medium and cells should be added on ice to prevent cells from attaching and adhering prematurely. There should be a positive meniscus bulging above the top of the well, as excess fluid is necessary to seal the well without any air bubbles.

5. Gently lay a piece of clear packing tape over the wells, and place the plate on a microtiter plate support. Apply the tape, starting from one end and squeegeeing the excess medium to spill over into the empty wells next to the test wells. Press down on the tape to make sure it adheres to the PVC surrounding each well.

The microtiter plate support is a rubber or metal template that holds the PVC plate while it is being sealed. A firm backing is necessary to support the pressure applied when sealing the tape. A metal plate can be made by a machine shop. Its thermal conductance keeps the wells cold during the manipulation and sealing procedures. If metal supports are unavailable, a 96-well plate can be used as a mold to make a plastic support device.

With a little practice, this maneuver can be done without introducing any bubbles into the sealed wells. If sealing is done correctly, each well should now be a completely filled, enclosed chamber, and should survive centrifugal forces up to $\sim 500 \times g$ without leaking. Some cells will be lost in the process of sealing, but that will not add an error factor to the analysis because of the way cells are counted against controls in the assay.

6. Centrifuge cells into contact with the substrate for ~ 3 min at $35 \times g$, 4°C , in a low-speed refrigerated centrifuge with a microtiter plate carrier.

Some larger cells can be allowed to settle onto the substrate at $1 \times g$. If cells are centrifuged onto the substrate, ~ 3 min of centrifugation will place 100% of the cells into contact with the substrate.

If this is done at 4°C , receptor-ligand combinations are brought into contact. As long as the plate is left at 4°C , this receptor-ligand affinity can be measured independent of cytoskeletal contributions.

One can substitute a big centrifuge bucket as a swinging bucket carrier if a microtiter plate carrier is not available.

7. *Optional:* To measure strengthening events in adhesion, simply move the plate, with cells in contact with the substrate, to a 37°C water bath and float the plate for the desired period of time.

For many cell types, incubations < 5 min are sufficient to convert the weak receptor-ligand binding into a highly strengthened adhesion that is strongly resistant to centrifugal shear forces.

8. Invert the plate and centrifuge the cells in the inverted position using a microtiter plate carrier, 5 min at the desired speed. Return the plate to ice, keeping it in the inverted position.

This should dislodge cells if enough centrifugation force is applied and if the cells are not strongly adhering. Obviously there are a number of parameters that can be controlled in this step: e.g., duration of contact at 37°C , rate of dislodgement, force of centrifugation, and rate of strengthening.

9. Place the wells on the stage of a compound microscope, and count cells using a $10\times$ objective. Subtract background binding from substrate binding, and determine the percent binding compared to the 100% attachment control.

Imaging software can be used to capture frames and automatically count the cells in each frame. To increase accuracy, set up the microscope so it will image a well prior to centrifugation, then return to the same field after centrifugation to assess adhesion.

COMMENTARY

Background Information

Early cell adhesion assays used cell aggregation as a semiquantitative measure. These assays were useful for studying some adhesive phenomena, but suffered from an inability to quantify the sequence of adhesion. Attempts to quantify adhesion have used viscometers, cell particle counters, spectrophotometers, radioactivity, and other approaches. In most cases, adhesion assays were limited in that they required cells to associate rather tightly with one another in order to survive unknown, or poorly defined, shear forces intrinsic to the assay.

This centrifuge assay was designed to use a single shear force of defined magnitude. The force operating on a cell can be easily calculated, providing an impression of the strength of an adhesion. This approach, though quantitative, is still not perfect. For example, one would really like to measure the “on rate” as an adhesion is formed, but this is an exceedingly difficult parameter to measure. Also, the assay described here measures the proportion of adherent cells within a population, rather than the behavior of individual cells. Single cell assays may offer advantages not offered by the assay given here.

The present assay is based on previously published versions (McClay et al., 1981; Lotz et al., 1989; Burdsal et al., 1991, 1994) in which centrifugal force is used as a dislodgement force (F_D) for the cells. This assay is highly modified and simplified compared to the original assays, but enjoys a better and more versatile capacity for quantification.

The following relationship is important for understanding where force fits into the adhesion process. As measured in the centrifugal adhesion assay, $F_D = (\rho_{\text{cell}} - \rho_{\text{medium}}) \times V_{\text{cell}} \times \text{RCF}$, where F_D is the dislodgement force (dynes/cell) tending to pull the cell from the substrate, $(\rho_{\text{cell}} - \rho_{\text{medium}})$ is the specific density difference between the cell and the medium (usually 1.07 g/cm³), V_{cell} is the volume of the cell, and RCF is the relative centrifugal force (McClay et al., 1981). Because of the small difference in specific density between the fluid and the cell, cells in culture are little affected by alterations at $1 \times g$, since in reality these cells are already experiencing microgravity.

The assay as presented above allows for very precise comparisons between cells that are only slightly different from one another. For example, if one cell population is transfected with a construct and the other population is a non-

transfected control, the two can be tested in the same well (with one of the populations fluorescently tagged) to accurately compare adhesive performance.

Most other cell-substrate assays have a certain unknown shear force that can confound quantification, especially when one wants simply to measure receptor-ligand interactions. The simple “stick-and-wash” assay, in which one allows cells to bind to the substrate for a period of time and then washes the wells, may be simpler to use than the assay presented above, but suffers from several problems. It has an undefined shear force (washing) that is difficult to control accurately. Stick-and-wash assays also cannot measure the initial receptor-ligand interactions, because cells must adhere at least somewhat tightly to the substrate in order to survive washing. Thus, the adhesion measured in a stick-and-wash assay is actually both adhesion and engagement of the cytoskeleton. Often such assays are completed hours after cells were added to wells, so that any number of postadhesion events could occur before measuring “adhesion.” In contrast, the present assay allows measurement of the progression of several adhesion events separately, and therefore has the capacity to examine several parameters in the sequence of forming an adhesion. It should be mentioned that for more than two decades the simpler “stick-and-wash” assay was successfully employed by a number of laboratories to find and characterize most of the known adhesion molecules. The present assay is useful for detailed structure-function analyses of those molecules.

An additional parameter that can be measured with the centrifugal adhesion assay is the relationship between adhesiveness and cell phenotype. Since the cells are counted by microscopy, one can also score cell phenotypic properties (e.g., spreading or motility). This adds to the versatility of the assay. The assay can be used, with minimal adaptation, for a wide variety of cell types, including cells from several phyla, tumor cells, and cells of all stages of embryonic development.

Initial binding

In practice there are many events in the establishment of an initial adhesion. There is an on rate by which the first receptor-ligand interactions occur. Then there is a rate of recruitment of additional cellular adhesion molecules to the site of adhesion. This parameter has

been measured, and is related to the rate of diffusion of the protein in the phospholipid bilayer. As long as no additional molecules are added to the bilayer, the total recruitment of receptor-ligand combinations is limited to the total population in the membrane at the time of the assay. Finally, since the interaction is not covalent, there is an off rate. Most cell types bind to substrates with a force that resists 10^{-6} to 10^{-5} dynes/cell of applied centrifugal dislodgement force. In reality, cells come off the substrate when the off rate (increased by centrifugal force) exceeds the on rate. Red blood cells fail to adhere to most substrates at around 10^{-8} dynes/cell. Some cells, such as macrophages, naturally adhere with a receptor-ligand avidity that is slightly higher than the 10^{-5} dynes/cell level. When measuring initial binding one must measure the adhesion at very low substrate concentrations. Once the well becomes supersaturated with multiple layers of substrate, the initial binding loses specificity for the receptor-ligand combination under investigation. This presumably results from non-specific effects of charge and substrate heterogeneity.

Cytoskeletal contribution to strengthening

After obtaining a measurement of initial binding (receptor-ligand affinity), a measurement can be made of the strengthening of that adhesive process. This cellular process is remarkably fast. Using this assay, most strengthening is completed within 5 min at 37°C. The degree of strengthening is usually more than 2 orders of magnitude, and may be as much as 5 to 6 orders of magnitude.

The limitations of this assay come into play during the strengthening process. Although we recommend a range of 1 to $500 \times g$ for the assay, the centrifuge plate carriers have a maximum safe speed that cannot be exceeded. Using large buckets as plate carriers can increase the centrifugation speed to ~ 4000 rpm. This means that the assay can be extended to forces between 1 and $\sim 2500 \times g$ in most centrifuges. In measurements using the ultracentrifuge to achieve very high forces, it was found that adherent fibroblast cells are not released by $15,000 \times g$, and only 30% are released at $\sim 59,000 \times g$ (Rich, 1978). Thus, for practical measurements of strengthening, one can quantify the early events to monitor the rate of strengthening, and one can study cells with modified cytoskeletons to experimentally address this mechanism.

Again it should be noted that the “stick-and-wash” assays that are employed in many labo-

ratories simply measure strengthened adhesion. In contrast, this is an accurate assay as long as the rate-limiting step in the adhesion sequence is the adhesive step of interest. The stick-and-wash assays can be misleading in such tests. For example, consider a laboratory with an interest in cytoskeleton contributions. Hopefully, if the stick-and-wash assay is used with cytoskeletally impaired cells, the data will indicate that no adhesion has occurred. This must assume that the initial binding occurs as normal, but that the cells then fail to strengthen due to the missing critical cytoskeletal component. However, if the initial cell-substrate binding step fails to occur properly, the investigator might be misled into thinking the cytoskeleton is at fault. With the centrifugal assay, it is possible both to separate initial adhesion from strengthening, and to determine the extent of strengthening. This application has been very useful in a variety of circumstances involving embryonic cells of different stages, or transfected cells with different adhesion deficiencies.

Biophysical analogy of initial binding versus strengthened adhesion

There are many biophysical models of adhesion that attempt to explain how the cytoskeleton works to accomplish an increase in adhesive strength by orders of magnitude. Based largely on the knowledge that cells are malleable, and knowing that cells adhere by noncovalent receptor-ligand interactions, Dembo and Bell (1987) showed that initial binding was relatively weak and was similar for most adhesion molecules. Strengthened adhesion, on the other hand is highly resistant to shear forces. In the Dembo and Bell model, cells adhering by receptor-ligand interactions alone simply peel away from the substrate breaking bond by bond. When adhesion is strengthened by recruitment of the cytoskeleton into the adhesion complex, there is a rapid and very large increase in adhesive strength. As a simple analogy of the initial binding versus strengthened adhesion, consider a piece of double-stick Scotch tape. The following test is a simple means of demonstrating this principle.

Stick a piece of double-stick Scotch tape onto a desk. The Mylar backing of the tape is malleable so that the force needed to initially start peeling one end of the tape away from the substrate will be fairly small, and will remain fairly constant until the entire strip of tape is peeled away from the substrate. This is because the peeling force is the force necessary to break

the adhesive bonds only at the immediate point of contact as the tape is progressively peeled away (Fig. 9.2.2A). The rate of peeling depends upon the applied force, the number of bonds, the strength of an individual bond, and the on and off rates of the bonds. As the peeling force is increased, a point is reached when the off rate exceeds the on rate causing the adhesion to fail at that peeled interface. In the centrifugal assay, the centrifugal force just below the threshold point of cell removal is a measure of the initial binding affinity of the cell.

To demonstrate how adhesive strengthening appears to work, place a microscope slide on top of the same piece of double-stick tape on the desk. The tape is the same, the adherence to the substrate is the same, but now instead of a malleable backing the glass provides a very stiff backing for the tape. An exposed end of the tape peels away rather easily until the edge of the slide is encountered. Then a force that is orders of magnitude larger must be used to

remove the tape from the substrate. The massive additional force needed to lift the tape from the substrate is a consequence of the hard backing provided by the glass slide. Because of that rigid backing, the entire piece of tape must be removed all at once. Thus, the entire adhesive surface of the tape under the glass slide simultaneously resists the removal force (Fig. 9.2.2B).

By analogy, this rigidity is a biophysical property provided by the cytoskeleton that enables the adhesion to be strengthened. By cooperatively linking multiple adhesive molecules into more of a unit, a vast increase in apparent adhesive strength can be accomplished without an increase in number of adhesion molecules. Mechanically, the cytoskeleton causes a local stiffening of the adhesion complex so that many more adhesion molecules are acting together to cooperatively strengthen the adhesion.

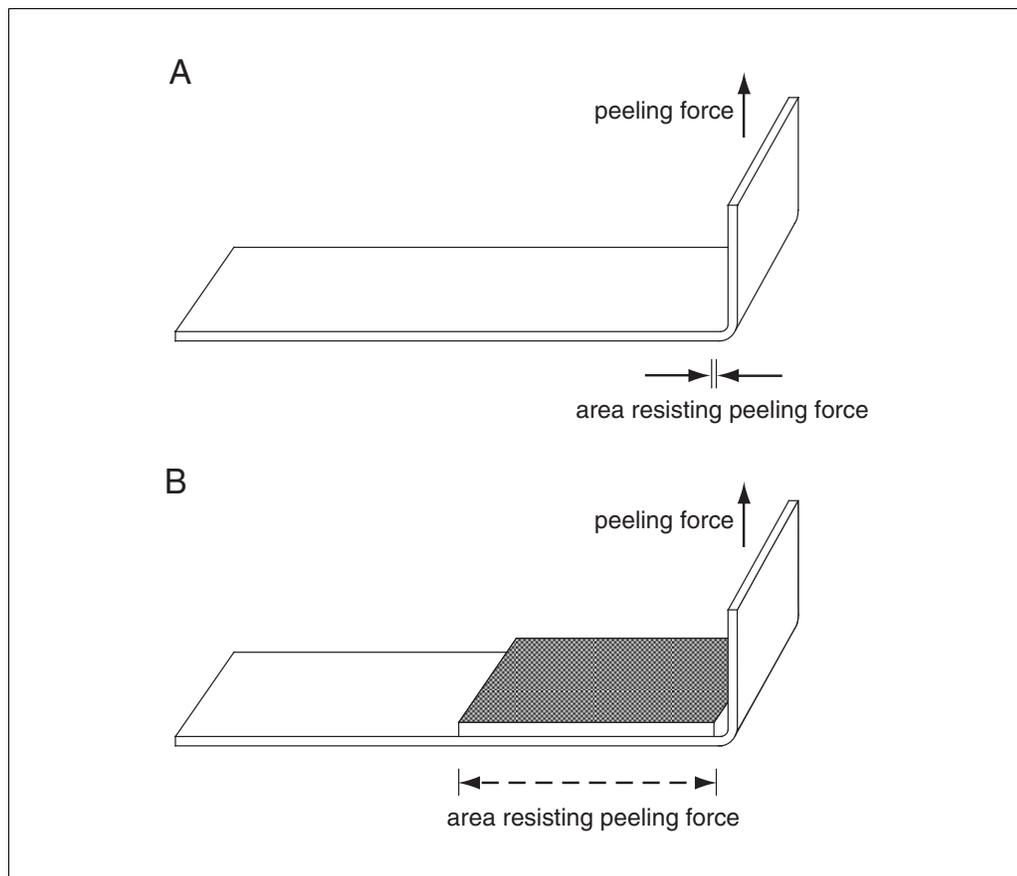


Figure 9.2.2 Tape analogy of two states of cell adhesion. (A) Double-stick tape applied to a surface is analogous to the adhesive behavior of cells that have only receptor-ligand association with the substrate. The adhesive area that resists the peeling force is small, limited to the area between the two arrows. (B) Adding a glass slide to the upper surface of the tape creates a situation analogous to the adhesive behavior of cells with cytoskeleton-supported cooperativity of cell-substrate association. The stiffened area of tape (membrane) increases the adhesive area that resists the peeling force.

Obviously, the cortex of the cell is not as rigid as the glass backing. Nevertheless, any coupling of the cytoskeleton to the group of adhesion molecules that allows these molecules to act as a unit rather than as freely diffusing molecules, provides an important cooperativity for strengthening. Conveniently, this allows the cell to control its own adhesiveness by controlling intracellular microcompartments (areas where adhesive molecules can couple with cytoskeleton and other areas where the cytoskeletal connections are released). An ability to measure many components of these dynamic associations is necessary to understand how adhesion molecules and cytoskeleton components work together to provide cells with a range of adhesive properties. The centrifugal adhesion assay can help with such analyses.

Critical Parameters

Concentration of substrate

As a rule, the substrate should be applied as a molecular monolayer on the plate. In the first set of experiments with a substrate protein or peptide, determine the threshold concentration for binding, and then operate just above that threshold in subsequent experiments. If one calculates the total surface area occupied by one substrate molecule, and calculates the total surface area of the well bottom, it is a simple calculation to figure out how much substrate would form a molecular monolayer. For example, $\sim 1 \mu\text{g}$ of fibronectin per well is about the right amount. Some overlapping is assumed, since the protein is filamentous, but the point is that it takes very little protein to completely coat the plate. At a concentration of $10 \mu\text{g}/\text{well}$, the assay loses specificity, presumably because many nonspecific factors can now contribute to the adhesion. Also, if the protein is layered on the plate, the effects of fibronectin peeling away from fibronectin may confound the assay.

Timing of adhesive strengthening

Significant strengthening is seen after even 1 min of incubation at 37°C . By 5 min at 37°C , the strengthening of most cells exceeds the ability of the cell centrifuge to provide enough force to dislodge them, because most bucket carriers or 96-well plates have a maximum RCF at which they can be centrifuged (usually on the order of $2500 \times g$). Note that this level of strengthening occurs well before any significant change can be seen in the cells, and before they establish visible focal contacts.

Increments in centrifugal force

For initial binding, cells can be centrifuged onto the substrate at $\sim 35 \times g$. For dislodgement of cells kept at 4°C , the force can vary from 1 to $\sim 2500 \times g$. Most cells will maintain adhesion to between 50 and $200 \times g$, but will be removed by higher g forces. For strengthened adhesions, a good guideline is to use a dislodgement force that is twice that necessary to remove the initially bound cells, and then ask how long it takes for the cells to strengthen at 37°C enough to remain adherent at that force. Usually that time is < 2 min.

Suitable media

The selection of medium depends on the cell type used, and several kinds of media are used for each assay. Cells should be dissociated using established protocols and media for each cell type. The cells are then maintained in a calcium-free buffer for counting and handling during the short time prior to adding them to the wells (see discussion of cations below). Finally, tissue culture medium without any fetal bovine serum (or equivalent) should be used as the cell adhesion test medium. If necessary, fetal bovine serum can be included, provided that proper controls are done for the contribution of cell adhesive substrates in the serum.

Phosphate buffered saline (PBS) containing 40 mg/ml BSA is used as a blocking solution for all wells pretreated with the substrate in question, as well as for background binding wells. In the authors' experience, the BSA eliminates almost all background binding to the well bottom. PBS without BSA serves as the washing reagent and as the buffer into which the substrates are diluted when initially coating the wells.

Role of cations

Many cell-cell interactions require calcium for some aspect of association. The actual function of calcium in these interactions has a rich literature. For the present case, keeping cells in calcium-free solutions is a useful way to control the experiment and to prevent adhesion from occurring prematurely. Thus, cells are added in calcium-free saline to the medium in the wells. There is enough calcium in most tissue culture media to allow this dilution of calcium in the well. Note that the calcium-free medium does not contain EGTA or any other calcium chelator. If the dissociation protocol uses calcium chelators, resuspend the cells in calcium-free medium without the EGTA after they are dissociated.

Recovery period after dissociation

If cells are enzymatically dissociated, they will need a recovery period to regenerate surface adhesion molecules prior to using the centrifugation assay. It is preferable to use nonenzymatic methods for dissociation so that cells are ready to use without recovery. If a recovery period is necessary, cells can be resuspended in complete culture medium with serum, and incubated in flasks at a rotation speed >70 rpm. This provides a chronic fluid shear force that prevents cells from adhering, yet allows them to recover from enzymatic treatments. The length of recovery varies according to cell type, but 4 hr at 37°C is a good first approximation. The cells should then be washed into calcium-free, serum-free medium for the assay.

Fluorescent molecules for labeling

Many fluorescent tags are available, though some work better than others. Rhodamine isothiocyanate (RITC) is the authors' first choice simply because it works on all cells and is slow to fade. The method of labeling is simple. Take ~1 mg of RITC (a bit on the end of a spatula) and add it to 50 µl dimethylsulfoxide (DMSO). Add the RITC/DMSO to calcium-free medium (without phenol red as a pH indicator) until the color of the medium barely turns pink. If the medium turns red, too much RITC has been added and the medium is toxic. Allow the cells to accumulate the RITC from the slightly pink medium, and they will be healthy. Usually 30 min or less is all that is necessary for labeling.

Several markers can be used as a second label. Fluorescein isothiocyanate (FITC) tends to be a bit difficult, as batches of FITC lose activity quickly. The fluorescein quenches easily as well. The easiest to use is Hoechst dye (33342 or 33258), which is easily loaded into cells, stains nuclei brightly, and is retained well. To label the cells, dissolve a small amount of Hoechst (or DAPI) dye in aqueous medium, add it to the cells at ~1:200, and incubate 30 min. Other dyes such as calcein AM or acridine orange can also be used to label other populations of cells. These dyes are easily loaded, and have good retention (i.e., do not bleed into unlabeled cells). To label cells, add 10 µl of a freshly prepared dye solution (up to 1 mg in 50 µl DMSO) to 100 ml cells in aqueous medium. Incubate 30 min, wash, and check labeling. Adjust as necessary, as labeling varies with cell type. High dye concentrations should be avoided because they may be toxic, and because

most dyes are cumulative (i.e., they will gradually be accumulated from a weak solution).

Troubleshooting

There are three potential pitfalls to be avoided in the assay. All three can be managed with relative ease, but some tips are offered below if these problems confound the assay.

Bubbles from improper sealing

The biggest problem encountered by the novice is the introduction of bubbles of air into the chamber when the wells are sealed. The bubble will act like a bulldozer and sweep cells off the substrate when the wells are inverted. To avoid this, it may take some practice sealing the wells. The easiest way to put the tape on is to place the wells in a rubber support, which acts as a backstop when the tape is pressed down. The tape is then rolled onto the wells, and any remaining medium is squeezed by rubbing the top either with fingers or a flat surface.

Bubbles generated during the warming incubation

If the sealed plate is incubated at 37°C for >5 min, air bubbles are often introduced. To avoid this, the plates can be sealed after the 37°C incubation. For long incubations, fill the wells with 100 µl of medium plus 100 µl of cell suspension (2/3 full). After the incubation, place the plates on ice and add 100 µl of medium to brim the wells. Add the tape and do the dislodgement step. In practice, this is really an easy step; however, it does introduce a potential error in that the late addition of medium adds a shear force other than centrifugal force to the system. One can easily control for this by examining the wells both prior to and after topping them off.

Quantifying cells

The cells will not be uniformly distributed on the bottom of the wells, which can potentially cause a sampling problem. This can be completely avoided by comparing an experimental population of cells, labeled with one dye, against a reference control population of cells, labeled with a different dye, in the same well. It is then possible to detect very small differences that might exist between the two cell populations. To count cells, it is simplest to capture images of the well bottoms, and either count the cells manually or use a computer-generated macro to automatically count the cells in the field.

Anticipated Results

With most cell types, two basic types of results will be obtained. Measuring adhesion as a function of g force will generate the two curves seen in Figure 9.2.3A. The cells adhering by a receptor-ligand interaction alone (4°C) will survive the dislodgement force until a certain force is reached and then there will be a rather sharp drop in their ability to resist the dislodgement force. On the other hand, if the cells are subjected to a brief incubation at 37°C with time held constant, they will tend to resist removal. Figure 9.2.3B shows an example of expected results when measuring adhesion as a function of time at 37°C, with the dislodgement force held constant.

Time Considerations

A typical assay requires 1 hr to complete. Extra time is required if the cells must recover after dissociation or if strengthened adhesion is measured.

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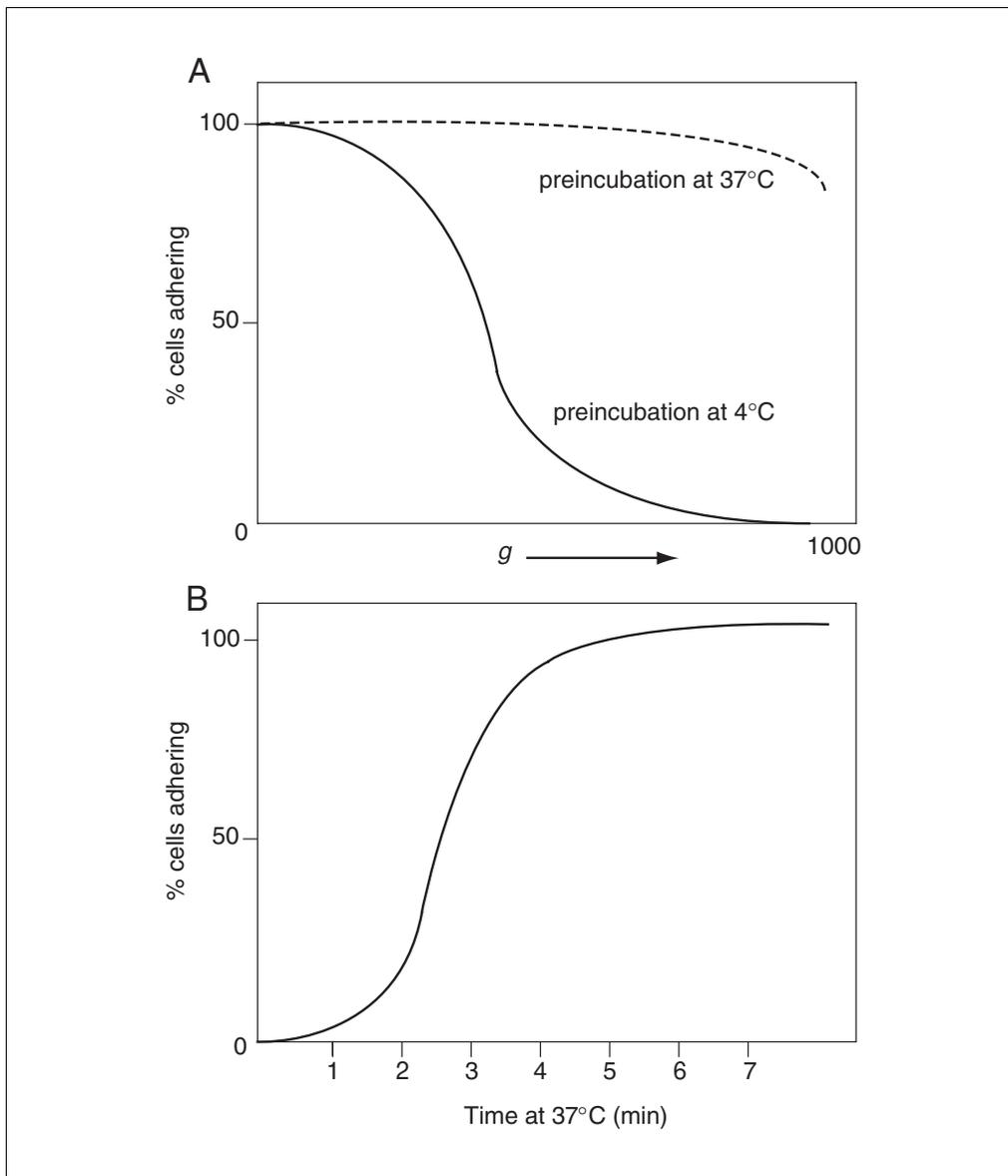


Figure 9.2.3 Theoretical data. (A) Force (in g) required to dislodge cells preincubated at 4°C and 37°C. (B) Time required to stabilize initial adhesion.

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Cell-cell adhesion mechanisms are subdivided into the Ca^{2+} -dependent system (CDS) and Ca^{2+} -independent system (CIDS), and cadherins are the major components of the former. In general, both systems are present in a single cell, and therefore these two adhesion systems must be discriminated for accurate assays of cell adhesion. This unit provides multiple protocols to assay cell-cell adhesion that is mediated by classic cadherins (see Background Information). Reaggregation of dissociated cells is described for measuring cadherin activity in a variety of cultures: short-term aggregation cultures (see Basic Protocol 1), long-term aggregation cultures (see Alternate Protocol), and mixed-cell aggregation cultures (see Basic Protocol 2). The methods for cell reaggregation should be modified depending on the purpose of the experiment. Two Support Protocols describe methods for dispersing cells while selectively preserving cadherins but removing CIDS. These protocols are optimized for using cultured fibroblastic cells (see Support Protocol 1) and embryonic tissue cells (see Support Protocol 2). Cells can also be dissociated by removing cadherins from the cell surface (see Support Protocol 3). The selection of a dissociation protocol is discussed in detail below (see Strategic Planning). Additional protocols describe how to detect and identify cadherins from cells (see Basic Protocol 3), how to inhibit cadherin-mediated cell adhesion (see Basic Protocol 4), and how to restore cadherin activity in cadherin- and catenin-deficient cell lines (see Basic Protocol 5).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

STRATEGIC PLANNING

Cells of multicellular solid tissues are connected to each other by various forms of cell-cell junctions—tight junctions, adherens junctions, desmosomes, and gap junctions—and by multiple classes of cell-surface molecules, each of which has distinctive functions. These structurally and molecularly complex adhesion systems can be subdivided operationally into CDS and CIDS (Takeichi, 1977, 1988). It is possible to selectively remove either of these two systems (or both) from cell surfaces by differential trypsin treatments (Takeichi et al., 1979; Urushihara et al., 1979). CDS is highly sensitive to trypsin even at low concentrations, but it can be protected from protease digestion by Ca^{2+} . On the other hand, CIDS can be removed only by treatment with relatively high concentrations of trypsin, and this proteolysis, in general, cannot be protected by Ca^{2+} . Accordingly, if cells are treated with a high concentration of trypsin in the presence of Ca^{2+} (TC treatment; see Support Protocols 1 and 2), CDS is left intact but CIDS is removed from the cell surface. If cells are treated with a low concentration of trypsin in the absence of Ca^{2+} (LTE treatment; see Support Protocol 3), CIDS is left intact, whereas CDS is digested and inactivated. Cells disaggregated by TC or LTE treatment can quickly reaggregate if placed in appropriate culture conditions, because adhesion molecules belonging to either CDS or CIDS are left intact on their surfaces.

As expected from the above properties of CDS and CIDS, treating cells with a high concentration of trypsin in the absence of Ca^{2+} (TE treatment; see Support Protocol 3) inactivates both adhesion systems. Thus, for most cell types, TE-treated cells temporarily lose all cell-cell adhesion activities, although they generally retain their cell-substrate adhesiveness. To have the TE-treated cells reaggregate, the adhesion molecules must be restored (see Basic Protocol 5).

Most cell-cell adhesion molecules identified thus far can be grouped into either CDS or CIDS. Classic cadherins (Ca²⁺-dependent cell-cell adhesion molecules) are the major components of CDS and, to the authors' knowledge, no other adhesion molecules are similar to the cadherins in their trypsin/Ca²⁺ sensitivity. Some of the other cell-cell adhesion molecules require Ca²⁺ for their activity, but are not preserved by Ca²⁺ during TC treatment. Therefore, CDS preserved from TC-treatment is represented virtually by a single family of adhesion molecules, the cadherins. CIDS includes multiple classes of adhesion molecules, especially members of the immunoglobulin superfamily; its entire biochemical profile remains to be clarified. Adhesion molecules left on LTE-treated cell surfaces may vary greatly according to cell type.

Cells retain a clustered state during and after TC treatment, because cadherins are active due to the presence of Ca²⁺. To dissociate them into single cells, they must be washed with a Ca²⁺-free medium. This strategy for cell disaggregation can be applied to many cell types, including fibroblasts (see Support Protocol 1), early embryonic cells (see Support Protocol 2), and neuroepithelial cells. However, it can be difficult to disaggregate certain cell types into single cells after TC treatment, especially differentiated epithelial cells. Some junctional structures seem to be resistant to the removal of Ca²⁺. For these cells, it is not possible to obtain healthy, single cells after TC treatment, and only TE treatment (see Support Protocol 3) is an effective way to obtain single cells. In the latter case, cadherins must be sacrificed to allow cell dissociation. Their activity can be studied, however, by letting the cells recover by long-term culture of the cells (see Alternate Protocol).

Each cadherin type can confer specific adhesiveness on cells. This nature of cadherins can be assayed by mixing cells expressing different cadherin types (Nose et al., 1988; Murphy-Erdosh et al., 1995; Nakagawa and Takeichi, 1995; see Basic Protocol 2). Cadherins are associated with cytoplasmic proteins, collectively called catenins, and this association is essential for cadherin activity (Aberle et al., 1996; Barth et al., 1997). On this molecular basis, manipulation of either cadherin or catenins can alter cadherin activity (see Basic Protocols 4 and 5).

BASIC PROTOCOL 1

SHORT-TERM AGGREGATION CULTURE

This protocol describes a method for allowing dispersed cells to reaggregate within short incubation periods. Cells are cultured for up to two hours in a simple balanced salt solution. This method should be chosen when adhesion molecules are assumed to be left intact on cell surfaces after trypsin treatments, and their activity is to be assayed. Physiological recovery of the trypsinized molecules is not expected in this simple culture system.

Materials

1% (w/v) BSA (see recipe)

HCMF (see recipe)

Cells of interest (see Support Protocols 1 to 3), suspended in ice-cold HCMF at 0.5–1 × 10⁵ cells/ml for cell lines, or at 2–5 × 10⁶ cells/ml for early embryonic neurons

100 mM CaCl₂ (APPENDIX 2A)

8% (w/v) paraformaldehyde in HCMF

24-well tissue culture plate (Falcon)

Gyrating shaker (New Brunswick Scientific, Model G2)

BSA-coated Pasteur pipets (see recipe)

Additional reagents and equipment for counting cell aggregates with a hemacytometer (UNIT 1.1) or Coulter counter

1. Precoat the wells of a 24-well tissue culture plate with 0.5 ml of 1% (w/v) BSA overnight at 4°C. Rinse the wells a few times with HCMF just before use.
2. Place the plate on ice and add 0.5 ml cells of interest to each well. Add 56 μ l of 100 mM CaCl₂ (1 mM final) to activate cadherins on TC-treated cells. Add an equal volume of water to controls wells to check the effect of Ca²⁺.

When desired, also add other substances, such as antibodies (Table 9.3.1) and inhibitors.

Cell density varies between cell lines and embryonic cells, because the latter are often smaller than the former.

Release of DNA from damaged cells tends to perturb cell aggregation processes (see Support Protocol 2). To avoid this, add DNase I (10 μ g/ml final) and MgCl₂ (1 mM final)

Table 9.3.1 Commercially Available Monoclonal Antibodies to Cadherins and Catenins

Antigen	Clone	Inhibitory activity ^a	Supplier ^b
<i>Cadherins</i>			
<i>Xenopus</i> C-cadherin	6B6		DSHB
Canine E-cadherin	rr1		DSHB
Human E-cadherin	HECD-1, SHE78-7	+	Takara, Zymed
Human E-cadherin			Transduction Labs
Human E-cadherin			Chemicon
Human E-cadherin	6F9		ICN, BIB, PGN
Human E-cadherin	67A4		Cosmo Bio
Mouse E-cadherin	ECCD-1	+	Takara, Zymed
Mouse E-cadherin	ECCD-2	-	Takara, Zymed
Mouse uvomorulin (E-cadherin)	DECMA-1		Sigma
<i>Xenopus</i> E-cadherin	5D3, 8C2		DSHB
Rat K-cadherin (cadherin-6)			Transduction Labs
Chicken N-cadherin	NCD-2	+	Takara, Zymed
Chicken ACAM (N-cadherin)	FA-5, GC-4, ID-7.2.3		Sigma
Human P-cadherin	NCC-CAD-299	+	Takara, Zymed
Human P-cadherin			Transduction Labs
Mouse P-cadherin	PCD-1	+	Takara, Zymed
Rat R-cadherin			Transduction Labs
Human VE-cadherin	BV6		BLD, BIB
Human cadherin-5			Transduction Labs
Human cadherin-5	TEA1/31		Cosmo Bio
Pan cadherin	CH-19		Sigma
Chicken LCAM	7D6		DSHB
<i>Catenins^c</i>			
α -Catenin			Transduction Labs
β -Catenin	CAT-5H10		Zymed
β -Catenin			Transduction Labs
β -Catenin	15B8, 6F9		Sigma
Plakoglobin (γ -catenin)	PG-11E4		Zymed
Plakoglobin (γ -catenin)			Transduction Labs
Plakoglobin (γ -catenin)	15F11		Sigma
Plakoglobin (γ -catenin)	PG5.1		PGN

^aInhibitory activity is indicated only for antibodies that have been tested directly by the authors. For other antibodies, refer to original descriptions.

^bBIB, Boehringer Ingelheim Bioproducts; BLD, BioLine Diagnostics; ICN, ICN Biomedicals; DSHB, Developmental Studies Hybridoma Bank; PGN, Progen Biotechnik GmbH. See SUPPLIERS APPENDIX for addresses.

^cSpecies name is omitted for catenins, as most antibodies can recognize a wide variety of species.

to the aggregation medium. The presence of DNase is often critical for accurate measurement of natural cell aggregation. Mg^{2+} might activate integrins, but this effect is generally negligible in cadherin assays. Cadherins themselves are insensitive to Mg^{2+} .

3. Place the plate on a gyrating shaker at ~80 rpm, and incubate 30 min to 2 hr at 37°C. Monitor the aggregation process every 30 min (or at shorter intervals during the initial 30-min period) with an inverted microscope, being careful not to disturb the central accumulation of cells.

The cells soon become concentrated at the center of each well, and will aggregate at this position. Accumulation can be perturbed by contaminating fibrous materials (such as cotton fibers), affecting the pattern and rate of cell aggregation. Therefore, it is very important to avoid such contamination.

TC-treated cells start to aggregate within 10 min in the presence of Ca^{2+} . Without Ca^{2+} , TC-treated cells should not aggregate. If they aggregate in the Ca^{2+} -free wells, it is possible that adhesion molecules other than cadherins are active in these cells. On the other hand, LTE-treated cells do aggregate in the absence of Ca^{2+} . TE-treated cells, in general, do not aggregate under any of these conditions. Sometimes, a low degree of aggregation may be observed for TE-treated embryonic cells, suggesting that some residual adhesion molecules are present.

Maximum aggregation of the cells is generally attained within 2 hr under the above culture conditions, and 30 to 60 min incubation is often sufficient to assess their aggregation properties. Longer incubations may cause cell death, as well as nonspecific cell aggregation due to various factors.

4. Place the plate on ice.

Cooling the cells stops cadherin-mediated aggregation. Ca^{2+} -independent aggregation may still proceed for LTE-treated cells.

5. Add an equal volume of 8% (w/v) paraformaldehyde to each well, mix, and incubate 15 min on ice.

Light fixation with an aldehyde-based fixative helps prevent redissociation of the aggregates. Quick mixing of the fixative with the cell suspension is essential to ensure that there is no artificial (fixation-induced) cell aggregation.

6. Gently stir the wells, obtain an aliquot of the cell suspension (or the whole suspension) using a BSA-coated Pasteur pipet, and count the number of cell aggregates and single cells with a hemacytometer or automated cell counter. Determine N_t/N_0 , where N_t is the total particle number (aggregates plus single cells) at incubation time t , and N_0 is the total cell number in the cell suspension (see Support Protocols 1 to 3, and the materials list above).

Be certain that cells or aggregates are uniformly distributed in the well when an aliquot of the cell suspension is removed for counting. See Background Information for details on how to quantify cell aggregation.

ALTERNATE PROTOCOL

LONG-TERM AGGREGATION CULTURE

As mentioned above (see Strategic Planning), for some cell types including most epithelia, complete disaggregation of cells cannot be achieved by TC treatment, and TE treatment (see Support Protocol 3) must be used. The long-term aggregation culture involves incubating cells for several hours to days, by maintaining cells in nutritious culture medium in place of HCMF. Under these conditions, cells undergo de novo protein synthesis, and therefore TE-treated cells gradually recover adhesion molecules, allowing them to aggregate. Cadherin-mediated aggregation begins within a few hours of culture. This aggregation can be inhibited with specific blocking antibodies to cadherins, if they are available. Cadherin activities of TE-treated cells can be studied by this method. TC- and LTE-treated cells do not require long-term cultures.

Additional Materials (also see *Basic Protocol 1*)

1% (w/v) agar (e.g., Noble agar, Difco) in standard culture medium

Cells of interest (see Support Protocol 3), suspended at $0.5\text{--}1 \times 10^5$ cells/ml for cell lines, or at $2\text{--}5 \times 10^6$ cells/ml for early embryonic neurons, in standard cell culture medium (e.g., supplemented Dulbecco's modified Eagle medium, DMEM; *APPENDIX 2A*) with 10% FBS (*APPENDIX 2A*)

1. Pour a minimum amount of 1% agar into the wells of a 24-well tissue culture plate (or other dishes) to cover the entire bottom surface. Allow the gel to set.

Agar is a powerful inhibitor of cell attachment to the substratum. For long-term cultures, BSA coating is not sufficient to prevent cells from attaching to the dishes.

2. Add cells of interest to agar-coated wells (see Basic Protocol 1, step 2). Incubate as in Basic Protocol 1 (step 3) for the desired period, placing the plate on a shaker in a 5% to 10% (v/v) CO₂ incubator if the medium is buffered with a NaHCO₃/CO₂ system.

The cultures can be maintained for several days if the culture medium is properly refreshed (e.g., by changing half of the medium every day). Cell aggregation also can be induced without rotating the plates. CO₂ levels should be adjusted to maintain pH 7.4.

3. Fix the cells and determine the extent of aggregation (see Basic Protocol 1, steps 4 to 6).

MIXED-CELL AGGREGATION CULTURE

To evaluate whether two given populations of cells can adhere to each other, they are mixed and allowed to reaggregate in one of the above culture systems. The resultant cell aggregates are analyzed to determine whether or not the cells have segregated. To do this, the two cell populations must be distinguishable. This can be achieved by staining one population of cells with a fluorescent dye or by labeling two populations with optically distinct dyes. Suitable dyes include 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO), Cell Tracker (Molecular Probes), PKH26 (ZYNAXIS), or Fluoro-gold (Fluorochrome).

Materials

Cell cultures

Standard cell culture medium (e.g., supplemented Dulbecco's modified Eagle medium, DMEM; *APPENDIX 2A*) with 10% FBS (*APPENDIX 2A*)

Fluorescent dye(s): e.g., 3 mg/ml 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) in dimethylsulfoxide

HEPES buffer (see recipe)

8% (w/v) paraformaldehyde in HCMF (see recipe for HCMF)

Glycerol-based mounting medium containing antifading reagents (e.g., 1 mg/ml *p*-phenylenediamine in 90% glycerol)

BSA-coated microcentrifuge tubes (see recipe)

Additional reagents and equipment for dispersing cultures (see Support Protocol 1 to 3), for counting cells (*UNIT 1.1*), and for short-term or long-term aggregation culture (see Basic Protocol 1 or Alternate Protocol)

1. Label one culture with a fluorescent dye, or label both cultures with optically distinct fluorescent dyes. For example, label a monolayer cell culture by incubating the cells overnight at 37°C with standard cell culture medium containing 15 µg/ml DiO, and wash 3 times with HEPES buffer. Disperse cultures into single cells (see Support Protocols 1 to 3) and resuspend.

Cells can also be labeled after the dissociation step.

BASIC PROTOCOL 2

Cell Adhesion

9.3.5

For other fluorescent dyes, follow the manufacturer's instructions. Make sure that all the cells have been equally labeled by examining with a fluorescence microscope.

- Count the cells (*UNIT 1.1*) and adjust the density with medium to be used for the following aggregation culture. Mix the two disaggregated cell cultures in a 1:1 ratio and incubate for aggregation (see Basic Protocol 1, steps 1 to 3, or see Alternate Protocol, steps 1 and 2).
- Add an equal volume of 8% paraformaldehyde to each well of the dish, and fix the cells 30 min at 4°C.
- Transfer the contents of each well into a BSA-coated microcentrifuge tube, and centrifuge the sample 1 min at 700 × g, 4°C.

The microcentrifuge tube should be coated with 1% BSA so that the cells will not stick to the wall of the tube.

- Discard the supernatant, and gently wash the cells three times with HEPES buffer.
- Mount the cells on a glass slide with a minimum amount of glycerol-based mounting medium containing antifading reagents.
- Analyze the aggregation pattern of the aggregates using a fluorescence microscope.

In mixed-cell aggregation assays, three kinds of pattern can be observed for the distribution of the two cell populations. (1) Cells of each population form separate aggregates, indicating that they can adhere only to like cells. (2) Two cell populations randomly intermix with each other, suggesting that they share common adhesion mechanisms. (3) Each cell aggregate contains the two cell types, but their distribution is not random, and they tend to segregate from each other within the aggregate, forming their own clusters. By analyzing these patterns, selectivity in the adhesiveness of two given cell populations can be evaluated.

SUPPORT PROTOCOL 1

DISSOCIATION OF FIBROBLASTS BY TC TREATMENT

This protocol describes cell dissociation employing a high concentration of trypsin in the presence of Ca²⁺ (TC treatment), which has been used successfully for cultured fibroblasts and similar cells. For other cell types, some modifications of the method may be necessary (see Support Protocol 2).

Materials

Semiconfluent monolayer of the cells of interest
HMF (see recipe)
TC solution (see recipe)
0.5% (w/v) soybean trypsin inhibitor solution (see recipe)
HCMF (see recipe), ice cold
BSA-coated 10-ml test tubes and Pasteur pipets (see recipe)

Additional reagents and equipment for disaggregation by TE treatment (see Support Protocol 3) and for counting (*UNIT 1.1*)

Prepare cultures

- Prepare semiconfluent monolayer cultures of cells by seeding them 1 or 2 days before use. To confirm successful TC treatment, prepare parallel cultures treated with TE (see Support Protocol 3).

Overcrowded or old cultures should not be used, as they are often difficult to dissociate into healthy single cells.

- Rinse the cultures three times with HMF.

Perform TC treatment

3. Remove HMF and add TC solution to the rinsed cultures (e.g., ~4 ml for a 5-cm dish).

The Ca^{2+} concentration can be modified, balancing protection against proteases and adequate cell disaggregation according to cell type (see Critical Parameters and Troubleshooting).

4. Incubate 15 to 30 min at 37°C on a rotary shaker, and then examine the cells with an inverted microscope.

The suspended cells should exist as tight clumps if the TC treatment is successful. In sharp contrast, TE-treated cells should be virtually dispersed as single cells. If TC treatment results in a nearly complete dispersion of the cells, either the cells do not have any cadherins, or something was wrong with the treatment procedure, resulting in inactivation of cadherins. To test the latter possibility, the Ca^{2+} concentration in the TC solution can be increased to 5 mM.

Dissociate cells

5. Transfer cells into a BSA-coated 10-ml test tube. If necessary, collect attached cells by flushing the solution with a BSA-coated Pasteur pipet.

In general, cells spontaneously detach from the dishes during trypsin treatment.

6. Centrifuge 3 to 4 min at $700 \times g$, 4°C, and remove the supernatant.
7. Add 100 μ l of 0.5% soybean trypsin inhibitor solution to the cell pellet, followed by 5 ml ice-cold HCMF. Resuspend the cells by brief pipetting with a BSA-coated Pasteur pipet and centrifuge them again. Aspirate the supernatant.
8. Add 5 ml ice-cold HCMF to the cells and disperse the cell clumps by gentle pipetting. Centrifuge as in step 6 and aspirate the HCMF. Repeat.

For complete dispersion of TC-treated cells, vigorous pipetting is generally required. After several repeats of pipetting, place a tiny aliquot of the cell suspension onto a glass slide and examine it under an inverted microscope. If cell clumps are still present, repeat the pipetting until essentially all cells become dissociated.

Some cell types, such as epithelial cells, may resist being dissociated into single cells after TC treatment. For such samples, prolonged pipetting might eventually kill them. In this case, other methods for cell dispersion should be employed (e.g., TE treatment; see Support Protocol 3).

9. Resuspend the cells in 2 to 5 ml ice-cold HCMF, and pipet them using a BSA-coated Pasteur pipet in order to obtain completely dissociated cells.
10. Count cell density (UNIT 1.1), centrifuge the cells as in step 6, and finally suspend the cells at $0.5\text{--}1 \times 10^5$ cells/ml in ice-cold HCMF (see Basic Protocol 1). Store on ice, and use as soon as possible.

Never leave the cell suspension at room temperature, as this causes cadherin degradation if Ca^{2+} is absent, and induces precocious cadherin-mediated aggregation if Ca^{2+} is present.

DISSOCIATION OF EMBRYONIC CELLS BY TC TREATMENT

This protocol describes how to modify TC treatment (see Support Protocol 1) for dissociating embryonic tissues. This protocol works nicely to dissociate early neural and fibroblastic tissues. For some tissues (e.g., early embryonic brains), Ca^{2+} concentration can be increased up to 10 mM to optimize preservation of cadherins without reducing the efficiency of cell disaggregation. On the other hand, it may be difficult to dissociate epithelial organs into single cells by the standard TC treatment (see Support Protocol 3 to resolve this problem).

SUPPORT PROTOCOL 2

Cell Adhesion

9.3.7

Additional Materials (also see Support Protocol 1)

Embryo(s)
HEPES buffer (see recipe), ice cold
DNase I
1 M MgCl₂ (APPENDIX 2A)
BSA-coated 5-cm petri dish (see recipe)

1. Remove tissue from embryos and place in ice-cold HEPES buffer. Cut the tissue into small pieces in HEPES buffer.
2. Transfer the collected tissue with a minimum amount of HEPES buffer to the edge of a 5-cm BSA-coated petri dish, and mince with a pair of fine-tipped scissors.
3. Add 4 to 5 ml TC solution, and incubate 20 to 30 min at 37°C on a rotary shaker.

Larger pieces of tissue may require longer incubation periods.

4. Lightly pipet the suspension with a BSA-coated Pasteur pipet to briefly disperse the tissue, and examine with an inverted microscope. If viscous DNA gels are present, digest them by adding an excess amount of soybean trypsin inhibitor (add 1/10th vol of a 0.5% solution) followed by 10 µg/ml DNase I and 10 mM MgCl₂ (final concentrations). Incubate without shaking at 37°C until the viscous material completely disappears (several minutes).

DNA gels can cause nonspecific cell aggregation (see Critical Parameters and Troubleshooting).

5. Transfer the cell suspension to a BSA-coated 10-ml test tube, and wash (see Support Protocol 1, steps 6 to 9).

If soybean trypsin inhibitor was added in step 4, it does not need to be added again here.

The trypsinized cell suspensions may contain undigested, large cell clumps or tissue debris, mainly derived from extracellular matrices. These should be removed by low-speed centrifugation or with a filter (e.g., Cell Strainer, Falcon), at some step in the washing process.

6. Count and resuspend cells (see Support Protocol 1, step 10), bringing embryonic brain cells, for example, to 2–5 × 10⁶ cells/ml.

**SUPPORT
PROTOCOL 3**

DISSOCIATION OF CELLS BY LTE OR TE TREATMENT

Treatment of cells at a low concentration of trypsin (LTE treatment) or at a high concentration of trypsin (TE treatment), both in the presence of 1 mM EDTA, was designed to obtain cells lacking cadherins. In LTE-treated cells, CIDS remains active, whereas in TE-treated cells, both cadherins and CIDS are inactivated. Thus, TE treatment produces temporarily null-adhesive cells, and this treatment is useful for complete dissociation of most cell types. As mentioned above, cells of certain tissues (e.g., epithelial tissues) cannot be completely dissociated by TC treatment. In this case, TE treatment is the only choice for obtaining single-cell preparations. Many aspects of the LTE and TE treatments are identical to those for TC treatment; only those steps that must be modified are present here.

Additional Materials (also see Support Protocols 1 and 2)

TE solution (see recipe)
LTE solution (see recipe)

- 1a. *For cultured cells:* Wash cells three times with HCMF.
- 1b. *For embryonic tissue:* Rinse tissue with HCMF and transfer to a 5-cm BSA-coated petri dish for trypsin treatment.

2. Add 4 to 5 ml LTE or TE solution to the dish and incubate 15 to 30 min at 37°C on a rotary shaker.

For LTE treatment, the best trypsin concentration should be determined for every tissue or cell line to ensure that a sufficient CIDS activity is preserved, that cadherins are fully inactivated, and that cells can be dissociated into single cells. For many cell lines, 0.0001% (w/v) trypsin gives satisfactory results; 0.0005% is suitable for embryonic brain.

3. Examine the cells with an inverted microscope. For cells from embryonic tissues, digest DNA gels as necessary (see Support Protocol 2, step 4).

In contrast to TC-treated cells, which are tightly clumped, LTE-treated cells should be more loosely clustered, and TE-treated cells should be virtually dispersed as single cells.

4. Transfer cells to a BSA-coated 10-ml test tube and wash (see Support Protocol 1, steps 6 to 9).

5. Count and resuspend cells (see Support Protocol 1, step 10), bringing cell lines to $0.5\text{--}1 \times 10^5$ cells/ml, and embryonic cells to $2\text{--}5 \times 10^6$ cells/ml.

For long-term culture, suspend cells in the appropriate culture medium (see Alternate Protocol).

DETECTION OF CADHERINS AND CATENINS

When working with cells for which no information is available concerning cadherin expression or activity, the following procedures should be helpful in determining their cadherin activity.

Examine morphology

Observe the morphology of cell colonies in relatively low-density cultures. If any cadherins are active, the cells should exhibit firm associations with each other at their margins. If the cells have no cadherin activity, individual cells should be independent (i.e., not forming any tight contacts with others). When these cells become confluent, they may temporarily form monolayer-like sheets, which are sometimes indistinguishable from cadherin-active cell sheets. However, upon further proliferation, overgrown cells tend to be excluded from the sheets if cadherins are inactive; many of the excluded cells may stay on the top of the sheets, exhibiting a round shape. This type of cell association pattern is generally not observed for cadherin-active confluent cell layers, as they maintain tight mutual associations even at high densities.

Examine catenin protein localization

Immunostain cell layers with fluorophore-conjugated antibodies to β -catenin, α E-catenin, or α N-catenin (UNIT 4.3; Table 9.3.1). If one of these molecules is concentrated at contact sites between the cells, it strongly suggests that a certain type of cadherin is present and colocalizes with the catenins.

Determine Ca^{2+} -dependent trypsin sensitivity

Treat the cells by the TC (see Support Protocols 1 or 2) and TE (see Support Protocol 3) methods. If cells are detached from the dish as tight clumps after TC treatment, but are dispersed into single cells by TE treatment, this is a strong indication that they have certain cadherins.

Examine cadherin expression

If anti-cadherin antibodies are available (Table 9.3.1), use them for immunoblotting (UNIT 6.2) or immunostaining (UNIT 4.3). If cadherins are active, immunostaining signals

should be concentrated at cell-cell contact sites. Note that most anti-cadherin antibodies can recognize only limited species.

If immunological strategies do not work, try reverse transcription PCR (RT-PCR) using primers that can amplify fragments of multiple cadherin types. Refer to Suzuki et al. (1991) and Sano et al. (1993) for appropriate PCR primers.

INHIBITION OF CADHERIN FUNCTION

There are at least three established ways for blocking cadherin activity: removal of Ca^{2+} , use of inhibitory antibodies, and expression of dominant negative cadherin constructs. Each is detailed below.

Inhibition by Ca^{2+} removal

Removal of Ca^{2+} is the most effective way for blocking cadherins. For most cell types, cadherins are inactivated at <0.1 mM Ca^{2+} . Incubation of cells with EGTA or EDTA (e.g., 1 mM) greatly facilitates Ca^{2+} removal and induces visible dissociation of the cells within several to 10 min at 37°C . Any cells treated with these chelators tend to round up, exhibiting formation of clear gaps between them. However, it should be stressed that many cells, especially epithelial cells, are not completely separated from each other by chelator treatment alone. They often maintain tight connections through fine processes. These chelator-resistant connections cannot be disrupted by a simple mechanical force. The molecular nature of these connections is not fully understood, and only TE treatment can disrupt them.

Inhibition with antibodies

Many antibodies raised against the extracellular domain of cadherins can inhibit their adhesion activities. In monolayer cultures, one can observe morphological changes in antibody-treated cells. These changes are not as drastic as those observed with Ca^{2+} removal, but careful investigators can detect clear differences between the antibody-treated and untreated cells. Most importantly, gaps often become visible between the antibody-treated cells. These changes can be recognized within a few hours after addition of the antibodies. High-density cultures should not be used for these observations, because it is difficult to detect morphological changes. When three-dimensional tissues are treated with anti-cadherin antibodies, their response is seen as a rounding-up of individual cells and a loss of the compact tissue appearance.

For anti-cadherin antibodies, the following points should be kept in mind. (1) Among available anti-cadherin antibodies, those with blocking activity are limited (Table 9.3.1). (2) Many antibodies show species-specific reactivity. (3) A single cell usually expresses multiple cadherin types. For complete inhibition of a cell's cadherin-mediated adhesion, all types must be blocked. Nevertheless, blocking the major cadherin expressed by the cell is often sufficient to induce a morphological response to the antibodies.

Inhibition with dominant negative cadherin constructs

The products of two groups of cadherin cDNA constructs are known to exhibit a dominant negative effect (Takeichi, 1995). One group (ΔN) encodes a molecule with a truncated extracellular domain and an intact cytoplasmic domain (Kintner, 1992; Fujimori and Takeichi, 1993). These molecules are not functional as adhesion molecules themselves, because they lack the extracellular domain. However, they can associate normally with the cytoskeletal system. Based on this nature, the ΔN molecules can compete with endogenous, intact cadherins for interactions with cytoplasmic components, thus interfering with the adhesion activity of the latter. ΔN can nonspecifically inhibit a wide variety of classic cadherins.

The other type of dominant negative cadherin construct (ΔC) was designed to encode a molecule consisting of the intact extracellular domain and a truncated cytoplasmic domain (Levine et al., 1994). Again, the products of these constructs are not functional as adhesion molecules, and they are assumed to interfere with the homophilic interactions at the extracellular domain of endogenous cadherins. In contrast with the use of ΔN , it is possible to specifically block the function of a particular cadherin type with these constructs, because each cadherin type has a unique sequence in its extracellular domain. ΔC has been used successfully when overexpressed in *Xenopus* embryos but not in cell lines. It may be that a large excess of the mutant molecule is required to fully compete with endogenous intact cadherins.

RESTORATION OF CADHERIN ACTIVITY IN CADHERIN- OR CATENIN-DEFICIENT CELL LINES

Cell lines with impaired cadherin function are useful materials for studying the mechanisms of cadherin-mediated adhesion. Cadherin dysfunction can be induced by various mechanisms, including loss or mutation of cadherin or catenin genes, or down-regulation of their expression. Cell lines that do not show any cadherin expression include L (Nagafuchi et al., 1987), Neuro 2a (Matsunaga et al., 1988), and S180 (Mege et al., 1988). α -Catenin-deficient lines include PC9 (Hirano et al., 1992) and HCT-8/R1 (van Hengel et al., 1997). HSC39 has a truncated β -catenin gene (Kawanishi et al., 1994; Oyama et al., 1995). In some lines, cadherin function is blocked despite the presence of all known components for the cadherin adhesion machinery. Such blocking is perhaps based on physiological mechanisms. Cadherin dysfunction is most frequently found in carcinoma cell lines. In many of them, not only cell-cell adhesion but also cell-substrate adhesion is often impaired, and they grow as suspended cells without forming tight clumps. If a cell line is found to grow as single cells, it is highly possible that the cells' cadherin activity is abnormal due to one of the above mechanisms.

In cadherin- or catenin-deficient cell lines, cadherin activity can be restored by transfecting with a cDNA encoding the affected protein products. Isolation of successfully transfected clones is relatively easy, because their morphology is dramatically changed from a dispersed to an adhesive type, allowing selection of positive colonies by microscopic observation. On the other hand, the physiologically blocked cadherin function cannot be rescued by a simple addition of cadherin or catenin cDNA.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

BSA-coated glass- and plasticware

Place 1% (w/v) BSA solution (see recipe) into test tubes, dishes, and microcentrifuge tubes to be used for collection of cells. Submerge Pasteur pipets in the BSA solution. Let sit at room temperature for at least 1 hr before the experiment. Rinse with HCMF (see recipe) before use.

Cells suspended in protein-free solutions readily attach to plastic or glass surfaces, causing loss of and damage to the cells. To avoid this, all plastic or glass dishes, tubes, and pipets to be used for cell preparation must be precoated with BSA.

If necessary, BSA can be left in tubes/pipets overnight at 4°C.

BSA solution, 1% (w/v)

1 g bovine serum albumin (Fraction V; Sigma)
HCMF (see recipe) to 100 ml
Adjust pH to 7.4 with NaOH
Filter sterilize through a 0.45- μ m filter
Store \leq 1 week at 4°C

HEPES buffer

1 liter HCMF (see recipe)
1 ml 1 M CaCl₂ (APPENDIX 2A)
1 ml 1 M MgCl₂ (APPENDIX 2A)
Filter through a 0.45- μ m pore-sized filter to remove any dusty materials
Store up to several months at 4°C

HEPES buffer, Ca²⁺- and Mg²⁺-free (HCMF), pH 7.4

8 g NaCl (0.137 M final)
0.4 g KCl (5.4 mM final)
0.12 g Na₂HPO₄·12H₂O (0.34 mM final)
1.0 g glucose
2.38 g *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; 10 mM final)
H₂O to 1 liter
Adjust pH to 7.4 with 1 N NaOH
Filter through a 0.45- μ m filter to remove any dusty materials
Store up to several months at 4°C

HEPES buffer, Mg²⁺-free (HMF)

1 liter HCMF (see recipe)
1 ml 1 M CaCl₂ (APPENDIX 2A)
Filter through a 0.45- μ m filter to remove any dusty materials
Store up to several months at 4°C

LTE solution

10 μ l 0.1% (w/v) trypsin solution (see recipe; 0.0001% final)
100 μ l 100 mM EDTA (APPENDIX 2A) or ethylene glycol bis(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) (1 mM final)
9.89 ml HCMF (see recipe)
Prepare fresh
EDTA or EGTA should give the same effect.

Soybean trypsin inhibitor solution, 0.5% (w/v)

50 mg soybean trypsin inhibitor (Sigma)
HCMF (see recipe) to 10 ml
Divide into 100- μ l aliquots
Stored \leq 1 month at -20°C

TC solution

1 ml 0.1% (w/v) trypsin solution (see recipe; 0.01% final)
100 μ l 100 mM CaCl₂ (1 mM final)
8.9 ml HCMF (see recipe)
Prepare fresh

TE solution

1 ml 0.1% (w/v) trypsin solution (see recipe; 0.01% final)
100 μ l 100 mM EDTA (*APPENDIX 2A*) or ethylene glycol bis(β -aminoethylether)-
N,N,N',N'-tetraacetic acid (EGTA; 1 mM final)
8.9 ml HCMF (see recipe)
Prepare fresh

EDTA or EGTA should give the same effect.

Trypsin solution, 0.1% (w/v)

10 mg crystalline trypsin (Sigma)
HCMF (see recipe) to 10 ml
Divide into 1-ml aliquots
Store \leq 1 month at -20°C

COMMENTARY

Background Information

The cadherins constitute a large superfamily of molecules with diverse primary structures (Takeichi, 1995). Among its members, those of the “classic cadherin” subfamily have most extensively been characterized as cell-cell adhesion molecules. More than 20 members of the classic cadherin subfamily are known, and they are subdivided into type I and II groups (Takeichi, 1995). All of them bind cells by homophilic interactions. Based upon specificity in their molecular interactions, cells tend to preferentially adhere to those expressing the same cadherin (Takeichi, 1995). Each cadherin is expressed in restricted tissues, and a single cell generally expresses multiple cadherin types. This expression profile should be kept in mind when identifying cadherins in a given cell. The protocols described in this unit should be applied only to the classic cadherins. Other cadherins such as desmosomal cadherins and protocadherins may show different biochemical and biological properties.

The cytoplasmic domain of classic cadherins is associated with catenins (Aberle et al., 1996; Barth et al., 1997). The catenins are subdivided into the β -catenin and α -catenin groups. The former includes β -catenin Armadillo in *Drosophila*), plakoglobin (γ -catenin), and p120^{cas}, and the latter consists of α E-catenin and α N-catenin. The β -catenin group directly associates with the carboxy terminal domain of cadherins. α -Catenins bind to β -catenin, and the cadherin/ β -catenin/ α -catenin complex formation is crucial for full activity of this adhesion machinery. Therefore, cadherin dysfunction can be brought about by loss of any of these three components. TE treatment cleaves only the cadherins.

The mechanism by which Ca^{2+} protects cadherins from proteolytic digestion is not fully understood, although the Ca^{2+} -binding sites have now been identified (Overduin et al., 1995; Shapiro et al., 1995). Although cadherins are generally resistant to TC treatment, this procedure can cleave the proximal portion of the cadherin extracellular domain if incubation is long (especially when isolated cell membranes are treated), resulting in the production of an extracellular fragment (Takeichi, 1988).

How the strength of cell-cell adhesion should be quantified is a classic controversial issue. The simplest way, which does not require sophisticated equipment, is to measure the decrease in the total particle number in cell suspensions. The degree of cell aggregation can be represented by the index N_t/N_0 , where N_t is the total particle number (aggregates + single cells) at incubation time t , and N_0 is the total cell number in the cell suspension. Counting of particle number can be done using a hemacytometer (*UNIT 1.1*) but is greatly facilitated if a Coulter counter or its equivalent is available.

Critical Parameters and Troubleshooting

Calcium

When preparing cells for cadherin assays, the presence or absence of Ca^{2+} affects the preservation of cadherins on the cell surfaces, even when exogenous proteases are absent. With physiological concentrations of Ca^{2+} , cadherins are always protected from protease digestion. In contrast, in Ca^{2+} -free media, cadherins tend to be gradually degraded by proteases released from lysed cells in a temperature-dependent manner. Therefore, when the preservation of cadherins is desired, it is highly

recommended that 1 to 2 mM Ca^{2+} be added to all solutions used for cell preparation or tissue dissection. Higher concentrations of Ca^{2+} are more effective in preserving cadherins against tryptic digestion, but they can cause difficulty in cell disaggregation in subsequent steps. For cells that disaggregate with relative ease, the Ca^{2+} concentration should be increased to 5 to 10 mM. Conversely, the Ca^{2+} concentration can be reduced to 0.2 to 0.5 mM for other cell types; this enhances cell disaggregation but reduces cadherins left intact on the cell surface. Ca^{2+} at <0.1 mM fails to protect cadherins. It should also be remembered that more Ca^{2+} is required when higher concentrations of trypsin are used. Ca^{2+} can protect cadherins against many classes of proteases.

To dissociate cells, however, they must be exposed to Ca^{2+} -free conditions to inactivate cadherins. At this time, cadherins can be protected by low temperatures. Ca^{2+} -free cell suspensions should always be kept at an ice-cold temperature. If Ca^{2+} is added to a suspension of cells with cadherins, it induces immediate cell aggregation at physiological temperatures. Under ice-cold conditions, however, cadherin-mediated aggregation is inhibited. Thus, placing cells in an ice-cold, Ca^{2+} -containing medium is the best way to both preserve their cadherin activity and keep them dispersed.

Nonspecific aggregation or adhesion

In cell aggregation assays, the cell suspension should be as clean as possible, as any noncellular contaminant can disturb natural cell aggregation. For example, one piece of cotton fiber can act as a core to induce artificial clumping of the cells.

DNA gels most effectively clump cells. During the trypsin treatments, DNA may be released from lysed cells. The DNA forms viscous gels, which trap cells in loose aggregates. These artificial aggregates should be distinguished from those mediated by real cell-cell adhesion molecules. The presence of DNA gels can also block cell pelleting in the washes that follow trypsin treatment. Therefore, if a significant amount of viscous material is observed in the cell suspension, it should be removed by DNase treatment before the cell washes (see Support Protocol 2). This pretreatment with DNase is generally not required when cell lines are used, because DNA release is not as extensive.

BSA precoating of the dishes, tubes, and pipets to be used for the rotation culture or for cell preparation is an absolute requirement for

the success of the experiments, as it prevents the attachment of cells to these surfaces. If cells attach to the dish during the cell aggregation assay, the aggregation process is severely affected, and such cultures should be discarded.

Anticipated Results

In suspension cultures, cadherin-mediated cell aggregation results in the formation of compacted cell masses, especially when the cells are cultured for a long period (e.g., overnight) in a nutritious medium. In the initial phase of the cadherin-mediated cell aggregation (e.g., during the first 2 hr after inoculation), cell aggregates often grow in a one-dimensional, branching pattern. Even in such aggregates, mutual cell-cell contacts are very tight. In principle, the cells in a pair, bound together by cadherins, tend to maximize their contact area by spreading onto one another. On the other hand, when only CIDS is active, cells are clustered maintaining their original round shape. This is the principal morphological difference between the cadherin-dependent and -independent cell-cell contacts.

Time Considerations

Short-term aggregation assays can be completed in <1 day; long-term assays require 1 to several days.

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Analyzing Integrin-Dependent Adhesion

UNIT 9.4

This unit describes methods for the analysis of integrin-ligand binding in both cell-based assays (see Basic Protocol 1) and solid-phase assays (see Basic Protocol 2). A major application of cell adhesion assays is in investigating whether a certain cell type can adhere to a specific adhesive substrate, and, if so, which receptors are involved. Particularly if the substrate is a matrix component (e.g., fibronectin), members of the integrin family are likely to play a dominant role in adhesion. Procedures are described here for assessing which integrins are involved in this process. A detailed analysis of ligand recognition by individual integrins can be performed using a solid-phase receptor-ligand binding assay. The unit also contains support protocols for integrin purification (see Support Protocol 1) and coupling of antibodies to Sepharose for use in this purification (see Support Protocol 2), as well as for biotinylation of integrin ligands to be used in the solid-phase assay (see Support Protocol 3).

ANALYZING INTEGRIN-DEPENDENT ADHESION IN CELL-BASED ASSAYS

BASIC
PROTOCOL 1

Cell-based assays for integrin-dependent adhesion are conducted in the same manner as the spreading and attachment assays described in *UNIT 9.1*, except that antibodies to integrins (see Table 9.4.1), peptides, or other reagents are included to identify the integrin(s) and characterize the molecular events associated with adhesion. Function-blocking monoclonal antibodies are the most useful reagents for these analyses because of their high degree of specificity. If only a single integrin is involved in mediating adhesion, antibodies to either the α or β subunit should abrogate cell adhesion. For example, antibodies to either $\alpha 5$ or $\beta 1$ should completely block HT-1080 fibrosarcoma cell attachment and spreading on fibronectin, showing that this interaction is mediated solely by $\alpha 5\beta 1$ (Akiyama et al., 1989). If multiple integrins are involved, a combination of monoclonal antibodies will be required to block adhesion (Mould et al., 1990, 1994). Although peptides and other inhibitors have, in general, less specificity than monoclonal antibodies, they can be useful for determining what amino acid sequence in the ligand is recognized by the integrin. For example, CS1 peptide inhibits A375-SM melanoma cell attachment to fibronectin, demonstrating that the CS1 sequence is involved in adhesion to this substrate.

Materials

Adhesion molecule of interest

Dulbecco's phosphate-buffered saline (DPBS, Life Technologies; also see *APPENDIX 2A*)

10 mg/ml heat-denatured BSA solution (*UNIT 9.1*)

Cells of interest

DMEM/HEPES: Dulbecco's modified Eagle medium (Life Technologies; *APPENDIX 2B*) with 25 mM HEPES, gassed with 5% to 10% CO₂

Inhibitor: integrin-specific monoclonal antibody (see Table 9.4.1) or peptide, dissolved in DPBS at appropriate concentration

96-well tissue culture microtiter plate

Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*) and spreading or attachment cell-substrate adhesion assays (*UNIT 9.1*)

1. Prepare the 96-well plate by coating the wells with the adhesion molecule of interest diluted to the appropriate concentration in DPBS and blocking with 10 mg/ml heat-denatured BSA solution (see *UNIT 9.1*, Basic Protocol 1, steps 1 to 4).

Cell Adhesion

9.4.1

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Supplement 15

Unless the level of adhesion is low, use a concentration of adhesion molecule that promotes 50% to 70% maximal adhesion for coating the wells.

2. Prepare a suspension of the cells of interest at a density of 2×10^5 cells/ml in warm DMEM/HEPES gassed with 5% to 10% CO₂. Incubate cells in an open tube 10 min at 37°C in a CO₂ incubator.
- 3a. *For cell spreading assay:* Remove BSA solution from the 96-well plate, wash the wells with 100 µl DPBS, and add 50 µl DPBS containing the inhibitor at twice the desired final concentration to each well. Add 50 µl cell suspension to each well.

Table 9.4.1 Monoclonal Antibodies Suitable for Use in Cell Adhesion Assays

Integrin subunit ^a	Ligand ^b	mAb ^c	Function-blocking	Supplier
α1	COL/LN	5E8D9	Yes	Upstate Biotechnology
		TS2/7	No	Serotec
α2	COL/LN	Gi9	Yes	Beckman Coulter
		16B4	No	Serotec
α3	FN/COL/LN	P1B5	Yes	Chemicon
		M-Kid2	No	Beckman Coulter
α4	FN/VCAM-1	HP2/1	Yes	Serotec
		44H6	No	Serotec
α5	FN	JBS5	Yes	Serotec
		VC5	No	Pharmingen
		mAb 16	Yes	K.M. Yamada ^d
α6	LN	GoH3	Yes	Serotec
		4F10	No	Serotec
α9	TN	Y9A2	Yes	Chemicon
		β1	4B4	Yes
αL	ICAM-1, -2, -3	K20	No	Beckman Coulter
		mAb 13	Yes	K.M. Yamada ^d
		MHM 24	Yes	Dako
αM	FG, ICAM-1	BV17	No	Chemicon
		ICRF 44	Yes	Serotec
β2	VN/FN/FG/TSP	LM11	No	Chemicon
		MHM 23	Yes	Dako
αV	VN/FN/FG/TSP	69-6-5	Yes	Beckman Coulter
		P3G8	No	Chemicon
β3		SZ21	Yes	Beckman Coulter
		PM6/13	No	Serotec
β4		ASC-3	Yes	Chemicon
		450-90	No	Serotec
β5		P1F6	Yes	Life Technologies

^aNote that β1 associates with α1-α9, β2 associates with αL and αM, β1, β3, and β5 associate with αV, and β4 associates with α6; β2 integrins are expressed only on leukocytes, but most other integrins are widely expressed.

^bAbbreviations: COL, collagen; FG, fibrinogen; FN, fibronectin; ICAM, intercellular adhesion molecule; LN, laminin; TN, tenascin; TSP, thrombospondin; VCAM, vascular cell adhesion molecule; VN, vitronectin.

^cAll mAbs, with the exception of GoH3, mAb 16, 69-6-5, and mAb 13, are mouse anti-human. GoH3, mAb 16, 69-6-5 and mAb 13 are rat anti-human. See above list of suppliers for antibodies to other species. Antibodies should be stored in small aliquots at -70°C.

^dAvailable by Materials Transfer Agreement from K.M. Yamada, National Institutes of Health.

- 3b. *For cell attachment assay:* Mix an aliquot of the cell suspension with an equal volume of DPBS containing the inhibitor at twice the final concentration. Incubate 15 to 30 min at 37°C. Remove BSA solution from the 96-well plate, wash the wells with 100 μ l DPBS, and add 100 μ l of the cell/inhibitor mixture to each well.

In the authors' experience, a final mAb concentration of 10 μ g/ml, or 1:500 dilution of ascites, gives a maximal inhibitory effect. However, the concentration of mAb to achieve a maximal level of inhibition should be tested using a range of mAb concentrations. An important consideration is that the mAbs should be azide-free. Many commercially available mAbs contain sodium azide as a preservative. This is toxic to all cells and will inhibit attachment and spreading. Therefore it is essential that the sodium azide be removed by dialysis into an appropriate buffer such as DPBS.

Peptides can be used in place of mAbs to analyze the contribution of integrins to cell adhesion. However, these are generally less useful than mAbs because only for a small number of integrins have specific inhibitor peptides been described. Examples are CS1 (DELPQLVTLPHPNLHGPEILDVPST) for α 4 β 1 and α 4 β 7, GACRRETAWACGA for α 5 β 1, and KQGADV for α IIb β 3. RGD peptides (e.g., GRGDS) are of broad specificity and inhibit α 5 β 1, α IIb β 3, and all α V integrins. However, RGD peptides may be useful as a "first screen" to determine if receptors of this class are important for adhesion, or alternatively to confirm their involvement if cell adhesion is inhibited by, for example, mAbs to α 5 or α V. Concentrations of peptides required to inhibit cell adhesion are generally higher than for mAbs: typically 0.1 to 1 mg/ml. Higher concentrations are normally required for inhibiting cell attachment than for inhibiting cell spreading. GRGDS, KQGADV, and CS1 peptides are available from Bachem.

4. Incubate, fix, and analyze for spreading assay (see UNIT 9.1, Basic Protocol 1, step 8 to 13) or for attachment assay (see UNIT 9.1, Basic Protocol 2, steps 8 to 18).

It is essential that the effect of each mAb on cell attachment or spreading be compared with appropriate controls. The best controls to use are mAbs that are noninhibitory against the integrins under test—e.g., K20 for β 1 (see Table 9.4.1). However, if these are not available, the following controls can be used: (1) wells to which no mAbs are added (DPBS only); (2) nonimmune mouse or rat IgG or ascites; (3) irrelevant isotype-matched mAb controls. Control peptides (e.g., GRGES) should be used in parallel with the authentic peptides to exclude any toxic or nonspecific effects.

If a partial inhibitory effect is observed with several different anti-integrin mAbs, the assay can be repeated using two or more mAbs in combination to analyze the relative contribution of each integrin to cell adhesion.

ANALYZING INTEGRIN-LIGAND INTERACTIONS IN SOLID-PHASE ASSAYS

This protocol describes a simple integrin-ligand binding assay in which the integrin is adsorbed to the wells of an ELISA plate. The plate is blocked using BSA (to reduce nonspecific binding of the ligand) and then biotin-labeled ligand is added. After washing to remove unbound ligand, bound ligand is detected by addition of an avidin-peroxidase conjugate followed by a colorimetric detection step. An example of an application of this procedure would be use of biotinylated fibronectin or fibronectin fragments as ligands for the integrin α 5 β 1.

Materials

- Purified integrin (see Support Protocol 1)
- Dulbecco's phosphate-buffered saline (DPBS, Life Technologies; also see APPENDIX 2A)
- Blocking solution (see recipe)
- Biotin-labeled ligand (see Support Protocol 3)
- Binding buffer (see recipe)

BASIC PROTOCOL 2

Cell Adhesion

9.4.3

ExtrAvidin peroxidase reagent (Sigma)
ABTS reagent (see recipe)
2% (w/v) SDS in water (APPENDIX 2A)
ELISA plate (e.g., Immulon 1B or 4HBX; Dynex Technologies)
Plastic film (e.g., Nescofilm, Parafilm, Saran wrap)
Multichannel pipettor
21-G hypodermic needle
Side-arm flask
Microtiter plate reader

Coat ELISA plate with integrin

1. Dilute purified integrin to ~1 $\mu\text{g/ml}$ with DPBS.

At least a 50-fold dilution is required, otherwise the detergent present in the purified integrin interferes with adsorption to the plate.

2. Add the diluted integrin to the wells of an ELISA plate (100 $\mu\text{l/well}$). Leave a set of wells empty for measuring binding of the ligand to BSA.

The authors normally perform the assay using 4 to 6 replicates for each sample.

Immulon 1B and 4 HBX ELISA plates (Dynex) are suitable. More recently, the authors have found that one-half area EIA/RIA plates (Costar) also work well, and have the advantage that similar results can be obtained with half as much integrin (i.e., 50 $\mu\text{l/well}$)

3. Wrap the plate in plastic film and store overnight at room temperature.

Alternatively, the plate can be stored for up to 1 week at 4°C.

Block the ELISA plate

4. Add 25 μl blocking solution to each integrin-containing well using a multichannel pipettor, then remove the solution by aspiration using a 21-G hypodermic needle attached by tubing to a side-arm flask connected to a vacuum source, or by inverting the plate over a sink and flicking out the liquid.

A small amount of blocking solution is added to the wells before aspirating the integrin solution because the authors have found that this renders the wells hydrophilic and prevents them from drying out when they are aspirated. Drying out of the wells destroys the activity of some of the integrin.

5. Add 200 μl blocking solution to each well (including those used for testing binding to BSA alone) using a multichannel pipettor. Leave the plate at room temperature for 1 to 3 hr, then aspirate or flick out the blocking solution.
6. Add 200 μl of binding buffer to each well using a multichannel pipettor. Remove the buffer by aspirating or flicking it out. Repeat two times.
7. Remove residual liquid by inverting the plate and striking it hard several times onto adsorbent paper towels.

Add biotin-labeled ligand

8. Dilute the biotin-labeled ligand in binding buffer to the appropriate concentration. Add 100 μl of this solution to each experimental well.

The appropriate concentration must be determined by pilot experiments. A concentration of 0.1 $\mu\text{g/ml}$ works well for biotinylated 80-kD fragment of fibronectin (Mould et al., 1995a).

Other reagents (e.g., mAbs, peptides, or synthetic compounds) can be added simultaneously with the ligand at this stage to test for their effects on ligand binding.

9. Cover the plate with plastic film and incubate 3 hr at 37°C.

A cell culture incubator is suitable for this incubation.

10. Aspirate solutions from the wells to remove unbound ligand.

11. Wash wells three times with 200 µl binding buffer (see step 6). Remove the residual buffer (see step 7).

Detect bound biotin-labeled ligand

12. Dilute ExtrAvidin-peroxidase reagent 1:500 in binding buffer. Add 100 µl of the diluted ExtrAvidin-peroxidase reagent to each well using a repeating pipettor. Incubate the plate 10 to 15 min at room temperature. During this time prepare the ABTS reagent.

13. Aspirate solutions from wells to remove unbound ExtrAvidin peroxidase reagent.

14. Wash wells two times with 200 µl of binding buffer and then two times with 400 µl of binding buffer. Remove the residual buffer (see step 7).

15. Add 100 µl of ABTS reagent to each well using a repeating pipettor. Allow the reaction to proceed until a strong (but not dark) green color is obtained (typically 10 to 30 min).

16. Stop the reaction by adding 100 µl of 2% SDS solution to each well using a repeating pipettor.

17. Read the plate using an automatic microtiter plate reader at 405 nm.

18. Calculate the mean and standard deviation of the absorbance readings for ligand binding to integrin, and for ligand binding to BSA alone, using the following equations.

net binding to integrin = (mean of absorbance readings for ligand binding to integrin) –
(mean of absorbance readings for ligand binding to BSA alone)

$$\text{standard deviation of net binding} = \sqrt{(\text{standard deviation of absorbance readings for ligand binding to integrin})^2 + (\text{standard deviation of absorbance readings for ligand binding to BSA alone})^2}$$

INTEGRIN PURIFICATION

This protocol describes the affinity purification of the integrin $\alpha 5 \beta 1$ from human placenta using anti- $\beta 1$ and anti- $\alpha 5$ mAbs. The procedure can be adapted to purify other integrins from different tissue sources or from pellets of cultured cells. If one is starting from cultured cells, typically enough cells should be used to give at least 10 ml of packed cells, and the volumes of reagents and resins should be scaled down 2- to 4-fold.

Materials

Human placenta (from maternity unit of local hospital; process within a few hours of delivery)

Homogenization buffer (see recipe)

1% (w/v) Virkon (Merck) in water

**SUPPORT
PROTOCOL 1**

Cell Adhesion

9.4.5

Extraction buffer (see recipe), 4°C
 Sepharose 4B resin (Sigma)
 Rat IgG–Sepharose resin (see Support Protocol 2)
 mAb 13 (anti-β1)–Sepharose and mAb 16 (anti-α5)–Sepharose (see Support Protocol 2)
 Wash buffer (see recipe), 4°C
 Elution buffer (see recipe), 4°C
 1 M Tris·Cl, pH 8.2 (APPENDIX 2A; store up to 6 months at 4°C), 4°C
 0.1 M Tris·Cl, pH 8.3 (APPENDIX 2A)/0.1% (w/v) Triton X-100 (Ultra grade, Sigma), 4°C (store up to 3 months at 4°C)
 Phosphate-buffered saline (PBS; prepare using 10× stock solution from Life Technologies) containing 0.05% (w/v) sodium azide (add from 20% w/v sodium azide stock in H₂O)
 PBS (Life Technologies)
 5× SDS-PAGE sample buffer (see recipe)
 6% SDS-PAGE gel (UNIT 6.1)
 Large scissors
 Blender
 Beckman J6-B centrifuge with JA-10 and JA-20 rotors (or equivalent refrigerated centrifuge)
 500-ml polycarbonate centrifuge bottles (Nalgene)
 50-ml polyallomer centrifuge tubes (Nalgene)
 Econo-Pac 20-ml disposable polypropylene columns (Bio-Rad)
 50-ml screw-top polypropylene tubes (Becton Dickinson Labware)
 Rotating platform (Cole-Parmer)
 1.6 × 20-cm C16 column (Amersham Pharmacia Biotech)
 Peristaltic pump
 Fraction collector
 0.8-cm diameter Poly-Prep 2-ml disposable polypropylene column (Bio-Rad)
 Additional reagents and equipment for SDS-PAGE and staining of gels (UNIT 6.1)

CAUTION: Human placenta should be treated as potentially biohazardous; take suitable precautions such as wearing latex gloves, eye protection, and a lab coat. The homogenization should be performed in a primary cell culture cabinet and any spillage of homogenate or extract should be treated with 1% Virkon. Centrifuge bottles and tubes should be soaked in 1% Virkon after use.

Homogenize placenta

1. Cut the placenta (minus umbilical cord and amniotic membranes) into small chunks using large scissors, and place the pieces in a blender with ~500 ml of cold homogenization buffer. Homogenize the placenta using a moderate speed for ~1 min. Pour the homogenate into 500-ml polycarbonate centrifuge bottles and store at –70°C until required (up to 2 years).

One placenta yields ~1 liter of homogenate. Commercially available laboratory homogenizers may be used, but a robust domestic blender is adequate for this purpose.

Extract the homogenate

2. Thaw the homogenate in a cold room (preferable) or at room temperature overnight.
Perform all subsequent operations at 4°C where possible.
3. Centrifuge the homogenate 10 min at 4400 × g (5000 rpm in a JA-10 rotor), 4°C. Discard the supernatant into a bucket containing 5 liters of 1% Virkon, and resuspend

the pellets in 600 ml of homogenization buffer. Centrifuge again under the same conditions and discard the supernatant as described.

Most of the soluble proteins have been removed at this stage. The next step uses detergent (Triton X-100) to solubilize proteins (including integrins) from cell membranes. The extraction buffer contains protease inhibitors and BSA to minimize proteolytic degradation of the integrins.

4. Extract pellet by adding 400 ml extraction buffer to the centrifuge bottle and keeping on ice for ~1 hr, shaking vigorously every few minutes to ensure that the pellets are fully resuspended.

The extract obtained will be red in color because not all of the hemoglobin has been removed in step 3.

5. Centrifuge the extract 10 min at $6400 \times g$ (6000 rpm in a JA-10 rotor), 4°C. Pipet the supernatant into 50-ml centrifuge tubes, then centrifuge 30 min at $48,000 \times g$ (20,000 rpm in a JA-20 rotor), 4°C.

Purify the $\beta 1$ integrins

6. Pack a 20-ml disposable column with 4 ml of Sepharose 4B by pouring 8 ml of a 50% suspension of Sepharose 4B into the column. Preclear and filter the supernatant from step 5 by passing it through the column and collecting the flowthrough.

Several columns can be used simultaneously to speed up this step.

7. Mix the flowthrough with 8 ml of a 50% suspension of rat IgG–Sepharose in screw-top 50-ml polypropylene centrifuge tubes and agitate for 2 hr on a rotating platform. Remove the rat IgG–Sepharose by pouring through 20-ml disposable columns (filtration is accomplished by the fritted disc at the bottom of the column).

This step removes proteins that bind to rat IgG. If a murine mAb is being used in the subsequent purification steps mouse IgG–Sepharose should be used in place of rat IgG–Sepharose. The authors normally discard the rat IgG–Sepharose at this stage.

8. Mix the flowthrough from step 7 with 8 ml of mAb 13–Sepharose in 50-ml polypropylene tubes and agitate for 2 hr on a rotating platform. Recover the mAb 13–Sepharose by pouring through a 20-ml disposable column. Retain the flowthrough and store at 4°C.
9. Resuspend the mAb 13-Sepharose in wash buffer, pack the suspension into a 1.6×20 -cm C16 column, and wash the column overnight at ~10 ml per hr with wash buffer, delivered via a peristaltic pump.
10. Elute integrin by passing elution buffer through the column at 45 ml/hr for 30 min. During this time collect 2-min (1.5-ml) fractions (using a fraction collector) into tubes to which 0.5 ml of 1 M Tris·Cl, pH 8.2, has been added. Mix the fractions with this buffer as they elute from the column to ensure prompt neutralization. Store fractions at 4°C.
11. Neutralize the column immediately with 20 ml of 0.1 M Tris·Cl (pH 8.3)/0.1% Triton X-100 at a flow rate of 45 ml/hr. Reequilibrate the column with PBS/0.05% sodium azide and store the mAb 13–Sepharose (removed from the column) at 4°C.

Because of the low pH used to elute the integrin, the mAb 13–Sepharose gradually deteriorates in its capacity for integrin purification. However, in our experience the column can be reused about ten times before replacement is necessary.

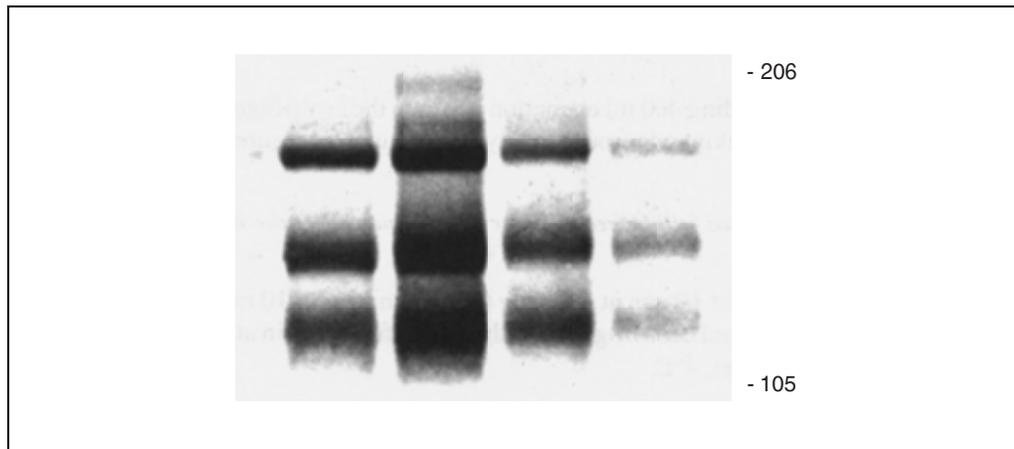


Figure 9.4.1 SDS-polyacrylamide gel of sequential fractions from low-pH elution of mAb 13 column. The major bands observed are (from top) $\alpha 1$ subunit, other α subunits, and $\beta 1$ subunit. The migration positions of molecular weight standards are indicated (in kDa).

Analyze the purified $\beta 1$ integrins

12. To 25- μ l aliquots of the fractions, add 25 μ l 1 \times PBS and 12.5 μ l of 5 \times SDS-PAGE sample buffer to each aliquot. Heat the samples in a boiling water bath for 3 min and run on a 6% SDS-polyacrylamide gel (UNIT 6.1).
13. Stain the gel with Coomassie blue for 1 hr (UNIT 6.1). Destain the gel and check for elution of integrin. A typical elution profile is shown in Figure 9.4.1.

The flowthrough from step 8 can be reapplied to the mAb 13–Sepharose, after any precipitate has been removed by centrifuging for 10 min at 4400 \times g (5000 rpm in a JA-10 rotor), 4°C (follow step 8 onwards; elute and neutralize the column before chromatographing the flowthrough a second time, then pool the $\beta 1$ integrin fractions; repeat until the yield of $\beta 1$ integrins is markedly lower than from the first purification). In the authors' experience, the mAb 13 purification step needs to be repeated several times before all the $\beta 1$ integrins have been depleted from the extract.

Purify the integrin $\alpha 5\beta 1$

14. Pool the fractions from step 10 that contain the purified $\beta 1$ integrin. Centrifuge the pooled fractions 10 min at 48,000 \times g (20,000 rpm in a JA-20 rotor), 4°C. Mix the supernatant with 2 ml of mAb 16–Sepharose for 2 hr on a rotating platform. Pack the suspension into a 0.8-cm diameter Poly-Prep 2-ml disposable column and wash with 12 ml of wash buffer.
15. Elute $\alpha 5\beta 1$ with 5 ml of elution buffer, added in 0.4-ml aliquots. Collect 0.4-ml fractions in 1.5-ml microcentrifuge tubes containing 0.1 ml of 1 M Tris-Cl, pH 8.2. Mix the fractions with this buffer as they elute from column to ensure prompt neutralization. Store fractions up to 2 years at -70°C .
16. Neutralize the column with 5 ml of 0.1 M Tris-Cl (pH 8.3)/0.1% (w/v) Triton X-100. Re-equilibrate the column with PBS/0.05% sodium azide and store the mAb 16–Sepharose at 4°C.

Analyze the purified $\alpha 5\beta 1$

17. Analyze 25- μ l aliquots of the fractions by SDS-PAGE as described in steps 12 and 13.

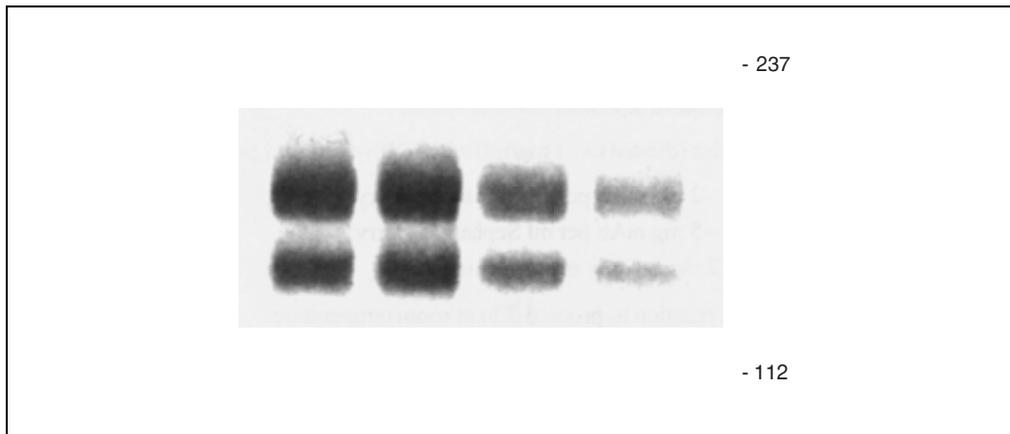


Figure 9.4.2 SDS-polyacrylamide gel of sequential fractions from low-pH elution of mAb 16 column. The major bands observed are (from top) $\alpha 5$ subunit and $\beta 1$ subunit. The migration positions of molecular weight standards are indicated (in kDa).

The major bands detected by Coomassie blue staining should be those corresponding to expected positions of the $\alpha 5$ and $\beta 1$ subunits (Fig. 9.4.2). The molecular weights are ~ 150 kDa for $\alpha 5$ and ~ 130 kDa for $\beta 1$.

COUPLING OF ANTIBODIES TO SEPHAROSE

Suitable mAbs for purification may be available in-house or can be generated using published protocols (e.g., Akiyama et al., 1989). Some anti-integrin hybridomas are available from cell culture collections (e.g., ATCC). The authors have found that the anti- $\beta 1$ mAb 13 and the anti- $\alpha 5$ mAb 16 are particularly suitable for affinity purification of $\alpha 5\beta 1$. Both mAbs are available from K.M. Yamada, National Institutes of Health.

Materials

Antibodies/IgG to be coupled: anti- $\beta 1$ and anti- $\alpha 5$ mAbs (e.g., mAb 13 and mAb 16) and rat IgG (Sigma)

Coupling buffer: 0.5 M NaCl/0.1 M NaHCO₃ (store up to 6 months at room temperature)

CNBr-activated Sepharose (Sigma)

1 mM HCl

1 M ethanolamine in H₂O (store up to 6 months at room temperature)

Acetate wash buffer: 0.1 M sodium acetate, pH 4 (adjust with glacial acetic acid)/0.5 M NaCl (store up to 6 months at room temperature)

Tris wash buffer: 0.1 M Tris·Cl, pH 8 (APPENDIX 2A)/0.5 M NaCl (store up to 6 months at room temperature)

Phosphate-buffered saline (PBS; prepare using 10 \times stock solution from Life Technologies) containing 0.05% (w/v) sodium azide (add from 20% sodium azide stock solution in H₂O)

PBS (Life Technologies)

Buchner funnel with medium-porosity fritted-glass disc

Conical flask with side arm

50-ml screw-top polypropylene centrifuge tubes

Rotating platform (Cole-Parmer)

Additional reagents and equipment for dialysis (APPENDIX 3)

SUPPORT PROTOCOL 2

Cell Adhesion

9.4.9

1. Dialyze the antibodies (if necessary) into an amine-free buffer (e.g., PBS or coupling buffer). Measure the absorbance of an aliquot of the dialyzed mAb at 280 nm. Dilute or concentrate antibodies to ~1 mg/ml

Dialysis is only necessary where the mAb is supplied in amine-containing buffer (e.g., Tris).

2. Pre-swell the CNBr-activated Sepharose in ~50 ml of 1 mM HCl. Wash with ~200 ml of 1 mM HCl per ml of resin in a Buchner funnel. Prepare a 50% resin slurry in PBS.

3 g of CNBr-activated Sepharose swells to ~10 ml.

3. Mix the antibodies (diluted to ~1 mg/ml) with CNBr-activated Sepharose as follows.
 - a. For mAb 13: ~2 mg mAb per ml Sepharose slurry
 - b. For mAb 16: ~5 mg mAb per ml Sepharose slurry
 - c. For rat IgG: 2 mg IgG per ml Sepharose slurry.

Allow coupling reaction to proceed 2 hr at room temperature.

4. Following the coupling reaction, add the mixture to a Buchner funnel attached to a conical flask with side arm and remove the supernatant by vacuum filtration. Determine the absorbance at 280 nm for an aliquot of the recovered supernatant and compare to the starting value according to the following equation, where D is the dilution factor (total volume divided by volume of starting solution).

$$\text{coupling efficiency} = \left(1 - \frac{A_{280} \text{ of filtrate} \times D}{A_{280} \text{ of starting solution}} \right) \times 100\%$$

Typical coupling efficiencies are 95% to 99%.

5. Wash the Sepharose in the Buchner funnel with 20 ml of coupling buffer and remove all buffer under suction. Remove the Sepharose from the funnel using a spatula, place it in a 50-ml conical polypropylene centrifuge tube with 20 ml of 1 M ethanolamine, and incubate 1 hr at room temperature on a rotating platform

The buffer for washing may be added in several aliquots if the capacity of the funnel is low.

6. Return the Sepharose to the Buchner funnel and wash with three alternating applications, 50 ml each, of acetate wash buffer and Tris wash buffer.

7. Wash the Sepharose with 20 ml of PBS and store in PBS/0.05% sodium azide at 4°C.

The buffer for washing may be added in several aliquots if the capacity of the funnel is low.

SUPPORT PROTOCOL 3

BIOTINYLATION OF INTEGRIN LIGANDS

This protocol describes labeling of ligands for in vitro integrin-binding assays (see Basic Protocol 2). Biotin is covalently conjugated to amino groups on the ligand to permit quantification of bound ligand.

Materials

Ligand of interest

Coupling buffer 0.5 M NaCl/0.1 M NaHCO₃ (store up to 6 months at room temperature)

Sulfo-NHS Biotin (Pierce)

Tris/saline: 25 mM Tris·Cl (pH 7.4)/150 mM NaCl

Tris/saline containing 0.05% sodium azide
Rotating platform (Cole-Parmer)

Additional reagents and equipment for dialysis (*APPENDIX 3*) and protein assay
(*APPENDIX 3*)

1. Dialyze ligand into 1 liter of coupling buffer (*APPENDIX 3*) for at least 2 hr at room temperature.

About 0.5 ml of ligand at a concentration of ~0.5 mg/ml gives sufficient material for a large number of assays.

2. Add an equal mass of sulfo-NHS-biotin (~0.25 mg) to the dialysate in a 1.5-ml microcentrifuge tube and mix on a rotating platform 30 min at room temperature.

For some proteins, sulfo-NHS-LC-biotin (Pierce) gives higher signals than sulfo-NHS-biotin in the solid phase assay.

3. Dialyze the solution against two changes of 1 liter Tris/saline, and once against 1 liter of Tris/saline, 0.05% azide (at least 2 hr per dialysis) at room temperature.

4. Microcentrifuge the dialysate for 15 min in a 1.5-ml microcentrifuge tube at maximum speed, room temperature.

This removes any large aggregates or precipitate from the solution.

5. Measure the concentration of biotinylated protein in the supernatant using, for example, the BCA assay (*APPENDIX 3*). Store up to 6 months at 4°C.

Alternatively, many biotinylated proteins can be stored in aliquots at -70°C. This procedure will typically provide sufficient material for >100 $\alpha 5\beta 1$ fibronectin assays. How much material will be required for any assay will depend upon the affinity of the integrin-ligand interaction.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

ABTS reagent

ABTS buffer:

0.05 M Na₂HPO₄

0.1 M sodium acetate

Adjust pH to 5.0 using concentrated HCl

Store up to 3 months at room temperature

ABTS reagent: Immediately before assay, dissolve 11 mg of 2,2'-azinobis(3-ethylbenzthiazoline)sulfonic acid (ABTS; Sigma) in 0.5 ml water. Mix 67 μ l of 30% H₂O₂ with 7 ml water. Add 0.5 ml of this ABTS solution to 10 ml ABTS buffer (see above) and 100 μ l H₂O₂ solution. Mix thoroughly.

This amount of reagent is sufficient for one full 96-well plate assay.

Binding buffer

Mix the following components in the order indicated:

150 mM NaCl

25 mM Tris-Cl, pH 7.4 (*APPENDIX 2A*)

1 mM MnCl₂

0.1% (w/v) BSA (fraction V; Sigma, 99% pure)

Prepare fresh

This is conveniently prepared from Tris/saline [25 mM Tris-Cl (pH 7.4)/150 mM NaCl] and a stock solution of 1 M MnCl₂. BSA is then added.

Blocking solution

Prepare a solution of 25 mM Tris·Cl containing 150 mM NaCl. Add sufficient 20% sodium azide stock for a final concentration of 0.05% (w/v). Add BSA (fraction V; Sigma, 98% pure) for a final concentration of 5% (w/v) and dissolve by vigorous stirring. Centrifuge the solution in 50-ml centrifuge tubes for 5 min at 2800 × g (4000 rpm in a JA-10 rotor), and filter the supernatant through a 20-ml disposable column. Store up to 3 months at 4°C.

This is conveniently prepared from Tris-saline [25 mM Tris·Cl (pH 7.4)/150 mM NaCl] to which sodium azide is added from a 20% stock solution. BSA is then added and dissolved by vigorous stirring. The solution is then centrifuged and filtered as above. Final concentrations are 150 mM NaCl; 25 mM Tris·Cl, pH 7.4, 5% (w/v) BSA, and 0.05% (w/v) sodium azide.

Elution buffer

10 mM sodium acetate, pH 3.25
1 mM CaCl₂
1 mM MgCl₂
0.1% (w/v) Triton X-100 (Sigma, Ultra grade)
Store up to 1 month at 4°C

Extraction buffer

150 mM NaCl
25 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
2% (w/v) Triton X-100 (Sigma, Ultra grade)
1 mM PMSF (100 mM stock solution prepared in isopropanol; also see APPENDIX 1B)
10 µg/ml leupeptin (1 mg/ml stock solution prepared in water; also see APPENDIX 1B)
2 mg/ml BSA (fraction V; Sigma, 98% pure)
Prepare fresh, then cool on ice

Homogenization buffer

150 mM NaCl
25 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
0.005% (w/v) digitonin
Store for up to 3 months at 4°C

SDS-PAGE sample buffer, 5×

25% (v/v) glycerol
125 mM Tris·Cl, pH 6.8 (APPENDIX 2A)
10% (w/v) SDS
0.1% (w/v) bromophenol blue
Store indefinitely at 4°C. Warm in a hot water bath and mix well before use.

Wash buffer

150 mM NaCl
25 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
1 mM CaCl₂ (add from 1 M stock)
1 mM MgCl₂ (add from 1 M stock)
0.1% (w/v) Triton X-100 (Sigma, Ultra grade)
Store up to 1 month at 4°C

This is conveniently prepared from Tris-saline [25 mM Tris·Cl (pH 7.4)/150 mM NaCl] and stock solutions of 1 M CaCl₂ and 1 M MgCl₂.

COMMENTARY

Background Information

Monoclonal antibodies were of crucial importance in the initial identification of cell surface receptors that mediated adhesion of cells to extracellular matrix components (e.g., Wayner and Carter, 1987). A wide range of well characterized anti-integrin mAbs are now commercially available for use in cell attachment and spreading assays. The choice of which anti-integrin mAbs to use is determined in part by the substrate. For example, if collagen type I is the substrate, anti- α 1 and anti- α 2 mAbs should be tested, as these are the likely integrins involved. The complement of integrins expressed by the cell also helps to determine which mAbs should be tested. If the profile of integrin expression is unknown, it can be determined by flow cytometry or by immunoprecipitation of surface-labeled cells. Conversely, if a mAb blocks cell adhesion, it is important to demonstrate that the corresponding integrin subunit is expressed by the cells.

Historically, the first purification of an integrin receptor was a major advance in understanding the molecular basis of cell-matrix interactions (Pytela et al., 1985). This and later methods employed a ligand affinity column as the major purification procedure. In particular, fibronectin fragments have been used to purify α 5 β 1, and RGD peptides have been used to purify α V β 3 and α IIb β 3 (Pytela et al., 1987; Smith and Cheresch, 1988; Yamada and Yamada, 1990). Ligand affinity columns remain the method of choice where mAbs are unavailable. However, the use of mAbs has the advantage that the purification is more specific (e.g., several different integrins bind to fibronectin affinity columns) and higher yields of integrins can be obtained. Multiple β 1 integrins can also be obtained from the same source. For example, other β 1 integrins from human placenta that are present in the flowthrough from the mAb 16 column can be purified using other specific anti- α mAbs. For some assays, and particularly where one β 1 integrin predominates in the tissue or cell extract, it may be sufficient to purify the total β 1 integrins and use this partially purified preparation in the solid-phase assay. For example, β 1 integrins purified from MOLT-4 cells (Newham et al., 1998) contain ~75% α 4 β 1, the remainder being α 5 β 1. The protocol described here can be adapted to purify other integrins from other tissues or from pellets of cultured cells.

The major advantage of assays using purified integrins, as compared to cell-based assays, is that integrin-ligand binding can be studied in isolation. Integrin clustering, signaling, and cytoskeletal interactions are all known to affect the strength of adhesion in cell-based assays. Furthermore, adhesion may be modulated by indirect effects (e.g., by signaling from other cell-surface receptors). Although doubts are often expressed as to whether plastic-adsorbed integrin is representative of integrin in its native environment, a number of careful studies have shown no significant differences between the behavior of integrins in solid-phase assays and on cell surfaces. Hence, this approach has been broadly validated. The first solid-phase integrin-ligand binding assay was described by Charo et al. (1991) for studying fibrinogen binding to α IIb β 3. The α 5 β 1-fibronectin assay developed by the author of this unit is both extremely sensitive and highly versatile. For example, the author has described how the assay can be used to investigate the effects on ligand binding of divalent cations, activating and inhibitory mAbs, peptide inhibitors, and mutations (Mould et al., 1995a,b, 1996, 1997). Another important area in which this type of assay is finding use is in the pharmacological screening of inhibitors of integrin-ligand interactions. This assay can give information about the inhibitory potency of a compound and whether it is a direct competitive or allosteric inhibitor of ligand binding. The attenuation of mAb epitopes can also provide data on the location of the binding site of an inhibitor on the integrin (Mould et al., 1997).

The author's preferred method for labeling of integrin ligands is biotinylation, because of its safety and simplicity. One potential drawback is that if one or more lysyl residues in the ligand are crucial for integrin binding, their modification may render the ligand inactive. In this case, a possible solution may be to reduce the amount of biotinylation reagent so that some of the lysyl residues remain unmodified. Other labeling methods such as radioiodination can also be used. Alternatively, if the ligand is a recombinant protein, a "tag" such as an epitope sequence or the Fc region of IgG can be incorporated for use in the detection of bound ligand.

Table 9.4.2 Troubleshooting Guide for Problems Encountered in Solid-Phase Assays

Problem	Possible cause	Solution
High background binding to BSA-coated wells	Insufficient blocking of wells Ligand concentration too high	Block for longer time (e.g., overnight) Test range of ligand concentrations for optimal signal/background
Spuriously high signal in some wells	Insufficient washing Tops of wells contaminated	Follow washing protocol carefully Add reagents to the center of wells
Wide variation in signal in experimental wells	Integrin added to plate insufficiently mixed Wells aspirated before adding blocking reagent Plate contaminated, e.g., by dust	Mix diluted integrin thoroughly before coating the plate Add blocking reagent before removing coating solution Use fresh, clean plates
Low signal above background binding to BSA	Insufficient integrin Ligand concentration too low Inactive ligand	Use lower dilution of integrin Use higher concentration of ligand Check activity of ligand in cell-based assay

Critical Parameters and Troubleshooting

For cell-based assays, as described in *UNIT 9.1*, the health of the cells and careful preparation are important for achieving optimal spreading or attachment. In order to optimize the sensitivity of cell attachment or spreading to inhibition, the author recommends that a concentration of substrate be chosen that gives 50% to 70% of maximal adhesion (unless this value is low, in which case a concentration of substrate that gives near maximal adhesion should be used). If the level of adhesion is near maximal, inhibitors are less effective at blocking adhesion.

Failure of a mAb to inhibit spreading or attachment can normally be taken to mean that the integrin it recognizes is not involved in adhesion. However, it is important to check that the antibody is effective in a system where inhibition should be observed (e.g., an anti- $\alpha 5$ mAb should block spreading of HT-1080 fibrosarcoma cells on fibronectin). Conversely, if an antibody does inhibit, it is important to test that it does not inhibit adhesion to an inappropriate ligand (e.g., an anti- $\alpha 5$ mAb should not perturb HT-1080 cell spreading on collagen). Most of the antibodies described in Table 9.4.1 are well characterized and should not show any non-specific effects. However, as described earlier, it is essential that the antibodies not contain sodium azide.

It is also important to bear in mind that inhibition of adhesion can be caused by “cross-talk”—i.e., where ligation of one integrin (e.g., by peptide) indirectly affects the activity of a

second integrin via intracellular signaling (Diaz-Gonzalez et al., 1996). While it is often difficult to rule out such effects, they normally only cause a partial reduction in adhesion, whereas adhesion is frequently totally ablated by specific antibody or peptide inhibition. Finally, if anti-integrin mAbs or peptides do not completely block cell adhesion, it is possible that non-integrin receptors may play some role. This may be observed particularly in cell attachment assays. For example, cell-surface proteoglycans contribute to melanoma cell attachment to the heparin-binding domain of fibronectin (Mould et al., 1994).

For solid-phase assays, the specificity of the assay must be tested carefully. The most important test for specificity is the ability of unlabeled ligand to compete with labeled ligand for binding to the integrin. Hence, in the presence of a large excess of unlabeled ligand, very little binding of labeled ligand should be observed. Nearly all integrin-ligand interactions are divalent-cation dependent. Hence, replacing the Mn^{2+} in the binding buffer with EDTA should reduce binding to levels similar to that observed for BSA-coated wells. Further tests for specificity can be carried out. For example: (1) mAbs that are inhibitory in cell-based assays should also inhibit ligand binding in solid-phase assays, (2) mutations known to affect integrin binding sites should perturb ligand recognition, and (3) known ligand mimetics (e.g., RGD peptides for $\alpha 5\beta 1$ or CS1 peptide for $\alpha 4\beta 1$) should block ligand binding. All of these tests have been performed for the $\alpha 5\beta 1$ -fibronectin

Table 9.4.3 Troubleshooting Guide for Problems Encountered in Integrin Purification

Problem	Possible cause	Solution
Large number of proteins copurify with integrins	Inadequate preclearing or filtration of extract, or precipitate forms during purification procedures	Recentrifuge after preclearing and filtering extract on Sepharose 4B, or when any precipitate is visible
Small amounts of integrins purified	Insufficient mAb coupled to Sepharose Affinity of mAb too low Column has been used many times	Couple more mAb to Sepharose Use mAb with higher affinity Replace with fresh mAb-Sepharose
Integrin degraded	Insufficient levels of protease inhibitors in extraction buffer Extraction or other manipulations performed at too high a temperature	Increase levels of protease inhibitors and BSA in extraction buffer Perform all manipulations at 4°C or on ice

assay. Table 9.4.2 is a troubleshooting guide for solid-phase ligand-binding assays.

For integrin purification, it is essential to have sufficient tissue or cell pellets for the extraction and enough mAb-Sepharose to perform a successful purification. Table 9.4.3 is a troubleshooting guide for integrin purification.

Anticipated Results

For cell-based assays, in most cases, it is possible to obtain levels of spreading or attachment of >50%. It should be feasible using anti-integrin mAbs, either alone or in combination, to reduce the level of spreading or attachment to close to that seen on BSA.

For integrin purification, yields of $\alpha 5\beta 1$ are ~2 mg per placenta (estimated by Coomassie blue staining). Each full 96-well-plate solid-phase assay uses <10 μg of integrin. Hence, this is enough to perform a large number (~200) of solid-phase assays. In these assays, a strong positive signal ($\text{OD}_{405} \sim 1$) is normally achieved in ≤ 30 min, whereas background binding to BSA is generally very low ($\text{OD}_{405} \sim 0.1$).

Time Considerations

Cell attachment and spreading assays are quick to perform. The actual assays can be carried out in about half a day or less; however, particularly if many different mAbs or peptides are being tested, quantification of spreading assays can take several hours.

Purification of $\alpha 5\beta 1$ from placenta takes ~3 days in total. Allow an additional day for preparation of buffers and mAb-Sepharose. The solid-phase assay can be performed in 5 to 8 hr but only requires 1 to 2 hr of hands-on time. However, the assay needs to be planned in advance so that the appropriate number of wells can be coated with integrin overnight. Plates

can be coated with integrin several days in advance.

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Analysis of Cell-Cell Contact Mediated by Ig Superfamily Cell Adhesion Molecules

The calcium-independent cell adhesion molecules (CAMs) constitute a large family of cell surface molecules. A major group among these are the immunoglobulin superfamily (IgSF) molecules. IgSF-CAMs may be composed of immunoglobulin (Ig) folds only, Ig folds linked to fibronectin type III (FnIII) folds, or Ig folds linked to protein modules other than FnIII folds. The IgSF is a large protein superfamily comprising >100 proteins involved in cell-cell adhesion. Its members are found in vertebrates, invertebrates, and also in yeast. Most of the molecules of the IgSF are cell surface molecules that are membrane-anchored either by a single transmembrane segment or by a glycosylphosphatidylinositol (GPI) anchor that is posttranslationally attached to the C-terminus. Some of the IgSF-CAMs also occur in soluble form, e.g., in the cerebrospinal fluid or the vitreous fluid of the eye, due to a cleavage of the GPI-anchor or the membrane-proximal peptide segment. In some cases, such as NCAM, various forms may be generated by alternative splicing.

This unit provides protocols for the purification of IgSF-CAMs from tissue extracts and tissue culture supernatants and for the analysis of the adhesive functions of IgSF-CAMs with isolated molecules and in the cellular context. Following personal expertise, the authors have added a few frequently used functional assays demonstrating the role of IgSF-CAMs in neural development, such as neurite outgrowth from cultured neurons, and the use of antibodies for the inhibition of IgSF-CAM functions in vitro. The first group of protocols describe affinity purification of IgSF-CAMs (see Basic Protocol 1), preparation of the affinity column (see Support Protocol 1), solubilization of membrane proteins (see Support Protocol 2), transient transfection of HEK 293 cells to express IgSF-CAMs (see Support Protocol 3), and detection of IgSF-CAMs by dot blot analysis (see Support Protocol 4). Assays using fluorescent microspheres with coupled proteins are used for one type of functional analysis based either on interactions between microspheres (see Basic Protocol 2) or on interactions between microspheres and cultured cells (see Basic Protocol 3). There are two protocols for coupling proteins to microspheres: coupling proteins to fluorescent microspheres (see Support Protocol 5) and coupling proteins to glutaraldehyde-activated amino beads (see Support Protocol 6). A second group of protocols analyze the functions in cell-based assays. *Trans*-interactions are studied using IgSF-CAM-transfected myeloma cells (see Basic Protocol 4). This protocol requires stable transfection of myeloma cells (see Support Protocol 7). *Cis*-interactions are detected by chemical cross-linking (see Basic Protocol 5) and antibody co-capping (see Basic Protocol 6). IgSF-CAMs and other substrates have the ability to promote neurite outgrowth (see Basic Protocol 7), which requires coating of the growth surface with IgSF-CAM (see Support Protocol 8), nitrocellulose as a binder for the substrate of interest (see Support Protocol 9), poly-D-lysine (see Support Protocol 10), collagen (see Support Protocol 11), or laminin (see Support Protocol 12). Differential fixation protocols are used for fluorescent immunohistochemistry samples (see Support Protocol 13) or for morphological analyses (see Support Protocol 14). Finally, there is a protocol for assessing the effect of inhibiting CAM-CAM interactions in vitro (see Basic Protocol 8).

PREPARATION OF IgSF-CAMs

Purification of IgSF-CAMs by Immunoaffinity Chromatography

The best method for the purification of native, functionally intact proteins is certainly the use of standard chromatography, such as ion exchange, hydrophobic interaction, and gel permeation columns. However, the establishment of such a standard purification protocol can be time-consuming and requires expensive equipment. As the specific characteristics exploited for chromatography differ from protein to protein, purification protocols cannot be generalized. A faster and less expensive way to purify IgSF-CAMs is to use affinity chromatography. Tissue homogenates, body fluids in the case of secreted proteins, or cell lines engineered to express a particular protein of interest, either transiently or stably, can be used as a source for IgSF-CAM purification. Affinity chromatography makes use of the specific binding properties of the proteins, e.g., receptors for their ligands, enzymes for their substrates, or antibodies for their antigens. For IgSF-CAMs, the purification by immunoaffinity is most commonly used. A disadvantage of affinity purification is the possibility of the loss of activity, as the protein is sometimes eluted from the column by rather harsh conditions. The protocol described below has been successfully used for the purification of functionally intact IgSF-CAMs (Stoeckli et al., 1991, 1996; Rader et al., 1993).

The general principle of immunoaffinity chromatography is the use of a resin-coupled monoclonal antibody directed against the protein to be purified. Generally, activated Sepharose resins are used. The resin is packed into a column connected to a peristaltic pump and to a UV-detector to monitor the elution profile of the column. The purity of the eluted protein is analyzed by SDS-PAGE (UNIT 6.1). Here, we describe a purification protocol for a membrane-bound IgSF from brain membranes (see Support Protocol 2 for membrane preparation and protein solubilization).

Materials

- CNBr-activated Sepharose 4B column (see Support Protocol 1)
- Loading buffer: 0.5% CHAPS in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$
- Elution buffer: 0.5% CHAPS in 50 mM diethylamine
- Protein solution (see Support Protocol 2)
- 1 M Tris·Cl, pH 7.0 (APPENDIX 2A)
- PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ (see recipe)
- 0.02% (v/v) merthiolate or equivalent bacteriostatic agent

Prepare column

1. Rinse the column extensively with loading buffer, especially for a column that was prepared earlier and has been stored for a while. Add 2 to 3 vol (i.e., 3 times the volume of the column) of elution buffer to the column to test the stability of the column under the elution conditions and to make sure that the column does not contain any contaminations, such as unspecifically bound proteins from previous use of the column.
2. Properly reequilibrate the column to loading conditions before loading the protein solution (see Support Protocol 2 for protein solution preparation).

As the binding affinity of antibodies is usually not temperature-sensitive, the authors recommend running the affinity column at 4°C rather than at room temperature. Keeping the column and the protein solution to be loaded at 4°C helps to prevent contamination and slows down degradation of the proteins.

Load the column

3. During loading of the protein solution onto the column, adjust the flow rate to a sufficiently slow rate (usually 0.1 ml/min is chosen for good yields) to allow good interaction between antigen and antibodies coupled to the sepharose beads.

The capacity of the column can be tested by collecting fractions of the flow-through and subsequent immunoblot analysis (see Support Protocol 4). For best yield, the capacity of the column should not be exhausted. Typically, column volumes of 1 ml are used.

Elute column

4. Incubate the column in 0.9 vol elution buffer for 10 min.

This will increase the elution efficiency (a higher concentration of the eluted protein in eluate, rather than a broad elution peak with a long tail). Do not expose the column to elution conditions for longer times than necessary, because the high pH of the buffer could be detrimental for the affinity column.

5. Elute the column at a rate of 1 ml/min. Collect the eluate in vials containing enough 1 M Tris·Cl, pH 7.0, to buffer the eluate at a neutral pH value.

For a 1-ml column, 1-ml fractions are collected in 1.5-ml microcentrifuge tubes.

The volume of 1 M Tris·Cl, pH 7.0, required for restoring the pH should be determined at 4°C, as the pH value of Tris is extremely temperature sensitive.

The flow rate for elution can be much higher than that for loading. However, check the maximal flow rate acceptable for a specific resin. For Sepharose resins a maximal flow rate of 30 ml hr⁻¹cm⁻² is recommended.

The eluate can be stored a few days at 4°C; for longer storage below -20°C is recommended. However, keep in mind that repeated thawing and freezing is detrimental to the protein. Furthermore, freezing of dilute protein solutions is not recommended.

6. Re-equilibrate the column to loading conditions for a second run, or prepare the column for storage.

Regenerate and store the affinity column

7. Immediately after elution re-equilibrate the column to neutral pH values with PBS with Ca²⁺/Mg²⁺. For storage, add 0.02% merthiolate or an equivalent bacteriostatic agent to the PBS to prevent bacterial growth. Store the column at 4°C.

Prevent drying of the column during storage. The column can be stored for several months at 4°C.

Preparation of the Affinity Column

This protocol only describes the preparation of an immunoaffinity column. Generally, because IgSF-CAMs have low binding affinities for their binding partners and have no enzymatic activity that could be used for substrate-based purification, the use of immunoaffinity columns is the method of choice.

However, a prerequisite is the availability of a monoclonal antibody against the protein to be purified. This antibody is covalently coupled to a Sepharose resin. Affinity columns are versatile, they can be used for tissue homogenates, solubilized membrane proteins, or culture supernatants from cell lines that are engineered to produce and release IgSF-CAMs. If stored appropriately, affinity columns can be reused many times over several months.

SUPPORT PROTOCOL 1

Cell Adhesion

9.5.3

Materials

CNBr-activated Sepharose 4B gel
1 mM HCl
Buffer I: 0.5 M NaCl in 0.1 M NaHCO₃, pH 8.3
Monoclonal antibody against the protein to be purified
0.2 M glycine, pH 8.0
Buffer II: 0.5 M NaCl in 0.1 M sodium acetate, pH 4.0
Loading buffer (see Basic Protocol 1)

Sintered glass filter connected to a vacuum pump
Column (e.g., Poly-Prep column, Bio-Rad)
U-bottomed polypropylene vial that can be closed tightly

1. Soak 1:2 (w/v) CNBr-activated sepharose 4B in 1 mM HCl for 15 min at room temperature.

For a 1-ml column, start with 350 to 400 mg Sepharose.

2. Transfer beads to a sintered glass filter connected to a vacuum pump, and wash with ≥ 25 vol of 1 mM HCl, followed by buffer I.

It is very important to prevent the beads from drying between the additions of buffer (one gram dry resin yields ~ 3.5 ml swollen gel).

3. Transfer the slurry to a U-bottomed polypropylene vial containing the antibody solution in buffer I. Carry out the reaction for 2 hr at room temperature. Close the vial tightly.

The final concentrations should be: 100 mg Sepharose (dry weight) and 5 mg antibody per ml coupling reaction mix.

Ideally, rotating the vial end-over-head is used to maximize the coupling efficiency.

Do not use a magnetic stirrer, which will damage the agarose beads.

4. Stop the reaction by gently centrifuging the Sepharose beads for 5 min at $2000 \times g$, room temperature.
5. Add 3 vol 0.2 M glycine, pH 8.0, to the pellet and continue to rotate the vial end-over-head for an additional 2 hr.

Collect the supernatant of the coupling reaction to check the coupling efficiency.

6. Pack the slurry into a column.

Typically, column volumes are ~ 1 ml with a column diameter of 0.5 cm.

7. Wash the column with 5 vol buffer I followed by 5 vol buffer II. Repeat wash procedure four times to remove excess uncoupled ligand.
8. Before loading the protein solution (see Support Protocol 2), wash the column thoroughly with 25 to 30 vol loading buffer.

An affinity column can be used repeatedly, if stored appropriately with an antibacterial agent (e.g., 0.02% merthiolate) at 4°C. Before using the column after storage, rinse the column extensively with loading buffer. Use 2 to 3 vol elution buffer to clean the column, restore loading conditions by rinsing thoroughly with loading buffer (≥ 10 vol).

Solubilization of Membrane Proteins

Most IgSF-CAMs are either glycosyl-phosphatidylinositol-anchored or transmembrane proteins, therefore, they have to be solubilized from cell membranes with detergents. In the authors' experience, the use of CHAPS has given the best results with respect to yield and functional integrity of the purified proteins. Keep in mind that for many functional assays, detergent removal from the protein solution is necessary after purification; the presence of detergents can also interfere with binding assays. Especially sensitive assays are those that involve neurons, such as neurite outgrowth assays (see Basic Protocol 7). For removal of detergents from protein solutions, the authors have used SM-2 beads from BioRad or Calbiosorb from Calbiochem. The use of high concentrations of detergents can interfere with the purification by immunoaffinity columns, therefore, dilution of the protein solution after the solubilization step, i.e., before the solution is loaded onto the affinity column, is recommended. The protocol given below has been successfully used for the purification of functionally intact L1/NgCAM from E14 chicken brain membranes (e.g., Stoeckli et al., 1991, 1996). It is adapted from the purification protocol described by Grumet and Edelman (1984). However, the authors have used the same protocol for the solubilization and purification of other IgSF-CAMs (Rader et al., 1993; Fitzli et al., 2000). For storage, membranes and proteins can be frozen at the indicated steps. However, repeated freeze-thaw cycles are detrimental for proteins and for high yields of intact proteins and should be minimized.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

14-day-old chicken embryo brains, freshly frozen in liquid nitrogen
Liquid nitrogen
Ca²⁺/Mg²⁺-free buffer (CMF buffer; see recipe)
0.8 M and 2.25 M sucrose in PBS
1 M and 2 M NaCl in PBS
50 mM triethylamine
0.5% and 1% CHAPS in PBS

Mortar and pestle
Dounce homogenizer
Centrifuge tubes for Sorval SS-34 or equivalent rotor
38-ml polycarbonate tubes for ultra high-speed centrifuge

Prepare membranes

1. Remove brains of 14-day-old chicken embryos and immediately freeze in liquid nitrogen.

If necessary, brains can be stored at -70°C for extended periods of time.

2. Cool a mortar and pestle of sufficient size with liquid nitrogen and grind frozen brains in batches to a fine powder. Add small volumes of liquid nitrogen to keep brains/brain powder frozen during grinding.

Carefully avoid thawing of the tissue at any time.

3. Add ~3 vol CMF buffer to 1 vol brain powder. Homogenize the brain powder in a Dounce homogenizer.
4. Centrifuge homogenate for 20 min at 45,000 × g (10,000 rpm in a SS-34 rotor), 4°C.

5. Resuspend the pellets in 1.5 to 2 vol of 2.25 M sucrose, use Dounce homogenizer to get homogenous suspension.
6. Transfer ~24 ml suspension to each polycarbonate ultracentrifuge tube, overlay with $\frac{1}{4}$ vol of 0.8 M sucrose.
7. Centrifuge for 60 min at $150,000 \times g$, 4°C .
8. Transfer the membranes that are accumulated in the interphase to the Dounce homogenizer, resuspend them in 25 vol PBS.
9. Centrifuge for 60 min at $150,000 \times g$, 4°C .
10. Decant supernatant and resuspend in PBS, centrifuge as in step 9, and resuspend pellets in smallest possible volume of PBS for storage at -20°C , or use directly for stripping.

Samples can be stored up to 1 month at -20°C , or at -70°C for longer storage.

Strip packed membranes

11. Add 1 vol of 2 M NaCl in PBS to packed membrane suspension (from step 10), homogenize suspension.
12. Add 1 M NaCl in PBS to a final volume of 4 to 5 times the volume of the packed membranes.
13. Stir suspension 1 hr on a magnetic stirrer at low speed, 4°C , to strip membranes from peripheral membrane proteins.
14. Centrifuge for 60 min at $150,000 \times g$, 4°C .
15. Resuspend pellets in 20 vol of 50 mM triethylamine, stir for 60 min at 4°C .
16. Repeat centrifugation step 14. Carefully remove the supernatant with a pipet, as the membranes do not form a stable pellet after the high pH extraction step.
17. Resuspend the pellets in PBS.
18. Centrifuge for 60 min at $150,000 \times g$, 4°C .
19. Repeat washing the membranes with PBS at least one time to restore a pH value between 7.2 and 7.6. Keep an aliquot for measuring the protein concentration.
20. Freeze the stripped membranes at -20°C or use directly for solubilization step.

For storage longer than 1 month, store at -70°C .

Solubilize integral membrane proteins

21. If frozen membranes are used, they should be washed once again with PBS (steps 17 and 18).
22. Transfer pellets into a Dounce homogenizer and resuspend in 1 vol of 1% CHAPS buffer. Add 4 vol of 0.5% CHAPS buffer.

For good solubilization the protein concentration should be adjusted to ~ 1 mg/ml.

23. Extract the membrane proteins by stirring the suspension for 60 min at 4°C .
24. Centrifuge for 60 min at $150,000 \times g$, 4°C .
25. Combine supernatants, measure the volume, and remove aliquot to determine the protein concentration.

Do not freeze the solubilized proteins, but use directly for affinity purification step.

Production of Recombinant CAM by Transient Transfection of HEK293 Cells with Calcium Phosphate

Transient transfection of HEK 293 cells with expression vectors containing cDNAs of IgSF-CAMs represents a fast and convenient method for the production of intermediate amounts (100 µg to 1 mg) of recombinant protein. The human embryonic kidney cell line 293 (HEK 293) is a well established, easily transfectable cell line that is widely used for the expression of recombinant proteins. The transfection method of choice with HEK 293 cells is always calcium phosphate transfection, an inexpensive, convenient technique that results in high efficiencies of transfection.

DNA can be introduced into a wide variety of cultured cell lines as a calcium phosphate complex (Graham and van der Eb, 1973; Wigler et al., 1977). The transfected DNA can either integrate into the genome of the recipient cell, resulting in stable transgene expression accompanied by a stably altered phenotype of the cell (stable transfection) or remain episomal resulting in only transient expression of the transgene (transient transfection). The following protocol can be used likewise for the generation of stable cell lines by subsequent selection for stable transfectants or for transient expression only.

Efficient transfection requires the formation of a fine precipitate of calcium phosphate in the presence of DNA. The formation of the DNA-containing calcium phosphate particles is initiated under defined chemical conditions, in the absence of cells or serum. The particle size is the most critical parameter regarding efficiency of transfection, that is uptake of DNA-containing calcium phosphate particles by the cells. The main determinants of particle size are calcium and phosphate concentrations, the concentration of DNA, size of DNA fragments involved, pH, temperature, and time of incubation. After initial formation of the DNA-containing calcium phosphate particles, the precipitate is added to the cells. During the incubation of the precipitate with the cells, the formation of DNA-containing calcium phosphate particles continues and preexisting particles grow in size. The particles adhere to the cells and are taken up by endocytosis. After a few hours of exposure, the medium is changed and the cells start to express the recombinant protein. The period of efficient transgene expression varies between different expression vectors but lasts generally for a few days.

Materials

- HEK 293 cells
- Cell culture medium for HEK 293 cells (see recipe)
- Purified DNA of interest
- CaCl₂ solution (see recipe)
- HBS solution (see recipe)
- 175-cm² tissue culture flasks
- Additional reagents and equipment for trypsinizing cells (*UNIT 1.1*)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO₂ humidified incubator.

1. For transient transfection, grow HEK 293 cells of low passage number (passage <40) and split cells every 2 to 3 days before 80% confluency is reached.

By frequent splitting and growth in subconfluent density, the cells remain in their exponential growth phase and are highly susceptible for transfection up to about passage number 40.

2. One day prior to the experiment, trypsinize cells briefly (*UNIT 1.1*) and prepare a single cell suspension. Plate 5×10^6 cells in 30 ml of medium per 175-cm² flask and incubate for 16 to 20 hr at 37°C, 5% CO₂ incubator.

For optimal transfection efficiency, 60% to 70% confluency of cells is required. This allows two additional cell divisions after transfection. Some 293 lines adhere only poorly to tissue culture plastic, especially after transfection. To achieve more stable adherence, tissue culture flasks can be coated with poly-D-lysine (see Support Protocol 10).

HEK 293 cells do not need a medium change prior to addition of the calcium phosphate precipitate.

3. Mix purified DNA (40 to 80 µg in a maximum volume of 100 µl) with 1.5 ml CaCl₂ solution and incubate for a few minutes at room temperature.

For efficient formation of the DNA-containing calcium phosphate particles, temperature is critical. The solutions should be kept at room temperature for a couple of hours before use. The optimal amount of DNA depends on the particular expression construct used for transient transfection and has to be determined individually for each system.

4. Add 1.5 ml of HBS solution and mix well. Incubate for 1 to 15 min at room temperature and then add the reaction mixture to the cells (from step 2).

The formation of the DNA-containing calcium phosphate particles starts immediately after mixing the two solutions and can be observed by a slightly opaque appearance of the solution. Longer incubation periods generally result in the formation of a precipitate with larger particles. The optimum time of incubation critically depends on the size of the plasmids used.

The growth rate of the calcium phosphate particles frequently correlates inversely with the size of the plasmids. For very large plasmids (>30 kb), preincubation of no longer than 1 min is recommended.

5. Incubate the DNA-containing calcium phosphate particles with the cells for 4 to 6 hr at 37°C in an incubator.

The formation of the calcium phosphate precipitate can be monitored under an inverted light microscope with a 63× objective. The precipitate should become visible as tiny particles (~300 nm in diameter) especially on the surface between the cells. However, long exposure to the atmosphere outside of the incubator should be avoided.

6. Change medium after the incubation period and continue incubation. Begin testing for expression of the recombinant protein 1 day after transfection.

The cells should now express the recombinant protein for a few days.

Uptake of the DNA-containing calcium phosphate particles by cells can be checked by examination of free surfaces between cells. The margins surrounding the cells should be cleared of particles, due to their uptake by the cells.

SUPPORT PROTOCOL 4

Detection of IgSF-CAMs by the Dot Immunoblot Method

A rapid and convenient method for the detection of IgSF-CAMs in eluates from chromatography or affinity columns are dot immunoblots. Similar to immunoblots, dot blots are a semi-quantitative method to detect proteins transferred onto a nitrocellulose membrane via a chromogenic reaction. For this purpose, secondary antibodies coupled to peroxidase or alkaline phosphatase are used to detect binding of the first antibodies directed against the protein of interest. However, in contrast to immunoblots, proteins are not separated on a polyacrylamide gel, therefore dot blots are a much faster way to demonstrate the presence of a specific protein in a given sample. Furthermore, as the proteins are directly applied to the nitrocellulose membrane for dot blots, there is no loss due to difficulties in

transfer from the gel to the membrane. The fact that only small volumes can be applied onto the membrane for dot blots may, however, limit its use for detection of proteins in dilute solutions.

The procedure described here can also be used to stain IgSF-CAMs after immunoblotting. Use manufacturer's manuals and protocols for sample preparation, SDS-PAGE, and blotting of proteins onto nitrocellulose (also see *UNITS 6.1* and *6.2*).

Materials

Protein solution of interest

TBS: 0.2 M NaCl in 50 mM Tris, pH 7.4

Blocking solution: 2% (w/v) milk powder in TBS with or without 0.1% (w/v)

Tween 20

Antibody against the protein of interest diluted in blocking solution

Secondary antibody coupled with horseradish peroxidase (HRP) diluted in blocking solution

4-chloro-1-naphthalene solution (see recipe)

0.2- μ m nitrocellulose membrane (e.g., Schleicher and Schuell)

96-well plates

Rotary shaker

1. Place matching round disks of 0.2- μ m nitrocellulose membrane into wells of a 96-well plate.

When large numbers of samples have to be analyzed, the use of a commercially available protein-dispersing device is advised (e.g., Dot Punch IM-96, Inotech AG). These devices allow the application of protein solutions to the membrane before they are put into the wells. This is more convenient and less time-consuming compared to the procedure described below.

2. Apply small volumes (<10 μ l) of the protein solution onto the membrane.

The solution should not cover the entire membrane but remain confined to a segment of the membrane (dot). This is important in order to ensure the attachment of sufficient amounts of proteins per area to reach detection limits. If the protein solution is very dilute, the dotting procedure can be repeated after drying the nitrocellulose after the first application. The repeated application of protein to the membrane increases the amount of the protein of interest.

3. Dry the membranes to optimize protein adherence to the nitrocellulose.
4. Wash membranes three times with 100 μ l TBS per well.
5. Block protein adsorbance of the membrane by incubating \geq 30 min with blocking solution, room temperature.

Generally, 2% to 5% milk powder in TBS with or without 0.1% Tween 20 works well as a blocking solution. In some cases, addition of 2% to 10% (v/v) serum in TBS improves the signal-to-background ratio.

6. Apply 1 to 10 μ g/ml first antibody diluted in blocking solution and shake for \geq 1 to 2 hr, room temperature, on a rotary shaker.

If necessary, incubate overnight.

Shaking the 96-well plate improves antibody/antigen interaction.

7. Wash thoroughly with TBS (\geq 3 times).
8. Incubate with blocking solution for \geq 30 min before applying the secondary antibody.

9. Apply 1 to 10 $\mu\text{g/ml}$ of secondary antibody coupled with horseradish peroxidase diluted in blocking solution to the membrane and incubate 1 hr at room temperature.

As a secondary antibody, the use of a peroxidase-coupled antibody is described, but good results can also be obtained with phosphatase-coupled antibodies. Generally, an antibody concentration of 1 to 10 $\mu\text{g/ml}$ is used to get good signal-to-noise ratios but batches or products from different suppliers vary.

10. Remove antibody solution and wash membrane thoroughly with at least three changes of TBS.
11. Visualize the antibody by adding 0.1 ml of 4-chloro-1-naphthalene solution.

Generally, the dark blue color develops immediately.
12. Stop the reaction by rinsing the membranes with water.

FUNCTIONAL ASSAYS WITH PURIFIED PROTEINS

Analysis of Protein Interactions with Fluorescent Microspheres

There are essentially two ways to analyze the binding properties of cell adhesion molecules, (1) binding assays based on purified proteins, and (2) binding assays based on heterologously expressed proteins in their physiological environment (cell aggregation assays). This protocol describes a method that utilizes biochemically purified proteins coupled to fluorescent microspheres, whereas Basic Protocol 4 is based on cell aggregation assays. If pure cell adhesion molecules are available, proteins can be coupled to polystyrene beads of different fluorescent colors. Coupling can be either by covalent linkage or by hydrophobic adsorption of the protein to the microsphere surface. After dispersion of the beads by ultrasonication, pairwise combinations of differently coupled beads aggregate during a particular incubation period. Binding activities are analyzed by the evaluation of aggregate formation both qualitatively, by inspection on a fluorescent microscope, and quantitatively, using a fluorescence-activated flow cytometer. Although this protocol is a reliable method to detect binding activities of IgSF-CAMs and has been used by several laboratories, one has to consider false negative results as well as discrepancies between results of bead and cell aggregation assays. Some discrepancies have been explained by differences in the orientation of proteins coupled to beads in comparison to proteins on the surface of cells.

Materials

Protein-conjugated fluorescent microspheres; stock solutions contain 10^{11} beads/ml in 0.5% (w/v) BSA (see Support Protocol 5; Duke Scientific; Bangs Laboratories; Polysciences)

0.5% (w/v) BSA solution (see recipe)

0.5 mg/ml Fab fragments of antibodies against proteins of interest in PBS (optional)

0.5% (w/v) trypsin (optional)

PBS (see recipe)

Water bath sonicator (Branson Ultrasonics)

Fluorescence microscope equipped with FITC and TRITC filters

0.5-ml microcentrifuge tube

Rotator

Glass microscope slides

Refrigerated microcentrifuge

Fluorescence-activated flow cytometer

BASIC PROTOCOL 2

Prepare mixtures of fluorescent microspheres

1. Sonicate test tubes containing stock solutions of protein-conjugated microspheres in a water bath sonicator for 2 min at room temperature. Use lowest sonicator output setting needed to achieve monodisperse bead solution to prevent protein structure damage.

Make relatively small aliquots of protein-conjugated bead stocks because repetitive ultrasonication may damage protein structure.

For the applications described here, fluorescent microspheres with a nominal diameter of 0.5 μm work best. Both covalent coupling of proteins via surface-exposed functional groups or hydrophobic adsorption may be used. The best mode of coupling may depend on the particular nature of the protein to be studied.

The authors have consistently used Covaspheres fluorescent microspheres from Duke Scientific (Kuhn et al., 1991; Suter et al., 1995; Rader et al., 1996; Kunz et al., 1998; Fitzli et al., 2000). Fluorescent Covaspheres with a nominal diameter of 0.5 μm were easy to handle, allowed covalent coupling of the proteins, and were readily detectable in the light microscope and cell sorter. However, Duke Scientific has discontinued the distribution of Covaspheres, but a new line of beads with well characterized fluorescence and charge density is available. Different functional groups for covalent coupling of proteins will be offered, including carboxylate and aldehyde groups.

Support Protocol 5 describes covalent coupling of proteins to Covaspheres as well as the hydrophobic adsorption of proteins to polystyrene beads. With some modifications, it may also be suited for other types of beads.

The authors have added a support protocol that can be used for coupling proteins to microspheres exhibiting amino groups on their surfaces (see Support Protocol 6).

2. Check monodispersity of bead stock solution by quick inspection of a 1:100 dilution in 0.5% BSA under a fluorescence microscope.
3. Immediately after ultrasonication, prepare different test mixtures by combining protein-coupled beads of different fluorescent colors. Add 2 μl of green-fluorescent beads conjugated with protein 1 and 2 μl of red-fluorescent beads conjugated with protein 2 into a 0.5-ml microcentrifuge tube and bring to a final volume of 20 μl with 0.5% BSA solution (the concentration of each species of beads in the test mixture is $10^{10}/\text{ml}$). Vortex beads.

Use as many controls as possible. Typical control incubations are:

- a. Each type of beads alone.
- b. Beads conjugated with control proteins that should not bind to protein of interest (e.g., BSA, non-immune IgG).
- c. Protein-conjugated beads that were either incubated in boiling water for 10 min or treated with 0.5% trypsin overnight.

These treatments should abolish specific protein interactions.

- d. Pretreat beads with Fab fragments that are specific to the conjugated protein of interest. For this purpose, incubate an appropriate volume of the bead stock solution with Fab from polyclonal IgG at a concentration of 0.5 mg/ml in PBS for 2 hr at room temperature. Remove unbound antibodies by three consecutive washes with PBS. For solution changes, centrifuge beads in a microcentrifuge tube for 10 min at 16,000 $\times g$, 4°C. Dissolve possible aggregates of beads by ultrasonication as in step 1.

4. Incubate by slowly rotating test mixtures protected from light for 1 hr at room temperature on a rotator.

If such a rotator is not available, invert tubes a few times every 15 min. Tubes can be light protected with aluminum foil.

Analyze by fluorescence microscopy

5. Analyze the test mixtures for aggregate formation by fluorescence microscopy. Prepare a 1:10 dilution of the different incubations in 0.5% BSA solution and place 20 μ l of this dilution on a glass slide for inspection.

Most fluorescent microspheres emit a relatively intense signal. Therefore, antifading agents are not necessary for inspection. After a while, aggregates will settle and pictures can be taken for documentation or quantification of aggregate sizes. Adhesive interactions between two different proteins coupled to beads labeled with FITC and TRITC, respectively, should result in aggregates consisting of up to 100 beads of both colors that are equally distributed throughout the clusters. Carefully check for bleed-through between the different fluorescence channels.

Analyze by fluorescence-activated flow cytometry

6. Use flow cytometric analysis for quantification of aggregate formation. Calibrate the flow cytometer correctly for nonaggregated beads of each color.
7. Dilute test mixtures 1:1000 in 0.5% BSA solution and inject into a fluorescence-activated flow cytometer equipped with appropriate FITC and TRITC filter sets (Kuhn et al., 1991).

Always use fluorescence microscopy in parallel to flow cytometer analysis to obtain information on the distribution of the beads in aggregates (see Critical Parameters and Troubleshooting).

8. Use uncoated, nonaggregating beads to determine relative fluorescence intensity (RFI) of individual beads of each color. Compensate for the spectral overlap of the FITC and TRITC emission electronically.
9. Record the data output for number, size, and composition of mixed aggregates.

Data can be presented as two-dimensional contour or dot plots with the RFIs in the FITC and TRITC channel as x- and y-coordinates indicating size and composition of aggregates (Kuhn et al., 1991; Suter et al., 1995). The signal intensity represents the number of aggregates of a certain size and composition. The RFI values of single beads will allow the investigator to set the boundaries between mixed aggregates and aggregates that consist only of one species of beads. Determination of these boundaries is necessary to calculate the percentage of beads found in mixed aggregates as an indicator for protein binding.

SUPPORT PROTOCOL 5

Coupling Proteins to Fluorescent Microspheres

This support protocol describes a method for the preparation of protein-conjugated fluorescent microspheres required for the protein interaction studies described in Basic Protocols 2 and 3. It is based on the procedure that the authors have used for the coupling of proteins to Covaspheres (Duke Scientific). However, the same procedure has successfully been used for the adsorption of proteins to polystyrene beads, which bind proteins by hydrophobic interactions.

Additional Materials (also see Basic Protocol 2)

- 10¹¹ unconjugated beads/ml fluorescent polystyrene microspheres, 0.5- μ m diameter (Duke Scientific; Bangs Laboratories; Polysciences)
 - Biochemically purified IgSF-CAMs (see Basic Protocol 1) in a phosphate-based buffer system (either PBS or 20 mM sodium phosphate, pH 7.0)
1. Sonicate tubes containing stock solutions of 10¹¹ unconjugated beads/ml fluorescent polystyrene microspheres in a water bath sonicator for 2 min at room temperature.

2. If bead solution is monodisperse, transfer 100 μ l of beads to a new tube and add 50 μ g of biochemically purified IgSF-CAMs in PBS to a total volume of 1 ml (10^{10} beads/ml during coupling). Vortex tubes.

Before coupling, test protein preparations carefully for purity with SDS-PAGE to avoid false positive, as well as false negative, results.

Store proteins under sterile conditions in PBS or 20 mM sodium phosphate, pH 7.0, 4°C. For highly stable proteins, storage for up to 1 year at 4°C under sterile conditions is possible.

Cell adhesion molecules with transmembrane and cytoplasmic domains (e.g., NgCAM and NrCAM) require the presence of detergents such as CHAPS and deoxycholate during purification. The authors found that biological activity, such as binding, is better preserved when these detergents are maintained during protein storage at 4°C and during coupling to beads.

3. Incubate the mixture for 1 hr in a 37°C water bath. Mix periodically by inverting tube.
4. Centrifuge for 10 min at $16,000 \times g$, 4°C. Remove supernatant containing unbound protein and save for coupling analysis. Resuspend beads in 1 ml of 0.5% BSA solution.

This step is necessary to block residual binding sites that could result in unspecific bead-bead interactions.

5. Sonicate beads as in step 1 and incubate 30 min at room temperature.
6. Centrifuge beads as in step 4 and resuspend pellet in 100 μ l of 0.5% BSA solution. Store beads at 4°C.

Protein-conjugated microspheres should never be frozen and can be kept up to 1 year at 4°C (depending on protein stability).

7. (Optional) Determine coupling yield by SDS-PAGE of serial dilutions of protein samples taken before and after coupling.

Densitometric analysis of bands after silver staining or immunoblot detection allows the determination of both coupling efficiency and the number of protein molecules bound per microsphere.

Typically, a coupling yield of >50% is obtained under the conditions described. In the case of the IgSF-CAMs axonin-1 and NgCAM, ~16,000 molecules were found to bind per Covasphere bead (Kuhn et al., 1991).

Covalent Coupling of Proteins to Glutaraldehyde-Activated Amino Beads

As an alternative method for coupling proteins to beads, this protocol describes the glutaraldehyde-activated coupling of proteins to microspheres with amino-functional groups. Purified IgSF-CAMs can be coupled directly (not oriented) to amino beads. Alternatively, Fc-containing recombinant proteins can be bound to protein A-conjugated microspheres in an oriented manner.

Additional Materials (also see Basic Protocol 2)

- Amino-functional microspheres (e.g., silica aminopropyl beads from Bangs Laboratories)
- 0.1 M NaOH or HCl (optional)
- 8% (v/v) EM-grade glutaraldehyde, newly opened bottle
- 400 μ g/ml biochemically purified IgSF-CAMs (see Basic Protocol 1) in a phosphate-based buffer system (either PBS or 20 mM sodium phosphate, pH 7.0)
- Blocking solution (see recipe)

SUPPORT PROTOCOL 6

Cell Adhesion

9.5.13

1. Prepare an appropriate volume (e.g., 0.5 ml) of 1% amino-functional microsphere stock solution in ultra pure water. Wash beads two times with 1 ml water. Centrifuge for 4 min at $16,000 \times g$, room temperature, to separate microspheres from wash solution. Remove supernatant and resuspend microspheres in 0.5 ml water.

The amino-functional microspheres are available as powder or 10% (w/v) solution. Keep microsphere stock solutions at 4°C and never freeze.

2. Before adding the glutaraldehyde solution, check under the microscope whether the bead solution is monodisperse. If the bead solution contains significant clumps, sonicate in the water bath sonicator for 5 min at room temperature. If clumps persist, use a Branson microtip sonicator at the lowest output power needed to disrupt the clumps. Check pH of bead solution with cut pH strips and, if necessary, adjust pH to 6.5 or 7.0 with 0.1 M NaOH or HCl, respectively.

Glutaraldehyde activation works best at pH 6 to 7.

3. If the bead solution is monodisperse and has a pH of 6.5 to 7.0, add an equal volume of a newly opened bottle of 8% EM-grade glutaraldehyde and mix. Incubate beads on rotator for ≥ 6 hr or overnight at room temperature.
4. Centrifuge beads as in step 1, remove supernatant, and wash activated beads at least three times with 1 ml water, and once with the buffer in which the protein is dissolved (PBS or 20 mM sodium phosphate).
5. Add 0.5 ml of 400 $\mu\text{g/ml}$ purified IgSF protein in PBS, pH 7.3, or in 20 mM sodium phosphate, pH 7.0, to bead pellet and resuspend beads in protein solution (1% beads during coupling reaction). Incubate on rotator for 4 hr at room temperature or overnight at 4°C.

If less protein is available, scale down amounts of protein and beads proportionally.

6. Centrifuge beads 4 min at $16,000 \times g$, room temperature, save supernatant for coupling analysis on SDS-PAGE, and resuspend beads in 0.5 ml blocking solution. Incubate beads in blocking solution for 30 min at room temperature on rotator.
7. Centrifuge beads again. Resuspend beads in 0.5 ml blocking solution (1% bead stock) and store at 4°C for months.

BASIC PROTOCOL 3

Binding of Protein-Conjugated Microspheres to Cultured Cells

This protocol determines binding specificity of a cell adhesion molecule to a receptor on a particular cell type. Both primary cell cultures (Kuhn et al., 1991; Suter et al., 1995) and cell lines (Buchstaller et al., 1996; Rader et al., 1996) can be incubated with protein-conjugated microspheres under live conditions to determine interactions between specific proteins and cells. Antibody preincubations can be used as a control but also for receptor identification. Furthermore, cells transiently transfected with mutated receptor forms can be used for the identification of extracellular protein domains necessary for binding of the cell adhesion protein (Rader et al., 1996; Kunz et al., 1998; Fitzli et al., 2000).

Materials

Primary cultures of neuronal and/or glial cells or other cells
Complete medium used for cell cultures
Serum-free BSA-containing cell culture medium
 10^{11} beads/ml protein-conjugated fluorescent polystyrene microspheres; stock solutions in 0.5% BSA (see Support Protocol 5)

0.5 mg/ml Fab against protein of interest in serum-free medium (optional)
Fixation solution (see recipe)
PBS (see recipe)
Mounting medium (see recipe)
Waterbath sonicator
37°C, 10% CO₂ humidified incubator
Glass microscope slides
Fluorescence microscope

Prepare and bind microspheres to cells

1. Cultivate primary neurons and/or glial cells in conditions that allow live cell incubations in relatively small volumes.

For example, grow cells on substrate-coated coverslips using a removable donut-shaped Teflon ring to limit the incubation volume to 200 to 250 μ l (Suter et al., 1995).

If culture conditions include the use of serum, prepare also a corresponding serum-free, BSA-containing medium as in Stoeckli et al., 1991; see recipe.

2. Wash cultured cells one time with complete culture medium and one time with serum-free, BSA-containing cell culture medium.

Medium exchanges are carried out carefully with a pipet.

3. Prepare 1:1000 dilutions of 10¹¹ beads/ml protein-conjugated fluorescent polystyrene microspheres in serum-free cell culture medium, sonicate dilutions for 2 min, room temperature and immediately add to cultured cells.

4. (*Optional*) To test whether a specific CAM binds to a particular cell type via a characterized receptor (to which antibodies are available), preincubate cells with 0.5 mg/ml Fab fragments in serum-free medium, before adding the beads, for 2 hr in a 37°C, 10% CO₂ humidified incubator. Remove unbound antibodies by washing two times with serum-free medium before proceeding to step 5. To test for the specificity of bead binding to cells, use control protein-conjugated beads as well as beads that were preincubated with the corresponding Fab fragments.

5. Incubate the cells with the bead solution (step 3) for 1 hr in a 37°C, 10% CO₂ humidified cell culture incubator.

6. Use a Pasteur pipet to carefully aspirate medium with unbound beads. Immediately add serum-free medium. Repeat this step three times.

Do not allow the cells to dry.

Beads bound to live cells can now be inspected. However, if more time is needed for analysis, the authors recommend fixing the cells. Fixation also allows processing of the cells for immunostaining of marker proteins or CAMs and, therefore, correlative analysis of CAM binding and CAM expression.

Fix cells

7. Fix the cells by adding 80 μ l of 4 \times fixation solution to the cultures, which are in 240 μ l of serum free medium. Gently mix and incubate 1 hr at 37°C.

8. Wash fixed cells three times with PBS.

9. For immunofluorescence staining of the cells, proceed as described in UNIT 4.3. Otherwise mount cells on a glass microscope slide in mounting medium for inspection under a fluorescence microscope.

Beads are generally intensely fluorescent. Therefore, relatively short exposure times are sufficient when taking pictures.

CELL-BASED ASSAYS FOR IgSF-CAM FUNCTION

BASIC PROTOCOL 4

Trans-Interaction Assay with Myeloma Cells

Interactions between IgSF-CAMs that have been determined either by a fluorescent microsphere assay or by binding of fluorescent microspheres to cells do not provide any information on whether the interaction occurs between molecules located on different cells (*trans*-interaction) or between molecules residing on the membrane of the same cell (*cis*-interaction). In order to distinguish between a *trans*- and a *cis*-interaction, the IgSF-CAMs need to be studied in a natural environment, i.e., as membrane-bound molecules residing in their proper orientation in a biological membrane. Expression in nonadherent myeloma cells (see Support Protocol 7) provides a means of assessing IgSF-CAMs for *trans*-interactions. Two populations of stably transfected myeloma cell clones are stained with optically distinct intracellular fluorescent dyes. The cells are dissociated, incubated, and examined under a microscope in order to determine whether re-aggregation has occurred.

To evaluate whether two populations of myeloma cell clones expressing the IgSF-CAMs of interest on their surface can adhere to each other, they are labeled, mixed, dissociated, and allowed to re-aggregate. Labeling with optically distinct fluorescent dyes is necessary to distinguish the two populations.

Materials

Two populations of myeloma cell clones expressing the CAMs of interest
Selection medium, e.g., 5 mM L-histidinol in DMEM supplemented with 10% (v/v) FCS
PBS with Ca²⁺/Mg²⁺ (see recipe)
Stock solution of green fluorogenic dye, e.g., 1 mM
2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (Molecular Probes) in DMSO
Stock solution of red fluorogenic dye, e.g., 7.5 mM
5-(and-6)-carboxynaphthofluorescein diacetate (Molecular Probes) in DMSO
1% (v/v) FCS in PBS with Ca²⁺/Mg²⁺
5 mg/ml *p*-phenylenediamine in 1% FCS in PBS with Ca²⁺/Mg²⁺
15-ml conical polypropylene centrifuge tubes
Hemocytometer
V-shaped 96-well microtiter plate (e.g., Costar, Corning)
Centrifuge and rotor for microtiter plates
22-G needle attached to 1-ml syringe
Glass microscope slide
Fluorescence microscope with appropriate filters for green and red fluorescence, e.g., FITC and Texas Red
Additional reagents and equipment for counting cells (*UNIT 1.1*)

Prepare cells

1. Grow transfected myeloma cell clones that express the CAM(s) of interest on their surface in selection medium to a cell density of $\sim 5 \times 10^5$ cells/ml. Use a hemacytometer to monitor the cell density (the aggregation assay requires 5×10^5 cells per population per sample; see *UNIT 1.1*).

Transfected myeloma cell clones that express high concentrations of a homophilically trans-interacting cell adhesion molecule can grow in very large cell aggregates consisting of thousands of cells that are easily visible by eye. The authors found that cultures of aggregating myeloma cell clones contain more dead cells than nonaggregating myeloma cell clones. This might, at least in part, be due to a limited oxygen and nutrition supply to

the center of the aggregate. In order to limit the number of dead cells, aggregating myeloma cell clones should be split more frequently avoiding large cell aggregates. The cell density can be determined with a hemacytometer after dissociating the cells by repeated pipetting through a 22-G needle.

2. Transfer 5 to 10 ml of cell culture into 15-ml conical polypropylene tubes and centrifuge for 3 min at $500 \times g$, room temperature. Aspirate the supernatant and resuspend the cells in 1 to 2 ml PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$. Determine the cell density with a hemacytometer (UNIT 1.1). Add PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ to give a cell density of 5×10^5 cells/150 μl . Pipet 150 μl /well to a V-shaped 96-well plate.

The aggregation assay requires two populations in distinct wells per sample. Use wells A1 and A2 for the first sample, B1 and B2 for the next sample, etc.

When desired, the cells can be pre-incubated with antibodies at this step by incubating the cells with Fab monoclonal or polyclonal antibodies in a concentration range of 10 to 500 $\mu\text{g}/\text{ml}$ in 1% FCS in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ for 1 hr at room temperature.

3. Centrifuge the microtiter plate for 2 min at $500 \times g$, room temperature, remove the supernatant by flicking plate into a sink (cells will remain in wells). Resuspend cells in 90 μl PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$.

Label cells

4. Prepare fresh working solutions of both, green and red, fluorogenic dyes by diluting 10 μl of the stock solution in 990 μl PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$. Add 10 μl of the appropriate working solution to the well with the appropriate 90- μl cell suspension. Use one column for one fluorogenic dye (e.g., stain A1, B1, etc. with green and A2, B2, etc. with red). Incubate 30 min at 37°C .

The end concentration of the fluorogenic dyes is 1 μM of 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester and 7.5 μM of 5-(and-6)-carboxynaphthofluorescein diacetate. The electrically neutral ester substrates freely diffuse through the cell membrane into the cell, where they are cleaved into fluorescent products by nonspecific intracellular esterases. The charged fluorescent products are retained by cells with intact plasma membranes. Serum of the cell culture medium contains esterases and has to be washed away prior to the incubation and avoided during the incubation.

5. Centrifuge microtiter plate and remove the supernatant as in step 3. Resuspend the cells in 150 μl PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$. Repeat step.
6. Centrifuge microtiter plate 2 min at $500 \times g$, room temperature and remove the supernatant by flicking plate into sink. Resuspend cells in 75 μl of 1% FCS in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$.

Dissociate cells and allow re-aggregation

7. Combine complementarily stained cells of one sample in one well (e.g., add A2 to A1, B2 to B1, etc.). Dissociate cells by slowly pipetting up and down ten times through a 22-G needle attached to a 1-ml syringe. Avoid foaming. Incubate 45 min at 4°C .

During re-association the plate should not be moved.

8. Centrifuge microtiter plate for 2 min at $500 \times g$, room temperature, and remove the supernatant by flicking plate into sink. Resuspend the cells in 150 μl of 1% FCS in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$. Repeat step.
9. Centrifuge microtiter plate and remove the supernatant as in step 8. Resuspend the cells in 40 μl of 1% FCS in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$.

Analyze cells

10. Immediately prior to microscopic analysis of an individual sample, add 10 μl of 5 mg/ml *p*-phenylenediamine in 1% FCS in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$. Pipet the sample several times up and down using a 200- μl pipet tip. Mount 10 μl of the sample on a glass slide.

The use of the antifading reagent p-phenylenediamine in an end concentration of 1 mg/ml markedly preserves the fluorescence intensity.

11. Analyze the aggregates with a fluorescence microscope using FITC and Texas Red filters.

Filters that allow the simultaneous detection of green and red fluorescence facilitate the analysis.

SUPPORT PROTOCOL 7

Stable Transfection of Myeloma Cells by Protoplast Fusion

This protocol describes the stable transfection of myeloma cells with a vector that allows the surface expression of IgSF-CAMs. A vector particularly suited for myeloma cell expression was described by Traunecker et al. (1991). Expression by this vector is driven by an Ig κ promoter and enhancer. The 3' end of the transcript of interest is spliced onto an exon encoding the Ig κ constant domain in order to mimic stable Ig transcripts. The vector contains a histidinol dehydrogenase gene that allows the selection of stable transfectants in the presence of L-histidinol. L-histidinol is a precursor of L-histamine and an inhibitor of protein synthesis. The vector has been stably transfected into the mouse myeloma cell line J558L for the production of soluble lymphocyte-derived cell-surface receptor proteins (Traunecker et al., 1991). The system has also been used for the surface expression of IgSF-CAMs (Rader et al., 1993; Buchstaller et al., 1996; Fitzli et al., 2000). Alternatively, other mammalian expression vectors and myeloma cells can be used.

Myeloma cell clones that stably express large amounts of IgSF-CAMs on their surface were generated by a transfection method known as protoplast fusion. Transfection by protoplast fusion is a highly efficient method for the direct transfer of mammalian expression vectors from bacteria to mammalian cells (Schaffner, 1980; Sandri-Goldin et al., 1981; Rassoulzadegan et al., 1982; Gillies et al., 1983). It involves digesting bacterial cell walls with lysozyme to produce protoplasts and then fusing the protoplasts to mammalian cells in the presence of polyethylene glycol. The following protocol is based on the myeloma expression system described by Traunecker et al. (1991) and can easily be adapted to other systems.

Materials

Glycerol stock of an *E. coli* strain 803 clone (ATCC #35581) transformed with a mammalian expression vector containing the cDNA of the IgSF-CAM of interest (store at -80°C)

LB agar/ampicillin plates (see recipe; store at 4°C)

DMEM supplemented with 10% (v/v) FCS

LB medium (see recipe), prewarmed to 37°C

50 mg/ml ampicillin (store at -20°C)

60 mg/ml chloramphenicol in ethanol (store at -20°C)

DMEM supplemented with 10% (w/v) sucrose and 10 mM MgCl_2 , prewarm

20% (w/v) sucrose in 50 mM Tris-Cl, pH 8.0, ice cold

1 mg/ml lysozyme (Roche Molecular Systems), freshly dissolved 10 mg in 10 ml of 250 mM Tris-Cl, pH 8.0, and filtered through 0.22- μm filter

250 mM EDTA, pH 8.0, ice cold

50 mM Tris-Cl, pH 8.0, ice cold

10 mg/ml DNase I (Roche Molecular Systems; store at -20°C)
DMEM
PEG 1500 in DMEM supplemented with DMSO (see recipe)
Mouse BALB/c myeloma cell line J558L (ECACC #88032902) or another
myeloma cell line
50 mg/ml kanamycin
50 mM L-histidinol (see recipe)
Polyclonal anti-IgSF-CAM antibody
Fluorescein-conjugated secondary antibody

25-ml cell culture flasks
12-ml and 50-ml polypropylene tubes
15- and 50-ml conical polypropylene centrifuge tubes
37°C bacterial shaker
500-ml Erlenmeyer flask
Refrigerated tabletop centrifuge
37°C water bath
Glass microscope slides
Microscope with 1000× magnification
Multipipet trays
24- and 96-well tissue culture plates
Multipipettor and tips
Plastic wrap (e.g., Saran)
96-well plates with V-shaped wells

Additional reagents and equipment for indirect immunofluorescence (*UNIT 4.3*) and
freezing cells (*UNIT 1.1*)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO₂ humidified incubator.

NOTE: The following protocol is written for one sample. It is not recommended to handle more than four samples in parallel.

Day 1: grow transformed bacterial strain

1. Streak a glycerol stock of an *E. coli* strain 803 clone containing the mammalian expression vector onto an LB agar/ampicillin plate. Grow overnight at 37°C.

E. coli strain 803 (also termed 1106) might be more efficient than other *E. coli* strains in producing stable protoplasts (Rassoulzadegan et al., 1982).

The antibiotic has to be adapted to the prokaryotic selection marker of the mammalian expression vector.

2. Grow myeloma cells in DMEM supplemented with 10% (v/v) FCS in 25-ml cell culture flasks. Aim at a high cell density of $\sim 1 \times 10^6$ cells/ml that is reached on day 3 (protoplast fusion requires 5×10^6 cells per sample).

Day 2: grow transformed bacterial cultures

3. Inoculate 2 ml LB medium prewarmed at 37°C in a 12-ml polypropylene tube with a single *E. coli* colony from the freshly streaked LB agar/ampicillin plate. Add 2 μl of 50 mg/ml ampicillin. Grow for 4 hr at 250 rpm in a 37°C bacterial shaker.

4. Dilute 100 μl of cell culture into a 500-ml Erlenmeyer flask with 100 ml of LB medium and 100 μl of 50 mg/ml ampicillin. Grow to an OD_{600} of ~ 0.6 . Start checking the optical density after 3 hr.
5. After $\text{OD}_{600} \sim 0.6$ is reached, add 200 μl of 60 mg/ml chloramphenicol to a final concentration of 120 $\mu\text{g/ml}$. Grow overnight at 250 rpm in a 37°C bacterial shaker.

*Plasmids carrying the *colE1* origin of replication can be amplified in the presence of chloramphenicol (Hershfield et al., 1974).*

Day 3: harvest bacterial cells and form protoplasts

6. Transfer the overnight culture into two 50-ml conical polypropylene centrifuge tubes and centrifuge 10 min at $2500 \times g$, 4°C.
7. In the meantime, prewarm 20 ml DMEM supplemented with 10% sucrose and 10 mM MgCl_2 in a 50-ml polypropylene tube in a 37°C water bath.
8. Pour off the supernatants of the spun culture.

Prepare protoplasts

9. From here on proceed in a sterile laminar flow bench. Vortex and combine the two bacterial pellets in 2.5 ml ice-cold 20% sucrose in 50 mM Tris-Cl, pH 8.0.
10. Add 500 μl ice-cold 1 mg/ml lysozyme in 250 mM Tris-Cl, pH 8.0, mix gently (swirl), and incubate 5 min on ice.
11. Add 1 ml ice-cold 250 mM EDTA, pH 8.0, gently swirl, and store on ice 5 min.
12. Add 1 ml ice-cold 50 mM Tris-Cl, pH 8.0, gently swirl, and incubate 10 min at room temperature. During this incubation period, mount 10 μl of the sample on a glass microscope slide and analyze protoplast formation under a microscope.

A microscope with 1000 \times magnification is required to distinguish between spherical protoplasts and rod-shaped bacteria. At the end of incubation, $\sim 90\%$ protoplasts should be formed.

13. Add 20 ml DMEM supplemented with 10% sucrose and 10 mM MgCl_2 very slowly to the protoplast preparation. To do this, swirl protoplasts gently, start adding drops of DMEM supplemented with 10% sucrose and 10 mM MgCl_2 , and slowly increase the added volume.

The prepared protoplasts are fragile and need to be handled with care. Protoplast lysis is indicated by an increasing viscosity of the preparation due to the release of genomic DNA. The preparation can be analyzed using a microscope as above.

14. Add 40 μl of 10 mg/ml DNase I and incubate 15 min at room temperature.

Addition of DNase I reduces the viscosity of the protoplast preparation.

Prepare for fusions

15. In the meantime, prewarm the following in separate 50-ml propylene tubes in a 37°C water bath:

15 ml DMEM
10 ml DMEM supplemented with 10% (v/v) FCS
50 ml DMEM supplemented with 10% (v/v) FCS.

Thaw at room temperature:

2 ml PEG 1500 in DMEM supplemented with DMSO.

16. Centrifuge protoplast preparation in the 50-ml polypropylene tube 30 min at $2500 \times g$, room temperature.
17. In the meantime, transfer 5×10^6 mouse BALB/c myeloma cells into 15-ml conical polypropylene centrifuge tubes and centrifuge 10 min at $500 \times g$, room temperature. Aspirate the supernatant and resuspend the cells in 5 ml prewarmed DMEM.

The myeloma cell preparation should be serum-free.

Other myeloma cell lines that have been transfected by protoplast fusion or electroporation include mouse P3-X63Ag8.653, mouse Sp2/0-Ag14, mouse NSO, and rat YB2/0 (Gillies et al., 1989; Nakatani et al., 1989; Bebbington et al., 1992; Shitara et al., 1994).

18. Pour off the supernatant of the centrifuged protoplast pellet.

The protoplast pellet should have a smooth surface.

Carry out fusions

19. Slowly layer the myeloma cell preparation on top of the protoplast pellet in the 50-ml conical polypropylene centrifuge tube. Centrifuge 10 min at $500 \times g$, room temperature.
20. Aspirate the supernatant. Mix cell and protoplast pellet by hand-flicking the tube and tapping it on the benchtop.
21. Add 2 ml PEG 1500 in DMEM supplemented with DMSO. Resuspend the pellet by pipetting up and down several times.
22. After addition of the PEG solution (~1 to 2 min), very slowly add 10 ml prewarmed DMEM. To do this, swirl protoplasts gently, start adding drops of DMEM, and slowly increase the added volume.
23. Add 10 ml prewarmed DMEM supplemented with 10% FCS, swirl gently, and centrifuge 10 min at $500 \times g$, room temperature.
24. Aspirate the supernatant, resuspend the pellet in 50 ml prewarmed DMEM supplemented with 10% FCS, and add 100 μ l of 50 mg/ml kanamycin.
25. Pour into a multipipet tray and distribute among five 96-well tissue culture plates by adding 100 μ l/well using a multipipettor. Wrap tissue culture plates in plastic wrap and incubate for 48 hr in a 37°C , 10% CO_2 humidified incubator.

Day 5: select transfected cells

26. After 48 hr, prepare selection medium by adding 10 ml prewarmed 50 mM L-histidinol to 40 ml prewarmed DMEM supplemented with 10% FCS. Add 100 μ l of 50 mg/ml kanamycin. Pour into a multipipet tray and add 100 μ l/well using a multipipet. Rewrap tissue culture plates in plastic wrap and continue incubation in a 37°C , 10% CO_2 humidified incubator.

The antibiotic, here L-histidinol, has to be adapted to the eukaryotic selection marker of the mammalian expression vector. Only transfected myeloma cells will survive the treatment with L-histidinol.

27. Examine plates visually for clones ~10 days after selection medium is added.

No medium change or any other treatment is necessary during this time

Identify IgSF-CAM expressing clones

28. Once a clone becomes clearly visible by eye, analyze it for the expression of IgSF-CAM by indirect immunofluorescence staining (UNIT 4.3). Transfer $\leq 50\%$ of the cloned cells to a well of a 96-well plate with V-shaped wells and perform indirect

immunofluorescence analysis with a polyclonal anti-IgSF-CAM and a fluorescein-conjugated secondary antibody. Identify clones that are expressing IgSF-CAM.

29. Expand positive clones into 24-well tissue culture plates.
30. Subclone positive clones by limiting dilution in 96-well tissue culture plates.
31. Maintain positive subclones in selection medium, e.g., 5 mM L-histidinol in DMEM supplemented with 10% FCS. Store backup cells frozen in liquid nitrogen using standard procedures (UNIT 1.1).

Cells from positive subclones are used in myeloma cell aggregation assays.

BASIC PROTOCOL 5

Detecting *Cis*-Interactions between IgSF-CAMs by Chemical Cross-Linking

The following basic protocols describe methods for the detection of *cis*-interactions, i.e., interactions between proteins that reside in the same membrane. In the chemical cross-linking methods described in this protocol, bifunctional reagents are used to establish covalent cross-bridges between associated proteins. The covalently linked protein complexes are then analyzed by SDS-PAGE (UNIT 6.1) and immunoblots (UNIT 6.2), using specific antibodies. In the antibody-induced co-capping methods (see Basic Protocol 6), a hypothesized *cis*-interaction between two cell surface proteins is evaluated by inducing a redistribution of one molecule and testing whether the putative binding protein follows. For both methods, the cells analyzed should be cultured at low density, in order to prevent contact between cells. Under these conditions, close associations of proteins are only possible between proteins residing in the membrane of the same cell.

Chemical cross-linking joins two molecules by means of a cross-linking reagent. The method critically depends on the cultivation of the cells of interest as single cells at low density to avoid the formation of cell-cell contacts. Moreover, the structural integrity of the cells should be maintained throughout the procedure. The use of hydrophilic, membrane-impermeable bifunctional cross-linking reagents restricts the cross-linking to extracellular domains of membrane proteins. Considering the close spatial association between proteins interacting within the same membrane, a high degree of specificity of chemical cross-linking is mandatory.

The specificity of chemical cross-linking is mainly determined by the chemical reactivity of the functional groups of the cross-linking reagent and the length of the spacer separating the reactive groups. The *N*-succinimidyl group combines efficient reactivity with a high selectivity for primary amino groups, thereby limiting the cross-linking to lysine side chains at the surface of proteins engaged in interactions. Since most IgSF-CAMs contain multiple lysine residues in their extracellular domains, the amino-group-specific homobifunctional *N*-succinimide-derivatives are suitable reagents for the detection of *cis*-complexes formed between such molecules. The following protocol will primarily focus on the application of hydrophilic homobifunctional di-*N*-succinimidyl derivatives with relatively short (0.6 to 1.2 nm) spacer sequences separating the reactive groups. In order to enhance the specificity of the chemical cross-linking reaction to stably associated proteins, lateral movement in the cell membrane is reduced by performing the cross-linking reaction on ice. Ideal spacer length of the cross-linking reagent, optimal concentrations of cross-linkers, and reaction times strongly depend on the specific system that is analyzed and must be evaluated empirically. The parameters described below turned out to be optimal in many experimental situations and represent a good starting point for further optimizations. After quenching the cross-linking reaction, cells are lysed and the cross-linked complexes ideally isolated by immunoprecipitation (UNIT 7.2) using specific antibodies against the molecule of interest. Immunoprecipitates are separated by SDS-PAGE and cross-linked complexes can be detected by immunoblot analysis. The presence of

known binding partners within the cross-linked complexes can be addressed by immunochemical techniques, which require only low amounts of proteins. However, immunochemical analysis clearly restricts the detection of cross-linked binding partners to known molecules against which antibodies are available. Scaling-up of the procedure may result in the isolation of sufficient amounts of cross-linked material for subsequent microsequencing allowing the detection of novel binding partners.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO₂ humidified incubator.

Materials

Cells of interest growing in tissue culture at low density
PBS with Ca²⁺/Mg²⁺ (see recipe)
100 mM cross-linking reagent (see recipe):
Bis(sulfosuccinimidyl)suberate (BS³) in water
Disuccinimidyl tartrate (DST) in water-free DMSO
Disulfo disuccinimidyl tartrate (Sulfo-DST) in water
3, 3'-Dithiobis(sulfosuccinimidyl propionate) (DTSSP) in water
5 mM EDTA in Ca²⁺/Mg²⁺-free PBS
1 M glycine solution in water, pH 8.0
Lysis buffer (see recipe)
Primary antibody: serum, purified immunoglobulin, or purified immunoglobulin immobilized on agarose or Sepharose matrix
Protein A or protein G coupled to agarose or Sepharose matrix (optional)
Wash buffer (see recipe)
Sample buffer for SDS-PAGE (*APPENDIX 2A*)

10-cm tissue culture dishes precoated with poly-D-lysine combined with additional substrates, such as laminin (see Support Protocols 10 and 12)
Horizontal shaker
Cell scraper
2-ml microcentrifuge tubes
End-over-end rotator (model 750)
100- or 200- μ l and 500- μ l Hamilton syringe and 22-G needle

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), immunoblot analysis (*UNIT 6.2*), and immunoprecipitation (*UNIT 7.2*)

Prepare cells

1. Prior to the experiment, seed cells onto 10-cm tissue culture dishes at a low density. Plate slowly dividing (division rate of <1 division in 48 hr) or nondividing cells, e.g., primary neurons, 12 to 16 hr prior to the experiment at a density of 10⁴ cells per cm² on cell culture dishes precoated with poly-D-lysine (Support Protocol 10) combined with additional substrates, such as laminin (see Support Protocol 12).

Fast dividing cells, especially fast-growing cell lines, are problematic in this experiment as extensive contacts are formed between the progeny of cell divisions. With COS or HEK 293 cells, cultures of mainly single cells can be obtained by plating the cells 4 to 6 hr prior to the experiment on poly-D-lysine coated cell culture dishes in a density of 1-2 \times 10⁴ cells per cm². For the final passage of cells, do not trypsinize cells. Most cell lines can easily be detached when cultured on untreated cell culture plastic by incubation for a few minutes in 5 mM EDTA in Ca²⁺/Mg²⁺-free PBS at room temperature. Care must be taken to generate single-cell suspensions without any cell aggregates by careful trituration using a large-bore

pipet tip. Glass pipets (Pasteur pipets) are not recommended, since the sharp glass edges and the strong shearing forces damage the cells resulting in the formation of clumps and aggregates.

2. Use one 10-cm tissue culture dish (containing $\sim 10^6$ cells) for each reaction. In the first set of experiments, include three different concentrations of cross-linking reagent and three to four different reaction times, resulting in a total of nine to twelve individual samples.
3. Prior to the experiment, check cells for homogenous distribution under an inverted microscope that allows the examination of living cells. Do not use cultures containing aggregates or clumps of cells.
4. Remove culture medium and wash the cells three times with PBS containing $\text{Ca}^{2+}/\text{Mg}^{2+}$. Add 10 ml PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ and incubate 5 to 10 min on ice.

Incubation on ice not only reduces lateral movements of proteins within the cell membrane but also decreases endocytosis of membrane proteins.

The cells are living and therefore, during this and subsequent steps, 5 through 8, are very fragile, especially primary neurons that have the tendency to detach spontaneously during the procedure. All steps must be carried out with the utmost care to protect the cultures from mechanical stress.

Cross-link cells

5. During the incubation period of step 4, freshly prepare 10 ml each of 0.1 mM, 1 mM, and 10 mM cross-linker solution per dish in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$, using the 100 mM DST stock solution in water-free DMSO.

The concentration of the cross-linking reagent is a very critical parameter. Most published protocols use concentrations of ~ 1 mM for efficient cross-linking. For a first set of experiments, the authors recommend 0.1 mM, 1 mM, and 10 mM for the reagents DST and DSSP.

The cross-linker solution in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ is unstable at room temperature and should be kept on ice for ≤ 5 to 10 min prior to adding to cells.

6. Remove PBS from cells and add the prechilled cross-linker solution carefully to the cells. Carry out the cross-linking reaction on ice with gentle shaking (20 to 30 rpm) on a horizontal shaker.

Tilt cell culture dish slight and add cross-linker solution at the edge of the dish with a pipet. Distribute the cross-linker solution carefully over the entire surface of the dish.

Quench reaction and lyse cells

7. Quench the reaction after 0, 5, 15, and 45 min by adding 0.5 ml of 1 M glycine solution, pH 8.0 (final glycine concentration is 50 mM). Perform the quenching reaction for 15 min.

The reaction time is a critical parameter regarding specificity and yields of chemical cross-linking. Quenching of the reaction in an initial experiment after 0, 5, 15, and 45 min results in a "kinetic profile" of the cross-linking reaction. This "kinetic profile" is often valuable, not only for the optimization of the reaction time as a parameter but also in the interpretation of the results.

8. Completely remove the reaction mixture from the cells without detaching them and add 2 ml of lysis buffer per dish. Remove cells from the culture dish with a cell-scraper and lyse them for 30 min on ice on a horizontal shaker.
9. Transfer cell lysate to 2-ml microcentrifuge tubes and microcentrifuge 15 min at 12,000 to 14,000 rpm, 4°C.

This centrifugation step clears the cell lysates from nuclei and cell debris. Still, the cleared lysates frequently have an opaque appearance. The lysates can be frozen and kept for several weeks at -20°C .

Immunoprecipitate cross-linked proteins

10. In 2-ml microcentrifuge tubes, prepare the primary antibody for immunoprecipitation.

Primary antibodies for immunoprecipitation can be used in the form of whole serum, purified immunoglobulins, or coupled to an agarose or Sepharose matrix (for coupling of purified immunoglobulins to cyanogen bromide agarose or Sepharose see Support Protocol 1).

Prior to the addition of the cell lysate, preincubate whole serum with Sepharose-coupled protein A (ideal for rabbit antisera) or protein G (for goat or sheep antisera) in a ratio serum/protein A/G Sepharose of 2:1 and purified immunoglobulins with protein A/G matrix in a ratio of 5 μg immunoglobulin/ μl matrix for 30 min at room temperature. For an efficient immunoprecipitation ~10 to 20 μg of specific immunoglobulin are required. A total matrix volume between 10 and 20 μl is recommended.

11. Add the cleared cell lysate to the primary antibody and rotate for 4 to 16 hr end-over-head at 4°C .
12. Microcentrifuge samples for 1 min at 14,000 rpm, 4°C and carefully remove the supernatant; keep supernatant and store at -20°C .
13. Resuspend matrix in 1 ml wash buffer, vortex for 20 sec, and microcentrifuge 30 sec at 14,000 rpm, 4°C . Repeat the wash step three times and carefully remove the supernatant after final wash step. Remove the residual fluid from the matrix pellet with a 100- or 200- μl Hamilton syringe without aspirating matrix material.
14. Elute material bound to the matrix in 50 to 100 μl sample buffer for SDS-PAGE by boiling 5 min. Microcentrifuge samples for 1 min at 14,000 rpm, room temperature.

After boiling, the protein sample in SDS-PAGE sample buffer can be stored several months at -20°C or years at -70°C .

15. Analyze protein samples by SDS-PAGE (UNIT 6.1) and detect the cross-linked complexes by immunoblot analysis (UNIT 6.2).

Detecting Cis-Interactions between IgSF-CAMs by Antibody-Induced Co-Capping

The lateral mobility of integral membrane proteins within the plane of the cell membrane allows the induction of large clusters of molecules by cross-linking their extracellular moieties with antibodies. This phenomenon is termed “antibody-induced capping”. Interactions between two cell surface proteins in the plane of the same membrane (*cis*-interactions) can be addressed by examining the effects of antibody-induced capping of one molecule on the distribution of the other molecule. Reciprocal co-capping or co-distribution of two molecules after induction of capping with antibodies against one of them strongly indicates a (direct or indirect) *cis*-interaction. The following protocol describes a basic method for the induction of capping by antibodies and the detection of co-capping by fluorescence microscopy.

Cells of interest are cultivated at low density to avoid the formation of extensive cell-cell contacts. Incubation with the primary antibody against the first molecule (A) is performed on intact, live cells in the cold to reduce lateral movement of membrane proteins. Following the incubation with primary antibody against molecule A, unbound antibody is removed. Bound primary antibody is detected by a fluorochrome-conjugated secondary

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Cell Adhesion

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antibody against the animal species in which the anti-A primary antibody was raised. Subsequently raising the temperature to 37°C restores the lateral mobility of proteins in the cell membrane and results in the formation of clusters of molecule A due to antibody-mediated cross-linking. The cells are washed, fixed, and counter-stained with a primary antibody against the putative binding partner of molecule A (molecule B). To this end, the primary antibody against molecule B must be raised in a different species than the anti-A antibody. The anti-B antibody is detected by a secondary antibody conjugated to a different chromophore than the secondary antibody against molecule A. Care should be taken that the two antibody combinations do not cross-react. Further, the emission wavelengths of the chromophores coupled to the secondary antibodies must be distinct to allow a complete separation of the signals by the use of narrow band-pass filters in the subsequent examination by immunofluorescence microscopy. Once the conditions for antibody-induced capping and for observing specific immunolocalization have been established, co-localization of the fluorescence signals elicited by the secondary antibodies allows the detection of co-clustering of molecules A and B on the cell surface. Quantitative analysis of the localization of the fluorescent signals requires a spatial resolution not normally achieved by standard light microscopes. To this end, due to its superior resolution power, confocal laser scanning microscopy represents a powerful technique for the quantitative assessment of co-distribution induced by antibody-induced capping.

Materials

- Cells of interest growing in tissue culture (e.g., nondividing cells or primary neurons)
- Cell culture medium (used for the cells of interest) without serum
- PBS (see recipe)
- Hank's Balanced Salt Solution (HBSS; *APPENDIX 2A*)
- HBSS/1% FCS: HBSS containing 1% (v/v) fetal calf serum (FCS)
- Primary antibodies (serum or purified immunoglobulin) for molecules A and B
- Secondary antibodies of the appropriate sources coupled to different fluorescent dyes
- 4× Fixative solution (see recipe)
- Vectashield mounting medium for fluorescence (H-1000, Vector Laboratories)
- 10-cm tissue culture dishes
- 12-mm no. 1 round glass coverslips, sterilized by autoclaving or soaking in 70% ethanol for 1 hr and precoated with poly-D-lysine (see Support Protocol 10)
- Watchmaker's forceps
- 24-well tissue culture plates
- Microscope slides
- Nail polish
- Fluorescence microscope with 63× and/or 100× oil immersion objectives

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO₂ humidified incubator.

Prepare cells

1. One day prior to the experiment, seed cells at low density onto 10-cm cell culture dishes, each containing 15 to 20 sterilized poly-D-lysine coated 12-mm round glass coverslips. Plate nondividing cells or primary neurons 12 to 16 hr prior to the

experiment at a density of 2×10^4 cells per cm^2 and fast-growing cell-lines 8 to 12 hr prior to the experiment at a density of 10^4 cells per cm^2 .

These cell densities should result in ~10% confluency at the time of the experiment.

Coating of glass coverslips with poly-D-lysine is always recommended, in order to prevent cell detachment during the procedure.

2. Two to 4 hr prior to the experiment, transfer the glass coverslips with watchmaker's forceps from the 10-cm cell culture dish to wells 24-well tissue culture plates containing 1 ml cell culture medium per well. Incubate at 37°C

Aseptic technique is recommended, but not mandatory.

3. After 2 to 4 hr of incubation, remove the culture medium and wash the cells three times with 1 ml HBSS at room temperature, each time by adding 1 ml of HBSS, incubating 1 min, and aspirating wash solution. Add 200 μl HBSS/1% FCS per well and incubate the 24-well plate for 10 min on ice.

Do not let the cells dry throughout the procedure. The incubation with HBSS/1% FCS on ice will block potential unspecific sites for antibody adsorption. Incubation on ice not only reduces lateral movements of proteins within the cell membrane but also decreases endocytosis of membrane proteins.

The cells are living and therefore, during this and subsequent steps are very fragile, especially primary neurons that have the tendency to detach spontaneously during the procedure. Carry out all steps with the utmost care to protect the cultures from mechanical stress.

Prepare and bind primary antibody for molecule A

4. Prepare the appropriate dilutions of primary antibody and control antibody to molecule A in HBSS/1% FCS in microcentrifuge tubes and centrifuge for 1 min at maximum speed. Transfer supernatants to new tubes and put primary antibody solutions on ice ≥ 5 min prior to use.

For co-capping experiments the primary antibodies are generally applied at higher concentrations than for standard immunofluorescence protocols. Titrate optimal antibody concentrations for each experimental system individually. As a rule, dilute affinity-purified antibodies to a range of 5 to 20 $\mu\text{g}/\text{ml}$ and antisera between 1:20 and 1:200. If using a commercially available antibody, multiply the concentration recommended for immunofluorescence staining by a factor of five. For a first set of experiments, try a range of dilutions and test for potential cross-reactivity of the primary antibodies in advance. Add negative controls to ensure specificity of antibody-induced capping. To this end, preimmune serum or purified antibodies from preimmune serum are required.

Antibody dilution is a critical parameter regarding cross-reactivity: this phenomenon may occur at the high concentrations that are used for co-capping experiments even if absent at concentrations based on own experience or recommended by manufacturers for standard immunofluorescence protocols.

5. Remove HBSS/1% FCS from cells and add 200 μl of cold anti-A antibody in HBSS/1% FCS to each well. Incubate with the primary anti-A antibody for 30 min on ice.

Prepare and bind secondary antibody for molecule A

6. Dilute fluorochrome-conjugated secondary antibody in HBSS/1% FCS in microcentrifuge tubes and centrifuge for 1 min at maximum speed. Transfer supernatants to new tubes and place secondary antibody solutions on ice ≥ 5 min prior to use.

Commercially available preparations of secondary antibodies must be tested for cross-reactivity with the primary and secondary antibodies against molecule B in advance. Even

if manufacturers assure that no such cross-reactivity occurs, consider that the concentrations of secondary antibodies used in co-capping experiments are well above those normally applied for immunofluorescence staining. Typical dilutions of commercial preparation are between 1:20 and 1:200 (~5 to 10 times higher than for standard immunofluorescence applications).

7. Aspirate primary antibody solution and wash cells two times with ice-cold HBSS. Add 250 μ l/well of secondary antibody and incubate for 30 min on ice. Protect from light.
8. Remove the solution containing the secondary antibody and wash the cells two times with ice-cold HBSS. Add 200 μ l of ice-cold cell culture medium without serum and put the 24-well cell culture plate for 30 min in a 37°C, 5% CO₂ humidified incubator.

Never use HBSS or PBS for this incubation step. The phosphate buffer system present in HBSS and PBS is insufficient to buffer the pH efficiently in a 5% CO₂ atmosphere. The pH would drop too much and lead to false results. Always use the cell culture medium normally applied for the cell cultures of interest without serum. When stored on ice, keep the cell culture medium in air-tight tubes.

Fix cells

9. Add 66.7 μ l of 4 \times fixative solution to each well and allow cells to fix for 15 min at 37°C in the dark.

A frequent source of experimental artifacts in co-capping experiments is fixation. It is necessary to block any further lateral movement of the capped proteins after fixation. The fixation in culture medium with 2% formaldehyde/0.1% glutaraldehyde results in total immobilization of membrane proteins without permeabilization of the cell membrane (for a detailed discussion, see Dubreuil et al., 1996).

10. Aspirate fixative and wash cells two times with PBS. Remove the PBS after the second wash step and add 500 μ l PBS/1% FCS. Incubate for 15 min at room temperature.

Prepare and bind primary antibody for molecule B

11. Prepare dilutions of the primary antibody for molecule B in PBS/1% FCS as described in step 4.

For the counter-staining (here of molecule B), the primary antibodies are generally applied in concentrations comparable to standard immunofluorescence protocols. Titrate optimal antibody concentrations for the counter-staining in a way that similar intensities of fluorescence signals result for both molecules. As a rule, dilute affinity-purified antibodies in a range of 1 to 10 μ g/ml, purified Fab fragments 5 to 20 μ g/ml, and antisera between 1:100 and 1:1000.

If using a commercially available antibody, the concentration recommended for immunofluorescence staining is a good starting point for the optimization of the counter-staining. For a first set of experiments, try a range of dilutions and test again for potential cross-reactivity of the antibodies in advance. Add negative controls to ensure specificity of the counter-staining. In addition to preimmune serum or purified antibodies from preimmune serum, include further primary antibodies against molecules for which no co-capping with molecule A is expected in the experiment. The specificity of co-capping is the most critical issue in the whole experiment.

12. Remove the PBS/1% FCS and add 200 μ l/well of diluted primary anti-B antibody in PBS/1% FCS. Incubate 1 hr at room temperature. Protect from light with aluminum foil or place 24-well plate in a drawer.

Prepare and bind secondary antibody for molecule B

13. Dilute the secondary antibody against anti-B antibody in PBS/1% FCS as described in step 6.

If commercially available preparations of secondary antibodies are used for the counterstaining, dilutions between 1:100 and 1:500 are recommended in most cases. These concentrations are similar to those used for standard immunofluorescence applications. The issue of cross-reactivity with the primary and secondary antibodies against molecule B is much less critical than in case of the antibody used for capping but have to be tested anyway to use the system in reverse order to detect reciprocal co-capping (see below).

14. Remove primary anti-B antibody solution and wash cells two times with PBS/1% FCS. Add 200 μ l/well secondary anti-B antibody in PBS/1% FCS and incubate 45 min at room temperature. Protect from light.

Fix cells

15. Wash cells two times with PBS/1% FCS and one time in PBS only. Add 250 μ l of 1 \times fixative solution (2% formaldehyde/0.1% glutaraldehyde in PBS) and let cells fix for 5 min at room temperature in the dark.

16. Remove fixative and wash cells two times with PBS.

The samples can be stored protected from light for up to 1 day at 4°C.

Mount and examine cells

17. Label microscope slides and place 1 drop of Vectashield mounting medium onto slide. Carefully remove each coverslip from the 24-well plate with watchmaker's forceps and blot excess fluid by touching edge with a paper towel. Invert coverslip, cell-side down onto mounting medium. Do not apply pressure. Blot excess mounting medium with a paper towel and allow slides to dry 5 min at room temperature in the dark. Seal around the rim of the coverslip with nail polish.

18. Examine specimen on a standard fluorescence microscope using a 63 \times or 100 \times oil immersion objective.

Due to its superior resolution power, confocal laser scanning microscopy represents the preferred technique for the quantitative assessment of capping and co-capping.

Neurite Outgrowth Assay

This protocol describes a method to quantify the growth of neurites from cultured neurons. Neurite outgrowth assays are used to determine a potential role of an IgSF-CAM as a substratum for neurite outgrowth. The authors describe here the use of embryonic chicken sensory neurons (Stoeckli et al., 1991), however depending on the substratum to be tested and the responsiveness of the cells, other neurons such as tectal, cerebellar, and spinal cord neurons, may be used as well. It is important that for these assays the neurons are plated at a very low density in order to prevent contact between neurons and their axons, as well as contact between neurons and non-neuronal cells. Only then does the measured neurite length reflect the neurite outgrowth-promoting activity of the substratum used.

Many IgSF-CAMs were found to be potent neurite outgrowth-promoting substrates for various types of neurites. This protocol is an example of the one used for low-density cultures of dorsal root ganglia neurons on an IgSF-CAM substrate. Low-density cultures (Stoeckli et al., 1991, 1996), rather than explants or high-density cultures, are used to minimize effects derived from cell-cell contacts. To achieve reproducible results for growth assays use serum-free, chemically defined media wherever possible.

BASIC PROTOCOL 7

Cell Adhesion

9.5.29

Many commercially available software packages (e.g., NIH Image, Metamorph) provide convenient methods for neurite length measurements. The goal of this protocol, therefore, is not the explication of a particular method of neurite length measurement, but rather the discussion of basic requirements and principles that need to be fulfilled to obtain reproducible values.

Materials

Chicken embryos (E8 to E10)
0.5% (v/v) glucose in PBS
0.25% (w/v) trypsin in PBS without Ca²⁺/Mg²⁺ (Life Technologies)
Serum-free culture medium (see recipe)
Tissue culture dishes coated with substrate of choice (see Support Protocol 8)
Sterile dissecting tools
15-ml centrifuge tubes
Fire-polished Pasteur pipet, ~0.3-mm diameter bore
Neubauer chamber for cell counting (Fig. 1.1.1)
35-mm cell culture dishes
Image analysis software and required equipment
Additional reagents and equipment for counting cells (*UNIT 1.1*)

Dissect dorsal root ganglia and process cells

1. Coat 35-mm tissue culture dishes with appropriate substrate according to Support Protocol 8, 9, 10, 11, or 12.
2. Dissect dorsal root ganglia (DRG) from 10-day-old chicken embryos (Sonderregger et al., 1985) with sterile dissecting tools and collect DRGs in a noncoated 35-mm tissue culture dish on ice in 1 ml of 0.5% glucose in PBS.
Younger embryos can also be used, but the dissection before E8 requires more skills and practice.
3. Transfer DRG to a 15-ml centrifuge tube and centrifuge 3 to 5 min at 300 to 500 × g, room temperature.
4. Carefully remove supernatant and add 2 ml of 0.25% trypsin solution to pellet. Resuspend ganglia and incubate 25 min in 37°C water bath.
5. Centrifuge DRG for 3 to 5 min at 300 to 500 × g, room temperature, decant trypsin solution supernatant, and add 1 ml serum-free culture medium.
6. Use a fire-polished Pasteur pipet with an opening of ~0.3-mm diameter to mechanically dissociate (triturate) the ganglia. Continue until no cell clumps are visible by eye.

Less than ten passages should be sufficient to get a single cell suspension.

Count and plate cells

7. Count an aliquot of the cell suspension in a Neubauer chamber (*UNIT 1.1*).
8. Obtain a cell suspension dilution of 150,000 cells/ml. Plate 1 ml of cell suspension per 35-mm cell culture dish.
9. Incubate cultures for 24 to 30 hr in a 37°C, 5% CO₂ humidified incubator.

Axons reach their maximal length after 24 to 30 hr. However, growth rate and onset of axon growth are substratum-dependent.

Measure and count neurites

10. At the appropriate time, measure neurite length for neurites extending from single cell bodies without contact to other neurites (see Fig.9.5.1 and legend).

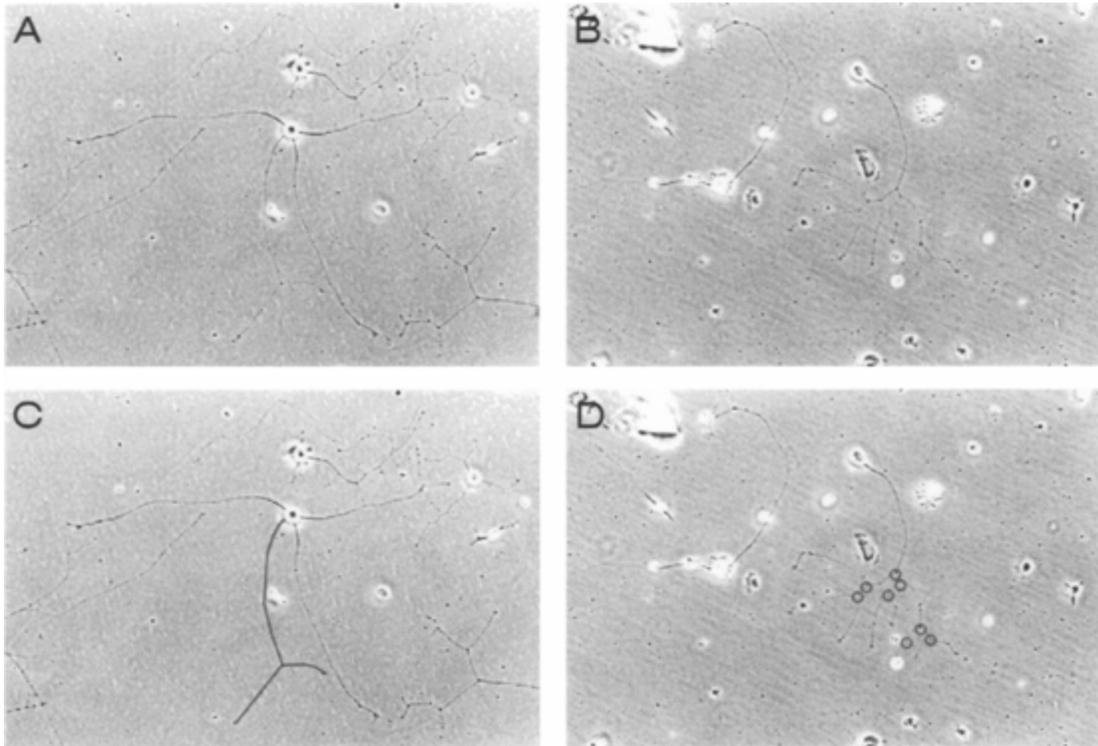


Figure 9.5.1 In order to be a valid assessment of the neurite outgrowth-promoting qualities of a test substrate, the evaluation of neurite outgrowth has to be carried out according to strict standards. Culture conditions have to be reproducible and identical. Therefore, chemically defined media should be used rather than serum containing media. Even minor changes in the composition of media can dramatically alter growth characteristics and the morphology of neurons (e.g., Savoca et al., 1995). The main criteria in the evaluation of neurite growth-promoting qualities of a test substratum is neurite length. However, keep in mind that only neurite lengths of single axons and not bundles of axons should be measured, as the latter would reflect a combination of neurite outgrowth promoted by the substratum and neurite growth along axons. Neurite length can be assessed either as length of the longest neurite (**C**) or as total neurite length of a neuron, in which case the lengths of all neurites of a neuron are added up and represented as one value. Different means can be used to measure the length of a neurite. The most convenient way is the use of a computerized system where one can trace the neurite with the joy stick and the computer will automatically determine its length. Make sure that the software allows the inclusion of the length of the side branches, as they have to be included into the measurements. The comparison between the neurons shown in (**A**) and (**B**) clearly demonstrates that the branching of a neurite is an important trait characteristic for a given substratum. The clear morphological differences between the neurons shown in (**A**) and (**B**) are reflected in the length plots (see Fig. 9.5.2). While the curves for the length of the longest neurite and the total length of all neurites of a neuron would greatly differ for neurons such as the one shown in (**A**), they would be superimposed for neurons like the one shown in (**B**). In other words, a strong deviation of the two curves indicates that neurons have, on average, multiple neurites, whereas a small deviation indicates that the majority of the neurons have only one neurite. Another way to quantify the morphological differences between neurons is the counting of branch points per neurite as shown in (**D**). While the neuron shown in (**A**) has 0 to 2 branch points per neurite, the neuron shown in (**B**) has 8 branch points (**D**).

A prerequisite for reliable neurite length measurements are low-density cultures of sufficient and reproducible quality. Care has to be taken to avoid the measurement of neurite fascicles rather than single neurites, as this confuses the results.

Fasciculation as well as cell-cell contact positively influences neurite length. Thus, for accurate determination of the neurite outgrowth-promoting capacity of a particular substrate or experimental condition, only single neurites extending from single cell bodies without contact to other neurites and especially without contact to non-neuronal cells should be included in the experiment. Cell numbers and culture conditions have to be chosen accordingly.

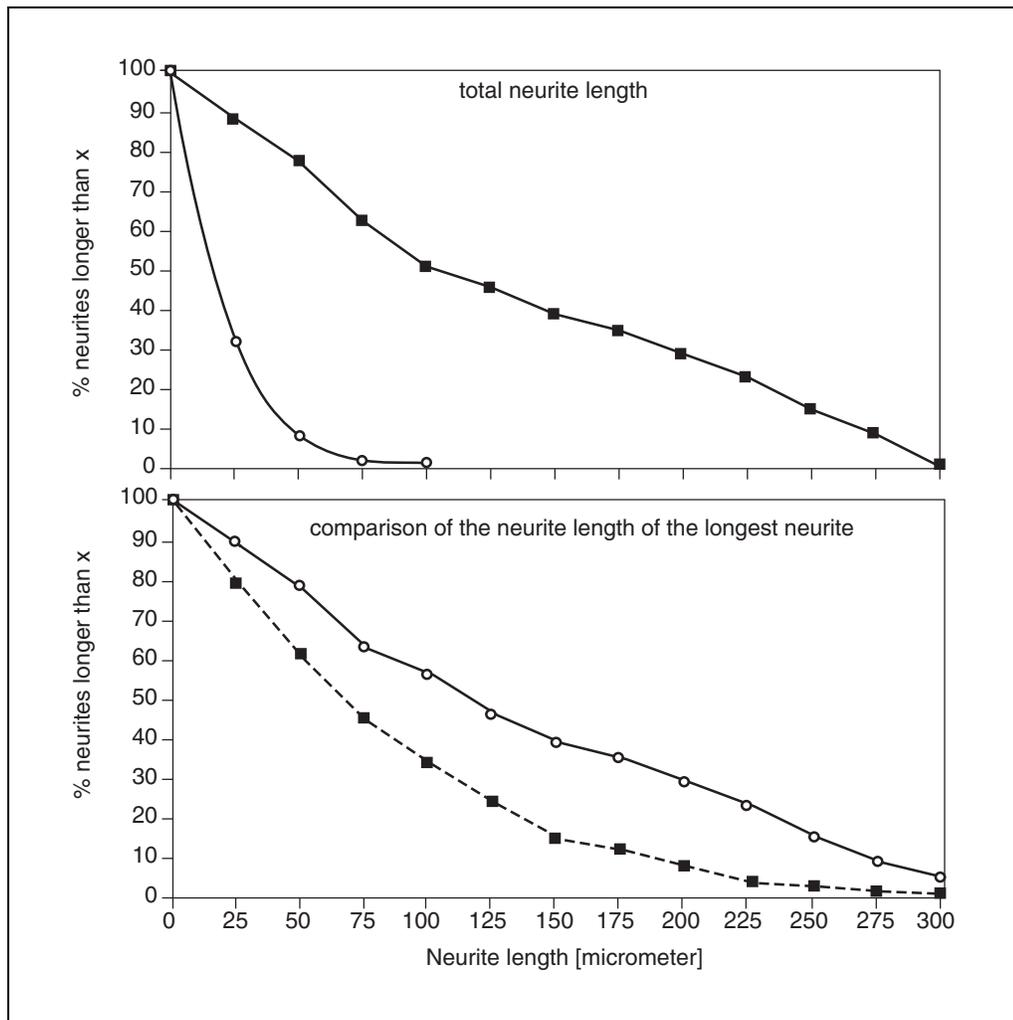


Figure 9.5.2 Graphic representation of neurite length. The neurite outgrowth-promoting activity of different IgSF-CAMs compared to each other or compared to a control protein can be represented in the type of plot that was introduced by S. Chang and colleagues (1987). In these plots the total length of all neurites extending from a neuron can be shown. The two curves in (A) represent a good (solid line) versus a poor substrate (dashed line). The same type of plot can also be used to represent a difference in neurite morphology (B). When the two curves representing the length of the longest neurite of a cell (dashed line with squares) and the total length of all neurites of a given cell (solid line with dots) differ a lot from each other, the neuron has most likely several long neurites, whereas cells with only one long neurite have plots where the two curves are almost the same.

11. Count the number of neurites per neuron, number of branches, and branching order.

Very often the morphology of neurites is substratum-specific. Therefore, the total neurite length per neuron may only give you a partial account of the differences between distinct substrates. Therefore, additional information should be collected, such as number of neurites per neuron, number of branches per neurite, and branching order (i.e., appearance of secondary or even tertiary branches).

12. Analyze the growth cone. Measure the area, number of filopodia, and length of filopodia.

Sometimes the analysis of the growth cone morphology is crucial. Characteristics, such as growth cone area, number of filopodia, or length of filopodia, can change dramatically depending on the substrate used. However, the culture conditions must be controlled carefully, because many of these characteristics can vary depending on the culture medium

as well as on the substratum used. Also, carefully control the origin of the neurons: only neurons from the same embryonic age can be compared. Neurites taken from adult animals can show completely different growth characteristics compared to embryonic neurons. This is especially true with respect to the dependence of neurons for trophic factors. Ideally, serum-free, chemically defined media are used in comparative studies of neurite length and morphology, as the content and concentration of serum components is subject to considerable batch-to-batch variability.

13. Represent the quantitative results.

A very common graphic representation of neurite length is the plot introduced by Chang *et al.* (1987). In these graphs the percentage of neurons with a total neurite length longer than x is easily visible (Fig. 9.5.2). Similarly, the length of the longest neurite per cell or the number of branches can be represented. The comparison between the curves for total neurite length per neuron and the length of the longest neurite can be used as a graphical representation of the difference between neurons with predominantly one neurite that is very long and neurons with many neurites per neuron that amount to the same total length when added together (Fig. 9.5.2B).

Inhibiting CAM-CAM Interactions In Vitro

One way to investigate the function of a particular protein is the analysis of changes resulting from blockage of function. This can be done with several methods both in vivo and in vitro. Whereas in vivo experiments are far more difficult and complex to perform, they more likely reflect the true function of the particular CAM because all parameters are the same, all binding partners are present, all interactions are possible, and even the most difficult parameter to test experimentally, the factor time, can be taken into account. However, there are also disadvantages; in vivo assays are far more complicated and time-consuming than in vitro studies. Most in vivo studies in vertebrates are done in “knock-out” mice, whereby homologous recombination in embryonic stem cells, a gene of interest is replaced by a construct that selects for the cells that have lost the expression of this gene. These “knock-out” mice can be analyzed for phenotypes resulting from the lack of expression of the gene of interest. While this is certainly a very potent method to analyze the function of CAMs, there are several major drawbacks; it is time-consuming, expensive, labor-intensive, and requires appropriate facilities with specially trained experimenters.

Furthermore, as the analysis of several knock-out lines has shown in the past, some mutations result in early embryonic lethality, in which case the organ of particular interest may not have developed by the time of death. This is especially true, when the function of a particular gene is to be analyzed in the nervous system. Alternatively, the lack of one gene may be compensated for, in which case, phenotypes are not detectable or only minor changes occur that are detectable only by very detailed analyses that often exceed the potential of one lab.

An alternative to these genetic loss-of-function analyses is an in vivo study where the blockage of function is done at the protein level (e.g., Stoeckli and Landmesser, 1995; Perrin and Stoeckli, 2000). Due to the relatively easy accessibility of the chicken embryo, it is the model of choice for this type of study. Obviously in contrast to the in vivo experiments described above, this is only possible for functional studies during embryonic development. Although less expensive and less dependent on an appropriate animal facility, in vivo studies using chicken embryos as a model system are time-consuming and require a considerable level of technical skills and training.

A description of in vivo loss-of-function studies would exceed the scope of this unit. For that reason, only in vitro methods for inhibition of IgSF-CAM function will be discussed.

The blockage of IgSF-CAM function in cultures of dissociated cells or tissue explants is much less complex than the inhibition of IgSF-CAM function *in vivo*; however, the results obtained *in vitro* may not reflect or only partially reflect the function of a particular IgSF-CAM *in vivo*. The advantages of *in vitro* experiments are: they are easier to perform, less time-consuming, and more suitable to study a particular question, due to lower degree of complexity. Assays as the one described in this protocol were used to study the role of NgCAM (Chang et al., 1987) and axonin-1 (Stoeckli et al., 1991) in the fasciculation of dorsal root ganglia axons.

Explants of intact dorsal root ganglia (DRG) are grown on a collagen substrate. Under these culture conditions axons extend from the explants in a radial fashion in the form of fascicles. The role of NgCAM as a candidate molecule mediating the axon/axon interaction under these conditions has been tested by the addition of Fab from anti-NgCAM antibodies to the culture medium.

Materials

10-day-old chicken embryos (E10)
0.5% (w/v) glucose in PBS
Chemically defined, serum-free cell culture medium (see recipe)
Control Fab
Fab against CAM of interest

15-ml centrifuge tubes
8-well-slide cell culture dishes (e.g., LabTek, Life Technologies) coated with IgSF-CAM (see Support Protocol 8)
Pasteur pipet or automatic pipettor with 200- μ l tips

1. Dissect DRG from E10 embryos, collect in 0.5% glucose in PBS on ice.
2. Transfer DRG to a 15-ml centrifuge tube, and centrifuge for 3 to 5 min at 300-500 \times g, 4°C or room temperature.
3. Decant supernatant and resuspend pellet in 1 ml serum-free cell culture medium by tapping the vial on the bench and swirling the tube.
4. Prepare 8-well-slide cell culture dishes coated with IgSF-CAM according to Support Protocol 8 by filling the wells with 300 μ l medium containing no Fab, control Fab, or Fab against CAM of interest in different concentrations.

*Antibody concentrations ranging from 100 to 500 μ g/ml for polyclonal and 10 to 500 μ g/ml for monoclonal IgGs have been found useful for *in vitro* studies.*

5. Plate DRG in the smallest possible volume either by using a Pasteur pipet or an automatic pipettor with a 200- μ l tip.

The DRG should be carefully placed on the surface of the dish rather than just put in the medium to facilitate attachment to the substratum. Take care not to scratch the coated surface of the cell culture dish.

Carefully avoid shear forces that can destroy the ganglia. For inexperienced investigators, it is best to use an automatic pipettor set at 20 μ l.

6. Grow DRG for 40 hr in a 37°C, 5% CO₂ humidified incubator.
7. Fix DRG as described in Support Protocol 13 by adding fixative directly to the culture medium to avoid detachment of the ganglia.

COATING OF CULTURE DISHES

To provide a suitable substratum for neurite growth, tissue culture dishes are coated with components of the extracellular matrix, such as laminin (see Support Protocol 12) or collagen (see Support Protocol 11). Alternatively, IgSF-CAMs can be used as substratum by either coating them directly onto tissue culture plastic (see Support Protocol 8) or on top of a nitrocellulose layer (see Support Protocol 9). The application of a nitrocellulose layer prior to coating of the protein substrate (Lagenaur and Lemmon, 1987; see Support Protocol 9) is useful to increase the coating efficiency. When glass has to be used instead of tissue culture plastic, precoating with nitrocellulose can be necessary. For coating with ECM components such as collagen (see Support Protocol 11) or laminin (see Support Protocol 12), glass is an acceptable surface even without precoating.

NOTE: All solutions and equipment must be sterile, and proper sterile technique should be used accordingly. Use filtration to sterilize solutions.

Coating with IgSF-CAM

Purified IgSF-CAMs can be used to coat culture surfaces for neurite growth assays. As purified IgSF-CAMs are usually available in limited amounts, draw a ~0.5-cm² circular area near the center of a 35-mm dish with a lab marker on the bottom outside surface of the dish. Then apply the protein to be tested to the marked area of the dish only. Coat the rest of the dish with bovine albumin (Albumax). This configuration provides better optical access to the cells than a 24 well plate. It also allows comparison of the growth-promoting effect of the protein of interest with that of bovine serum albumin in the same dish.

Materials

Protein to be coated
PBS (see recipe)
10 mg/ml bovine serum albumin (e.g., Albumax, Life Technologies) in PBS

1. Dissolve the proteins in PBS at concentrations of 10 to 100 µg/ml.

Dilute protein solutions should not be stored for extended periods of time, no more than a few days at 4°C and never frozen. If freezing is required, add 100 µg/ml BSA as a carrier protein. Check for possible interference with the assay.

2. Pipet 20 µl protein solution per 0.5-cm² area of the culture dish and spread over marked area or use sufficient solution to cover entire surface.

If glass is used, the volume per area should be doubled to reach sufficient dissipation of liquid.

3. Incubate for 2 hr in a 37°C humidified incubator.
4. Aspirate protein solution and rinse the entire surface of the dishes three times with PBS.
5. To saturate the protein adsorbance capacity of the tissue culture plastic or glass coverslip, incubate dishes for 30 min with a 10 mg/ml BSA solution. Cover the entire surface of the dish (1 ml per 35-mm culture dish).

The use of BSA purified as fraction V from bovine serum is not advised, as most batches contain contaminations of endotoxins that are detrimental for cell survival, especially for neurons. The bovine serum albumin sold as Albumax from Life Technologies has given very satisfactory results.

6. Rinse dishes three times with PBS. Remove PBS immediately before plating cells. For reproducible results, do not allow the coated proteins to dry.

Dishes should be coated immediately before use; storage is not recommended.

SUPPORT PROTOCOL 8

Cell Adhesion

9.5.35

Pre-Coating Glass Surfaces with Nitrocellulose

IgSF-CAMs do not adhere well to glass surfaces unless those surfaces have previously been coated with nitrocellulose.

Materials

Methanol
Nitrocellulose (e.g., BA-83, Schleicher and Schuell), 0.2- μ m pore size
Coverslips, 22-mm diameter (preclean glass with acetone before coating, dry, and autoclave)

1. Dissolve a 5-cm² piece of 0.2- μ m pore size nitrocellulose membrane in 17 ml methanol.

Make sure membrane is completely dissolved. The membrane becomes translucent and is difficult to see. The solution has to be prepared immediately before use. Do not store.

2. Dilute 300 μ l of nitrocellulose membrane/methanol solution with 3.7 ml sterile water to obtain a coating solution.
3. Coat 22-mm glass coverslips by incubating with 200 μ l coating solution for 2 hr in a laminar flow hood. Remove excess coating solution and let coverslips dry before use. Do not store. Proceed with coating with IgSF-CAM (Support Protocol 8).

The same procedure has been used to precoat Thermanox coverslips for electron microscopic use.

Tissue culture plastic can be coated with undiluted nitrocellulose solution, using 450 μ l of the stock solution per 35-mm culture dish.

Pre-Coating with Poly-D-Lysine

The neurite outgrowth-promoting capacity of laminin is enhanced by precoating culture surfaces with poly-D-lysine. Alternatively, poly-D-lysine coating alone provides a good substrate for attachment of neurons and nonneuronal cells (also see Basic Protocol 5).

Materials

0.5 mg/ml poly-D-lysine (see recipe)

1. Make a stock solution of 0.5 mg/ml poly-D-lysine in sterile 150 mM sodium borate, pH 8.4. For coating tissue culture plastic, prepare a working solution immediately prior to use.

Stock solution may be stored for a few weeks at 4°C. The sodium borate buffer can be autoclaved or filtered before poly-D-lysine is added.

Depending on the type of culture, the coating concentrations range between 10 and 500 μ g/ml. Dilute the appropriate volume of the poly-D-lysine stock solution with sodium borate.

2. Incubate dishes with 1 ml coating solution per 35-mm cell culture dish overnight in a 37°C humidified incubator.
3. Aspirate coating solution and rinse dishes at least three times with sterile water. Incubate the dishes overnight with sterile water in a 37°C humidified incubator.

The dishes can be used immediately or dried and stored for several weeks.

Coating with Collagen

Collagen is a good substrate for many non-neuronal cells, but can also be used for axon growth assays e.g., for motor neurons. For most neuronal populations, laminin is the better neurite outgrowth promoting substrate than collagen.

Materials

2 mg/ml collagen in 0.1% (v/v) acetic acid, sterile
35-mm cell culture dishes

1. Dilute a 2 mg/ml stock solution of collagen in 0.1% acetic acid to get a final concentration of 0.25 to 0.5 mg/ml.
2. Coat 35-mm cell culture dishes with 750 μ l of 0.25 to 0.5 mg/ml collagen solution. Evaporate the solution by incubating the dishes at 60°C overnight or until dry.

The dishes can be kept for a few weeks in a dry place at room temperature.

Coating with Laminin

Laminin is a very potent substrate for most neuronal and non-neuronal cells. It can be coated directly to tissue culture plastic and glass surfaces. However, laminin reveals best neurite outgrowth-promoting capacities when coated on dishes precoated with poly-D-lysine (see Support Protocol 10).

Laminin should not be stored in diluted solutions. For best results, keep small aliquots at -20°C , thaw slowly at 4°C , and prepare coating solution immediately before use. Use 750 to 1000 μ l of 10 to 20 $\mu\text{g/ml}$ solutions of stock per 35-mm cell culture dish. Coating is done as described in Support Protocol 8 for IgSF-CAMs. Dishes coated with laminin can be stored.

FIXATION OF CELLS FOR IMMUNOHISTOCHEMICAL STAINING PROCEDURES USING FLUORESCENT ANTIBODIES

In order to avoid the detachment of axons or the collapse of growth cones, the fixative should be added directly to the culture medium. For staining procedures involving fluorescent antibodies, glutaraldehyde can only be used in a very limited concentration, as it is autofluorescent. A concentration of 0.1% (v/v) is compatible with the use of fluorescent secondary antibodies. However, some antigens do not tolerate fixation by glutaraldehyde.

Materials

Paraformaldehyde solution (see recipe)
Cell culture medium

Add 350 μ l of concentrated paraformaldehyde solution to 1 ml cell culture medium to get a final concentration of 2% paraformaldehyde and 0.1% glutaraldehyde. Fix cells for 30 min to 1 hr at 37°C or 2 hr to overnight at 4°C .

**SUPPORT
PROTOCOL 11**

**SUPPORT
PROTOCOL 12**

**SUPPORT
PROTOCOL 13**

Cell Adhesion

9.5.37

**FIXATION FOR MORPHOLOGICAL ANALYSIS, NEURITE LENGTH
MEASUREMENTS, AND FOR IMMUNOHISTOCHEMISTRY WITH
NON-FLUORESCENT SECONDARY ANTIBODIES**

If the autofluorescence of glutaraldehyde does not matter, higher concentrations than 0.1% (v/v) can be used. Glutaraldehyde has a higher crosslinking activity than formaldehyde and therefore, is the fixative of choice for good preservation of morphology. Final concentrations of up to 1% can be used. Add $\frac{1}{3}$ vol 4 \times fixative solution (see recipe) to culture medium. Fix cells 30 min to 1 hr at 37°C or 2 hr to overnight at 4°C. For non-neuronal cells that are more strongly attached to the culture dish than neurons with axons, aspirate the culture medium and add 1 \times fixative solution.

NOTE: The preservation of antigens may restrict the use of high concentrations of glutaraldehyde, even if not used in combination with fluorescent secondary antibodies.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Blocking solution (for amino bead coupling)

0.5 g bovine serum albumin (Fraction V; Sigma)
0.02% (w/v) NaN₃
50 mM Tris, pH 8.0, to 100 ml
Sterilize by filtering through a 0.45- μ m filter
Store up to 1 month at 4°C

BSA solution, 0.5% (w/v)

0.5 g bovine serum albumin (Fraction V; Sigma)
0.02% (w/v) NaN₃
PBS (see recipe) to 100 ml
Sterilize by filtering through a 0.45- μ m filter
Store up to 1 month at 4°C

Ca²⁺/Mg²⁺-free buffer (CMF buffer)

137 mM NaCl
4 mM KCl
0.4 mM Na₂HPO₄·2 H₂O
0.18 mM KH₂PO₄
12 mM NaHCO₃
11 mM glucose, pH 7.2
Filter sterilize through 0.22- μ m filter
Store <2 weeks at 4°C

CaCl₂ solution

250 mM CaCl₂
Dissolve in ultrapure water
Sterilize by filtering through a 0.22- μ m filter
Store at room temperature

Cell culture medium for HEK 293 cells

DMEM containing:

- 2 mM glutamine
- 2.4 g/liter sodium bicarbonate
- 10 mM HEPES, pH 7.5
- 10% (v/v) FCS
- Filter sterilize through 0.45- μ m filter
- Store no more than a few days
- Warm to 37°C before adding to cells

Chemically defined, serum-free medium

- MEM containing
- 5 mg/ml lipid-rich bovine serum albumin with 0.1% IgG (e.g., AlbuMAX I, Life Technologies)
- 2 mM L-alanyl-L-glutamine or dipeptide glycyl-L-glutamine (e.g., GlutaMAX I, Life Technologies)
- 100 μ g/ml transferrin
- 10 μ g/ml insulin
- 20 ng/ml triiodothyronine
- 40 nM progesterone
- 200 ng/ml corticosterone
- 200 μ M putrescine
- 60 nM sodium selenite
- 20 ng/ml nerve growth factor (NGF)
- Prepare fresh

4-chloro-1-naphthalene solution

Stock solution: Dissolve 4-chloro-1-naphthalene in ethanol to give a 3 mg/ml solution. Store several weeks at -20°C .

Working solution: Immediately before use, dilute stock solution in TBS (APPENDIX 2A) and add 30% H_2O_2 to give final concentrations of 0.5 mg/ml chloronaphthalene and 0.01% H_2O_2 in TBS.

Cross-linking reagents

100 mM stock solution of cross-linker in water (BS^3 , Sulfo-DST, and DTSSP) or water-free DMSO (DST). Stocks in DMSO can be stored at -20°C under dry nitrogen or argon for 1 to 2 months.

- Bis(sulfosuccinimidyl)suberate (BS^3 , Pierce)
- Disuccinimidyl tartrate (DST, Pierce)
- Disulfo disuccinimidyl tartrate (Sulfo-DST, Pierce)
- 3, 3'-Dithiobis(sulfosuccinimidyl propionate) (DTSSP, Pierce)

These reagents can be stored under water-free nitrogen or argon for up to 1 year at -20°C .

Working solution: Dilute stock solution of cross-linker in PBS with 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+} to a concentration of 1 mM. Prepare fresh and do not keep for >1 hr on ice.

Fixation solution, 4 \times

- 40 ml concentrated paraformaldehyde solution (see recipe)
- 5 ml 20 \times PBS
- 0.4 ml 50% (v/v) glutaraldehyde (reagent grade) in water
- H_2O to 50 ml

For best results, prepare fresh. If necessary, the fixative can be stored 2 to 3 days at 4°C .

HBS solution

50 mM HEPES
1.5 mM Na₂HPO₄
140 mM NaCl
Dissolve in ultrapure water
Adjust pH to 7.05 with 1 M NaOH or 1 M HCl
Sterilize by filtering through a 0.22- μ m filter
Store for months at 4°C

L-histidinol, 50 mM

Dissolve 535 mg L-histidinol dihydrochloride (Sigma) in 50 ml of 100 mM HEPES, pH 7.4, pass through a 0.22- μ m filter, and store in aliquots for up to 1 year at -20°C.

LB medium

Dissolve 10 g NaCl, 10 g bacto-tryptone (Difco), and 5 g yeast extract (Difco) in 1 liter water, autoclave, and store at 4°C.

LB/ampicillin agar plates

Dissolve 15 g bacto-agar in 1 liter LB medium (see recipe) by autoclaving, cool down to 50°C, add 1 ml of 50 mg/ml ampicillin, mix, and pour into 10-cm polystyrene plates. Let sit at room temperature overnight and store protected from light at 4°C.

Lysis buffer

1% (w/v) CHAPS
0.1% (w/v) SDS
50 mM Tris-Cl, pH 7.6 (APPENDIX 2A)
150 mM NaCl
5 mM EDTA
10 μ g/ml leupeptin
10 μ g/ml aprotinin
10 μ g/ml pepstatin A
1 mM PMSF

Protease inhibitors except PMSF are stable in lysis buffer at -20°C for several months. However, PMSF has to be added fresh. Prepare a 100 mM PMSF stock solution in isopropanol and <5 min before use add to lysis buffer to a final concentration of 1 mM. PMSF stock solution can be stored several weeks at -20°C.

Mounting medium

10 ml 1M Tris-Cl, pH 9.0 (APPENDIX 2A)
70 ml glycerol (70% v/v final)
5.09 g *n*-propyl-gallate (0.24 M final)
H₂O to 100 ml
Adjust pH to 9.0
Make 1-ml aliquots and store at -20°C

Paraformaldehyde solution, 10% (w/v)

Add 5 g paraformaldehyde to 45 ml water, add 75 μ l of 1 M NaOH and dissolve by heating to 60°C in a water bath. Let solution cool to room temperature, add water to a final volume of 50 ml, check that pH does not exceed 7.4. For best results prepare fixative immediately before use. Do not store >2 to 3 days at 4°C.

PBS

8.00 g NaCl (137 mM final)
0.20 g KCl (2.7 mM final)
2.16 g Na₂HPO₄ × 7 H₂O (8 mM final)
0.20 g KH₂PO₄ (1.5 mM final)
990 ml H₂O
Adjust pH to 7.4 with 1 N HCl or 1 N NaOH
Adjust volume to 1 liter with water
Filter sterilize through a 22-mm filter
Store at 4°C

PBS with Ca²⁺/Mg²⁺

8.00 g NaCl (137 mM)
0.20 g KCl (2.7 mM)
2.16 g Na₂HPO₄ × 7 H₂O (8 mM)
0.20 g KH₂PO₄ (1.5 mM)
0.10 g CaCl₂ (0.9 mM)
0.10 g MgCl₂ × 6 H₂O (0.5 mM)
990 ml H₂O
Adjust pH to 7.4 with 1 N HCl or 1 N NaOH
Adjust volume to 1 liter with water
Filter sterilize through a 0.22-μm filter
Store at 4°C

PEG 1500 in DMEM supplemented with DMSO

Melt 42.5 g polyethylene glycol 1500 (PEG 1500) in a microwave oven, add 50 ml DMEM and 10 ml DMSO, pass through a 0.22-μm filter, and store in aliquots up to 1 year at -20°C.

Poly-D-lysine solution

Dissolve 0.5 mg/ml poly-D-lysine (Sigma) in sterile 150 mM sodium borate, pH 8.4. Store a few weeks at 4°C.

Wash buffer

0.1% (w/v) CHAPS
0.1% (w/v) SDS
50 mM Tris·Cl, pH 7.6 (APPENDIX 2A)
150 mM NaCl
5 mM EDTA
1 mM PMSF
Add <5 min before use

COMMENTARY

Background Information

IgSF-CAMs have been characterized as crucial molecules for both the development and the normal function of multicellular organisms. They exert their function by mediating the interactions between cells under various circumstances. Interactions mediated by IgSF-CAMs may determine whether a cell remains where it is or whether it moves somewhere else; they may be involved in determining the path of cell migration and the time and the location of the

end of the migratory path. Other IgSF-CAMs have been implicated in processes such as the extravasation of white blood cells, inflammation, wound healing, as well as tumor invasion and metastasis. In the developing nervous system, IgSF-CAMs play important roles in cellular migration, in the regulation of axonal outgrowth and pathfinding, and in synaptogenesis. In the adult nervous system, IgSF-CAMs play a role in the regulation of neural plasticity and in nerve regeneration after injury. Defective

genes of IgSF-CAMs have been found to result in severe cerebral malformations and mental retardation.

Recent advances in the characterization of IgSF-CAMs have revealed that in several cases IgSF-CAMs not only mediate cell-cell adhesion by binding to each other, but that they are often capable of eliciting intracellular signals upon binding an extracellular ligand. Intracellular signals elicited by extracellular ligand contact of IgSF-CAMs comprise many signal pathways, including the production of second messengers and the activation of transmembrane or intracellular kinases and phosphatases.

The enormous complexity of binding partners is the most striking difference between the calcium-dependent cell adhesion molecules and the calcium-independent cell adhesion molecules of the IgSF. Many IgSF-CAMs have been found not only to bind to their own kind in so-called homophilic interactions, but also to engage in several heterophilic interactions. Therefore, the determination of ligands plays a very important role in the characterization of IgSF-CAMs. Only by identifying the macromolecular binding partners of IgSF-CAMs can their functional roles in living organisms be elucidated. Some of these very basic studies demonstrating interactions between IgSF-CAMs and other binding partners may be applicable to the study of cell surface molecules in general, including molecules specifically expressed in the immune system, virus receptors, tumor growth markers, or growth factor receptors. However, the analyses of the specific functional roles of these proteins is beyond the scope of this unit. For these, the reader is referred to more specialized applications.

Trans-interactions

IgSF-CAMs are composed of at least one Ig fold, which is also the molecular building block of antibodies (Rader and Sonderegger, 1998). It is therefore conceivable to express IgSF-CAMs in myeloma cells that are specialized in the production of antibodies. An expression system based on myeloma cells was established for the production of soluble lymphocyte-derived cell-surface receptor proteins by Traunecker et al. (1991). This system has also been used for the expression of IgSF-CAMs on the surface of myeloma cells (Rader et al., 1993). By studying the IgSF-CAMs in a natural environment, i.e., as membrane-bound molecules residing in their proper orientation in a biological membrane, *trans*-interactions between molecules residing in different mem-

branes can be readily detected through cell aggregation. Nonadherent myeloma cells provide an established natural environment for these studies. The myeloma cell aggregation assay described here has contributed significantly to the elucidation of the complex interaction pattern of two IgSF-CAMs, axonin-1 and NgCAM, which were found to *trans*-interact homophilically and *cis*-interact heterophilically (Rader et al., 1993; Buchstaller et al., 1996; Rader and Sonderegger, 1998; Sonderegger et al., 1998). In addition, the myeloma cell aggregation assay can be used for the structure-to-function analysis of a *trans*-interaction. Monoclonal antibodies that have been mapped to particular epitopes on the IgSF-CAMs can be analyzed for interference with myeloma cell aggregation. Defined mutants of IgSF-CAMs, e.g., engineered domain deletion mutants or pathological point mutations, can be expressed in myeloma cells and assessed for their effect on *trans*-interactions (Freigang et al., 2000). Furthermore, the influence of soluble molecules, e.g., ligands or toxins, on *trans*-interactions of IgSF-CAMs can be studied using myeloma cell aggregation. The expression in myeloma cells has also facilitated the production of soluble variants of IgSF-CAMs and fragments thereof (Rader et al., 1993, 1996).

Chemical cross-linking

Chemical cross-linking is a special form of chemical modification that results in the covalent coupling of molecules by a cross-linking reagent. In contrast to the simple conjugation of two molecules, cross-linking refers to the covalent coupling of two molecules that undergo an interaction with each other. The interacting molecules involved can be proteins, peptides, nucleic acids, or carbohydrates. Cross-linkers for proteins are bifunctional molecules containing two reactive groups that undergo reactions with side chains of amino acids. Cross-linkers can be generally classified into two main families, the homobifunctional cross-linkers, which contain two identical functional groups, and the heterobifunctional cross-linkers, which contain different types of reactive moieties. Simple homobifunctional cross-linkers, such as dialkyl halides and bis-imidoesters, were introduced in the 1950's. Since then >300 different cross-linking reagents have been synthesized and a large number of these are commercially available (Ji, 1983; Staros and Anjaneyulu, 1989). Recent developments led to the design and synthesis of cleavable bifunctional compounds that allow the recovery of the

individual compounds present within a cross-linked complex after its isolation.

Using bifunctional reagents for cross-linking of macromolecules, such as proteins, two different reaction products are generated resulting from either intramolecular or intermolecular cross-linking. For the study of protein-protein interactions as described in the protocol, intermolecular cross-linking is of interest. In the past, intermolecular cross-linking was instrumental in the investigation of antigen-antibody complexes, membrane protein structures, and protein-protein interactions at the quaternary structural level, e.g., in receptor-ligand or multienzyme complexes. The application of chemical cross-linking to identify membrane receptors for macromolecular ligands has become an established technique that can be successfully applied to the analysis of interactions between IgSF-CAMs (Staros, 1988).

The choice of reagents for cross-linking membrane proteins must take into consideration the particular physicochemical properties of biological membranes. A major problem is represented by the unspecific, random-collision dependent cross-linking, due to the lateral mobility of membrane proteins. Chemical cross-linking of membrane proteins should, therefore, generally be carried out in the cold to reduce lateral movements of proteins. A second major factor to be considered is the hydrophobicity of the membrane core. Hydrophobic and hydrophilic reagents have the potential to probe different regions of integral membrane proteins for interactions with binding partners. To study interactions between extracellular domains of IgSF-CAMs, hydrophilic, membrane-impermeable cross-linkers that bear hydrophilic or charged groups can be used (Buchstaller et al., 1996; Kunz et al., 1998). The limitation of cross-linking to closely associated binding partners within complexes of membrane proteins requires reagents with short spacers between their reactive groups. However, cross-linkers with spacer lengths of <0.5 nm result in poor yields of intermolecular coupling, whereas extensive cross-linking with decreasing specificity is generally observed with reagents with spacer lengths of >1.1 nm (Midgaugh et al., 1983). The use of a homologous series of cross-linking compounds is recommended for the evaluation of the system.

For efficient cross-linking between extracellular domains of membrane proteins, two functional groups that differ in reactivity and chemical selectivity are predominantly used, (1) the *N*-succinimidyl group and (2) the photoactivat-

able aryl azide group. The *N*-succinimidyl group combines efficient reactivity with a high selectivity for primary amino groups, thereby limiting the cross-linking to lysine side chains and free N-termini of proteins. Disuccinimidyl derivatives of variable spacer length and hydrophilicity are currently commercially available. Popular hydrophilic disuccinimidyl reagents are the disuccinimidyl suberate (DSS) derivative bis(sulfosuccinimidyl)suberate (BS³) with a spacer length of 1.14 nm, disuccinimidyl tartrate (DST) and its disulfo-variant disulfo disuccinimidyl tartrate (Sulfo-DST) with a spacer length of 0.64 nm. A water-soluble, membrane-impermeable, thiol-cleavable disuccinimidyl reagent with a spacer length of 1.2 nm is represented by 3, 3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP).

The aryl azide group undergoes a UV light-induced chemical reaction resulting in the generation of a nitrene, a highly reactive, short-lived intermediate containing a nitrogen electron sextet (aza-analogon to a carbene). Analogous to carbenes, the nitrenes react immediately with their direct environment. The major reaction of interest is the insertion into C-H and N-H bonds resulting in the formation of a new covalent bond. This broad specificity allows the nitrenes to react with virtually all chemical groups present on a protein surface, making this type of cross-linking extremely efficient. Common nitrene precursors are arylazides with absorbance maxima in the long UV region. Nitro-substitution of the aromatic ring results in a further shift of absorbance towards longer wave lengths, away from the absorbance maxima of proteins. Photosensitive heterobifunctional cross-linkers containing one amine-reactive *N*-succinimidyl group with the photoactivatable aryl azide group are widely used reagents for the cross-linking of membrane proteins with their receptors (Hermanson, 1996). Commercially available products of this group are *N*-hydroxysulfosuccinimidyl-4-azidobenzoate (Sulfo-HSAB, spacer length of 0.9 nm) and the highly versatile thiol-cleavable reagent sulfosuccinimidyl 4-(*p*-azidophenyl)dithio) propionate (Sulfo-SADP, with a spacer length of 1.39 nm). For protocols regarding the use of these photoreactive cross-linkers see Jung and Moroi (1983) and Wood and O'Dorisio (1985).

Co-capping

The detection of *cis*-interactions by antibody-induced co-capping is based on the lateral mobility of integral membrane proteins within

the plane of the cell membrane. The freedom of lateral movement allows the induction of large clusters of these molecules at the cell surface by cross-linking their extracellular moieties with antibodies, so-called capping. Molecules that undergo (direct or indirect) interactions with the capped molecules in the plane of the cell membrane are co-clustered and exhibit co-capping when examined by immunofluorescence microscopy.

Antibody-induced co-capping has been an established technique, in particular in the field of immunology, for the last decades and was instrumental for the detection of many interactions of leukocyte surface molecules that are involved in antigen presentation and recognition (Rojo et al., 1989; Zhou et al., 1993). The co-capping approach allows detection of interactions between membrane proteins at the level of individual intact cells. The availability of novel fluorescent dyes allows the detection of three or four different fluorochromes simultaneously. This allows the examination of the cellular distribution of, for example, effector molecules of signal transduction or cytoskeleton components as a function of co-clustering of cell surface proteins by a combination of classical co-capping protocols with intracellular immunofluorescence staining.

Classical biochemical techniques, for example co-immunoprecipitation or chemical cross-linking (see Basic Protocol 5), require a homogeneous sample. In contrast, co-capping studies examine interactions between proteins at the level of individual cells and can, therefore, be performed on heterogeneous samples, like mixed primary cell cultures derived from animal tissue, if appropriate markers are available that allow one to distinguish between different cell types. A further major advantage is the requirement for only a limited number of cells for co-capping studies. This opens the possibility to study specific, rare cell types, which can be isolated only in small quantities. A powerful combination is represented by the prior isolation of a specific cell type, e.g., by fluorescence-activated cell sorting (FACS), using appropriate combinations of cell surface markers with subsequent co-capping studies.

The crucial prerequisite of the co-capping approach is the availability of suitable antibodies. This limits application of the technique to already described molecules against which antibodies are available. In addition, the antibodies used must meet the following criteria, (1) absence of any cross-reaction with other cell surface proteins on the cells of interest; and (2)

no interference of antibody binding with the interaction that is studied (this point often represents an unknown factor that may influence the result). In addition, the two primary antibodies used must be raised in two different species.

Critical Parameters and Troubleshooting

Preparation of IgSF-CAMs

For the functional analysis of an IgSF-CAM, it is extremely important to have a highly purified protein sample. Due to the high complexity of their interaction pattern, a contamination of the protein sample to be analyzed by another IgSF-CAM could confound the results of binding assays (see Basic Protocols 2 and 3) or neurite outgrowth assays (see Basic Protocol 7). For this reason, protein samples purified with affinity columns should always be analyzed by SDS-PAGE followed by silver staining to confirm the absence of contaminating proteins. When possible contaminants are known, for instance based on their high expression level in the tissue used as a source for the purification, or when the absence of a particular IgSF-CAM is important for the experimental procedure, their absence can best be documented by immunoblot analysis of the purified fractions. As a quick alternative, dot blots described in Support Protocol 4 can be used. If contaminants are found, the following possibilities should be considered.

Affinity column was not rinsed properly after loading or has run dry. Make sure absorbance is back to baseline before starting elution. Take an aliquot from the wash fraction to confirm the absence of protein. It is extremely important that the column never runs dry.

Antibody used to make column is not specific or antibody is denatured because column is too old or was not stored properly. When the quality of the purified protein samples decreases, it is time to prepare a new affinity column. It is possible that the antibody is very sensitive to high pH conditions used for elution. Try an elution buffer with low pH (0.1 M glycine/HCl, pH 2.7) as an alternative. Change concentration and/or type of detergent used.

Because of the large number of IgSF-CAMs expressed in the nervous system and based on the enormous complexity of their interactions, the specificity of the antibodies used for function-blocking assays and the purity of IgSF-CAM preparations have to be tested carefully. It is important to include appropriate controls,

such as the use of preimmune IgG purified according to the same protocols as the specific IgGs to rule out toxicity of the antibody preparations. Assess the dose-dependence of the effect. A very convincing control for the specificity of the assay is the absence of an effect in the presence of a specific antibody against another IgSF-CAM that is expressed by the same cell.

Transfection

The most critical parameter determining the efficiency of the calcium phosphate protocol described is the 293 cells that are used. Cells of low passage numbers (not more than 40 passages) exhibit a much higher transfection efficiency than later passages.

The formation of the precipitate prior to the addition to cells is mainly influenced by the following parameters: concentrations of calcium, phosphate, and DNA, the nature of the DNA construct, pH, temperature, and time of incubation. The calcium and phosphate concentrations given in this protocol are optimized for this system. Optimal DNA concentration depends on the construct and must be determined for each system individually. A good starting point for optimizations when dealing with medium-sized plasmids of 5 to 10 kb is 40 to 80 μ g DNA. Plasmids of different sizes vary considerably in behavior in calcium phosphate transfection. The protocol described works best for average sized expression plasmids (5 to 10 kb). Very large plasmids (>30 kb) frequently result in the formation of much larger precipitates, reducing the efficiency of transfection considerably. A reaction at pH 7.0, room temperature (20° to 25°C), and 1 min reaction time are good starting conditions for optimizations. The formation of precipitate after mixing CaCl₂ solution with HBS solution can be monitored by measuring the absorbance at 320 nm (e.g., for the testing of new batches of solutions).

After addition to the cells, the stability of the DNA-containing calcium phosphate particles is the most critical factor for the efficiency of transfection. One source of instability is a reduction in medium pH due to the metabolic activity of cells. It is therefore important to include 10 mM HEPES (final concentration) in the 293 cell culture medium. The reduction of the CO₂ partial pressure to 3% is another option to raise the pH value, if necessary. Increased time of exposure to the precipitate can further enhance the efficiency of transfection. An incubation time of 4 to 6 hr given in the protocol is a good starting point and can be extended up

to 24 hr in cases where no cytotoxicity is observed. However, the combination of exposure to the precipitate with an osmotic shock, e.g., by adding 10% (w/v) glycerol to the cell culture medium, as suggested by some authors for the calcium phosphate transfection of Chinese hamster ovary (CHO) cells, is not recommended for 293 cells.

Immunoblotting

No positive dots. The concentration of the protein of interest may be below the detection limit. Use repeated applications of protein solution to the nitrocellulose membrane. Apply the purified protein onto the membrane as a positive control. If the total protein concentration of the solution applied to the membrane is high, even repeated applications of protein solution to the membrane may not give a satisfactory result.

High background. Make sure that the volumes of protein solution applied to the membrane are small. Let the dot dry before the next step. Wash membranes more thoroughly. Change the blocking solution and/or blocking time. For instance, try blocking with serum from the same species that was used to raise the secondary antibody.

Microsphere assays

False negative results in the bead aggregation assay. Coupling reaction results in protein orientation on the bead surface in a way that binding sites are not accessible. Alternatively, proteins could not be physiologically active after purification (e.g., elution after immunoaffinity column).

False positive results in the bead aggregation assay. Protein preparations that are not pure and contain another CAM could result in false positive binding results. Furthermore, if each of the two test proteins has homophilic binding properties, weak unspecific aggregation of preformed homophilic aggregates could result in false positive results with respect to heterophilic interactions. Therefore, check for unevenly distributed beads in mixed aggregates under the microscope (bead distribution in aggregates is not detectable in a flow cytometer).

Discrepancies between results of bead and cell aggregation assays. The orientation of proteins on the beads is likely to be random and not uniform as in a biological membrane. This has to be considered when interpreting and comparing the results with the ones of the cell aggregation assay. Furthermore, the orientation problem has to be taken into account when

analyzing *cis*- and *trans*-interactions (Kuhn et al., 1991; Buchstaller et al., 1996; Rader et al., 1996; Sonderegger et al., 1998).

Orientation of proteins on beads can be better controlled when using proteins with domains or tags that tightly bind to a linker protein that is coupled first to the microsphere. For example, recombinant Fc-fusion proteins can be coated on protein A-conjugated beads in an oriented manner. One has to consider that this linkage includes a noncovalent protein interaction.

Trans-interactions

The stably transfected myeloma cell clones may differ from the parental myeloma cell line in more than only the expression of the IgSF-CAM. The upregulation of endogenous cell adhesion molecules, such as integrins and IgSF-CAMs, can result in myeloma cell aggregation (Kawano et al., 1991). It is therefore essential to run a series of control experiments that address the question whether any cell aggregation is caused by a *trans*-interaction of the expressed IgSF-CAMs or by other factors. For this, follow these guidelines:

1. Confirm the functional surface expression of the IgSF-CAM using, for example, monoclonal antibodies directed against conformational epitopes.
2. Each aggregation assay should be performed in duplicate with cross-wise exchange of the fluorescent dyes.
3. Myeloma cell clones that express an IgSF-CAM should not form mixed aggregates with the parental myeloma cell line.
4. Independent myeloma cell clones that express the same IgSF-CAM should give the same aggregation pattern.
5. Independent myeloma cell clones that express different quantities of the same IgSF-CAM should be analyzed for a correlation between expression and aggregation.
6. Pre-incubation with polyclonal Fab directed to the IgSF-CAM should prevent aggregation.
7. Pre-incubation with an enzyme that removes the IgSF-CAM selectively from the surface should prevent aggregation. A very useful enzyme for this is phosphatidylinositol-specific phospholipase C, which selectively cleaves glycosyl-phosphatidylinositol-anchored proteins.

Chemical cross-linking

The application of cross-linking reagents on intact, live cells may result in possible artifacts due to the perturbation of the architecture of the

cell membrane by the chemicals. Very often, the reagents are used at millimolar or higher concentrations (0.1 to 10 mM) and must be dissolved in organic solvents prior to use. It is therefore very important to assure that the reagents by themselves and especially organic solvents, if used, do not affect the structural integrity and viability of the cells tested. Water-soluble reagents like BS³, Sulfo-DST, and DTSSP that carry sulfonyl groups can be used to circumvent the solvent problem.

Successful detection of *cis*-interactions between IgSF-CAMs requires a high specificity of chemical cross-linking on the one hand, and sufficient yields of cross-linked materials on the other hand, for subsequent biochemical characterization of cross-linked partners. As described above, the specificity and efficiency of the cross-linking reaction is determined by the following parameters: length of the spacer separating the reactive groups; chemical reactivity of the functional groups of the cross-linking reagent; concentration of cross-linking reagent; and reaction time.

Cross-linking reagents with short spacers between the reactive groups such as DST and Sulfo-DST restrict chemical coupling to closely associated molecules and are therefore preferable for the detection of *cis*-complexes between membrane proteins. However, due to the spatial proximity of their functional groups, these reagents exhibit an enhanced tendency for intramolecular cross-linking, i.e., coupling lysine side chains of the same molecule with each other, which may result in very low yields of cross-linked material. The application of reagents with longer spacers between the reactive groups such as BS³ and DTSSP generally results in higher yields, but bears the risk of unspecific reactions. As previously discussed, the chemical reactivity of the functional groups present in commercially available cross-linking reagents range from highly selective, like the *N*-succinimidyl group, to rather unselective photoactivated groups, like aryl azides, which generate highly reactive, unstable intermediates that undergo reactions with a wide variety of chemical structures within a protein. Although the *N*-succinimidyl group is most frequently used for modifications and cross-linking of cell surface proteins, its selective reactivity with primary amino groups (mainly lysine side chains) excludes this class of cross-linkers from extended hydrophobic interfaces through which IgSF-CAMs may interact. Upon photoactivation, reagents containing aryl azide groups exhibit a high reactivity towards ali-

phatic hydrocarbon groups, like RCH_2R and R_2CHR , that allows cross-linking within a strongly hydrophobic environment. However, the high reactivity and low selectivity of photoactivated aryl azides frequently results in a high degree of unspecific cross-linking, not only among proteins, but also extensive protein-lipid cross-linking. For the detection of specific interactions among the closely associated proteins on the surface of intact cells, these reagents are, therefore, not recommended as first choice, but represent an option in cases where no cross-linking products are obtained using more specific reagents.

Optimal concentrations of cross-linking reagents must be evaluated empirically for every experimental system. A more detailed discussion of the chemical background can be found in Lomant and Fairbanks (1976), Lewis et al. (1977), and Smith et al. (1978). For homobifunctional *N*-succinimidyl derivatives, like DST and DSSP, optimal concentrations for cross-linking on intact cells are consistently in the range of 0.1 to 10 mM in published protocols. This range of concentrations represents a good starting point for optimizations. Cross-linking reagents with higher reactivity, like e.g., aryl azides, are generally applied in much lower concentrations, usually between 10 μM and 1 mM, for cross-linking on intact cells.

A further critical parameter is reaction time. Longer reaction times generally result in better yields of cross-linked products, but also in more unspecific reactions. Using homobifunctional *N*-succinimidyl reagents, quenching of the cross-linking reaction after 0, 5, 15, and 45 min results frequently in a "kinetic profile" of the process. Specifically, a different pattern of cross-linked products is observed during the time course of the cross-linking reaction. This reflects the tendency of many membrane molecules, such as IgSF-CAMs, to form oligomeric or even multimeric aggregates in the membranes of living cells. The appearance of initial cross-linked molecules, after a few minutes, is often followed by the appearance of further cross-linked complexes with higher molecular masses, generated from the coupling of the initial complexes with additional molecules. Initial cross-linked products that are generated within the first minutes of the reaction may correspond to the first assembly units from which larger oligomeric or multimeric complexes are formed. The "kinetic profile" of the cross-linking reaction may, therefore, give some information about the nature of the com-

plexes or aggregates formed by molecules like IgSF-CAMs.

Co-capping

The demonstration of a *cis*-interaction by antibody-induced co-capping of cell surface molecules critically depends on many factors. To allow the induction of caps by antibody cross-linking and co-capping mediated by a *cis*-interaction, the molecules of interest must have a minimal lateral mobility within the plane of the cell membrane. In addition to mobility, the relative stoichiometry of the molecules of interest is critical for the detection of co-capping after antibody-induced capping. Ideally, comparable levels of expression at the cell surface allow reciprocal co-capping of two molecules that interact with each other in *cis* with sufficient affinity. However, large stoichiometric excess of one molecule due to different expression levels results in asymmetric results in reciprocal co-capping experiments; capping of the more abundant component is followed by clearly detectable co-capping of the less abundant molecule. In contrast, capping of the molecule expressed at lower level results in only partial co-capping of the more abundant component. For the detection of partial co-capping, confocal laser scanning microscopy is a powerful technique that allows the reliable detection of locally enhanced fluorescence signals (co-capping) versus a relatively high homogeneous background signal (uncapped monomers of the molecule present in stoichiometric excess).

In addition to the factors described above, the availability of specific primary antibodies against the molecules of interest, raised in different species, is an essential prerequisite for co-capping experiments. For antibody-induced capping, polyclonal or monoclonal antibodies can be used. Polyclonal antibodies are able to cross-link the cell surface antigen to some extent in the absence of secondary antibody, whereas in the case of monoclonal primary antibodies, cross-linking of bound primary antibody with the secondary antibody is required for the induction of caps. The most critical factors of every co-capping experiment is the specificity of the primary antibodies used. A major source of experimental artifacts is the potential of cross-reactivity of primary antibodies, either between the molecules of interest or with other, unidentified molecules expressed on the cell. It is therefore of pivotal importance to exclude any cross-reactivity under the conditions (antibody concentrations, temperature,

incubation times, etc.) used for co-capping and counter-staining. It is not recommended to test for cross-reactivity with other immunochemical techniques like immuno-blot or ELISA, because the antigens are presented in a different form on nitrocellulose and on plastic surfaces than on live cells. The absence of cross-reactivity of the partially or totally denatured proteins present in such immunochemical assays does, therefore, not necessarily exclude cross-reactivity of the native proteins present on the live cells under the conditions used for co-capping experiments. In situations where the proteins of interest are co-expressed in recombinant form in a cell type that does not normally express them, the test for cross-reactivity is straightforward. Specificity of detection can be checked by immunostaining of single transfectants and mock-transfected cells (as a negative control) with both antibodies (see, e.g., Buchstaller et al., 1996; Kunz et al., 1998). It should be noted that cross-reactivity of antibodies is a phenomenon that depends on the antibody concentrations used. Even in cases where no cross-reactivity is reported in the literature (e.g., based on experience with standard protocols for immunofluorescence), cross-reactivity may occur at the higher antibody concentrations that are normally applied in co-capping experiments. Cross-reactivity of the secondary antibodies represents only a minor problem, since highly specific preparations of fluorochrome-labeled secondary antibodies against a wide variety of species are commercially available. The use of phylogenetically more distant species is desirable since the potential of cross-reactivity between the secondary antibodies is lower. Combinations that are frequently documented in the literature are mouse/rabbit, mouse/goat (or sheep), rabbit/goat (or sheep). A considerable risk of cross-reactivity exists especially for the combination mouse/rat. In this case the choice of secondary antibodies has to be made with care.

Several controls must be included to ensure specificity of antibody-induced capping. The use of preimmune serum, or purified IgG from preimmune serum, is an essential negative control in cases where complete sera or total IgG fractions, respectively, are used as a source of primary antibodies. Additional controls should include the detection of unrelated molecules expressed by the cell of interest that are not expected to co-distribute with one of the molecules tested for *cis*-interaction.

Apart from false-positive results due to cross-reactivity of antibodies, false-negative

results can be due to potential interference of antibody binding with the interaction between the molecules of interest. Polyclonal antibodies directed against a variety of epitopes can perturb molecular interactions. It is therefore worthwhile to test several different antibodies, polyclonal as well as monoclonal, if available. The potential interference of antibody binding with the interaction between the molecules of interest can be prevented by using heterologously expressed recombinant proteins. Specifically, different *N*-terminal peptide tags, like the myc-tag or the influenza hemagglutinin-tag can be introduced by molecular cloning into the polypeptide sequences of the studied proteins. Subsequent capping with antibodies specific for the *N*-terminally localized tag sequences will reduce the risk of interference with a binding site on the surface of the molecule.

It should always be kept in mind, that the detection of antibody-induced co-capping between two molecules is no proof of a direct molecular interaction between the two components. In order to demonstrate such a direct binding, additional experimental techniques, like chemical cross-linking (see Basic Protocol 5) or biochemical binding assays using isolated, purified proteins (see Basic Protocol 2) are required.

Neurite outgrowth

Many IgSF-CAMs have a neurite outgrowth-promoting activity, therefore, neurite growth assays are widely used for functional analysis. The preparation of neuronal cultures takes some practice and should best be learned in a laboratory where culture techniques are established. The reproducibility of the cultures is very important to get results. Keep in mind that the growth and the morphology of neurites depend on the substrate and the medium. As the quality and the components of serum differ considerably from batch to batch, the lot of serum used should not be changed during analysis. For best reproducibility, the use of a chemically defined, serum-free medium is recommended. All the solutions and the purified proteins to be tested for their neurite outgrowth-promoting activity have to be of high quality. Especially, the absence of endotoxins is extremely important for the survival of neurons. Make sure that detergents are removed carefully from protein solutions before using them in tissue culture.

The growth characteristics of neurites are age-dependent. In particular, the dependence on specific growth factors may change dramati-

cally. Therefore, it is essential not to mix different ages of tissue. Similarly, the response of neurons to different substrates may be species-specific. Remember that neurite length is not the only criterion that can be assessed in neurite growth assays. Closely monitor neurite and growth cone morphologies and branching patterns.

Anticipated Results

Transfection

For transient transfections with the protocol described, reporter constructs can be used to assess the efficiency of transfection and calculate the percentage of transfected cells. Using reporter constructs that express green fluorescent protein (GFP) as a reporter under the control of the human cytomegalovirus (CMV) immediate early promoter (plasmid size of 5 to 10 kb, DNA amounts for transfection between 40 and 80 μg), the authors repeatedly observed transfection efficiencies of 30% to 40% based on the detection of GFP expression 24 to 48 hr after transfection. Expression of recombinant protein under the control of strong viral promoters lasts, generally, for 48 to 96 hr after transfection.

Microsphere assays

With the appropriate controls, all the protocols described here that involve microsphere and cell aggregation techniques are powerful methods to study binding properties of IgSF-CAMs. Furthermore, these assays are not very time-consuming and enable processing of several tests in parallel.

Trans-interactions

Myeloma cell clones that were stably transfected to express IgSF-CAMs form aggregates when the IgSF-CAMs interact *trans*. Homophilic *trans*-interactions are indicated by aggregates of cells that express the same IgSF-CAM. Heterophilic *trans*-interactions are indicated when two populations of cells that express distinct IgSF-CAMs form mixed aggregates.

Transfection-protoplast fusion

On average, 100 clones are obtained per 96-well tissue culture plate, i.e., 500 clones per 5×10^6 transfected myeloma cells. Thus, the transfection efficiency is in the range of 1×10^{-4} . Even with an optimized electroporation procedure, the transfection efficiency was ~ 20 times lower (Rader et al., 1993). While the majority of myeloma cell clones expresses

moderate amounts of the IgSF-CAM, a small percentage typically reveals very high expression. Thus, the higher the transfection efficiency, the higher becomes the likelihood to obtain a myeloma cell clone with very high expression. This makes protoplast fusion the method of choice for the transfection of myeloma cells.

Chemical cross-linking

During optimization of the cross-linking protocol, the emphasis should be placed on the specificity of the reaction. As described, there is generally an inverse relationship between specificity of a cross-linking reaction and its efficiency. A protocol that ensures a high degree of specificity often has the drawback of low yields of cross-linked material. Based on published results and experience in the authors' laboratory, yields of cross-linking protocols, like the one described, range from 0.1% to 1%, corresponding to a few hundred nanograms of cross-linked material from a reaction performed on 10^6 cells. This amount of protein is normally sufficient for immunochemical characterization of the cross-linked molecules, e.g., by immunoblot analysis or re-immunoprecipitation (Buchstaller et al., 1996; Kunz et al., 1998).

The appearance of only one or a few complexes of a molecule of interest indicates some degree of specificity of the reaction. The cross-linked complexes isolated by immunoprecipitation can be separated by SDS-PAGE (ideally two-dimensional; UNIT 6.4). The detection of presumed binding partners in the cross-linked complexes by immunoblot analysis should always include controls, that is the detection of a membrane protein present in the cell used for cross-linking that is not expected to associate with the molecule of interest.

Co-capping

Antibody-induced co-capping can be observed between molecules that undergo direct or indirect *cis*-interactions. Very clear results can be obtained in cases where the two components are expressed at comparable levels and directly interact with each other with relative high affinity, as demonstrated in the example shown in Figure 9.5.3. Co-capping of molecule A (NgCAM) with molecule B (axonin-1) was studied on stably double-transfected CV-1 cells. Capping of molecule A (NgCAM), induced by the subsequent incubation with a mouse monoclonal primary and a rabbit-anti mouse secondary antibody (Fig. 9.5.3A), re-

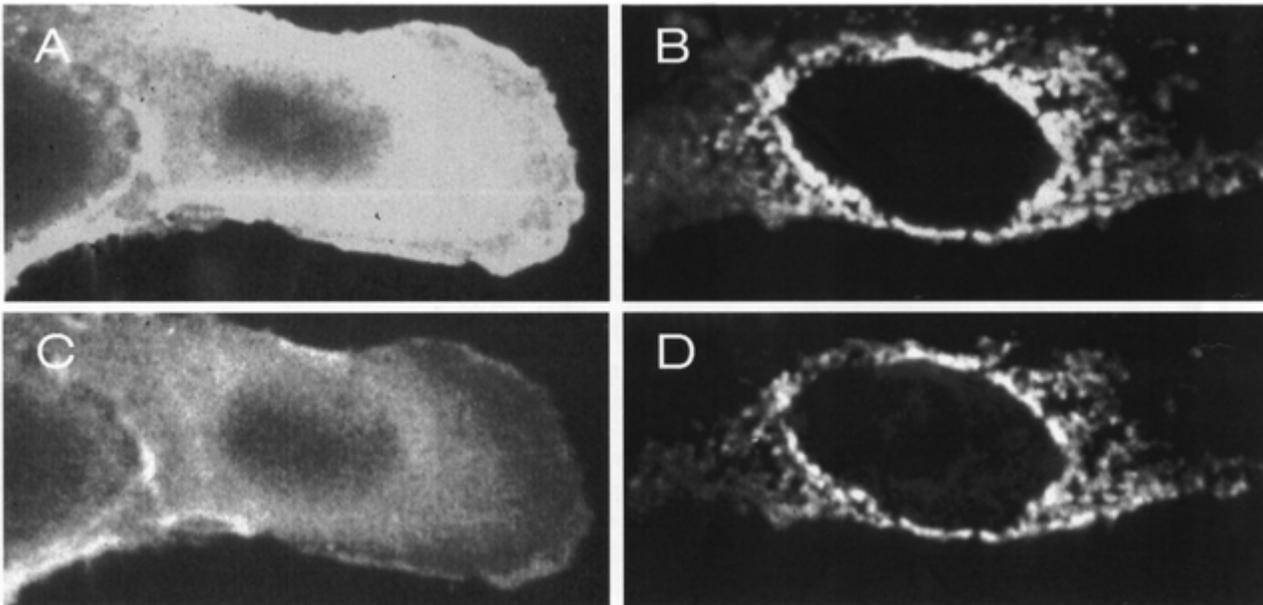


Figure 9.5.3 Antibody-induced co-capping of axonin-1 with NgCAM on stably double-transfected CV-1 cells. In double-transfected CV-1 cells, NgCAM (**A**) and axonin-1 (**C**) were randomly distributed. Capping of NgCAM was induced by a mouse monoclonal antibody and a rabbit anti-mouse IgG. NgCAM caps were detected by a Texas Red–labeled donkey-anti-rabbit IgG (**B**). The distribution of axonin-1 was detected by counter-staining with goat anti-axonin-1 Fab fragments and a FITC-labeled donkey anti-goat IgG (**D**). For examination of the cells, a confocal laser-scanning microscope equipped with an argon/krypton laser was used. Texas Red was detected using the 568-nm band-pass excitation filter (**A,B**) and FITC with the 488-nm band-pass excitation filter (**C,D**), minimizing cross-talk between the two fluorochromes.

sults in extensive co-capping of molecule B (axonin-1), as shown by counter-staining with goat anti-B (axonin-1) Fab fragments on the same cell (Fig. 9.5.3C). As expected, no capping or co-capping is detected in the absence of primary antibody against molecule A (NgCAM).

Time Considerations

Preparation of affinity column

The preparation of an affinity column takes 1 working day. Because affinity columns have to be loaded slowly, it is convenient to load large volumes of protein solution overnight to have the column ready for elution the following day. However, ensure that the column never runs dry.

Transfection

The entire transfection procedure can be performed in <1 hr for up to 12 samples.

Trans-interactions

Stable transfections of myeloma cells including selection, subcloning, and analysis require 4 to 6 weeks. Myeloma cell aggregation assays can be completed in <1 day.

Chemical cross-linking

The entire procedure can be performed within 2 days. The cross-linking reaction, including the making of the cell lysates, can be carried out within 3 to 4 hr. The lysates can be stored for several weeks at -20°C . Immunoprecipitation can be performed either with an incubation of 4 hr with the first antibody or with an incubation overnight.

Co-capping

The entire procedure can be performed in 4 to 5 hr. Incubation periods given here for antibody-induced capping are based on the authors' own optimized and published protocols. The time periods given for counter-staining can be extended for the primary antibody from 1 hr at room temperature to overnight at 4°C , if desired or necessary for higher sensitivity. The protocol can be interrupted after step 11 and 16 and the fixed coverslips stored at 4°C under light protection for up to 1 day before the subsequent steps are carried out.

Neurite outgrowth

The time required for neurite growth assays depends on the type of neurons used. Dissociated dorsal root ganglia neurons, for instance, will extend long neurites on many IgSF-CAMs

in <24 hr. DRG explants may take an additional day to reach their full length. Coating of IgSF-CAMs as a substrate is best done immediately before neurons or explants are plated, whereas dishes coated with poly-D-lysine or collagen can be stored. Dishes coated with laminin or IgSF-CAMs should not be stored and must not be dry.

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Measurement of Adhesion Under Flow Conditions

UNIT 9.6

This unit describes the analysis of dynamic cell adhesion using a flow chamber assay. The flow chamber enables the researcher to reconstruct cell systems in the presence of shear stress to assay adhesion under well-defined forces. These assays are most commonly used to study leukocyte adhesion, either to cultured endothelial cell monolayers or to purified substrates, simulating physiological interactions of leukocytes with endothelial cells. The assay described (Basic Protocol 1) utilizes commercially available parallel-plate flow chambers, in which cells are introduced under conditions of laminar flow between two flat surfaces, allowing for visualization of dynamic adhesion on a microscope (Lawrence and Springer, 1991). Flow cell assays are particularly useful to investigate adhesive events that occur very rapidly, on a time scale shorter than those assayed by static assays (Alon et al., 1995; Smith et al., 1999). Some adhesive events occur only in the presence of shear and, thus, cannot be characterized under static conditions (Finger et al., 1996; Lawrence et al., 1997). In addition, the flow cell assay allows visualization of the subprocesses of adhesion, including rolling, firm arrest, adhesion strengthening, spreading, and migration (Cinamon et al., 2001). Methods of data analysis are also discussed (Basic Protocol 2).

This assay can be also be used to characterize transient adhesive events or adhesion strengthening even for cells that do not normally experience shear stress, because contact time between cells and substrates and anti-adhesive forces can be closely regulated by stopping and starting the flow (Kassner et al., 1995). Flow chamber assays are also useful for measuring bacterial adhesion under flow (Mohamed et al., 2000; Poelstra et al., 2000).

FLOW ASSAY FOR CELL ADHESION

Commercial flow chambers incorporate the surface of either a culture dish or a coverslip as one of the two parallel plates between which laminar flow occurs. This surface should be coated with either endothelial cells or purified extracellular matrix proteins as an adhesive substrate.

Although several flow chambers are available, the same basic methods are used to conduct flow cell adhesion experiments regardless of the particular flow chamber apparatus used. Until recently, investigators often constructed their own laminar flow chambers, but with the advent of relatively inexpensive commercial models, this is not worthwhile unless special characteristics are required. The following method usually assumes that a GlycoTech flow chamber is used, but this protocol can easily be adapted for use with other chambers. Simply modify the assembly procedure according to the manufacturer's instructions and use cell- or ligand-coated glass coverslips instead of coated dishes if required.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

Materials

Substrate coating solutions:

- Fibronectin, gelatin, or other extracellular matrix proteins, either alone or in combination, for cell monolayers only
- 10 µg/ml matrix protein, such as fibronectin or collagen, in PBS (APPENDIX 2A), pH 7.4, for dishes and coverslips coated with purified ligand only

**BASIC
PROTOCOL 1**

Contributed by Dennis F. Kucik

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Cell Adhesion

9.6.1

Supplement 18

Cultured endothelial cells (*UNIT 1.1*) growing as a confluent monolayer culture in tissue culture dish or on a coverslip of a size appropriate for the chamber used

1% (w/v) BSA in PBS (*APPENDIX 2A*), pH 7.4, for dishes and coverslips coated with purified ligand only

PBS (*APPENDIX 2A*), pH 7.4, for dishes and coverslips coated with purified ligand only

100 mg/ml poly-L-lysine (PLL; e.g., Sigma), for coverslips coated with purified ligand only

1% (v/v) glutaraldehyde, for coverslips coated with purified ligand only

Leukocytes or other cells of interest

HBSS (*APPENDIX 2A*), room temperature and 37°C

2 mM 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Molecular Probes) in DMSO

35-mm plastic tissue culture dishes for GlycoTech chamber or glass coverslips of a size appropriate for the flow chambers that require them, sterilized by autoclaving, UV light, or flaming with ethanol

35-mm tissue culture dishes or 6-well tissue culture plates, for glass coverslips only

Permanent marker or diamond stylus, for dishes coated with purified ligand only

25-ml syringes

Programmable syringe pump

Flow chamber (e.g., GlycoTech) and appropriate tubing

Inverted phase-contrast microscope with low-power (~10 to 20×) objective, fluorescence optics (recommended but not required), and stage incubator set to 37°C

Video camera (e.g., CCD-300T-RC; Dage-MTI) and other recording equipment (e.g., VCR, television monitor, video cables)

Prepare substrates

For cell monolayer preparation

- 1a. Incubate a 35-mm sterile plastic tissue culture dish or glass coverslip with 1 ml substrate coating solution of fibronectin, gelatin, or other extracellular matrix proteins, either alone or in combination (Table 9.6.1) for 30 min at 37°C.

There are a number of commercially available flow chambers that require glass coverslips of different sizes. In general, only a small portion of the surface area of the coverslip is used for the assay. The size of the coverslip required has more to do with the geometry of the chamber than the actual surface area used.

- 2a. Remove excess coating solution and, if using coverslip, place the coverslip in a 35-mm dish or in the well of a 6-well tissue culture plate.

Table 9.6.1 Endothelial Cells and Culture Conditions

Cell type ^a	Dish coating ^b	Plating density (cells/cm ²)	Days to confluence
HUVEC	0.05% (w/v) gelatin 10 µg/ml fibronectin	5000	3-4
HAEC	0.05% (w/v) gelatin 10 µg/ml fibronectin	5000	3-4
MAEC	1% (w/v) gelatin	6000	8-10

^aAbbreviations: HAEC, human aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; MAEC, mouse aortic endothelial cells.

^bThe buffer used for the coating solution is HBSS (Hanks' balanced salt solution).

- 3a. Add 2 ml endothelial cell suspension (see Table 9.6.1 for cell density). Culture at 37°C.
- 4a. Feed cells every 2 to 3 days with an appropriate medium until confluent. Continue with step 9.

Coating density and the time to reach confluency depend on the cell type (Table 9.6.1). Optimal conditions for endothelial cell types other than those listed in Table 9.6.1 should be worked out empirically. Higher seeding densities result in less time to confluency, but lower densities and more time generally result in a more uniform monolayer. Depending on the cell type, cells are usually suitable for assays for 2 to 3 days after they reach confluency.

Endothelial cell adhesion to tissue culture plastic is generally superior to adhesion to glass coverslips, even if both are coated with extracellular matrix materials. Optical properties of plastic, however, are inferior to glass, because of a relatively high fluorescent background. Birefringence of plastic can also be a problem if Nomarski imaging is used. The optical properties of plastic are, however, adequate for the level of resolution needed for visualization and analysis of adhesion under flow, and they can provide better visualization of rolling cells than, for example, ex vivo preparations of blood vessels.

Leukocytes interact more with endothelial cells that have been stimulated. Interleukin 1 (IL-1), tumor necrosis factor α (TNF- α), and lipopolysaccharide (LPS) have been considered good paradigms for pro-inflammatory mediators and are often used experimentally on cultured endothelial cells to simulate inflammation (Cines et al., 1998). Stimulation results in expression of a number of adhesion molecules, in addition to other effects. Whether and how the endothelial cells should be stimulated will depend on the physiologic conditions that are being mimicked.

For plastic dishes coated with purified ligand

- 1b. Use a permanent marker or diamond stylus to outline a 5-mm diameter area of a sterile plastic tissue culture dish for coating.

If a diamond stylus is used, the scratches should be shallow enough that they do not prevent the flow chamber gaskets from sealing.

The size of the area covered depends less on the size of the dish or coverslip than on the area of observation required. The authors find that a 5-mm diameter circular area is sufficient to provide several fields of view for independent measurements of cell-substrate adhesion.

- 2b. Add 25 μ l of 10 μ g/ml matrix protein in PBS to the marked area and incubate 1 hr at room temperature.
- 3b. Remove coating solution and add 1 ml of 1% BSA in PBS. Incubate 1 hr at room temperature.
- 4b. Remove BSA solution. Wash twice with 5 ml PBS each. Continue with step 9.

The coated dish can be stored for 1 day at 4°C.

For glass coverslips coated with purified ligand

- 1c. Place sterile glass coverslip in a 35-mm tissue culture dish or in the well of a 6-well tissue culture plate.
- 2c. Add 0.2 ml of 100 μ g/ml poly-L-lysine (PLL) to the dish. Incubate 10 min at room temperature.

The amount of PLL used should be scaled up as needed depending on the coverslip size (e.g., 0.5 ml should be used for a 22 \times 22-mm coverslip). The authors routinely use PLL with a molecular weight of 70 to 150 kDa.

- 3c. Wash coverslips three times with 5 ml PBS each.

- 4c. Add 1% glutaraldehyde and incubate 30 min at room temperature.
- 5c. Wash coverslips three times with 5 ml PBS each.
- 6c. Add 25 μ l of 10 μ g/ml matrix protein and incubate 1 hr at room temperature.

For a 22 \times 22-mm coverslip, 100 μ l coating solution should be used.

- 7c. Add enough 1% BSA in PBS to completely cover the coverslip to block. Incubate 30 min at room temperature.
- 8c. Wash coverslip with 5 ml PBS. Add fresh PBS to keep moist until used for flow assay. Use within 1 day.

This protocol, in which matrix molecules are covalently linked to adsorbed PLL, results in tighter, more uniform binding of matrix materials to coverslips than direct adsorption of matrix molecules to the glass. If, however, PLL needs to be avoided, direct adsorption of matrix materials, as in the plastic dish protocol, may provide an adequate substrate.

Coverslips precoated with PLL are also commercially available, but the authors have not tried them.

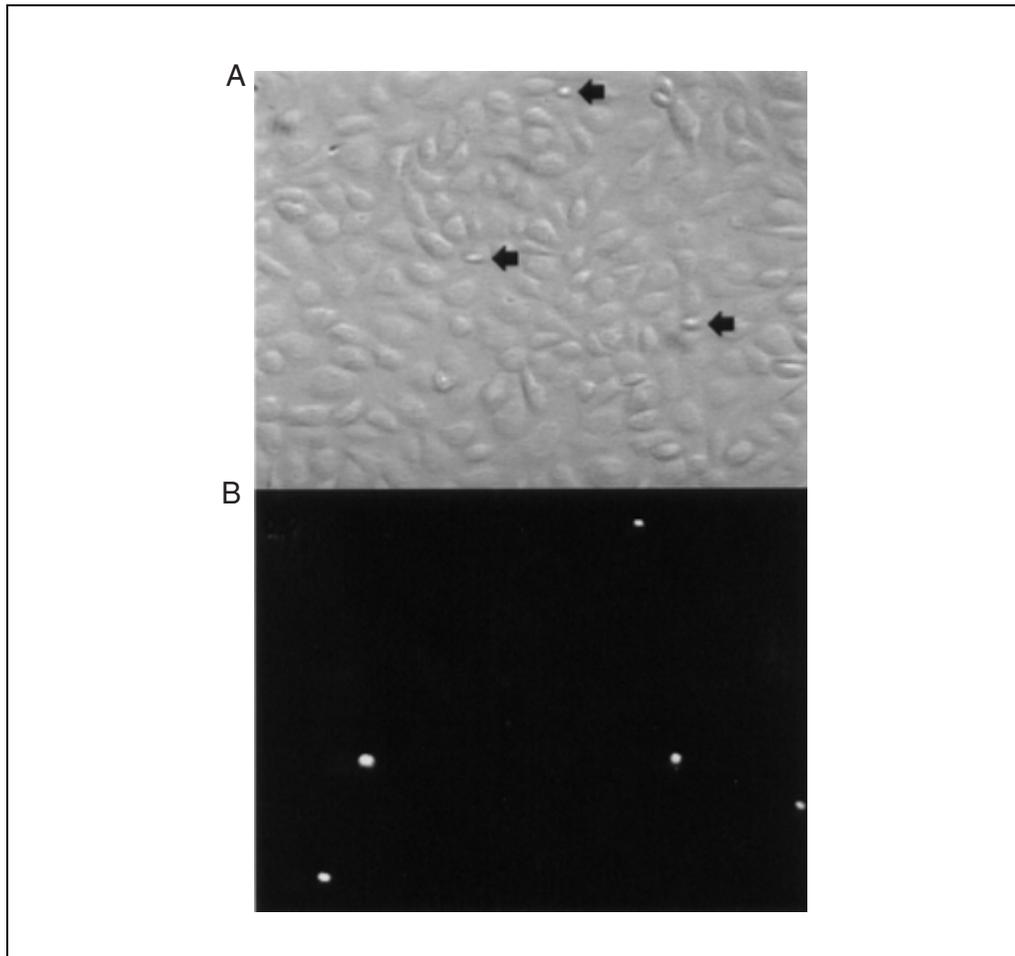


Figure 9.6.1 Visualization of endothelial cells and leukocytes using (A) phase contrast and (B) fluorescent optics. Arrows in (A) indicate leukocytes. Fluorescent optics provide high-contrast images that facilitate manual counting and are especially useful for computer tracking. When using endothelial cells as the substrate, however, it is important to observe the system with phase contrast or similar optics to assess the integrity of the monolayer before switching to fluorescence.

Load cells with fluorescent dye (optional)

9. Wash leukocytes or other cells of interest in HBSS and then resuspend them at $1\text{--}2 \times 10^6/\text{ml}$ in 25 ml HBSS.

Although rolling and adherent cells can be visualized without fluorescence, the increase in contrast afforded by labeling the perfused cells greatly facilitates observation and counting (Figure 9.6.1) and is sometimes a requirement for object-tracking software.

10. Add 12.5 μl of 2 mM BCECF-AM and incubate 30 min at room temperature in the dark.
11. Wash cells three times with 35 ml HBSS each.

Carry out flow assay

12. Resuspend leukocytes in prewarmed HBSS to a concentration of 0.5×10^6 cells/ml

Cell concentration should be high enough that sufficient events are observed in the few minutes of recording, but not so high that cell behavior is dominated by collisions with other rolling or adherent cells. The authors find that 0.5×10^6 cells/ml works well. The number of cells required will depend on the rate of flow and the length of observation needed to gather sufficient data to answer the particular experimental question. The authors usually use $3\text{--}5 \times 10^7$ cells/experiment (including controls).

In general, it is a good idea to perform experiments at 37°C (this necessitates a stage warmer), although useful data can sometimes be obtained at room temperature, depending on the experimental question.

13. Load leukocytes into a 25-ml syringe, if using the pushing method, or into a centrifuge tube or other reservoir, if using the pulling method.

There are two ways to use a syringe pump to perfuse cells through a flow chamber. The syringe can be filled with cells and the pump can be used to push them through the chamber. Alternatively, some investigators prefer to attach the syringe to the outlet side of the system to pull fluid and cells through the chamber.

14. Attach the tubing of a flow chamber to the syringe and remove any air bubbles from the system.
15. Assemble a flow chamber according to the manufacturer's instructions, using a tissue culture dish containing the monolayer of cultured endothelial cells (step 4a) or coated with purified ligand (step 4b or 8c). Again, work out any bubbles in the system.

Flow chambers come in a wide variety of sizes. Generally, the smaller the chamber, the more economical the experiment is in terms of cells used per experiment. The dimensions of the GlycoTech flow chamber are determined by the size of the gasket used because the gasket forms the side walls of the chamber and also determines its height. GlycoTech supplies four gaskets with its basic kit; these range in width from 0.25 to 1.0 cm in width and in thicknesses ranging from 0.005 to 0.010 in. The length is fixed at 2 cm.

Increasing the dimensions of the flow chamber will decrease the wall shear stress for a given flow rate. This has the disadvantage of requiring more volume (and thus more cells) to be perfused per unit time for a given shear. It can be advantageous, however, to use a larger chamber when low shears are desired because the syringe pump flow rates are less accurate and more pulsatile as flow is decreased. A wider chamber has the further advantage of providing more observation area if many fields of view are desired.

Significant shear stresses can be created during assembly, which can sometimes tear endothelial cell layers loose from the dish. Therefore, care must be taken to assemble the system slowly and gently.

16. Program the syringe pump to give the desired flow according to the manufacturer's instructions.

To determine the desired flow, flow rates must be converted to shear stresses. The manufacturer of the flow chamber will usually provide a handy conversion chart or table.

Alternatively, shear stress can be calculated from the dimensions of the flow chamber and the rate of fluid flow (Lawrence et al., 1987). The shear stress desired will depend on the physiological condition to be mimicked. For example, simulation of arterial flow will require higher shear rates than simulation of venous or postcapillary venule conditions. The authors usually do a range of shears to determine the shear dependence of any phenomena observed. As a rule of thumb, physiologic shear stresses are typically thought to be in the range of 0.5 to 5.0 dynes/cm².

17. Mount the flow chamber on an inverted phase-contrast microscope with a prewarmed stage incubator. Focus the system and adjust the optics, video camera, and any other recording equipment as needed.

If using endothelial cells, it is important to visualize the area of observation adequately to assess the integrity of the monolayer. Rips or other defects in the monolayer will confound interpretation of the results, because the data will reflect interactions of flowing cells not only with endothelial cells but also with bare plastic or glass.

It is advisable to do a sample recording of a minute or so at this point and to play it back to be sure that all of the equipment is working properly.

18. Choose an area to observe, start the recording equipment, and begin the flow.

The authors record on sVHS videotape for later digitization. Images can also be captured directly to computer. This may be necessary if the camera used does not have a video output.

19. Record a few minutes of data at each shear rate.

The authors generally record ~3 to 6 min of data at each shear rate from each area of observation. For preliminary experiments they use at least three or four shear rates. Once the optimal shear and the shear dependence are determined, one or two shear rates may be sufficient. Considerations include the number of cells available (e.g., primary mouse cells are more precious than cell lines) and any time dependence of treatment regimens. It is important to record diligently the points on the videotape where shear rates are changed so that data can be interpreted when it is time to do the analysis.

20. Move to a new field of view and repeat steps 18 to 19. If using the pushing method, with the cells in the syringe, rotate the syringe 180° to compensate for any settling. If using the pulling method, with the cells drawn from a tube or other reservoir, make certain that the cells are still suspended.

Most flow systems are large enough to allow a number of areas of observation for each preparation. It is important to observe multiple areas to control for any peculiarities in density of substrate coating or properties of endothelial cells. The number of fields to observe for each preparation will again depend on the number of cells available, any time dependence of treatment regimens, and the statistical power required.

21. Perform data analysis (see Basic Protocol 2).

DATA ANALYSIS

Quantifying Adherent Cells

The raw data from flow cell adhesion experiments will be videos of rolling and firmly adhering cells. The method of analysis will depend on the scientific questions to be answered. A simple count of adherent cells per minute can be obtained by visual review of videotapes. Just choose an appropriate time period (e.g., 1 min) and roll the tape to count the number of cells that become immobilized in the field of view during that time. It may be helpful to use two people to do this, one who counts and one who watches the time. Because the number of cells recorded will depend on the size of the field observed, it should be normalized for the area of observation. Area can be calculated using a stage micrometer, available from many scientific supply companies (e.g., Fisher), as a known

BASIC PROTOCOL 2

Measurement of Adhesion Under Flow Conditions

9.6.6

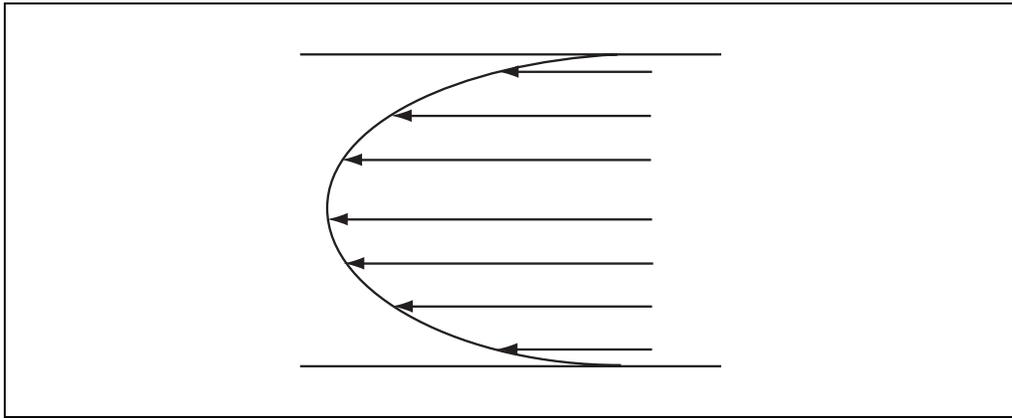


Figure 9.6.2 Velocity profile for fluid flow in a parallel-plate laminar flow apparatus. Longer arrows correspond to higher velocities. The velocity profile is parabolic, with a maximum velocity midway between the two plates and a slowest fluid flow near the plates.

length standard. A stage micrometer (also called a calibration plate) is a glass slide with accurate distance scales etched into the surface. By imaging this size standard, the size (in microns) of the video image or monitor screen can be determined.

An important consideration for meaningful comparisons of experimental conditions is that the number of cells perfused per minute must be the same under all experimental conditions. This requires that cells are loaded into the syringe or reservoir at a consistent density and that cells are not allowed to settle out of suspension.

Quantifying Rolling Cells

The number of rolling cells per minute can also be determined by visual review, but care must be taken in identification of rolling cells. By definition, under laminar flow there will be a parabolic hydrodynamic velocity profile, with the lowest velocity of the fluid occurring nearest the flow chamber wall (Figure 9.6.2). Thus, it may not be obvious which cells are moving slowly because of their position in the flow field and which are retarded by adhesive interactions (Goldman et al., 1967). Whereas under some optical conditions rolling cells can be distinguished by plane of focus, it is a good idea to calculate a critical velocity to verify that cells counted as rolling do indeed have adhesive interactions with ligand. The critical velocity is an objective measure that has been developed to identify rolling cells (Ley and Gaehtgens, 1991). A simple calculation for critical velocity is $V_c = \beta r \gamma$, where V_c is the critical velocity, β is a drag factor, r is the radius of the cell, and γ is the shear rate. A reasonable estimate of β that can be used for cells in a flow chamber is 0.5. Cells moving more slowly than the critical velocity can be confirmed as being rolling cells. Thus, even if the experimental question deals only with the number of rolling cells, and not their velocity, a few velocities should be measured to ensure that what appears to be rolling cells are moving more slowly than the critical velocity and, thus, are indeed rolling. With some practice, though, rolling cells can often be identified visually.

Rolling Velocities

The calculation of cell position in each digitized image can be done by comparison with a known size standard, again using a stage micrometer. A known length (in microns) can then be marked on the monitor screen with a laboratory marker. With a known distance established, velocities can be determined by simply counting the number of video frames required for a cell to travel this known distance. Alternatively, more accurate velocity determinations can be made using more sophisticated image processing software that can accurately localize objects in each image and convert pixels to distances. A variety of

commercial software packages are available. NIH Image, which is available online at no cost, will also track object motion (see Internet Resources).

COMMENTARY

Background Information

Leukocyte rolling and adhesion *in vivo* has been observed microscopically for more than 100 years (Cohnheim, 1889). More recently, investigators have used parallel-plate flow chambers to mimic the environment of the blood vessel under more controlled conditions. Much of what we know today about inflammation and leukocyte homing has been learned by examining leukocyte interactions with either endothelial cell monolayers or purified ligands in flow chambers (Butcher, 1991; Rossiter et al., 1997; Smith, 2000; Orsello et al., 2001). In addition, adhesion assays using flow chambers have provided important information about bacterial adhesion to surfaces. Today, adhesion under flow can readily be assayed economically using a variety of commercially available flow chambers. When mounted on a microscope and coupled to a video camera and a VCR, simple analysis of adhesion under shear is possible. More detailed analysis of rolling cell motion can be achieved by digitization of images coupled with motion analysis software.

Commercially available flow chambers, in general, work very well and make it easy to produce conditions that mimic physiological cell interactions with well-defined and tightly controlled shear stresses. Increased use of these assays will provide a fuller understanding of physiologic cell adhesion than could ever be achieved with static adhesion assays.

Critical Parameters and Troubleshooting

Flow experiments require some diligence to ensure that the system is working properly. The experimenter should watch the monitor carefully during the experiment to be sure that the flow is smooth and that perfused cell concentrations are uniform. Although a programmable syringe pump is capable of providing very steady perfusion, flow can become pulsatile when the syringe is almost empty or if the syringe is not mounted securely. Other sources of variability in flow are leaks or bubbles anywhere in the system. It is also a good idea to isolate the system from vibration.

If fluorescent dyes are used to label perfused leukocytes, it is a good idea to develop a protocol that labels a high percentage of the cells (>97%), because unlabeled cells will likely be

missed during analysis, confounding quantification of the number of cells interacting with the endothelium or substrate. Care should be taken, however, not to overload the cells, because fluorescent dyes can be somewhat toxic. Too much label interferes with cell motility and may affect regulation of adhesion in general. The optimal dye-loading protocol can be determined by varying both dye concentration and incubation times and should be worked out for each new cell type.

When using object tracking software, the experimenter should observe the tracking closely. Although most available programs do a very good job and provide temporal resolution not obtainable by cruder methods, they also make occasional errors, briefly locking onto an object other than the cell of interest. The operator should confirm visually for each cell track that the software has faithfully tracked the object of interest and has not misidentified the cell at any point.

Uniformity and integrity of the substrate are also of vital importance. This is especially true of endothelial cell monolayers. High shear forces associated with assembly of the chamber or with manual flushing of bubbles can dislodge portions of the monolayer. Thus, to avoid mistaking leukocyte interactions with the chamber itself for interactions with the endothelium, the importance of a visual inspection of each recorded field of view cannot be overemphasized. Purified protein substrates present less of a problem, but care should be taken to ensure uniform distribution and density of adsorbed matrix materials through use of good experimental technique.

Cameras

The authors recommend cameras with a video (rather than digital) output, because storage of images is more economical on videotape than on digital media. This allows the experimenter to record continuously throughout the experiment. This can be an important consideration if one is working with valuable perfused cells (e.g., leukocytes purified from gene-targeted mice). An economical recording system also encourages collection of the maximum amount of data from each experiment, avoiding the need to repeat experiments simply to increase statistical power. In addition, higher time

resolution (30 frames/sec) is possible with video than with most digital systems. Images can be digitized later for computer analysis using a variety of commercially available systems.

If fluorescent cells are to be visualized, a highly light-sensitive camera must be used. This is because when rolling cells are visualized, the time to acquire the image is limited. Extended exposures cannot be used, as the cells will appear blurry from their movement. Several camera companies offer cameras that are sensitive enough for this application, but the authors know of few with both adequate sensitivity and video output. The authors have found the CCD-300T-RC (Dage-MTI) to work well. Equivalent cameras may be available from other manufacturers as well.

It is also possible to do flow cell experiments without fluorescently labeling the cells. Although this results in less contrast between rolling cells and underlying endothelial cells or substrate, the images can be perfectly acceptable (Fig. 9.6.1). Imaging without fluorescence also avoids possible deleterious effects of dye loading on the cells. In addition, the time required for each experiment is shortened by the amount of time needed to load the cells with fluorescent dye, which may be useful if short-acting treatments are used. A major determinant of whether fluorescence can be omitted is the quality of the microscope optics. Another factor to consider, however, is whether any tracking software to be used depends on cell intensity for tracking motion. Although some programs can track objects based on their size and shape, other packages require the tracked object to be brighter than its surroundings.

When imaging without fluorescence, it is especially important to avoid fingerprints and other smudges on the culture dish or coverslip and other optical elements of the flow chamber, to avoid degrading the image.

Video recording

It is recommended that video be recorded using an sVHS, rather than a VHS (the typical home-recording format), VCR. Not only will the sVHS spatial resolution be better, but the recordings are more resistant to degradation with repeated playback. Alternatively, images can be recorded directly to computer memory or hard drive. With some cameras, this may be the only option.

Image digitization and compression technologies are progressing so rapidly that any hardware recommendations are likely to be out

of date within months. Therefore, it is recommended that the investigator research available products when setting up a system. An important factor to consider is the amount of compression necessary with each system, because increased compression can lead to decreased image quality and, consequently, a decrease in accuracy of cell position determination.

Anticipated Results

In general, leukocytes perfused over stimulated endothelial cells at physiological flow rates will be expected to interact with both rolling and firm adhesion. The amount and type of interaction will depend on the leukocyte type and its state of activation as well as the endothelial cell type (venous versus arterial, mouse versus human). Endothelial cells can be stimulated by TNF- α (e.g., 10 to 100 ng/ml for 8 hr). This induces expression of a number of adhesion molecules and should dramatically increase adhesive interactions with almost any leukocyte type. Human umbilical vein endothelial cells (HUVEC; *UNIT 2.3*) have been used in a number of flow chamber adhesion studies (Munn et al., 1994; Macconi et al., 1995; Thelilmeier et al., 1999; Patel, 1999; Kaur et al., 2001), are widely available, and make a good test system to validate experimental technique.

Time Considerations

The time required to prepare a culture monolayer is 1 to 2 hr with several days required for cells to reach confluency (Table 9.6.1). Preparing a pure ligand substrate requires 5 to 6 hr for either culture dishes or coverslips. The flow assay requires about 3 hr to do two cell types and three conditions, and at least 1 day, depending on the analysis performed is needed to analyze the data.

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Internet Resource

rsb.info.nih.gov/nih-image/about.html

Web site for NIH Image.

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CHAPTER 10

Extracellular Matrix

INTRODUCTION

Most cells of multicellular organisms interact routinely with extracellular matrix (ECM) molecules. The ECM provides structural support, as well as important regulatory signals governing cellular growth, metabolism, and differentiation. Epithelial cells of all types generally require various ECM components in the basement membranes to which they adhere for maintaining their characteristic polarized organization, differentiated state, and specific gene expression. Connective tissue cells nestle within matrices of collagens, proteoglycans, and other ECM components. Even circulating blood cells such as lymphocytes can interact extensively with ECM as they extravasate from blood vessels and localize in tissues during recirculation and inflammation. Two particularly dynamic tissue-remodeling processes in which ECM becomes critically important are cell movements during embryonic development and wound repair. In addition, tumor cells often must invade through basement membranes and connective tissue in order to metastasize (see *UNIT 19.1*).

Throughout these various cell-biological processes, ECM can act both as a structural scaffolding for cell adhesion and migration and as a trigger for signaling through ECM receptors. Binding of specific ECM molecules to their plasma-membrane receptors activates signal-transduction responses that can include activation of various tyrosine and serine-threonine kinase families, MAP kinase systems, ion fluxes, or phosphoinositide and arachidonic acid pathways. The study of these complex processes has blossomed recently due to the availability of individual purified ECM molecules, along with the realization that ECM modulates many crucial cell-biological functions.

UNIT 10.1 reviews key ECM functions, as well as the biochemistry of the major classes of ECM molecules. This comprehensive review provides a solid framework for understanding the initially somewhat bewildering complexity of ECMs, which range from basement membranes to loose connective tissue, cartilage, and ligaments.

Basement membranes are crucial for normal epithelial cell biology. *UNIT 10.2* describes the preparation of the basement-membrane extract termed Matrigel and the isolation of two important components, laminin and type IV collagen. Although these proteins are also available commercially, preparing them within the laboratory is much more economical for larger-scale studies. These purified proteins can be used in cell adhesion assays (*UNITS 9.1 & 9.2*) and for studying other cell-biological responses such as migration and differentiation.

Because extracellular matrices are generally three-dimensional, and their effects can be greater than the sum of their isolated components, cell biologists also use ECM gels. *UNIT 10.3* details the preparation and use of gels of purified collagen and of Matrigel basement-membrane extracts. Collagen gels and Matrigel can be used to study the three-dimensional behavior of epithelial, fibroblastic, and other cells. Matrigel can also be used in animals for angiogenesis assays (*UNIT 10.3*) and in tumor cell invasion assays (*UNIT 12.2*), as well as to promote the survival and growth of primary tumor cells that would not otherwise grow *in vivo* (*UNIT 10.3*).

An alternative approach is to use intact extracellular matrices assembled by living cells in culture; the cell monolayers are extracted away to leave just the three-dimensional extracellular matrix. *UNIT 10.4* describes the preparation of two forms of such complex matrices, which can be used as substrates for cell biological studies in vitro.

A particularly widely used approach involves the use of purified extracellular matrix molecules as substrates. Both fibronectin and vitronectin can be purified by affinity chromatography from human plasma, providing large quantities of an individual adhesive protein useful for cell culture studies using many types of cells. *UNIT 10.5* presents simple, reliable methods for purifying substantial quantities of fibronectin from plasma. Also included are two different protocols for purifying the cellular form of fibronectin, by extraction from cell surfaces or from fibronectin secreted by cells into serum-free medium; this form of fibronectin contains additional peptide sequences and has moderately enhanced adhesive activity. *UNIT 10.6* describes a simple protocol for the isolation of vitronectin, which is a cell adhesion protein used by many cells—e.g., by most adherent cells cultured in medium that contains 10% serum.

Even though proteoglycans are a major class of diverse extracellular and cell-surface molecules with important roles in a myriad of regulatory and structural functions, many researchers have hesitated to study them because they are technically difficult to analyze. These large, highly charged, readily aggregated molecules are now known to be present not only in extracellular matrices, but also linked to membranes by phospholipid, transmembrane (e.g., the syndecans), or even in intracellular locations in some cells. *UNIT 10.7* provides numerous protocols and valuable tips for isolating and analyzing these widely distributed molecules implicated in signal transduction, adhesion, and ECM structure.

The glycoproteins and proteoglycans of the extracellular matrix are not static, but are instead in a state of dynamic balance between synthesis and degradation. The continuous turnover and active remodeling of the extracellular matrix in embryonic development, growth, and tissue repair depend critically on carefully regulated degradation by proteolytic enzymes, particularly by the matrix metalloproteinases (MMPs). The MMPs are regulated by activation and by their inhibitors (the TIMPs, as well as $\alpha 2$ -macroglobulin). *UNIT 10.8* provides a series of methods for studying MMPs and their inhibitors, including a live-cell collagen degradation assay, analysis by either direct or reverse zymography, and enzyme-capture techniques.

The ECM of living organisms is often organized into a fibrillar, three-dimensional matrix. Although collagen gels and Matrigel provide valuable three-dimensional cell culture model systems, recent findings suggest that some cells require a matrix that more closely mimics their natural microenvironment in vivo in order to form normal cell adhesions; such matrices also enhance cell attachment, migration, and proliferation. *UNIT 10.9* provides methods for generating a fibroblast-derived three-dimensional ECM that closely resembles in vivo matrices. It also describes protocols for assessing cell adhesive and morphological responses to such matrices, as well as methods for generating two-dimensional substrate controls.

ECM proteins in a group termed ‘matricellular’ proteins play complex and interesting roles in regulating the interaction of cells with other ECM molecules. These proteins include SPARC (also known as osteonectin; *UNIT 10.11*), thrombospondin (*UNIT 10.10*), tenascin, and other molecules that function to modulate a wide range of biological responses to the ECM. *UNIT 10.11* provides comprehensive protocols for isolating and purifying SPARC, a matricellular protein that can regulate cell adhesion, proliferation, and other processes. This unit presents methods for its purification from a cell line or

platelets, as well as for purifying recombinant SPARC from *E. coli* or insect cells. Finally, it also describes assays for its inhibitory effects on cell proliferation and adhesion.

The units in this chapter span topics from individual purified ECM proteins to complex three-dimensional matrices consisting of many molecules interacting to form gels. They provide the opportunity to study the functions and mechanisms of the whole range of types of cell-ECM interactions, which are now recognized to play crucial roles in cell biology and pathology.

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In all multicellular organisms, development is influenced by the interactions between cells and their extracellular matrix (ECM). Information contained in the ECM provides the cell with temporal and positional clues, such as where it is, where it should be going, how old it is (in terms of cellular differentiation), and in some instances, when it is time for it to die (through apoptosis). It is not surprising, then, that there has been a great deal of interest in defining the extracellular signals as well as the cell surface receptors that interact with these molecules and interpret the information. Now more than ever, understanding cell biology requires understanding the ECM.

Studying the ECM, however, is not for the faint of heart! In most instances, the functional form of matrix macromolecules is a large, sparingly soluble aggregate that cannot easily be solubilized or dissociated into component units. Even when dissociated matrix components are obtained, the biological properties of the constituent chains often differ from the intact form. To complicate matters, most ECM macromolecules participate in supramolecular assemblies where their biological properties are modified by the molecules with which they interact.

These unusual physical properties create serious problems for matrix characterization using a standard “wet chemistry” approach. They also create some, though not many, unique advantages. For example, the multimeric, cross-linked nature of ECM imparts an element of stability that is not found in other proteins. This is most obvious if one takes a historical look at techniques used for matrix purification (Partridge, 1962; Piez, 1997). In the early days of matrix biology, “connective tissue” was purified using extraction protocols that relied on the ability of the target matrix component to withstand relatively harsh conditions: acid solutions were used for purifying collagen, chaotropic agents for mucopolysaccharides (now called proteoglycans), and, the harshest of them all, boiling sodium hydroxide for purifying elastin. It is quite remarkable that so much of what we know about these three matrix classes resulted from experiments using products purified in this way. Although purification strategies are now a bit more sophisticated, modifications of these basic protocols are still used today. The use of molecular biology and mouse genetics has quickened the pace of matrix char-

acterization and opened the door to functional studies of complex matrices that were unthinkable several years ago.

One of the most important properties of ECM is its functional diversity (Kleinman, 1993). Some components are designed to be rigid, others elastic; some wet, others sticky. All have modular designs that impart diverse roles, yet allow for highly specialized functions. The formation of a basement membrane, for example, requires the assembly of ECM molecules that have significant tensile strength (collagen), can act as charged molecular sieves (proteoglycans), and facilitate cell attachment (laminin). These molecules are woven together through processes that involve self-assembly and interactions with molecules that are specifically designed to serve as molecular bridges or linkers (nidogen/entactin; Yurchenco, 1994).

It is not possible to ascertain the functional properties of a complex matrix such as basement membrane without studying its individual components. At the same time, however, it is also clear that the functional complexity of the assembled basement membrane is greater than the sum of its component parts. To comprehend this greater sum requires shifting one’s view away from a reductionist biochemical approach to one focused on cell and developmental biology. Here the cell becomes the reagent, interpreting informational signals contained in the ECM and adjusting its physiology accordingly. The researcher’s task is to understand the readout.

The sections below contain an overview of the major classes of ECM. Molecules have been selected to illustrate specific functional or structural properties that are common to a matrix class or to ECM macromolecules generally. Where possible, recent reviews with references to more detailed literature are cited. Although somewhat dated, the text *Cell Biology of Extracellular Matrix* (Hay, 1991) provides an excellent overview of ECM biology. More detailed reviews can be found in various volumes of the *Biology of Extracellular Matrix* series, published by Academic Press.

COLLAGENS

Structure of Collagens

Collagen is the most ubiquitous ECM protein and is designed to provide structure and resiliency to tissues. It is defined by the pres-

ence of a triple-helical domain containing peptide chains with repeating Gly-Xaa-Yaa triplets, and by the presence of hydroxyproline and hydroxylysine (Kühn, 1987; Prockop and Kivirikko, 1995). To date, nineteen distinct genetic collagen types have been identified. The characteristic molecular form of collagen is a triple helix made up of three polypeptides, called α chains, that coil into a right-handed triple helix. Collagens exist either as homotrimers composed of three identical α chains ($\alpha 1$)₃ or as heterotrimers consisting of two ($[\alpha 1]_2\alpha 2$) or three ($\alpha 1\alpha 2\alpha 3$) α chains.

The nomenclature for the collagen superfamily consists of an indication of their genetic type (a Roman numeral that generally denotes the chronological order in which the collagens were characterized) together with the α -chain composition. Type I collagen, for example, is a heterotrimer of two $\alpha 1$ chains and one $\alpha 2$ chain, and is indicated as ($\alpha 1$ [I])₂ $\alpha 2$ (I). Type II collagen is a homotrimer of three $\alpha 1$ chains and is written ($\alpha 1$ [II])₃. Other collagens consist of three different α chains and (using type IX as an example) are written in the form $\alpha 1$ (IX) $\alpha 2$ (IX) $\alpha 3$ (IX). It is important to note that each α chain within a collagen type is a distinct gene product; that is, an $\alpha 1$ chain in one collagen type is not the same protein as the $\alpha 1$ chain in any other collagen type. It is critical, therefore, to indicate the collagen type when referring to a particular α chain (e.g., the $\alpha 1$ chain of type I collagen).

Synthesis of Collagens

Collagen α chains are synthesized on membrane-bound ribosomes (ER) as large precursors,

called pre-pro- α chains. In addition to the signal peptide (the “pre” part of the name) required for transport into the ER, each collagen precursor has extension peptides (the “pro” part) on both its N- and C-terminal ends (Fig. 10.1.1). Each pro- α chain combines with two others in the lumen of the ER to form the triple-helical molecule. The extension peptides are required for correct triple helix formation and remain with the triple-helical unit throughout the secretory pathway.

In the triple helix, the side chain of every third α -chain residue is directed towards the center of the helix, shifted by 30° from the preceding central residue of the same chain (Brodsky and Ramshaw, 1997). Steric constraints dictate that the center of the helix be occupied only by glycine residues; side chains of any other amino acid would perturb the triple-helical conformation.

Hydroxylation of proline residues in the Yaa position occurs as a post-translational modification in the lumen of the ER. The side-chain hydroxyl group of hydroxyproline stabilizes the helix through the formation of intermolecular hydrogen bonds. In fact, hydroxylation of ~100 prolyl residues is essential for the three pro- α chains of fibrillar collagens to form a triple helix that is stable at body temperatures. Hydroxylation of α chains is catalyzed by prolyl 4-hydroxylase, a tetrameric enzyme consisting of two α and two β subunits ($\alpha 2\beta 2$; Kivirikko and Myllyharju, 1998). Interestingly, the β subunit is protein disulfide isomerase, an ER protein that catalyzes thiol-disulfide interchange during protein folding (Koivu et al., 1987). The hydroxylation reaction catalyzed by

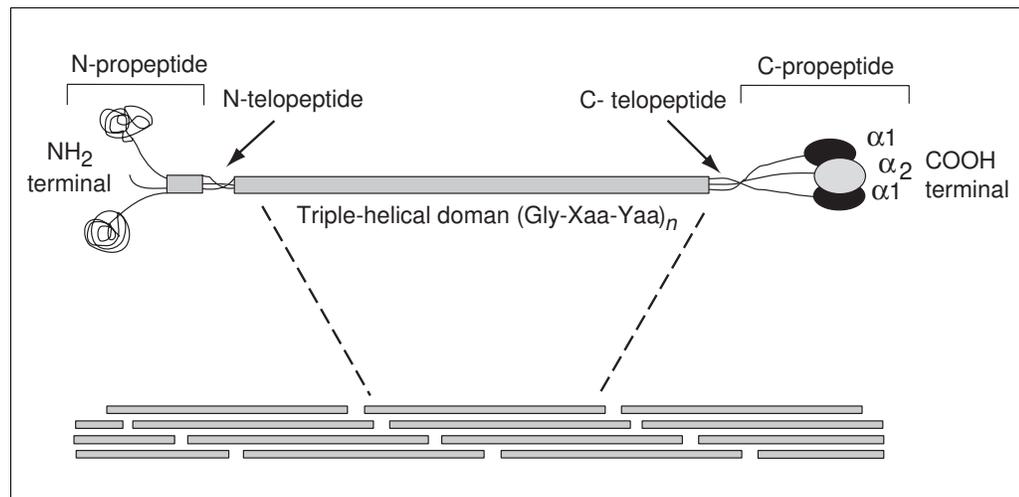


Figure 10.1.1 Functional domains of the type I procollagen molecule. Following cleavage of the propeptide domains in the extracellular space, collagen units assemble in a quarter-stagger arrangement to form a fibril.

prolyl 4-hydroxylase requires Fe^{2+} , 2-oxoglutarate, O_2 , and ascorbate. Conditions that prevent proline hydroxylation (such as nutritional deficiency of iron or of vitamin C) affect helix formation or stability. In scurvy, a human disease caused by a dietary deficiency of vitamin C, the nonhydroxylated pro- α chains are unstable and the skin and blood vessels become extremely fragile.

A second post-translational modification of procollagen that is crucial to its function is the hydroxylation of lysine residues. This reaction, which also occurs in the ER, is catalyzed by the enzyme lysyl hydroxylase. The active enzyme is a homodimer and, like prolyl hydroxylase, requires Fe^{2+} , 2-oxoglutarate, O_2 , and ascorbate. Hydroxylysine residues have two important functions: their hydroxy groups act as attachment sites for carbohydrate units, and they are essential for the stability of the intermolecular collagen cross-links that occur in the extracellular space after secretion. The glycosylation of hydroxylysine is unusual, consisting of a single galactose residue or a glucosyl-galactosyl disaccharide attached to the hydroxyl group. The amount of carbohydrate added to procollagen varies greatly among different types of collagen, and its function is unknown.

Assembly of Collagens

After secretion into the extracellular space, the extension peptides of procollagen are removed by specific proteolytic enzymes. Both the N and C proteinases are members of the zinc metallopeptidase family and contain domains that suggest the ability to interact with cells and other matrix components (Kessler et al., 1996; Colige et al., 1997). Removal of the extension peptides converts the procollagen molecules to collagen (once called tropocollagen). Triple-helical collagen units then come together in the extracellular space to form the much larger collagen fibrils. The process of fibril formation is driven, in part, by the tendency of the collagen molecules to self-assemble. The fibrils form close to the cell surface, however, and it seems likely that the cell regulates the sites and rates of fibril assembly. The nonfibrillar collagens (see below) undergo only limited proteolytic processing prior to assembly. Here it is important to distinguish between collagen and gelatin. As stated above, collagen is the triple-helical form of the protein and can exist as single triple-helical units or triple-helical units polymerized into fibrils. Gelatin is denatured collagen. The individual α chains are no longer in

a triple helix but can nevertheless polymerize into a random gel under appropriate conditions of temperature and ionic strength.

Collagen fibrils are greatly strengthened by covalent cross-links within and between the constituent collagen molecules. The types of covalent bonds involved are unique to connective tissue and are formed through deamination of certain lysine and hydroxylysine residues to yield highly reactive aldehyde groups. The aldehydes then undergo classical condensation reactions to form covalent bonds with each other or with other lysine or hydroxylysine residues. The extent and type of cross-linking varies from tissue to tissue, depending on tissue requirements. For example, collagen is highly cross-linked in tendons, where tensile strength is crucial. Lysyl oxidase, the enzyme that catalyzes cross-link formation, requires copper and molecular oxygen. If cross-linking is inhibited, collagenous tissues become fragile, and structures such as skin, tendons, and blood vessels tend to tear.

Collagen Classification

The polymeric structures formed by members of the collagen family vary depending on collagen type (Prockop and Kivirikko, 1995). The structures formed result, in large part, from the nontriple-helical “modules” found within many of the nonfibrillar collagens (Brown and Timpl, 1995). Based on structural similarities, the collagen superfamily can be divided into the following classes.

Fibril-forming collagens: types I, II, III, V, and XI. These collagens (Kühn, 1987; Kadler, 1994) all share a long triple-helical segment with a continuous Gly-Xaa-Yaa repeat over its entire length. They assemble into cross-striated fibers upon cleavage of N and C propeptides, with the individual units adopting a one-quarter stagger relative to their neighbors in the fibril. Types II and XI collagen undergo alternative splicing, and hybrid molecules containing both types V and XI collagen have been identified in some tissues.

Network-forming collagens: types IV, VIII, and X. α chains in the type IV collagen family (Hulmes, 1992; Kühn, 1994; Yurchenco, 1994) contain a large collagenous domain that is frequently interrupted by short noncollagenous sequences (i.e., something other than Gly-Xaa-Yaa). Noncollagenous domains are also found at the N and C termini of the chain, with the C-terminal domain being the larger of the two. Monomers associate at the C termini to form dimers and at the N termini to form tetramers.

The triple-helical domains intertwine to form supercoiled structures, resulting in a net-like structure. Type VIII collagen is found in Descemet's membrane in the eye and forms a stack of hexagonal lattices. A similar structure is formed by type X collagen synthesized by hypertrophic chondrocytes in the deep-calcifying zone of cartilage.

Fibril-associated collagens with interrupted triple helices (FACIT): types IX, XII, XIV, XVI, and XIX. These collagens (Mayne and Brewton, 1993; Olsen et al., 1995) are characterized by short triple-helical segments interrupted by short noncollagenous domains. They attach to the surface of fibril-forming collagens and do not form fibrils themselves. Type IX collagen is found on the surface of type II collagen, to which it is covalently bound. An unusual property of this collagen is the presence of a glycosaminoglycan (GAG) chain attached to a noncollagenous domain of the $\alpha 2(\text{IX})$ chain. Types XII and XIV collagen show structural similarities to type IX, including an attached GAG side chain. Types XVI and XIX have not been fully characterized but show similarities in structure to other members of the family.

Beaded filaments and anchoring fibrils: types VI and VII. Among the collagens of this family (Burgeson, 1993; Timpl and Chu, 1994), type VI collagen is characterized by α chains containing large N- and C-terminal globular domains separated by a small triple-helical segment. Alternative splicing produces variants of the $\alpha 2(\text{VI})$ and $\alpha 3(\text{VI})$ chains. Type VI collagen forms small beaded filaments in the ECM. Type VII collagen forms anchoring fibrils that link basement membranes to anchoring plaques of type IV collagen and laminin in the underlying ECM. Type VII collagen contains the longest triple helix of any known collagen, with only small interruptions throughout. The NC1 domain of type VII collagen binds to collagen types I and IV, fibronectin, and laminin 5.

Collagens with a transmembrane domain: types XIII and XVII. Types XIII and XVII collagen (Li et al., 1996) are unique in having a transmembrane domain with its N terminus predicted to be in the cytoplasm. Type XIII collagen undergoes extensive alternative splicing. Type XVII collagen is found primarily in the hemidesmosomes of the skin and is one of the antigens that produces the autoimmune disease bullous pemphigoid.

Other nonfibrillar collagens: types XV and XVIII. Types XV and XVIII collagen (Rehn and Pihlajaniemi, 1994) have large N- and C-

terminal globular domains and a highly interrupted triple helix. Their large number of potential N- and O-linked glycosylation sites suggests that both types have the potential to be highly glycosylated.

ELASTIN AND MICROFIBRILLAR PROTEINS

Elastin

During evolution, with the advent of the closed circulatory system, came the requirement for blood vessels to accommodate the pulsatile blood flow of the heart. Vessels made mostly of collagen were too stiff, so in its place, we see the emergence of a matrix protein that has the properties of elastic recoil. This protein, elastin, is the predominant protein component of the elastic fiber that is of particular importance to the structural integrity and function of tissues in which reversible extensibility or deformability are crucial, such as the major arterial vessels, lungs, and skin.

In contrast to the genetic diversity evident in the collagen gene family, elastin is encoded by only one gene. Like collagen, elastin maturation in the ECM involves the assembly of a soluble precursor molecule (tropoelastin) into a highly cross-linked polymer. This assembly process is more complex than that for collagen, however, because the ability to self-assemble does not appear to be an intrinsic property of tropoelastin. Instead, elastin assembly requires helper proteins to align the multiple cross-linking sites on elastin monomers (Mecham and Davis, 1994).

Two functional domains repeat along the tropoelastin molecule (Fig. 10.1.2). One domain, related to cross-link formation, is an α helix containing alanine and lysine. The other, related to extensibility, is enriched in glycine, valine, and proline. The hydrophobic amino acids in this domain are arranged in repeating sequences that form a succession of β turns. The stacked β turns form a β spiral with a hydrophobic core. Stretching the elastin polymer exposes the hydrophobic core to water. Recoil occurs when the leaves of the β spiral contract to shield the hydrophobic amino acids from the aqueous microenvironment. Mature, cross-linked elastin is extremely hydrophobic and insoluble under most conditions (including when boiled in sodium hydroxide; Partridge, 1962). Its unusual physical properties make insoluble elastin one of the most stable proteins in the body—lasting the lifetime of the organism. Two polyfunctional cross-links, desmos-

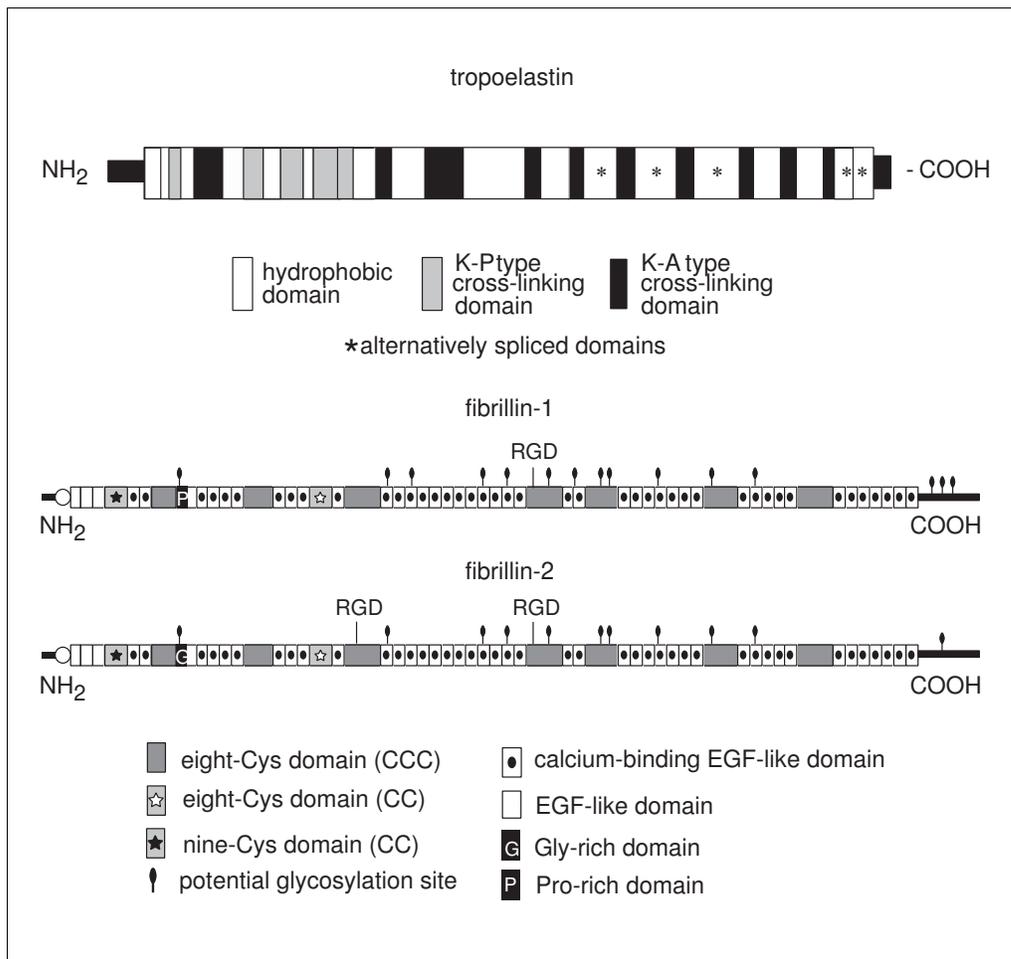


Figure 10.1.2 Domain map of tropoelastin and the fibrillins. Tropoelastin is secreted as a peptide of ~70 kDa and undergoes extensive covalent cross-linking during incorporation into the elastic fiber. Fibrillin-1 and fibrillin-2 each have a molecular weight of ~350 kDa and are the major structural elements of 10- to 12-nm-diameter microfibrils. Abbreviations: K-A, alanine-rich cross-linking domain; K-P, proline-rich cross-linking domain; RGD, Arg-Gly-Asp; CC, Cys-Cys sequences; CCC, Cys-Cys-Cys sequences; EGF, epidermal growth factor.

ine and isodesmosine, are unique to elastin and can be used as specific markers for this protein.

Fibrillin

Microfibrils were first identified as components of elastic fibers. They are found in greatest abundance in elastic tissues or in the ciliary zonules of the eye, although their distribution is widespread. Fibrillin-1 and -2 play key roles in microfibrillar architecture. These 350-kDa glycoproteins are highly homologous (Fig. 10.1.2), with modular structures consisting of repeating calcium-binding epidermal growth factor (EGF)-like domains interspersed between 8-cysteine domains similar to those found in the latent transforming growth factor- β (TGF- β)-binding protein family (Lee et al., 1991). Tandemly arranged EGF domains form a structural motif found frequently in ECM

macromolecules (e.g., laminin, fibulin, latent TGF- β -binding protein, nidogen). When stacked together, these tightly folded, disulfide-bonded loop structures form a rigid, rod-like arrangement stabilized by interdomain calcium binding and hydrophobic interactions (Downing et al., 1996). The precise function of microfibrils is unclear, although their association with developing elastic fibers suggests a role in elastin assembly. Both fibrillin-1 and fibrillin-2 interact with the α v β 3 integrin through an Arg-Gly-Asp (RGD) sequence (see Adhesive Glycoproteins).

ADHESIVE GLYCOPROTEINS

Most, if not all, ECM macromolecules interact with binding proteins on the surface of cells. In many instances, this is through a unique sequence motif that is accessible as part

of the protein's folded functional structure, or cryptic and exposed only when the protein undergoes a conformational change induced by binding to another protein or as the result of degradation or denaturation. One such "recognition motif" is the well-known RGD sequence that is recognized by several members of the integrin family.

Fibronectin

A great deal of biochemical work has led to a model of the fibronectin molecule in which the protein's binding functions and its structure are clearly correlated (Hynes, 1990). The molecule is secreted as a dimer consisting of two similar subunits joined together at the C terminus by disulfide bonds (Fig. 10.1.3). Each chain has a molecular weight of ~220 to 250 kDa and is subdivided into a series of tightly folded domains. Each domain is responsible for one of fibronectin's binding functions. In plasma, fibronectin exists as a soluble dimer, but in the ECM it is found as an insoluble multimer.

Amino acid sequence analysis of fibronectin shows that the molecule is made up mostly of three repeating motifs, referred to as types I, II, and III repeats. These repeats are organized into functional domains that contain binding sites for ECM proteins and cell surface receptors (see Fig. 10.1.3). For example, there are two fibrin-binding domains consisting of multiple type I repeats on each subunit of the protein. Type I repeats are also found in the collagen-binding domain, and the first five type I repeats play an important role in matrix assembly. The

N-terminal domain of fibronectin also mediates fibronectin's binding to gram-positive bacteria through type I modules. The type I module contains ~45 amino acids with four cysteines forming two disulfide bonds (Potts and Campbell, 1994). This module has also been found in a number of other proteins. In addition to type I repeats, the collagen-binding domain contains the only type II repeats found in fibronectin. Like type I repeats, these motifs contain two disulfide bonds, but they are larger than type I motifs.

The predominant structural feature of fibronectin consists of type III repeats, accounting for more than 60% of the sequence. No disulfide bonds are present in this structure, although two of the repeats contain a free cysteine. The cell-binding RGD sequence is located in the tenth type III repeat. This sequence is recognized by many members of the integrin family, including $\alpha 5\beta 1$, $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha IIb\beta 3$, and $\alpha 8\beta 1$. Other cell-binding regions include the C-terminal heparin-binding domain and the type III-connecting segment (IIICS), including the CS1 region. The type III consensus sequence is frequently found in other proteins.

Only one gene for fibronectin has been identified, but mRNAs for fibronectin have been shown to give rise to multiple versions of the protein through variable patterns of RNA splicing during gene transcription. Alternative splicing occurs predominately at three sites, termed extra type III domain A (EDA or EIIIA), extra type III domain B (EDB or EIIIB), and the

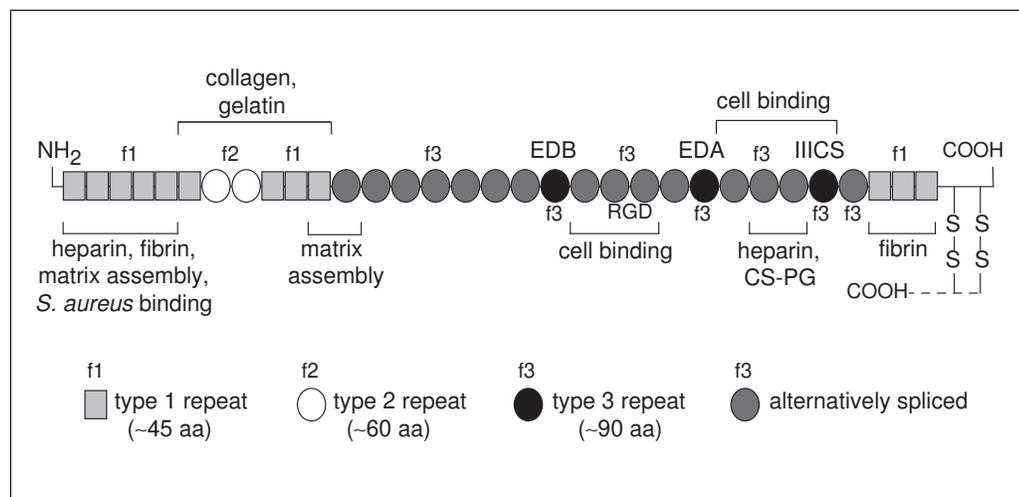


Figure 10.1.3 Domain map of fibronectin. The subunits of fibronectin vary in size between ~235 and 270 kDa. Alternative splicing occurs at three positions: EDA, EDB, and IIICS. Binding sites for other molecules and cells are indicated. Abbreviations: EDA, extra type III domain A; EDB, extra type III domain B; IIICS, connecting segment between the fourteenth and fifteenth type III repeats; RGD, Arg-Gly-Asp; CS-PG, chondroitin sulfate proteoglycan; aa, amino acids.

connecting segment between the fourteenth and fifteenth type III repeats (IIICS or V). Splicing within the IIICS segment produces five variants, such that twenty different fibronectin subunits can result from splicing within the three segments.

Subunits of plasma fibronectin produced by adult hepatocytes contain neither EDA nor EDB segments, and one subunit lacks the entire IIICS domain. Cultured fibroblasts, however, typically produce a form of fibronectin, referred to as cellular fibronectin, that contains the EDA and/or EDB segments. Fibronectins expressed in fetal and tumor tissues contain a greater percentage of EDA and EDB segments than those expressed in normal adult tissues. The biological functions of fibronectin isoforms are only poorly understood, despite having been studied extensively. Differences in solubility have been demonstrated, but it has been difficult to detect functional differences between plasma and cellular fibronectin in their ability to promote cell adhesion and spreading.

Vitronectin

Vitronectin (also called serum spreading factor, S-protein, and epibolin) is a multifunctional protein found in plasma and ECM. It is synthesized as a single chain that undergoes N glycosylation, tyrosine sulfation, and phosphorylation prior to secretion. In plasma, vitronectin circulates in two forms: a single chain of ~75 kDa and a proteolytically cleaved, two-chain form that dissociates into 65- and 10-kDa fragments upon reduction. It is present in fibrillar form in the ECM of a variety of tissues, where it sometimes colocalizes with fibronectin and elastic fibers. While little vitronectin immunoreactivity is detectable in most normal tissues, increased deposition has been observed in areas of tissue injury and necrosis. Tissue vitronectin was believed to be plasma derived, but recent studies indicate that extrahepatic cells have the biosynthetic potential to produce vitronectin and that its synthesis can be regulated under inflammatory conditions (Seiffert, 1997).

The cell attachment activity of vitronectin results from an RGD sequence that is recognized by a wide variety of integrins. Most of the cell adhesive activity of serum used for tissue culture can be attributed to vitronectin.

Laminin and Basement Membranes

Like fibronectin, the laminins are modular proteins with domains that interact with both cells and ECM (Eklblom and Timpl, 1996). They constitute a family of basement mem-

brane glycoproteins that affect cell proliferation, migration, and differentiation. Eleven different laminins have been identified, each containing an α , β , and γ chain (Fig. 10.1.4). Electron microscopy has revealed that all laminins have a cross-like shape with three short arms and one rod-like long arm, a shape well suited for mediating interactions between sites on cells and components of the ECM (Beck et al., 1990; Maurer and Engel, 1996). The rod-like regions separating the globular units of the short arms are made up of repeating EGF-like domains. The long arm is formed by all three component chains folding into an α -helical coiled-coil structure, and is the only domain composed of multiple chains. It is terminated by a large globular domain composed of five homologous subdomains formed by the C-terminal region of the α chain.

Along with type IV collagen, laminins are a major structural element of the basal lamina (Timpl, 1996). The molecular architecture of these matrices results from specific binding interactions among the various components. The structural skeleton is formed by type IV collagen chains that assemble into a covalently stabilized polygonal network. Laminin self-assembles through terminal domain interactions to form a second polymer network. Nidogen (Mayer and Timpl, 1994) binds laminin near its center and interacts with type IV collagen, bridging the two. A large heparan sulfate proteoglycan (HS-PG), perlecan, binds laminin and type IV collagen through its GAG chains and forms dimers and oligomers through a core-protein interaction. Perlecan is important for charge-dependent molecular sieving, one of the critical functions of basement membrane. Other components that are sometimes found associated with basement membranes but may not be intrinsic components include fibronectin, type V collagen, fibulin, osteonectin (also known as BM-40 or SPARC), and chondroitin sulfate proteoglycans.

Cells attach to laminin through specific interaction sites created by its multidomain structure. For example, sites for receptor-mediated cell attachment and promotion of neurite outgrowth reside in the terminal region of the long arm. A second cell-attachment site and a cell-signaling site with mitogenic action are localized in the short arms. Cell binding to laminin occurs via a variety of receptors, including non-integrins (Mecham and Hinek, 1996) and integrins (Aumailley et al., 1996). The β 1 family includes most of the laminin-binding integrins (α 1 β 1, α 2 β 1, α 3 β 1, α 7 β 1, α 9 β 1). Other

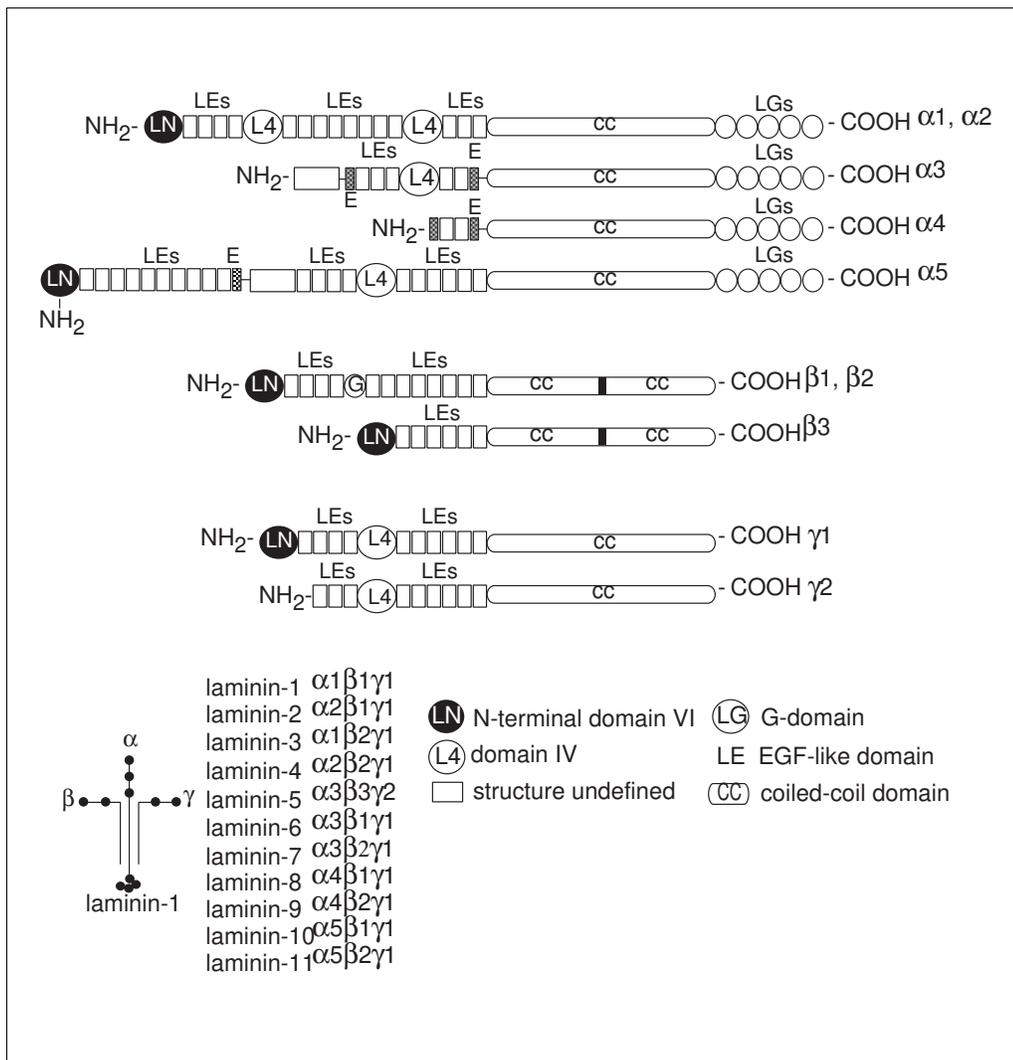


Figure 10.1.4 Domain map of laminin chains. Three polypeptide chains (α , β , and γ) form the laminin cross. The chain composition of known laminin types is shown in the insert. Abbreviation: EGF, epidermal growth factor.

integrins that bind laminin include $\alpha v \beta 3$ and $\alpha 6 \beta 4$. Basement membrane can also have an indirect effect on cells by binding and sequestering growth and differentiation factors, such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and TGF- β .

The importance of laminin to cell differentiation and migration has been demonstrated in developmental studies. Isoforms of laminin assembled from different chains are focally and transiently expressed and may serve distinct functions at early stages of development even before being deposited as components of basement membranes. Laminin is present at the two-cell stage in the mouse embryo, making it one of the first ECM proteins detected during embryogenesis.

MATRICELLULAR PROTEINS

The term “matricellular” has been applied to a group of extracellular proteins that function by binding to matrix proteins and to cell surface receptors, but do not contribute to the structural integrity of the ECM (Bornstein, 1995). Proposed members of this group include the thrombospondins, members of the tenascin protein family, SPARC/osteonectin (Lane and Sage, 1994), and osteopontin. These proteins are frequently called “antiadhesive proteins” because of their ability to induce rounding and partial detachment of some cells in vitro (Sage and Bornstein, 1991). Their ability to interact with many different matrix proteins and cell surface receptors may explain their complex range of biological functions.

ment sites suggests that the overall antiadhesive properties of the glycoprotein are effected by separate domains that override the attachment domains.

PROTEOGLYCANS

The proteoglycans (once called acid mucopolysaccharides) constitute a number of genetically unrelated families of multidomain proteins that have covalently attached GAG chains. To date, more than 25 distinct gene products have been identified that carry at least one GAG chain (Iozzo and Murdoch, 1996). Like other matrix components discussed in this review, proteoglycans exist as structural variants, further increasing their functional and structural diversity.

For historical reasons, proteoglycans are named based on the type of attached GAG chain(s): (1) chondroitin sulfate and dermatan sulfate, consisting of a repeating disaccharide of galactosamine and either glucuronic acid or iduronic acid; (2) heparin and heparan sulfate, consisting of a repeating disaccharide of glucosamine and either glucuronic acid or iduronic acid; and (3) keratan sulfate, consisting of a repeating disaccharide of glucosamine and galactose. Hyaluronate is also a repeating disaccharide but is not sulfated and not bound to a core protein. GAG chains are usually attached through *O*-glycosidic linkages to serine residues in the proteoglycan core protein. A characteristic feature of GAG chains is that at physiological pH they contain one to three negative charges per disaccharide due to carboxylate and sulfate groups.

Knowledge of the structure and function of proteoglycans increased dramatically when molecular biology was used to study the core proteins (Hassell et al., 1993). The heterogeneity of this family of matrix proteins also became evident with the finding that there are no structural domains common to all proteoglycans. There are, however, distinguishing characteristics that allow them to be grouped into four broad categories.

Large Proteoglycans that Form Aggregates by Interaction with Hyaluronan

These proteoglycans interact with strands of hyaluronate to form a very-high-molecular-weight aggregate. A structural trait shared by these proteoglycans is the presence of three functional domains: a globular hyaluronan-binding domain at the N terminus, a central extended region that carries most of the GAG

chains, and a modular C-terminal domain containing two EGF repeats, a C-type lectin domain, and a complement-regulatory-protein-like motif (Iozzo and Murdoch, 1996).

The largest member of this family is versican (Zimmermann and Ruoslahti, 1989), a major proteoglycan in blood vessels that is also expressed in nonvascular tissues. Aggrecan, the large aggregating proteoglycan of cartilage, has a smaller core protein than versican but contains nearly 3-fold more GAG chains (Fig. 10.1.6). The high charge density of aggrecan results in each monomer occupying a large hydrodynamic volume. Aggrecan's GAG chains result in a high density of fixed charge in cartilage, producing an osmotic swelling pressure that is balanced by tension in the collagenous network. The reversible redistribution of proteoglycan-bound water under loading gives cartilage the ability to absorb compressive loads (Wight et al., 1991). Two other members of this family include neurocan (Rauch et al., 1992) and brevican (Yamada et al., 1994), both found in brain tissues.

Basement Membrane Proteoglycans

HS-PGs appear to be ubiquitous components of all basement membranes. Perlecan is the largest basement membrane proteoglycan, with a modular core protein of 467 kDa (Fig. 10.1.6; Iozzo et al., 1994). It provides the basement membrane with a negative charge that is important to its sieving properties. The heparan sulfate chains of perlecan also bind growth factors and cytokines and sequester them into the basement membrane, where they may function as a reserve to be released during tissue repair. The interaction of heparan sulfate with the FGFs has been extensively studied (Aviezer et al., 1994). Perlecan interacts with other components of the basement membrane, particularly laminin and nidogen. The multidomain structure of perlecan core protein is reminiscent of other ECM proteins, and includes EGF repeats and repeats of structures found in the low-density lipoprotein receptor, laminin chains, and neural cell adhesion molecule.

Agrin was originally isolated from torpedo ray electric organ and was found to induce acetylcholine receptor aggregation. It is secreted by motor neurons and deposited in the synaptic cleft basement membrane. Agrin may also play a role in the sequestration of growth factors in the basement membrane. Like perlecan, agrin is a multidomain protein with regions of EGF and laminin G-domain homology. Agrin is found predominantly in the brain, but

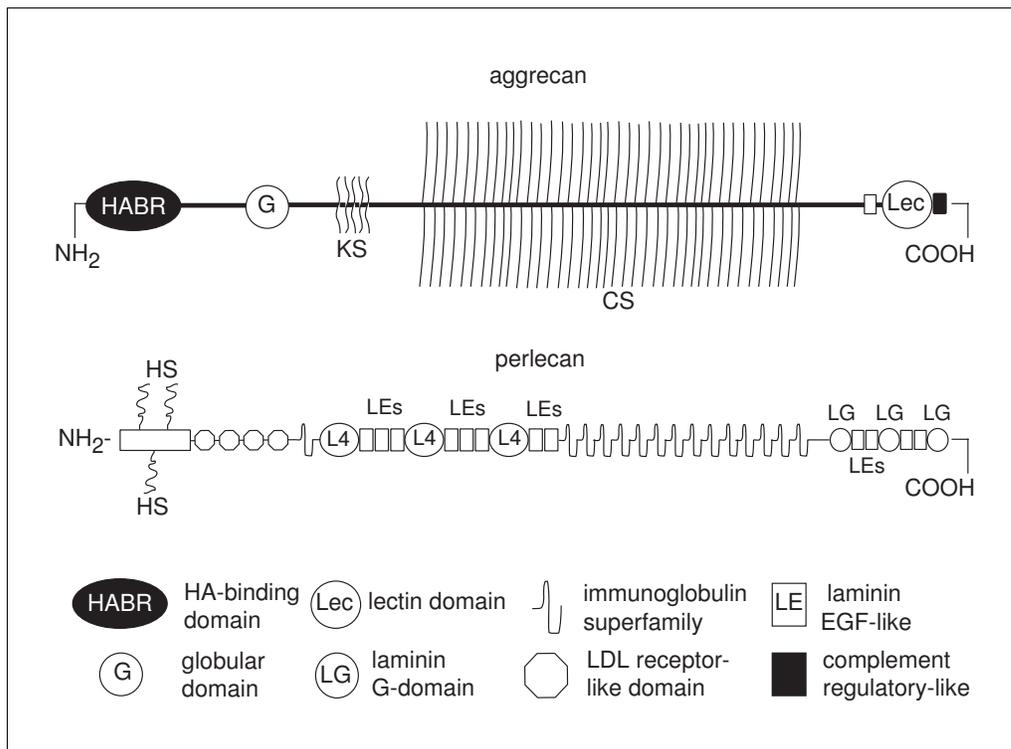


Figure 10.1.6 Domain map of two representative large proteoglycans. Aggrecan is the core protein of the aggregating proteoglycan found in cartilaginous tissues. The molecular weight of the aggrecan core protein is 210 to 250 kDa. There are 100 to 150 keratan sulfate chains and many more chondroitin sulfate chains that contribute to the 2500-kDa molecular weight of the mature proteoglycan. The glycosaminoglycans are attached to repetitive sequences in the middle two-thirds of the molecule, including several types of repeats containing Ser-Gly, the linkage site for chondroitin sulfate. Perlecan is the largest of the basement membrane proteoglycans and has two or three attached heparan sulfate side chains. Removal of heparan sulfate side chains by heparatinase produces a core protein of 400 to 450 kDa on SDS-PAGE. Abbreviations: HA, hyaluronate; KS, keratan sulfate; CS, chondroitin sulfate; HS, heparan sulfate; LDL, low-density lipoprotein; EGF, epidermal growth factor.

has also been localized to smooth and cardiac muscle.

Cell Surface Heparan Sulfate Proteoglycans

HS-PGs on the cell surface influence several important biological functions, including cell adhesion; the sequestration of heparin-binding ligands on the plasma membrane; and the promotion of dimerization/oligomerization of bound ligands, which enhances activation of primary signaling receptors.

Cell-associated HS-PGs have been divided into two major families, syndecan-like integral membrane HS-PGs (SLIPs) and glypican-related integral membrane HS-PGs (GRIPs; David, 1993; Carey, 1997). The SLIPs are transmembrane HS-PGs with a conserved intracellular domain that likely interacts with cytoskeletal and regulatory proteins. The GRIPs are linked to the cell surface by glycosyl

phosphatidyl inositol in the outer leaflet of the lipid bilayer.

The syndecans, the principal form of cell-surface HS-PG, are synthesized by many cells. Syndecans bind a variety of extracellular ligands via their covalently attached heparan sulfate chains and are thought to play important roles in cell-matrix and cell-cell adhesion, migration, and proliferation. To date, four homologous syndecan core proteins have been cloned from vertebrate cells. All syndecans are type I transmembrane proteins, with an N-terminal signal peptide, an ectodomain that contains several consensus sequences for GAG attachment, a single hydrophobic transmembrane domain, and a short C-terminal cytoplasmic domain. The majority of GAG chains added to syndecan core proteins are of the heparan sulfate type, although syndecan-1 and syndecan-4 have chondroitin sulfate chains attached as well. Syndecans act as cell surface

receptors for a number of matrix molecules, thereby mediating cell attachment and tissue organization. They influence the interactions of basic FGF and other growth factors with their receptors on cells and are responsible for the maintenance of a nonthrombogenic surface on endothelial cells.

Small Leucine-Rich Proteoglycans

Small leucine-rich proteoglycans (SLRPs) comprise a class of secreted proteoglycans that include five structurally related members: decorin, biglycan, fibromodulin, lumican, and epiphycan (see Fig. 10.1.7). Each has a leucine-rich core protein that assumes an arch-shaped

structure with a concave surface capable of interacting with various other proteins. The N-terminal region contains one (decorin) or two (biglycan and epiphycan) GAG chains that can be either dermatan or chondroitin sulfate. Instead of GAG chains, fibromodulin and lumican have tyrosine sulfate in the N terminus, which provides an analogous negatively charged domain. These two SLRPs also contain N-linked keratan sulfate chains in their central domain.

SLRPs interact with numerous ECM proteins (e.g., fibronectin, TSP, fibrillin, microfibril-associated glycoprotein) and act to orient and order collagen fibers during development

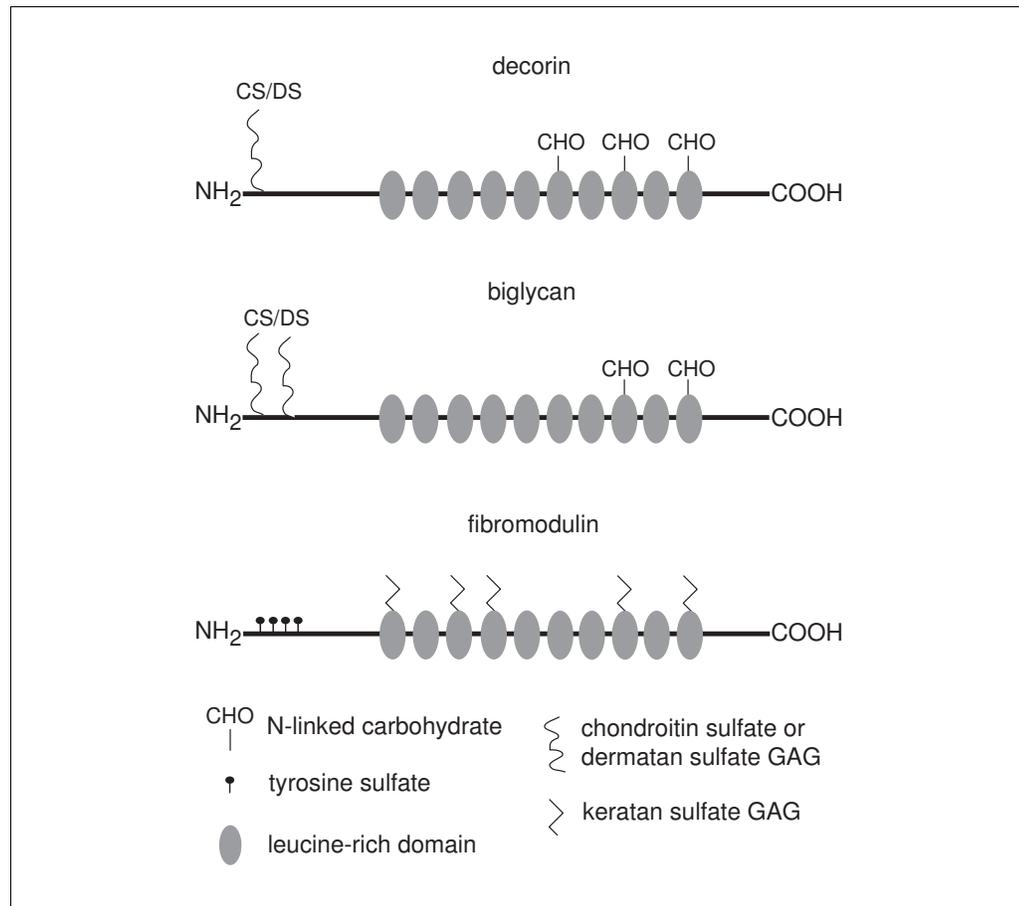


Figure 10.1.7 Domain map of representative members of the small leucine-rich proteoglycans. Decorin contains a single chondroitin or dermatan sulfate chain attached near the N terminus. The core protein is ~38 kDa. Decorin is heterogeneous with respect to glycosaminoglycan (GAG) chain size, such that the secreted proteoglycan shows a range of molecular weights centered between 100 and 250 kDa. The core protein of biglycan is similar in size to that of decorin, except biglycan contains two chondroitin or dermatan sulfate chains. The GAG chains are also heterogeneous in size, resulting in a broad band on SDS-PAGE centered anywhere from 200 to 350 kDa. Removal of GAG chains with chondroitin ABC-lyase results in a 45-kDa band. Fibromodulin has a core protein size of 42 kDa. Four of the five potential N-glycosylation sites in the leucine-rich region of the molecule are substituted with keratan sulfate chains. Five to seven closely spaced tyrosine sulfate residues are found in the N-terminal domain. Abbreviations: CS, chondroitin sulfate; DS, dermatan sulfate.

and tissue remodeling. Interactions with matrix proteins occur through the leucine-rich core which, in the case of type I collagen, influences collagen fibrillogenesis by binding to the surface of the collagen fibril at the d-band with the highly charged GAG chain extending out to regulate interfibrillar distances. Like other proteoglycans, SLRPs bind to growth factors (e.g., TGF- β) and thereby likely influence cellular differentiation and matrix synthesis. Decorin has recently been shown to directly regulate cell growth by activating the EGF receptor (Moscatello et al., 1998).

CONCLUSIONS

The furious pace of advances in the molecular biology of ECM has greatly expanded the knowledge of individual matrix components. The structure of many matrix macromolecules, for example, was determined from cloned cDNAs or genes long before complete protein information was available. With this increased knowledge as background, there is a growing realization that the information contained in the ECM is not a monosyllabic message encoded by individual molecules, but a complex and intricate arrangement dictated by the combinatorial organization of the supramolecular structure. As the focus of biological research changes from the letters to the message, understanding how cells read and interpret this information will undoubtedly reveal more about the letters in the code.

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Preparation of Basement Membrane Components from EHS Tumors

This unit describes methods for passaging and harvesting the basement membrane matrix-producing EHS tumor and for the subsequent isolation of a crude mixture of basement membrane components termed Matrigel (see Basic Protocol 1), which promotes the differentiation of a variety of epithelial, endothelial, and neuronal cells. Procedures for the isolation of the adhesive glycoprotein laminin-1 (see Basic Protocol 2) and of type IV collagen (see Basic Protocol 3) are also included.

All epithelial and endothelial cells produce a basement membrane matrix, but in most tissues they do so only in small amounts. The approach described here, using tumor cells, enables one to obtain basement membrane material of high purity in significant quantities. The material isolated from EHS tumors is structurally and antigenically identical to material from normal tissue basement membranes. The isolated material is native, intact, and homogeneous. It can be used in biological assays and for studying extracellular matrix assembly. The isolation methods for Matrigel and laminin-1 can also be applied to nontumor tissues, but yields are generally low and additional purification may be needed.

Type IV collagen is normally highly cross-linked; therefore it is difficult to extract it intact from tissues in quantity unless the animals are placed for several weeks on a lathrogenic diet, which makes collagens soluble by blocking cross-link formation. Support protocols cover the maintenance and harvesting of EHS tumors in mice (see Support Protocol 1) and maintenance of mice on a lathrogenic diet (see Support Protocol 2).

Procedures for preparing laminin-1 and type IV collagen are similar to that for Matrigel but require additional steps and different extraction buffers. Both laminin-1 and type IV collagen can be prepared from the same batch of tumor if the mice are lathyritic, but only laminin-1 can be prepared from nonlathyritic tissue. Once Matrigel is prepared from the tumor, the residue cannot be used for large-scale preparations of laminin-1 or type IV collagen because most of the material has been extracted.

PREPARATION OF MATRIGEL

Matrigel significantly promotes the differentiation of a variety of cell types, including endothelial, smooth muscle, fat, and neuronal cells. Untransformed cells generally do not proliferate on Matrigel, whereas transformed cells continue to proliferate. This preparative procedure requires 100 g of tumor tissue and the volumes used are important.

Materials

- EHS tumor (see Support Protocol 1)
- 3.4 M NaCl buffer (see recipe), 4°C
- 2 M urea buffer (see recipe), 4°C
- Tris-buffered saline (TBS; *APPENDIX 2A*), 4°C
- Chloroform
- Tissue culture medium (e.g., DMEM, RPMI)
- 70% ethanol
- Electric homogenizer (e.g., Polytron)
- Centrifuge and rotor, 4°C
- Sterile hemostat

Additional materials and equipment for dialysis and protein assays (see *APPENDIX 3*)

BASIC PROTOCOL 1

Extracellular Matrix

10.2.1

Contributed by Hynda K. Kleinman

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NOTE: All reagents and equipment should be prechilled to 4°C, and all procedures must be performed at 4°C.

1. If frozen, thaw the EHS tumor (100 g) at room temperature in 200 ml of 3.4 M NaCl buffer for ~1 hr. Homogenize the tumor until dispersed with an electric homogenizer.

A Polytron homogenizer works well. Do not homogenize for too long, as it is important not to warm the tissue.

2. Centrifuge the homogenate 15 min at 8000 × g, 4°C, to pellet the tumor tissue. Discard the pink supernatant and repeat homogenization (step 1).

The centrifuge and rotor must be cold before and during use for all manipulations.

3. Add 100 ml of 2 M urea buffer to the tumor pellets and homogenize to disperse the tumor. Stir overnight at 4°C.

4. Centrifuge the urea homogenate 20 min at 23,000 × g, 4°C, and decant the supernatant gently. Save the supernatant on ice.

Pour off the thick supernatant gently, as the pellet may detach from the bottom of the tube.

5. Homogenize the pellets in 50 ml of 2 M urea buffer and centrifuge as in step 4. Combine the supernatants and discard the pellets.

6. Dialyze the supernatant against 2 liters TBS containing 10 ml chloroform/liter for ≥4 hr (see APPENDIX 3). Manually rotate the bags after 2 hr to assure dispersion of the contents.

This step sterilizes the inside of the bag; chloroform will kill bacteria and spores. Subsequent steps remove the urea and chloroform. When discarding the chloroform-containing dialysis buffer, it is best to decant the buffer and discard the remaining chloroform, which settles to the bottom of the vessel, in a chemical waste container.

7. Dialyze twice more, for ≥2 hr each time, against 2 liters TBS and then once more against 2 liters tissue culture medium.

Manually rotate the bags between changes to facilitate the exchange of solutions.

8. Working in a sterile hood and holding the dialysis bag with a sterile hemostat, spray it with 70% ethanol, cut it with sterile scissors, and empty the contents into a sterile container on ice.

9. Determine the protein concentration using a conventional protein assay (e.g., Lowry assay; see APPENDIX 3). Divide the preparation (Matrigel) into aliquots and store up to 12 months at -20°C.

Matrigel concentration should be 10 to 15 mg/ml.

It is not desirable to freeze and thaw Matrigel too many times, so generally 1- and 10-ml aliquots are most useful. The size of the aliquots should be based on need.

PREPARATION OF LAMININ-1

Laminin-1 promotes the adhesion, differentiation, migration, and growth of many cells in culture. In addition, most neuronal cells tested extend long neuritic processes when grown on laminin-1. Laminin-1 cannot be prepared from the same batch of EHS tissue as Matrigel, but it can be prepared from the same batch of tissue as type IV collagen (see Basic Protocol 3).

Materials

EHS tumor (see Support Protocol 1)

3.4 M NaCl buffer (see recipe), 4°C

0.5 M NaCl buffer (see recipe), 4°C

Ammonium sulfate

TBS (*APPENDIX 2A*), 4°C

NaCl

Chloroform

Electric homogenizer (e.g., Polytron)

Centrifuge and rotor, 4°C

Sterile hemostat

Additional materials and equipment for dialysis and protein assays (see *APPENDIX 3*)

NOTE: All reagents and equipment should be prechilled to 4°C, and all procedures must be performed at 4°C.

1. Thaw 100 g of EHS tumor in 200 ml of 3.4 M NaCl buffer for ~1 hr at room temperature. Homogenize tumor with an electric homogenizer until dispersed.

The volumes given in all the protocols are based on 100 g of tissue. Larger or smaller amounts of tumor can be used with proportional volumes of buffers.

A Polytron homogenizer works well. Do not homogenize for too long, as it is important not to warm the tissue.

2. Centrifuge the homogenate 15 min at 8000 × g, 4°C, to pellet the tumor tissue. Discard the pink supernatant and repeat homogenization (step 1).
3. Add 200 ml of 0.5 M NaCl buffer to the tumor pellets and homogenize. Stir overnight at 4°C.
4. Centrifuge the homogenate 15 min at 8000 × g, 4°C. Save the supernatant in a container on ice. Repeat step 3 and repeat centrifugation.

The tumor homogenate pellet can be used to prepare type IV collagen (see Basic Protocol 3) if the tumor tissue was taken from lathyritic animals. Otherwise, the pellet should be discarded.

5. To the combined 0.5 M NaCl supernatants, slowly add ammonium sulfate to 30% saturation (16.4 g/100 ml) with vigorous stirring. Stir 1 hr after all of the salt has dissolved.
6. Centrifuge 15 min at 8000 × g, 4°C.

The pellets should be large and white, with a reasonably clear supernatant. The supernatant should be saved unless it is certain that all the laminin-1 precipitated.

7. Dissolve the laminin-1 pellets in 300 ml TBS and dialyze for ≥2 hr against 2 liters TBS (see *APPENDIX 3*). Repeat dialysis twice against 2 liters TBS each time.

**BASIC
PROTOCOL 3**

8. Empty the dialysis bags, measure the volume of the sample, and slowly add NaCl, with stirring, to a final concentration of 1.7 M. Centrifuge 15 min at $8000 \times g$, 4°C .

The solution is already 0.15 M NaCl, so 1.55 M NaCl or 90.6 g should be added per liter.

The pellet should be very small. This step removes the contaminating type IV collagen. If the pellet is large, repeat step 7 and omit step 8.

9. Dialyze the supernatant against TBS for three changes (as in step 7). Optionally, include 5 ml/liter chloroform to TBS in the first dialysis step to sterilize the laminin-1. If chloroform is added, then perform an additional dialysis with TBS.
10. Working in a sterile hood and holding the dialysis bag with a sterile hemostat, spray it with 70% ethanol, cut it with sterile scissors, and empty the contents into a sterile container on ice.
11. Determine the amount of protein with a standard protein assay (e.g., Lowry assay; see *APPENDIX 3*). Divide the preparation (laminin-1) into 1-ml aliquots and store at -70°C for 24 months.

The protein concentration should be 3 to 6 mg/ml.

PREPARATION OF TYPE IV COLLAGEN

Type IV collagen is used for cell adhesion and migration studies. Type IV collagen can be prepared from the same tumor tissue as laminin as long as the animals have been on a lathrogenic diet for ≥ 2 weeks prior to tumor harvest (see Support Protocol 2).

Materials

Tumor homogenate (see Basic Protocol 2, step 4), prepared from animals fed a lathrogenic diet (see Support Protocol 2)

0.5 M NaCl buffer (see recipe), 4°C

2.0 M guanidine-HCl buffer (see recipe), 4°C

2.0 M guanidine-HCl buffer containing 32 mg/liter dithiothreitol (DTT), 4°C

0.5 M acetic acid (28.5 ml acetic acid/liter water), 4°C

Centrifuge and rotor, 4°C

Additional materials and equipment for dialysis and protein assays (see *APPENDIX 3*)

NOTE: All reagents and equipment should be prechilled to 4°C , and all procedures must be performed at 4°C .

1. Prepare tumor homogenate in 300 ml of 0.5 M NaCl buffer. Centrifuge 15 min at $8000 \times g$, 4°C . Discard the supernatant and repeat centrifugation.

These steps and the subsequent ones remove contaminating laminin-1.

2. Homogenize pellets in 2.0 M guanidine-HCl buffer and centrifuge 15 min at $8000 \times g$, 4°C . Discard the supernatant and repeat the wash.
3. Homogenize pellets in 250 ml of 2.0 M guanidine-HCl buffer/DTT solution and stir overnight at 4°C .
4. Centrifuge tumor homogenate 15 min at $8000 \times g$, 4°C . Save the supernatant in a container on ice.
5. Homogenize pellets in 250 ml of 2.0 M guanidine-HCl buffer/DTT. Centrifuge immediately for 15 min at $8000 \times g$, 4°C . Discard pellet and combine supernatant with that from step 4.

6. Dialyze supernatants against four changes of 0.5 M acetic acid for ≥ 2 hr each (see *APPENDIX 3*).

If the collagen concentration is >0.8 mg/ml, it will precipitate, and it is difficult to recover the precipitated material. To ensure that the entire batch does not precipitate, dialyze one-tenth of the sample through three changes before processing the remainder. If the collagen does precipitate, the remaining supernatant should be diluted by half with cold distilled water and then dialyzed. The material that has precipitated can be collected by centrifugation, redissolved in twice the original volume of 2.0 M guanidine-HCl buffer/DTT solution, and then dialyzed against 0.5 M acetic acid.

7. Determine the amount of protein by amino acid analysis or by lyophilizing 10 ml and weighing the sample. Divide the preparation (type IV collagen) into 1-ml aliquots and store at -20°C for 24 months.

The solution should be clear. The presence of a precipitate indicates that collagen has come out of solution. The precipitate can be removed by centrifugation, leaving considerable type IV collagen in the supernatant.

MAINTENANCE AND HARVEST OF EHS TUMORS

This protocol describes a method for maintaining and harvesting the EHS tumor. In addition, information is provided on the lathrogenic diet that is necessary when the tissue is used for isolating type IV collagen (see Support Protocol 2). The tumor passage protocol requires live dispersed EHS tumor tissue or single cells, which can be obtained from American Type Culture Collection (see *SUPPLIERS APPENDIX*). A cell line can also be obtained from the developers of the line (R.V. Iozzo and J.R. Hassell). One 4-g tumor can be passaged into ten mice by subcutaneous or intramuscular injection. The tumor is ready for harvest in 3 to 4 weeks.

Materials

Fresh EHS tumor
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
PBS containing 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin
C57BL/6 mice
Anesthetic (e.g., 3.2% Avertin)
Dimethylsulfoxide (DMSO)
70% ethanol

50-ml plastic tubes
20- to 30-ml and 3-ml syringes
16-G needles
Centrifuge and rotor, 4°C

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

1. Mince fresh EHS tumor (~ 4 g) from each animal as much as possible with scissors. Add 7 ml PBS and transfer tissue chunks into a 20- to 30-ml syringe without a needle. Force tissue chunks through the syringe several times until the suspension goes through easily. Place a 16-G needle on the syringe and repeat the process.

Have several ice buckets handy to keep the tumor cold. Handle each tumor separately, using clean tubes and syringes, in case there is too much bacterial contamination with blood or pus in one of the tumors. Number the tubes and respective syringes when working with large numbers of tumors. This step can be messy; gloves, lab coats, and masks are recommended.

SUPPORT PROTOCOL 1

Extracellular
Matrix

10.2.5

2. Add 40 ml PBS and gently shake the tube to disperse the tissue. Allow tumor tissue to settle for a few minutes, or centrifuge for 5 to 10 sec to speed up the process. Discard supernatant and dislodge and disperse pellet by tapping the tube.
3. Repeat step 2. Add PBS containing penicillin and streptomycin to a final volume of 10 ml.

At this stage the preparation should be a thick slurry.

4. Lightly anesthetize mice by intraperitoneal injection of 200 μ l Avertin. Inject 1 ml cell slurry into each mouse, either subcutaneously or intramuscularly, using a 16-G needle with a 3-ml syringe.

It is possible to use two injection sites per animal to increase the yield. Watch the animals carefully as the tumors grow to be sure that the animals can obtain food, etc.

Female mice are more docile and easier to handle than males, and may be preferred as tumor recipients. The cages should be marked with the tumor number to allow tracking in case of contamination in the tumor.

Dispersed tumor can also be frozen and stored for future use: add PBS containing 5% DMSO to dispersed tumor; transfer 1-ml aliquots to freezing vials, place in a -70°C freezer for 2 hr, and then store in liquid nitrogen. It generally takes >3 weeks for the initial tumor to become fully grown when frozen material is injected, probably because some cells die during the freezing and thawing process. Where possible, it is best to work with fresh tumor tissue.

5. Harvest the tumors after 3 weeks. Euthanize mice by CO_2 asphyxiation and dip each one in 70% ethanol. Carefully excise the tumor to remove any capsule-like material. Place each tumor in a separate 50-ml tube if the tumor is to be passaged, or in a freezer bag if it is to be stored for preparation of basement membrane components.

Tumor size should not exceed 3 cm in length, height, or width as larger tumors will become necrotic. Tumors that are very bloody or full of yellow pus should be discarded. For convenience, freeze tumors at 100 g/bag and label the bags with tumor weight and date.

If not used immediately, fresh tumor can be frozen in powdered dry ice or immersed directly in liquid nitrogen and then stored for ≤ 1 year at -70°C in freezer bags.

SUPPORT PROTOCOL 2

MAINTENANCE OF MICE ON A LATHROGENIC DIET

The lathrogenic diet prevents collagen from cross-linking; hence type IV collagen derived from lathyrotic mice is more soluble and easily extractable, which increases the yield of material obtained. If mice are maintained too long on the diet (>3 weeks), they develop spontaneous bone fractures. It is important to begin monitoring the health of the mice after 2 weeks, and it may be necessary to euthanize mice at slightly different times to maximize the yield of tumor.

Materials

Lathrogenic chow (see recipe)

CAUTION: Lathrogenic chow contains toxic materials; gloves, mask, and lab coat should be worn when handling it.

1. Feed mice regular chow until 6 days after injection of tumor cells. Replace regular chow with lathrogenic chow (placed in containers inside the cage) after 6 days.

There is no need to feed mice lathrogenic diet until the tumor starts to grow. Animals may spread the food around the cage, so the levels of food should be checked daily.

2. Keep mice on the diet for ≤ 3 weeks, at which time the tumor should be harvested.

After 2 weeks on the diet, mice should be monitored for bone fractures, which are observed with this diet. Mice in distress should be sacrificed and the tumors harvested.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Guanidine-HCl buffer, 2.0 M

191.0 g guanidine-HCl (2 M final)
12.1 g Tris base (0.05 M final)
H₂O to 1800 ml
Adjust pH to 7.4 with 2 M HCl
Add H₂O to 2 liters
Prepare fresh as needed

Lathrogenic chow

100 g β -aminopropionitrile (Sigma)
2 g iproniazid (Sigma)
0.2 g pargyline (Sigma)
5 kg standard ground chow

Dissolve the first three ingredients in 300 ml distilled water and mix with the ground chow for 20 min in a commercial mixer. Store 6 months at 4°C.

When adding the solution, sprinkle it on different areas of the chow to help the mixing process.

CAUTION: Gloves, mask, and lab coat should be worn when handling these toxic materials.

NaCl buffer, 0.5 M

7.5 g NaCl (0.5 M final)
6.05 g Tris base (0.05 M final)
H₂O to 900 ml
Adjust pH to 7.4 with 2 N HCl
Add H₂O to 1 liter
Store 6 months at room temperature

NaCl buffer, 3.4 M

397 g NaCl (3.4 M final)
3 g EDTA (0.005 M final)
5 g *N*-ethylmaleimide (NEM; 0.02 M final)
12.1 g Tris base (0.05 M final)
H₂O to 1800 ml
Adjust pH to 7.4 with 2 N HCl
Add H₂O to 2 liters
Prepare fresh as needed

Urea buffer, 2 M

240 g urea (2 M final)
12.1 g Tris base (0.05 M final)
H₂O to 1800 ml
Adjust pH to 7.4 with 2 N HCl
Add H₂O to 2 liters
Store 6 months at 4°C

COMMENTARY

Background Information

The EHS tumor produces an abundant basement membrane matrix (Orkin et al., 1977). Basement membranes are thin extracellular matrices that underlie epithelial and endothelial cells and surround muscle, fat, and nerve cells (Martin and Timpl, 1987; Kleinman et al., 1993). Preparation of basement membrane and its major components, laminin-1 and type IV collagen, from tissues is difficult because they are present in normal tissues only in small amounts and are poorly soluble. These components have many uses in cell biology research and are generally needed in large quantities. Preparation of these components from EHS tumors is advantageous because the components are highly soluble and type IV collagen cross-linking can be prevented by passaging the tumor in mice fed a lathrogenic diet. In the past, type IV collagen could be isolated only in degraded forms.

The EHS tumor is a spontaneous tumor that was propagated from a wild mouse in the 1940s. Its tissue of origin is not known. It was initially characterized as a chondrosarcoma because of the large amount of extracellular material observed by histology. In 1977, the EHS tumor was defined as a basement membrane tumor when type IV collagen was discovered to be a major component (Orkin et al., 1977). The availability of basement membrane components from this tumor has greatly accelerated research on the structure and function of the basement membrane. All of the components isolated from this tumor are chemically, structurally, and antigenically identical to authentic tissue basement membrane components (Beck et al., 1990; Yurchenco and Schittny, 1990; Timpl and Brown, 1994).

The laminin isolated from the EHS tumor is composed of three chains, designated $\alpha 1$, $\beta 1$, and $\gamma 1$, and is termed laminin-1 (Timpl et al., 1979; Burgeson et al., 1994). Five α , three β , and two γ chains of laminin have been described to date in various tissues. Eleven variant forms of laminin, composed of different combinations of these chains, have been described. Good tissue or tumor sources of these latter laminins have not yet been described. A fragment of laminin-2 (merosalin composed of $\alpha 2\beta 1\gamma 1$) is commercially available. Many of these laminins have tissue-specific locations and are expressed at specific times in these tissues.

Type IV collagen isolated from the EHS tumor is composed of two different chains,

present in a 2:1 ratio, with the formula $\alpha 1(IV)_2\alpha 2(IV)$ (Orkin et al., 1977; Kleinman et al., 1982). Additional chains of type IV collagen have also been identified, including $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\alpha 6$. Like laminin, these molecules are tissue specific in their localization, and good sources for their isolation have not yet been found.

The components of the EHS tumor are important because of their biological activities. Matrigel, a mixture of basement membrane components, promotes the differentiation of various cells and is also active with tissue explants (Carey et al., 1986; Kleinman et al., 1986). The response to Matrigel depends on the cell type. Endothelial cells form capillary-like structures when cultured on Matrigel, while mammary cells form cyst-like structures that show an 80-fold induction of casein (Li et al., 1986; Kubota et al., 1988). Explants of dorsal root ganglia extend long neuritic processes that are surrounded by Schwann cells producing myelin (Carey et al., 1986).

Matrigel can be used to coat culture dishes, with cells plated directly onto the gelled material. Cells can also be mixed with the Matrigel to form a suspension when warmed (Hadley et al., 1985; UNIT 10.3). In vivo, Matrigel has been used to assess angiogenesis (Passaniti et al., 1992) and to increase the incidence of tumor take and the rate of growth (Fridman et al., 1991). Matrigel has also been used to coat filters in migration assay chambers to assess tumor cell invasion (Albini et al., 1987; UNIT 12.2). Laminin-1 has been used mainly as a culture substratum. It promotes cell adhesion, growth, migration, and neurite outgrowth (Evercooren et al., 1982; Kleinman et al., 1993). It also reduces fibroblast growth. Type IV collagen promotes the adhesion and growth of many cells and is also used to coat filters in migration assays.

Critical Parameters and Troubleshooting

EHS tumor. A major problem with propagating EHS tumors is bacterial infection. Infection is easy to recognize as the tumors become watery, yellow in areas, and full of pus. Infected tumors should be discarded and not used for passage. Infection can be minimized by using antibiotics and by keeping all of the tumors separate when passaging. It is also important not to let the tumors become too large, as necrosis will reduce the yield of usable mate-

rial. Generally tumors >4 g are necrotic. For preparation of matrix components, tumors can be stored frozen at -70°C for ≤ 1 year and then thawed as described in the protocols.

Matrigel. Matrigel should be prepared as quickly as possible, as activity is lost if the preparation is left too long at any of the steps. The entire isolation and dialysis can be completed in ≤ 4 days. It is also critical that Matrigel be kept cold at all stages of the preparation. Once isolated, it is important to keep Matrigel at 4°C , because the material cannot be recovered in the liquid form after it has gelled. Using cold pipets and syringes helps to keep Matrigel liquid. The Matrigel protein concentration should not be < 9 mg/ml; at lower protein concentrations, Matrigel will not form solid gels. Diluted Matrigel can be recovered by 40% ammonium sulfate precipitation, followed by suspension in a smaller volume and dialysis.

Laminin-1. It is safest not to throw anything away until the laminin-1 preparation is complete. It is also very important to keep all solutions cold during the preparation of laminin-1.

Type IV collagen. A major problem with the collagen preparation is having too much type IV collagen in the extraction buffer. The collagen will precipitate out of solution at concentrations > 0.8 mg/ml when dialyzed against acetic acid in the final step, and the precipitated material is difficult to recover. The yield of collagen can vary, depending on how lathyritic the animals are; therefore the final dialysis step should be performed first on a small batch to be sure that the collagen will stay in solution. Type IV collagen is best stored in acetic acid. For culture, type IV collagen is used as a dried substratum. This generally requires overnight drying in a sterile hood.

Anticipated Results

In general, 1 g of tumor tissue yields ~ 1.5 ml of Matrigel or 10 mg of laminin-1 and 5 mg of type IV collagen.

Time Considerations

EHS tumors generally reach ~ 4 g and can be harvested 3 weeks after injection of the cells into mice. It is important to begin monitoring the health of the animals at 2 weeks. It may be necessary to sacrifice the animals at slightly different times to maximize the yield. The preparations of Matrigel, laminin-1, and type IV collagen should all be performed in ≤ 1 week. Arresting the preparation at intermediate stages can result in some degradation.

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Preparation of Gelled Substrates

This unit describes the use of type I collagen as a gelled substrate for cell culture, as well as various ways to handle and manipulate the basement membrane matrix preparation Matrigel for use in cell culture and *in vivo*. Both matrix components form gels at high concentrations under physiological conditions. Type I collagen gels have been used mainly to promote cell growth and differentiation. Most cells in culture proliferate at a high rate and lose their differentiated phenotype, but do not proliferate when cultured on Matrigel. Matrigel is advantageous as a culture substratum because it promotes the differentiation of a variety of epithelial and endothelial cells; the response of the cells depends on the cell type (see Table 10.3.1). Differentiated cells *in vitro* have a variety of uses, including determining how genes are regulated and which factors control differentiation. Matrigel has been used *in vivo* to assess angiogenesis, increase tumor growth, and promote the survival of explanted cells and tissues.

The basic protocols outline the most common uses of type I collagen (see Basic Protocol 1) and Matrigel (see Basic Protocol 2) as gelled substrates. Gelled type I collagen can also be used as a floating substrate. Dried type I collagen substrates have been used in cell culture. Additional protocols describe how to grow cells inside Matrigel (see Alternate Protocol 1) and how Matrigel can be used *in vivo* to assess angiogenesis and to initiate and increase tumor growth (see Alternate Protocol 2).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

PREPARATION OF TYPE I COLLAGEN SUBSTRATES

Gelled collagen substrates should be prepared on the same day that they will be used for cell culture. Gelled type I collagen substrates are used for a variety of cell types, including hepatocytes and endothelial cells. Dried collagen substrates can be prepared the day before use and are often used to culture skeletal muscle cells. Type I collagen substrates allow for better growth, survival, and differentiation of various cell types, including skeletal muscle and hepatocytes, than does plastic or glass alone.

Materials

- 3 to 5 mg/ml type I collagen in dilute acid (e.g., Vitrogen 100, Collagen), ice cold
- 10× phosphate-buffered saline, pH 7.4 (10× PBS; APPENDIX 2A) or 10× medium salts (e.g., Medium 199, Life Technologies), ice cold
- 0.1 M NaOH, ice cold
- 0.1 M HCl, ice cold
- Dilute acetic acid: 28.5 ml glacial acetic acid/liter
- Sterile deionized H₂O
- Cells
- Tissue culture medium, 37°C
- Wet ice in a bucket
- Culture dishes

Preparing gelled type I collagen substrates

- 1a. Using prechilled solutions and keeping solutions on ice, mix 8 ml type I collagen with 1 ml of 10× PBS, pH 7.4, and 1 ml of 0.1 M NaOH (all solutions should be prechilled). Adjust pH to 7.4 with additional drops of 0.1 M NaOH or 0.1 M HCl as needed, using pH test paper or a pH meter to assess pH, and using aseptic technique.

BASIC PROTOCOL 1

Type I collagen commercially obtained at 3 to 5 mg/ml in dilute acid should not be further diluted more than necessary or the final gel will be too weak. Below 3 mg/ml, the gel will be very weak and will not withstand the usual manipulations of plating and feeding the cells. Type I collagen obtained as a powder can be solubilized at 3 to 5 mg/ml by stirring overnight at 4°C in dilute acetic acid.

Alternatively, 10× medium salts can be used in place of the PBS.

When a dried film of fibrillar collagen is desired, coat the dish with a small amount of collagen and air dry the material, usually overnight, in a sterile culture hood. Skeletal muscle cells are cultured on dried collagen to encourage cell differentiation (i.e., fusion to myotubes).

- 2a. Pipet the desired amount of collagen onto the culture dish and warm the dish at least 1 hr in a 37°C incubator.

The collagen should completely cover the surface of the dish; 1 ml is generally sufficient for a 35-mm dish. The matrix usually turns white when it gels.

CO₂ is not important for gelling of the collagen, but subsequent incubations with cells should be performed in a humidified 37°C, 5% CO₂ incubator.

- 3a. Before use, rinse dried gels and collagen films with sterile distilled water to remove the salts. Add cells and medium as usual and culture.

Warm all solutions and gently pipet onto the culture dish.

In some cases, one may want to grow cells inside a type I collagen gel. This can be done by overlaying the collagen after the cells have attached, or by mixing the cells in the liquid type I collagen prior to gelation (see Alternate Protocol 1).

Floating collagen gels can be obtained by gently agitating around the edge of the collagen gel with a spatula after the cells have attached. When type I collagen gels are used as floating gels with fibroblasts attached, some contraction of the gel will occur. Hepatocytes have also been successfully cultured on floating collagen gels.

Preparing nongelled type I collagen substrates

- 1b. Dilute 3 to 5 mg/ml collagen solution to 0.1 mg/ml with either dilute acetic acid or 0.1 M HCl.

When directly drying collagen on tissue culture dishes without gelation, usually much less material is needed than for gelled substrates. Generally 10 to 20 µg collagen provides good activity on 16-mm-diameter-well (24-well) dishes.

The collagen can be diluted with 0.1 M HCl as an alternative to acetic acid.

- 2b. Plate the desired amount of 0.1 mg/ml collagen in acetic acid onto the culture dish. Allow to air dry overnight.

For example, 200 µl of a 0.1 mg/ml collagen solution can be used for each 16-mm-diameter well of a 24-well dish; the solution should completely cover the bottom of the well.

- 3b. Before use, rinse collagen films with sterile distilled water (to remove salts). Add cells and medium as usual and culture.

Warm all solutions and pipet gently onto the culture dish.

PREPARATION OF GELLED MATRIGEL SUBSTRATES

Matrigel is a crude basement membrane preparation that provides an excellent substrate for the differentiation of a variety of cells (see *UNIT 10.2*). Epithelial, endothelial, smooth muscle, fat, and Schwann cells are in contact with a basement membrane in vivo. These cells do not differentiate well on tissue culture plastic, but show high degrees of differentiation on Matrigel in vitro.

Materials

Matrigel (*UNIT 10.2*; Matrigel can also be obtained commercially from Sigma or Becton Dickinson Labware)
Medium salts, 4°C (optional, for making weak gels)
Cells
Tissue culture medium, 37°C
Wet ice in a bucket
Culture dishes

1. Thaw Matrigel at 4°C.

Thawing can be accomplished by leaving the Matrigel in the refrigerator on ice overnight or by rolling the bottle in the hands until it is thawed. Do not allow the solution to become warm, as it will gel and cannot be recovered. It is not desirable to thaw and refreeze Matrigel too many times, so it should be stored in appropriate-sized aliquots based on the quantities generally needed.

It is best to always have fresh wet ice in a bucket when working with Matrigel to keep it cold.

2. In a sterile hood, pipet desired amount of Matrigel onto culture dish.

The Matrigel should be at ≥ 9 mg/ml for maximum gelling. Concentrations below that will form weak gels that may fall apart. Matrigel can be diluted to 4 mg/ml and will still gel, but the gel will be weak. In some cases, however, a weak gel is needed. Dilute the stock Matrigel with cold medium salts and mix well.

If there are bubbles in the Matrigel, which can cause problems once it gels, let stand for a few minutes on ice to allow them to dissipate. Keeping the dish level, quickly pipet the Matrigel into the dishes; when performing multiple pipetting steps, do these quickly, and change the pipets if the Matrigel starts to gel inside them. For 16-mm-diameter wells, use 320 μ l per well (this volume is necessary because a large meniscus forms at the edge of the well and distorts the surface). For larger dishes, a thinner coating works well, but one must be aware of the curve in the gel at the edges of the dish. For ≥ 100 -mm-diameter dishes, pipet the Matrigel so that the bottom of the dish is covered, then pour off the excess and use it for another dish.

3. Cover the culture dish and incubate 30 to 60 min at 37°C to allow gelling of the Matrigel.

Since Matrigel will gel at room temperature, place dishes in incubator immediately.

4. Gently add warm medium and cells to the culture dish as for normal culture. Return the plate to the incubator as usual and check the culture often for changes in cell behavior. Change the medium as usual, taking care not to disturb the gel layer.

For example, for in vitro assays of angiogenesis (tube formation), incubate 40,000 endothelial cells in 100 μ l complete medium in 16-mm-diameter wells containing 320 μ l gelled Matrigel. Fix the culture and stain with Diff-Quik (Baxter) using only the fix and solution II (use of solution I will stain the Matrigel and make it difficult to distinguish the cells). The assay can be quantified with an image processor (e.g., NIH Image).

GROWTH OF CELLS INSIDE MATRIGEL

Various cells may differentiate to different extents depending on whether the cells are plated on top of a Matrigel substrate or inside of Matrigel. For example, Sertoli cells are highly columnar on a Matrigel substrate but form cord-like structures when grown inside of Matrigel.

Materials

Matrigel (UNIT 10.2; Matrigel can also be obtained commercially from Sigma or Becton Dickinson Labware)

Cells

Tissue culture medium, 37°C

Wet ice in a bucket

Culture dishes

1. Thaw Matrigel at 4°C.

Thawing can be accomplished by leaving the Matrigel in the refrigerator on ice overnight or by rolling the bottle in the hands until it is thawed. Do not allow the solution to become warm, as it will gel and cannot be recovered. It is not desirable to thaw and refreeze Matrigel too many times, so it should be stored in appropriate-sized aliquots based on the quantities generally needed.

It is best to always have fresh wet ice in a bucket when working with Matrigel to keep it cold.

2. Prepare the cells as usual for cell culture. Centrifuge 5 min at 170 × g, room temperature, to collect cells. Decant supernatant, tap pellet to disperse cells, and put tube of cells in wet ice. Add desired amount of thawed Matrigel, mix well, and pipet onto culture dish.

Take care to fully mix the cells into the Matrigel without causing bubbles. This can be accomplished by gently tipping the tube 180° several times. Approximately 320 μl of Matrigel in a 16-mm-diameter well works well with endothelial cells.

The number of cells and volume of Matrigel needed for cell differentiation vary among cell types. It may be necessary to test various numbers of cells in a constant volume of Matrigel. A good range to test initially is 10,000 to 100,000 cells per 320 μl.

The cells will be trapped inside the Matrigel when it gels. Previous studies have found that some cells will migrate towards each other and form differentiated structures. Histological sectioning and/or electron microscopy is necessary to fully observe these structures.

3. Cover the dish and incubate 30 to 60 min at 37°C to allow gelling of the Matrigel.

Since Matrigel will gel at room temperature, place dishes in incubator immediately.

4. Gently add warm medium to the culture dish as for normal culture. Return the plate to the incubator as usual and check the culture often for changes in cell behavior. Change the medium as usual, taking care not to disturb the gel.

USE OF MATRIGEL IN VIVO FOR ANGIOGENIC ASSAYS AND TUMOR GROWTH

Matrigel implanted subcutaneously in an animal remains as a plug with very few cells invading for several months. If certain angiogenic factors are mixed with the Matrigel before injection, however, considerable endothelial cell invasion into the plugs is observed within 1 week. This migration of endothelial cells and subsequent formation of vessels can be quantified and used as an assay for measuring the activity of angiogenic and antiangiogenic compounds.

If tumor cells or minced pieces of biopsy material are premixed in the cold with liquid Matrigel and injected, an increase in the incidence of tumor take and growth can be observed. This technique is highly useful for tumors that are hard to grow and for human biopsy specimens.

Materials

Matrigel (*UNIT 10.2*; Matrigel can also be obtained commercially from Sigma or Becton Dickinson Labware)

C57BL6 mice for angiogenesis assay *or* athymic nude mice for human tumor growth

Test compound for angiogenesis assay *or* tumor sample

Proteases (e.g., trypsin or collagenase) for dissociating tumor pieces, if necessary
Hemoglobin assay kit (e.g., Drabkin Reagent Kit, Sigma) or additional materials and equipment for histology, including image processor (e.g., NIH Image)

Wet ice in a bucket

Culture dishes

1- to 3-ml syringes

23- or 25-G needles

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Care and Use Committee (IACUL) and must follow officially approved procedures for the care and use of laboratory animals.

1. Thaw Matrigel at 4°C.

Thawing can be accomplished by leaving the Matrigel on ice in the refrigerator overnight or by rolling the bottle in the hands until it is thawed. Do not allow the solution to become warm, as it will gel and cannot be recovered. It is not desirable to thaw and refreeze Matrigel too many times, so it should be stored in appropriate-sized aliquots based on the quantities generally needed.

It is best to always have fresh wet ice in a bucket when working with Matrigel to keep it cold.

2. Mix Matrigel on ice with the test substance for an angiogenesis assay or with cells or dispersed tumor pieces for the subcutaneous growth of tumors.

See Alternate Protocol 1, step 2, for details of mixing cells and Matrigel. The final injected volume should not exceed 1 ml. It is best not to reduce the concentration of Matrigel below 9 mg/ml.

Angiogenic factors for testing and tumor cells/pieces should be suspended in as small a volume as possible. Basic fibroblast growth factor at 1 to 5 ng/ml should be included with Matrigel in some plugs as a positive control.

If using tumor biopsy pieces, the material must be finely minced or dissociated with proteases, including trypsin or collagenase, so that it will pass through the syringe.

3. Cool a 1- to 3-ml syringe fitted with a 23- or 25-G needle by placing it in a beaker on ice. Load the syringe with 1 ml of the Matrigel/test compound or Matrigel/tumor cell mixture. For the angiogenesis assay, hold each mouse firmly in one hand and inject the material subcutaneously on the lower ventral side near one or both of the lower legs. For tumor-growth studies, restrain mice in a box and inject material subcutaneously in the upper dorsal area. After injecting, hold the needle in place for ~30 sec until the Matrigel starts to gel. Pull the needle out slowly and turn it to minimize leakage of Matrigel out of the injection site (some leakage may occur anyway).

Female mice are easier to handle than male mice. Mice do not need to be anesthetized.

If doing multiple animals, it may be necessary to change needles often, as the Matrigel will clog the needle.

- 4a. *Subcutaneous angiogenesis assay*: Sacrifice mice after 1 week. Gently cut the skin away and excise the Matrigel pellet, which will be clearly visible attached either to the undersurface of the skin or to the underlying muscle layer. Measure angiogenesis either using a hemoglobin assay kit (if there is an obvious abundance of vessels) or by histology.

For histology, fix the plugs, stain them with Masson trichrome, and quantify angiogenesis using an image processor by counting the number of vessels and/or measuring the area occupied.

- 4b. *Tumor growth*: Tumors are usually observable by 2 weeks but some cells, such as NIH 3T3, require >2 months.

COMMENTARY

Background Information

Most cells in tissues are in contact with an extracellular matrix. The use of collagen substrates in vitro enhances the growth and differentiation of many cell types over that observed with glass or plastic substrates (Elsdale and Bard, 1972; Emerman et al., 1979; Kleinman et al., 1981). Cells adhere directly to collagen through integrin receptors or via adhesion proteins such as fibronectin, which bind to both the cells (via integrin receptors) and the collagen. Collagen substrates are particularly useful for the differentiation of hepatocytes and breast epithelial cells. Dried collagen substrates have been used successfully for the culture of muscle cells (Hauschka and Konigsberg, 1966).

The important role of the basement membrane in cell differentiation was not realized until the crude basement membrane preparation known as Matrigel was developed and became widely used as a cell growth substrate (Kleinman et al., 1986, 1987). Most untransformed cells in culture form a monolayer and proliferate until confluence is reached. Cells cultured on Matrigel differentiate and their proliferation is arrested (Table 10.3.1). The cellular response depends on the cell type. For example, endothelial cells form capillary-like structures with a lumen, and melanoma cells produce melanin (Kleinman et al., 1986; Kubota et al., 1988). Salivary gland cells form acini (Hoffman et al., 1996) and bone cells form canaliculi (Vukicevic et al., 1990). Explanted neural tissue, such as dorsal root ganglion, shows extensive neurite outgrowth and myelin production (Carey et al., 1986). Another advantage of using Matrigel in culture is that fibroblasts (which can overgrow many primary cultures) do not grow well in its presence.

Attempts have been made to use whole natural basement membranes, such as the lens capsule or amnion, for cell culture and differentia-

tion studies, with limited success. Lack of reproducibility and difficulty in handling the natural materials have restricted their use. Type I collagen (the most abundant collagen, comprising some 30% of the dry weight of vertebrates) at 3 to 5 mg/ml gels when it is brought to neutral pH and warmed. Type I collagen gels have been used as culture substrata, but most cells do not differentiate as well on collagen as they do on Matrigel.

Matrigel can be used with the cells either plated on top of the gel or entrapped inside it (Hadley et al., 1985). Although limited data are available, there is evidence that cells behave differently under the two culture conditions. For example, Sertoli cells remain as a monolayer on plastic, but become 15-fold more columnar with polarized nuclei when plated on Matrigel. When cultured inside Matrigel, Sertoli cells migrate into cord-like structures that resemble an immature testis with polarized nuclei. In some cases, the amount and type of growth factors in the Matrigel have been altered either by their removal, antibody binding, or exogenous addition (Taub et al., 1990; Vukicevic et al., 1992). Manipulation of growth factor levels greatly affects differentiation for both kidney cells and bone cells. Other cell types have not been fully evaluated for the effects of growth factors and Matrigel.

The differentiation of cells in culture has provided some useful assay models. For example, although tube formation by endothelial cells on Matrigel does not mimic all of the steps in angiogenesis, tube formation has been used as an assay to define angiogenic and antiangiogenic agents. For example, the sera of vasculitis patients contain angiogenic activity as determined by the tube assay (Cid et al., 1993). Purification of the causative factor, haptoglobin, was monitored using the tube assay, and

Table 10.3.1 Effect of Matrigel on Cell and Explant Differentiation

Cell/explant	Biological effect	Reference
Bone cells	Formation of canaliculi	Vukicevic et al., 1990
Dorsal root ganglion	Outgrowth and myelination	Carey et al., 1986
Endothelial cells	Formation of capillary-like structures with a lumen	Kubota et al., 1988
Hair follicle	Shaft formation	S. Yuspa and N. Martinet, pers. comm.
Hepatocytes	Maintenance of albumin synthesis and cytochrome P ₄₅₀	Ben-Ze'ev et al., 1988
Mammary cells	Formation of ducts and lumina, increased casein production	Li et al., 1986
Melanoma cells	Rapid pigmentation	Kleinman et al., 1986
Notochord	Outgrowth	Bilozur and Hay, 1988
Olfactory neuroblast	Odorant responsiveness	Coon et al., 1989
Oviduct cells	Formation of tubes with a lumen with polarized secretion	Joshi, 1991
Pancreatic acinar cells	Acinar formation with secretory vesicles near lumen	Bendayan et al., 1986
Salivary gland cells	Acinar formation	Hoffman et al., 1996
Sertoli cells	15-fold columnar form, germ cell survival and differentiation	Hadley et al., 1985
Thyroid cells	Thyroglobulin production	Greenberg and Hay, 1986
Type II pneumocytes	Cuboidal cells, phosphatidylcholine synthesis	Blau et al., 1988

the activity was subsequently confirmed in several *in vivo* assays.

Critical Parameters and Troubleshooting

Matrigel and type I collagen must be maintained at 4°C when being manipulated. For long-term culture of >1 week, denser substrates may be needed, as the gels may begin to break apart over time in culture with certain types of cells. It is usually not desirable to significantly reduce the protein concentration of Matrigel. When testing new cell types, it may be necessary to try the cells on all the possible substrates (collagen gels, dried collagen films, on Matrigel, inside Matrigel) to determine the best configuration. Do not become discouraged if cells do not have a defined appearance before histological sectioning. Histology often reveals polarity and other indications of differentiation.

Anticipated Results

Histological analysis is recommended to assess the morphological differentiation of most cells, because the three-dimensional nature of the response is difficult to observe using a standard microscope. In some cases, however, the appearance of the cells *in vitro* may be obvious. Defined structures are observed for some cell type/substrate combinations, such as hepatocytes on type I collagen and endothelial cells on Matrigel; the latter form capillary-like structures with a lumen (Kubota et al., 1988). Normal cells will not continue to grow when cultured on Matrigel but rather will differentiate. The growth rate of tumors, on the other hand, generally increases 3- to 6-fold on Matrigel (Fridman et al., 1991). For tumor cells, the incidence of tumor take is increased several-fold: ~5% of most tumors grow in the absence of Matrigel and >50% grow in the presence of Matrigel.

Time Considerations

Type I collagen and Matrigel form gels within 30 min in the culture dish, but it is recommended that the dishes be kept at 37°C for a full hour before warm medium is added. The time required for differentiation to occur in vitro on Matrigel varies depending on the cell type. Endothelial cells require 18 hr to form capillary-like structures and should not be left more than 24 hr (Kubota et al., 1988). In contrast, salivary gland cells require several days to form acinar-like structures (Hoffman et al., 1996). The in vivo angiogenesis assay also shows some time variation depending on the factors being tested. Fibroblast growth factor is generally fully active by 5 days; other factors require more time (Passaniti et al., 1992). Generally the assay is quantified 7 days after injection.

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Preparation of Extracellular Matrices Produced by Cultured Corneal Endothelial and PF-HR9 Endodermal Cells

Cell behavior is regulated by the substratum upon which cells attach, migrate, and proliferate. Tissue culture surfaces are therefore coated with extracellular matrix (ECM) extracts or with single ECM components in order to better resemble the microenvironment of cells *in vivo* and promote cell adhesion, proliferation, and expression of differentiated functions. This unit outlines the coating of tissue culture surfaces with a natural ECM produced and deposited by cultured bovine corneal endothelial (BCE) cells or PF-HR9 mouse endodermal cells. BCE-ECM closely resembles the ECM of subendothelium *in vivo*, whereas ECM from PF-HR9 cells more closely resembles the composition of basement membranes of other epithelia *in vivo* (see Commentary).

The basic approach is to allow the cells to produce their own ECM, while avoiding extraction procedures and purification of single components that may alter the relative proportions of native ECM constituents, alter the ECM's supramolecular organization, and/or denature its active molecules. For this purpose, the ECM-producing cells are maintained in culture at a confluent cell density. Five to twelve days after seeding, cellular elements are removed by solubilization of the cell layer with Triton X-100 and NH_4OH , leaving the underlying ECM intact, free of cellular debris, and firmly attached to the entire area of the culture surface. The ECM is then washed and covered with PBS and stored at 4°C . Under these conditions there is no loss of biological activity (i.e., induction of cell attachment, proliferation, and differentiation) for up to 6 months.

The Basic Protocol describes the preparation of BCE-ECM-coated dishes. Alternate Protocols describe modifications for preparation of metabolically radiolabeled ECM (which can be used to study matrix degradation associated with cell invasion and metastasis) and ECM derived from PF-HR9 cells (HR9-ECM). To evaluate the quality of the ECM, Support Protocols present straightforward procedures for assessing the induction of cell attachment, cell proliferation, and cell differentiation by the ECM.

NOTE: All culture incubations should be performed in a humidified 37°C , 10% CO_2 incubator unless otherwise specified.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

PREPARATION OF BOVINE CORNEAL ENDOTHELIAL CELL ECM (BCE-ECM)

This protocol describes the initiation, establishment, and maintenance of stock primary and early-passage bovine corneal endothelial cell (BCE) cultures, as well as the preparation and storage of tissue culture surfaces coated with ECM produced by these cells. This ECM closely resembles that of the subendothelium *in vivo*, and is used to initiate primary cultures; to study the effects of local environment on cell adhesion, proliferation, differentiation, and survival; and to study various aspects of cell motility. Major constituents include collagens, proteoglycans, laminin, fibronectin, entactin, and elastin (see Background Information).

Although this procedure can be modified to coat any type of tissue culture dish, the best ECM-coated dishes (i.e., those with a homogeneous matrix that coats the entire surface of the dish) are obtained by plating the cells at a high density in 35-mm dishes. Thus, the strategy is to have the main extent of cell growth in 10-cm dishes (the primary stock

BASIC PROTOCOL

Extracellular Matrix

10.4.1

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cultures), followed by two to three population doublings in 35-mm dishes (the early-passage cultures, yielding ECM-coated dishes).

Materials

10 to 20 eyes of freshly slaughtered cows
95% (v/v) ethanol
PBS (*APPENDIX 2A*)
Supplemented low-glucose DMEM-10 (see recipe)
Recombinant human basic fibroblast growth factor (bFGF; Sigma)
Trypsin/EDTA solution (see recipe)
Dextran T40 (Sigma)
Triton/NH₄OH cell lysis solution (see recipe)
Gentamicin (Life Technologies)
Fungizone (amphotericin B; Life Technologies)

18-G needles
10-cm, 6-cm, and 35-mm tissue culture dishes
Groove director (curette or spoon-headed spatula), sterile
37°C, 10% (v/v) CO₂ incubator with 100% humidity
10-cm gelatin-coated tissue culture dishes (see recipe)

Establish and maintain stock BCE cell cultures

1. Obtain 10 to 20 eyes of freshly sacrificed cows from a local slaughter house. Transport at 4°C or room temperature and process within 24 hr.

The eyes can be stored at 4°C.

2. Ensure that eyes have unscratched, thin, crystal-clear corneas. Wash eyes with 95% ethanol to sterilize the outside of the corneas and destroy the outer epithelial cell layer.
3. Puncture the edge of each cornea at the junction of the cornea and the sclera with an 18-G needle. Insert dissecting scissors into the hole, dissect out the cornea, and place it endothelial side up in a 10-cm tissue culture dish.
4. Wash one cornea gently with PBS to remove traces of iris, which appear as small black fragments and may have adhered to the endothelium during its dissection.
5. Gently scrape the endothelium with a sterile groove director, being careful not to apply pressure.

Applying pressure while scraping the endothelium may cause one to penetrate the Descemet's membrane and pick up stromal cells.

6. Transfer tissue fragments by dipping the groove director into a 6-cm tissue culture dish containing 5 ml supplemented low-glucose DMEM-10. Repeat steps 5 and 6 two to three times.

Visual examination of the plate should reveal small whitish tissue fragments floating in the medium. When examined under a phase-contrast microscope, the tissue fragments appear to be composed of closely apposed hexagonal cells organized into a honeycomb pattern.

7. Repeat steps 4 to 6 for all remaining corneas, preparing a separate primary culture for each one.
8. Incubate plates at 37°C in a 10% CO₂ incubator with 100% humidity. Leave them untouched for 5 days, except for occasional examination under a phase-contrast microscope.

After 3 to 5 days, several tissue fragments attach to the plastic, and cells can be seen migrating out of the explants.

9. On day 5 to 6, replace medium with fresh medium and start adding recombinant bFGF every other day at a final concentration of 1 ng/ml.
10. On day 8 to 12, aspirate medium from 6-cm primary culture dish, add 2 ml trypsin/EDTA solution, and incubate at 37°C for 5 min or until the cells have rounded up but are not detached.

By day 8 to 12, well-developed colonies are clearly visible and each 6-cm dish contains $\sim 1 \times 10^6$ cells.

One primary culture is selected for preparation of stock culture dishes. Other primary culture dishes can be processed in parallel or maintained in the incubator for up to 3 weeks.

11. Aspirate trypsin/EDTA solution and resuspend cells in 5 ml supplemented low-glucose DMEM-10.
12. Transfer the entire 5-ml cell suspension ($\sim 1 \times 10^6$ cells) into one regular or gelatin-coated 10-cm tissue culture dish containing 5 ml supplemented low-glucose DMEM-10. Add bFGF at 1 ng/ml every other day until the plate is nearly confluent ($5\text{--}7 \times 10^6$ cells/dish).

Gelatin-coated dishes are used, if necessary, to improve the plating efficiency of BCE cells.

13. On day 4 to 6 of the first subculture (at near confluency), aspirate medium, add 5 ml trypsin/EDTA solution, and incubate at 37°C for 5 min or until the cells have rounded up but are not detached.

It is best to passage the cells when subconfluent, because after reaching confluence the cells produce a large amount of ECM and hence become less susceptible to trypsinization and prone to damage in the process. This will be reflected in poor plating efficiency and cell growth, as well as in aberrant cell morphology.

14. Aspirate trypsin/EDTA solution and resuspend cells in 11 ml supplemented low-glucose DMEM-10.
15. Add 1 ml cell suspension to each of eleven 10-cm regular or gelatin-coated culture dishes, each containing 10 ml supplemented low-glucose DMEM-10 with 1 ng/ml bFGF. Add bFGF at 1 ng/ml every other day until the plates are nearly confluent ($5\text{--}7 \times 10^6$ cells/dish).

The surface area of a 10-cm stock dish is approximately equal to that of eight 35-mm dishes. Therefore, to prepare a batch of two hundred 35-mm dishes for production of ECM (see step 19), it is advisable to use the cells from stock dishes. A group of eleven 10-cm stock dishes can be used so that one dish is kept as a stock dish, and cells of the remaining ten 10-cm dishes are used to produce two hundred 35-mm ECM-coated dishes.

If ECM is produced on a routine basis, try to prepare primary cultures once a month. Maintain confluent BCE cultures for up to 3 weeks in the CO₂ incubator with no medium change. The cells remain viable and can be readily subcultured at any time. The cells can also be frozen in liquid nitrogen using 10% (v/v) dimethyl sulfoxide (DMSO) in supplemented low-glucose DMEM-10, but the results are often not as good as those obtained with freshly isolated cells.

Prepare ECM-coated 35-mm dishes

16. Prepare 500 ml supplemented low-glucose DMEM-10 with 4% (w/v) dextran T40 and 1 ng/ml bFGF.

Dextran T40 is added to the growth medium to increase its viscosity and hence the phagocytosis/exocytosis activity of the BCE. As a result, the amount (thickness) of ECM deposited is at least two-fold higher with dextran T40.

17. Trypsinize cells from ten 10-cm stock culture dishes by aspirating the medium, adding 5 ml trypsin/EDTA solution to each dish, and incubating at 37°C for 5 min or until the cells have rounded up but are not detached.

Best production of ECM in terms of thickness and homogeneity is obtained with secondary or early passage BCE. Do not use cells beyond their sixth passage in culture.
18. Aspirate trypsin/EDTA solution and resuspend the cells of each dish in 5 ml supplemented low-glucose DMEM-10. Pool the cell suspensions and add to 500 ml supplemented low-glucose DMEM-10 with 4% dextran and 1 ng/ml bFGF.
19. Distribute 2-ml aliquots ($\sim 2 \times 10^5$ cells) into each of two hundred 35-mm plastic tissue culture dishes. Add 1 ng/ml recombinant bFGF every other day until the cultures are confluent (ordinarily within 4 to 6 days).
20. Incubate cultures for an additional 5 to 8 days without medium change.

Remove cells

21. Warm Triton/NH₄OH cell lysis solution 10 to 20 min at 37°C. Aspirate the culture medium from each 35-mm culture dish and add 1 ml cell lysis solution.

In certain experiments, Triton X-100 and NH₄OH should be avoided (see Critical Parameters). In this case, cells are removed with 2 M urea in supplemented low-glucose DMEM-10, for 10 to 20 min at 37°C (Gospodarowicz et al., 1983).

22. Incubate dishes for 3 to 5 min with gentle shaking at room temperature and examine one of the dishes under a phase-contrast microscope.

Generally, complete cell lysis occurs within 2 to 3 min and the ECM remains firmly attached to the tissue culture plastic. If cell nuclei remain bound to the bottom of the dish, incubate for an additional 5 to 10 min at 37°C to obtain complete solubilization of the cell layer.

ECM produced by BCE cells appears as a delicate uniform network of amorphous material closely associated with the plastic. The homogeneous nature and thickness of the ECM lattice can be appreciated by scratching the plate with a needle, which may detach and lift the ECM membrane along the edge of the scratch. A more fibrillar, nonhomogenous type of ECM is less active and is often produced by late-passage cells.

Detached ECM can be used for purification or biochemical analysis; however, purification of a single component from bound or detached ECM is not straightforward.

23. Wash each plate four times with 2 ml PBS.
24. Cover ECM-coated plates with 2 ml PBS supplemented with 50 µg/ml gentamicin and 0.25 µg/ml Fungizone. Store up to 4 months at 4°C.

**ALTERNATE
PROTOCOL 1**

PREPARATION OF METABOLICALLY LABELED ECM

The procedure described in the Basic Protocol can also be applied to prepare radioactive, metabolically labeled ECM. Metabolically labeled ECM can be prepared by adding labeled sulfate (Na₂³⁵SO₄), amino acids ([³H]proline or [¹⁴C]glycine), or sugars (i.e., [¹⁴C]glucosamine) to the tissue culture medium. The labeled ECM synthesized in this manner can be utilized as a substrate to study degradation of specific ECM constituents by certain purified enzymes or invasive cells, such as metastatic tumor cells or activated cells of the immune system. For example, sulfate-labeled ECM-coated dishes are useful as a natural basement membrane-like substrate for measurement of heparanase activity expressed by highly metastatic tumor cells and activated cells of the immune system (Vlodavsky et al., 1983, 1992, 1995; Naparstek et al., 1984).

To prepare sulfate-labeled BCE-ECM, add 40 µCi Na₂³⁵SO₄ (540 to 590 mCi/mmol; Amersham) per 35-mm dish on days 2 and 5 after seeding the early-passage cultures (see

Basic Protocol, step 19), and incubate without medium change. Ten to twelve days after seeding, prepare ECM as described (see Basic Protocol, steps 21 to 24). To increase the specific activity of the labeled ECM, use Fischer's medium (which does not contain sulfate) rather than DMEM for the 35-mm ECM-producing cultures. Supplement Fischer's medium (Life Technologies) as for supplemented low-glucose DMEM-10 and add 4% (w/v) dextran and 1 ng/ml bFGF. Maintain stock cultures as described above (see Basic Protocol). About 80% of the ECM-associated sulfate radioactivity is incorporated into sulfated proteoglycans, primarily heparan sulfate proteoglycans.

PREPARATION OF HR9-ECM

Initiation of BCE cultures requires certain skills and is time consuming. It may, therefore, be more convenient to work with a permanent cell line capable of producing ECM. Such a cell line (PF-HR9) was derived from a differentiated mouse endodermal carcinoma (Chung et al., 1977) and can be obtained from ATCC or any of the many laboratories that are working with it (Kramer and Vogel, 1984; Rogelj et al., 1989). Unlike BCE cells, PF-HR9 cells are epithelial in nature and, as such, they produce ECM that better resembles epithelial basement membranes (rather than the subendothelial ECM produced by BCE). While the BCE-ECM provides an excellent model for basement membranes of blood vessels, the HR9-ECM provides a model for basement membranes underlying epithelial cells in a variety of organs and tissues. Major constituents of this ECM are laminin, entactin, collagen type IV, and heparan sulfate proteoglycans. Unlike the BCE-ECM, HR9-ECM contains little or no fibronectin, collagen type III, or dermatan sulfate proteoglycans, and no detectable bFGF (Gospodarowicz et al., 1984; Rogelj et al., 1989). It induces cell attachment and flattening, and promotes growth and differentiation of kidney tubule epithelial cells (Gospodarowicz et al., 1984), but fails to induce endothelial cell proliferation and neurite outgrowth in PC12 cells (Rogelj et al., 1989).

This procedure is described for 35-mm tissue culture dishes, but can easily be modified to coat other tissue culture plates and flasks. In fact, all available culture dishes and multiwell plates have been successfully coated with BCE-ECM and HR9-ECM.

Additional Materials (also see Basic Protocol)

PF-HR9 cells (ATCC; Chung et al., 1977; Kramer and Vogel, 1984)
Supplemented high-glucose DMEM-10 (see recipe)
Ascorbic acid (Sigma)
Penicillin
Streptomycin
Fibronectin-coated tissue culture dishes (see recipe)

1. Maintain stock cultures of PF-HR9 cells in supplemented high-glucose DMEM-10 in 10-cm tissue culture dishes at 37°C in a humidified, 10% CO₂ incubator. Dissociate stock cultures with trypsin/EDTA solution (5 min at 37°C) and subculture weekly at a 1:10 split ratio.

PF-HR9 cells can be frozen in liquid nitrogen in high-glucose DMEM containing 10% (v/v) FBS and 10% (v/v) DMSO.

2. Dissociate one 10-cm stock culture of PF-HR9 cells with trypsin/EDTA solution (5 min at 37°C), rinse cells, and resuspend in 100 ml supplemented high-glucose DMEM-10.

One stock culture provides enough cells for fifty 35-mm dishes.

3. Seed 2×10^5 cells per 35-mm fibronectin-coated tissue culture dish in supplemented high-glucose DMEM-10 with 4% (w/v) dextran T40.

ALTERNATE PROTOCOL 2

Extracellular Matrix

10.4.5

These dishes will be coated with HR9-ECM. Precoating with fibronectin enforces a firm adhesion of the HR9-ECM to the plastic substratum.

4. Incubate for 5 or 6 days, adding ascorbic acid at 50 µg/ml to the culture medium on days 2 and 4.
5. Warm Triton/NH₄OH cell lysis solution 10 to 20 min at 37°C. Aspirate the culture medium from each 35-mm culture dish and add 1 ml cell lysis solution.

In certain experiments, denudation with Triton X-100 and NH₄OH should be avoided (see Critical Parameters).

6. Incubate the dishes for 3 to 5 min with gentle shaking at room temperature and examine one of the dishes under a phase-contrast microscope.

Generally, complete cell lysis occurs within 2 to 3 min and the ECM remains firmly attached to the tissue culture plastic. If cell nuclei remain bound to the bottom of the dish, incubate for an additional 5 to 10 min at 37°C to obtain complete solubilization of the cell layer.

The appearance of the HR9-ECM is similar to that of BCE-ECM (see Basic Protocol, step 22) except that it often contains cellular debris and is slightly more fibrillar.

7. Aspirate the solubilized material and wash each plate four times with 2 ml PBS.

If needed, remove cellular debris by pipetting. If plates have not been precoated with fibronectin, the HR9-ECM may come off the plates during washing.

8. Add 1 ml PBS containing 50 µg/ml gentamicin and 0.25 µg/ml Fungizone to each 35-mm HR9-ECM plate. Store at 4°C for up to 4 months.

SUPPORT PROTOCOL 1

CELL ATTACHMENT ASSAY

A human colon carcinoma cell line, isolated from the liver and designated HS703T, is used to evaluate the cell adhesion-promoting activity of the ECM. The cells can be obtained from the Cell Culture Laboratory (Oakland, CA), under the auspices of the Office of Naval Research and the Regents of the University of California. HS703T cells have previously been shown to utilize laminin but not fibronectin in order to attach and spread (Vlodavsky and Gospodarowicz, 1981). On uncoated or fibronectin-coated tissue culture plastic, the cells grow as loosely attached aggregates of spheroid cells. In contrast, as early as 5 min after plating on ECM, the cells migrate out of the cell aggregates and become firmly attached and flattened (Vlodavsky et al., 1980; Vlodavsky and Gospodarowicz, 1981; Fig. 10.4.1). Attachment and spreading of HS703T human colon carcinoma cells therefore provide a rapid, convenient, and sensitive assay for the presence of a uniform ECM coating on plastic surfaces. This protocol can be used to assess BCE-ECM as well as HR9-ECM.

Materials

HS703T cells (Vlodavsky et al., 1980; Vlodavsky and Gospodarowicz, 1981)
Supplemented high-glucose DMEM-10 (see recipe)
Trypsin/EDTA solution (see recipe)
35-mm ECM-coated tissue culture dishes (see Basic Protocol; see Alternate Protocol 2)
PBS (APPENDIX 2A)
35-mm tissue culture dishes
Coulter counter

1. Maintain stock cultures of HS703T cells in supplemented high-glucose DMEM-10 in a humidified 37°C, 10% CO₂ incubator.

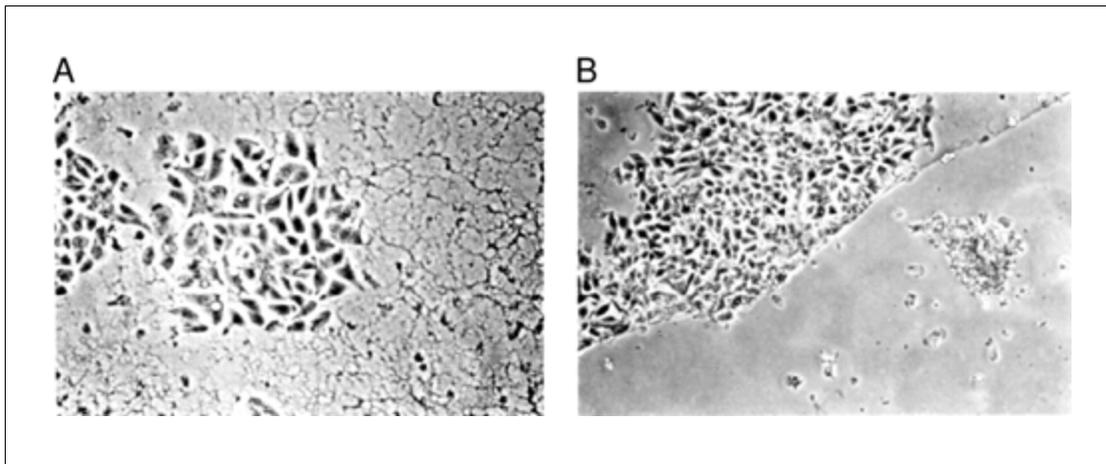


Figure 10.4.1 Adhesion and flattening of human colon carcinoma cells (HS703T) on BCE-ECM. (A) Intact ECM; (B) “wounded” ECM, where the ECM coating was scraped from part of the dish. Loosely attached aggregates of spheroid cells are seen on plastic (wounded ECM) as compared to firmly attached and flattened cells on ECM. Phase-contrast micrographs (180 \times) were taken 6 hr after seeding.

2. Dissociate cells with trypsin/EDTA solution (incubate 5 min at 37°C, then rinse with medium) or by repeated pipetting.
3. Seed 1×10^5 cells each onto duplicate 35-mm regular tissue culture dishes and duplicate ECM-coated tissue culture dishes.
4. At various times ranging from 5 to 60 min after seeding, remove unattached cells by gentle trituration and rinse the dishes twice with 2 ml PBS.
5. Dissociate remaining firmly attached cells with 1 ml trypsin/EDTA solution as above. Count the cells of duplicate dishes with a Coulter counter according to manufacturer’s instructions.

Alternatively, prelabel stock cultures with [3 H]thymidine (1 μ Ci/ml, 20 hr) and proceed as described above. Instead of counting cells, solubilize the firmly attached cells with 0.1 N NaOH (20 min, 37°C) and measure the radioactivity by liquid scintillation spectroscopy (Fridman et al., 1985).

CELL PROLIFERATION ASSAY

Bovine aortic vascular endothelial cells (BAEC; prepared according to Gospodarowicz, 1976) are applied to evaluate the growth-promoting activity of the BCE-ECM. When seeded in complete medium at a clonal cell density (300 cells/35-mm dish), the cells proliferate and form colonies on ECM (Fig. 10.4.2B), but not on regular tissue culture plastic (Fig. 10.4.2A; Gospodarowicz et al., 1980a,b; Rogelj et al., 1989). This protocol is not suitable for HR9-ECM.

Materials

- Aortic tissue from freshly sacrificed cows
- DMEM/BCS: supplemented low-glucose DMEM-10 (see recipe) in which FBS and newborn calf serum are replaced with 10% (v/v) bovine calf serum
- Trypsin/EDTA solution (see recipe)
- 35-mm regular and BCE-ECM-coated tissue culture dishes (see Basic Protocol)
- 3.7% formaldehyde (prepared from commercial 37% formalin)
- 0.125% (w/v) crystal violet solution (see recipe)

SUPPORT PROTOCOL 2

Extracellular Matrix

10.4.7

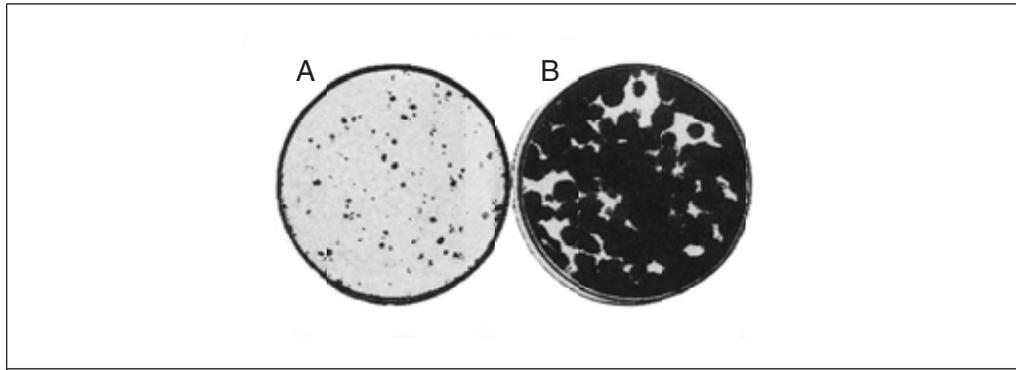


Figure 10.4.2 Clonal growth of bovine aortic endothelial cells on (A) plastic or (B) ECM. Cell colonies were fixed and stained with 0.125% crystal violet ten days after seeding (300 cells/35-mm dish).

1. Initiate clonal populations of bovine aortic endothelial cells (BAEC) from freshly obtained aortic tissue as described (Gospodarowicz et al., 1976), and maintain cells in DMEM/BCS in a humidified 37°C, 10% CO₂ incubator.
2. Dissociate a confluent stock culture into a single-cell suspension by incubating 5 min at 37°C with trypsin/EDTA solution. Rinse with medium.
3. Add 2 ml DMEM/BCS to duplicate 35-mm tissue culture dishes and duplicate BCE-ECM-coated tissue culture dishes, seed each with 300 cells, and incubate in CO₂ incubator.
4. At 10 to 12 days after seeding, aspirate medium and fix cells 1 hr at room temperature with 1 ml of 3.7% formaldehyde.
5. Stain cultures for 5 min with 1 ml of 0.125% crystal violet solution and rinse with tap water.

At least 100 cell colonies, each containing >70 closely apposed, polygonal cells, should be seen on ECM. In contrast, uncoated dishes should have no cell colonies or very small and lightly stained colonies composed of senescent cells.

SUPPORT PROTOCOL 3

CELL DIFFERENTIATION ASSAY

The ability of PC12 cells, originally established from a transplantable rat pheochromocytoma, to extend neurites is used to evaluate the differentiation-promoting activity of BCE-ECM (Vlodavsky et al., 1982; Rogelj et al., 1989). This protocol is not suitable for HR9-ECM.

Materials

PC12 cells (ATCC)
 Supplemented high-glucose DMEM-10 (see recipe)
 Trypsin/EDTA solution (see recipe)
 35-mm regular and BCE-ECM-coated tissue culture dishes (see Basic Protocol)

1. Maintain PC12 cells in supplemented high-glucose DMEM-10 in a humidified 37°C, 10% CO₂ incubator.

For PC12 cells, FBS in the medium should be heat inactivated (APPENDIX 2A).

2. Dissociate cells into a single-cell suspension by incubating 5 min at 37°C with trypsin/EDTA solution and rinse with medium.

3. Add 2 ml supplemented high-glucose DMEM-10 to each of the triplicate 35-mm tissue culture dishes triplicate ECM-coated tissue culture dishes, seed each with 1×10^5 cells, and incubate in a CO₂ incubator.
4. Examine cultures daily for neurite outgrowth.

Extension of neurites to at least five-fold the length of the cell body is seen within 2 to 4 days on ECM. In contrast, there is almost no outgrowth on regular tissue culture plastic.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Crystal violet solution, 0.125% (w/v)

Dissolve 2.5 g crystal violet in 100 ml methanol. Stir overnight and filter through Whatman no. 3 paper. Store up to 4 months at room temperature. Dilute 1/20 in H₂O immediately before use.

Fibronectin-coated tissue culture dishes

Prepare a 25 µg/ml solution of human plasma fibronectin (Sigma) in PBS (APPENDIX 2A). Add 1 ml per dish to 35-mm tissue culture dishes and incubate 1 hr at room temperature. Remove fibronectin solution and rinse once with PBS. Store covered with PBS up to 1 month at 4°C. Rinse again after storage.

Gelatin-coated tissue culture dishes

Prepare a 0.2% (w/v) gelatin solution in PBS (APPENDIX 2A). Autoclave the solution, cool, and filter through a 0.2-µm filter. Add 5 ml solution per dish to 10-cm tissue culture dishes, and incubate ≥3 hr at 4°C. Remove the gelatin solution and wash the dishes once with 10 ml PBS. Store covered with PBS up to 1 month at 4°C. Rinse again after storage.

Supplemented low-glucose DMEM-10

Dulbecco's modified Eagle medium (DMEM) containing 1 g/liter glucose, supplemented with:

- 10% (v/v) FBS (APPENDIX 2A)
 - 5% (v/v) newborn calf serum
 - 50 µg/ml gentamicin
 - 0.25 µg/ml Fungizone (amphotericin B; Life Technologies)
- Store up to 1 month at 4°C

Supplemented high-glucose DMEM-10

Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/liter glucose, supplemented with:

- 10% (v/v) FBS (APPENDIX 2A)
 - 50 U/ml penicillin
 - 50 µg/ml streptomycin
- Store up to 1 month at 4°C

Heat inactivation of FBS is only required for PC12 cells (see Support Protocol 3).

Triton/NH₄OH cell lysis solution

PBS (APPENDIX 2A) containing:

- 0.5% (v/v) Triton X-100
 - 20 mM NH₄OH
- Store up to 3 months at 4°C

Trypsin/EDTA solution

2.5 g trypsin
0.2 g EDTA
8 g NaCl
0.4 g KCl
1 g glucose
0.35 g NaHCO₃
0.01 g phenol red
H₂O to 1 liter
Store up to 3 months at -20°C

COMMENTARY

Background Information

The ECM is an organized complex of collagens, proteoglycans, and glycoproteins, all interacting to produce a highly stable structure upon which cells migrate, proliferate, and differentiate in vivo (Kleinman et al., 1981). Historically, ECM was regarded as a relatively inert scaffolding that stabilizes the physical structure of tissues. Subsequent studies indicated that the ability of cells to respond to various growth and differentiation factors is determined to a large extent by their shape and orientation, and that these are modulated by components of the ECM through interaction with specific transmembrane cell-surface integrin receptors (Gospodarowicz et al., 1978; Ingber, 1990; Hynes, 1992; Damsky and Werb, 1992). Studies with mammary gland epithelial cells led not only to the identification of genes that are dependent upon the ECM for their transcription, but also to the discovery of ECM response elements (Boudreau et al., 1995). It was also proposed that mechanical forces generated through cell-ECM interactions produce an altered cytoskeleton, altered nuclear morphology, and ultimately changes in the pattern of gene expression (Chen et al., 1997). Based on these and other observations, it is recognized that the ECM plays an active and complex role in regulating the morphogenesis of cells that contact it, influencing their development, migration, proliferation, survival, and metabolic functions (Meredith et al., 1993; Vlodavsky et al., 1993; Aharoni et al., 1997).

It appears that cellular responses to ECM are mediated by the combined action of basement membrane (BM) macromolecules (i.e., collagen IV, laminin, nidogen/entactin, proteoglycans) and active molecules (i.e., growth factors, enzymes) that are immobilized and stored in the ECM by means of binding to its macromolecular constituents, primarily to heparan sulfate proteoglycans (HSPG; Ruoslahti and

Yamaguchi, 1991; Vlodavsky et al., 1993). Heparan sulfate also contributes to the assembly and integrity of the ECM through binding to various ECM molecules, including the fibrillar interstitial collagens (types I, III, and V), fibronectin, laminin, thrombospondin, and tenascin.

Elucidating specific components of the ECM involved in controlling cell proliferation and differentiation either in vivo or in vitro is a difficult task, mostly due to its intricate nature. Because the correct in vitro reconstruction of the ECM from its isolated native components into the highly ordered structure that it represents in vivo would be a formidable task, advantage is taken of the fact that cultured bovine corneal endothelial cells (BCE) and PF-HR9 endodermal cells have the ability to produce, underneath their basal surface, a thick ECM that adheres strongly to plastic (Vlodavsky et al., 1980; Gospodarowicz et al., 1984). The ECM produced by cultured BCE cells closely resembles the subendothelium in vivo in its morphology and molecular composition. It contains primarily collagens (mostly types III and IV, with smaller amounts of types I and V), proteoglycans (mostly heparan sulfate and dermatan sulfate proteoglycans, with smaller amounts of chondroitin sulfate proteoglycans), laminin, fibronectin, entactin, and elastin. Vascular endothelial cells and other cell types plated in contact with the subendothelial ECM no longer require the addition of soluble basic fibroblast growth factor (bFGF) and/or other growth-promoting factors in order to proliferate and express their differentiated functions (Gospodarowicz et al., 1980a,b; Vlodavsky et al., 1982). Unlike cells maintained on plastic (e.g., vascular smooth muscle cells), cells cultured in contact with BCE-ECM resemble their in vivo counterparts and proliferate in response to plasma factors (Gospodarowicz and Ill, 1980).

PF-HR9 cells are epithelial in nature. As such, they produce ECM composed primarily of characteristic epithelial basement membrane components such as collagen type IV, heparan sulfate proteoglycans, laminin, and entactin (Timpl et al., 1979; Leivo et al., 1982). This ECM resembles epithelial basement membranes better than the subendothelial ECM produced by BCE. While the BCE-ECM provides an excellent model for basement membranes of blood vessels, the HR9-ECM provides a model for basement membranes underlying epithelial cells in a variety of organs and tissues. Unlike the BCE-ECM, HR9-ECM contains little or no fibronectin, collagen type III, dermatan sulfate proteoglycans, and no detectable bFGF (Rogelj et al., 1989).

Although many cell types are capable of producing ECM *in vitro*, the ECM is most often removed together with the cells when the cell layer is solubilized with Triton/NH₄OH. This is due primarily to secretion of ECM components by the cultured cells (i.e., fibroblasts, smooth muscle cells) both in between the cells and toward the bottom and top of the cell layer. In contrast, the corneal endothelial cells and, to a lesser degree, the PF-HR9 cells deposit their ECM in a polar fashion, almost exclusively underneath the cell layer. This enables a preferential removal of the cell layer while leaving the ECM bound to the tissue culture plastic. In particular, this property and mode of polar deposition is characteristic of BCE cells whose ECM is more firmly bound to the tissue culture dish than that produced by PF-HR9 cells. Thus, BCE-ECM can be washed extensively to efficiently remove cellular debris.

The polar secretion of ECM by BCE and the firm interaction of this ECM with the tissue culture plastic provide an appropriate system to obtain a naturally produced substrate, free of cellular elements (Vlodavsky et al., 1980; Gospodarowicz et al., 1980a,b). The presence of nuclei or cytoskeletal elements could not be detected on the denuded ECM when coated plates were examined by phase-contrast microscopy, scanning electron microscopy, or indirect immunofluorescence, using anti-actin and anti-vimentin antibodies, or the benzimidazole derivative Hoechst 33258 for nuclear staining (Gospodarowicz et al., 1983). Moreover, ECM prepared after a 24-hr exposure of subconfluent BCE cultures to [³H]thymidine was devoid of labeled material. Likewise, no labeled cell surface components remained associated with the ECM after solubilization (Triton/NH₄OH) of lactoperoxidase-iodinated

BCE that were plated on ECM for 24 hr. No serum proteins could be identified in the ECM (Gospodarowicz and Ill, 1980).

Unlike the BCE-ECM, cellular debris is always left adhering to the HR9-ECM. This is mostly due to the fact that part of the PF-HR9 cell population dies while proliferating. Cellular debris is therefore trapped in the ECM that is continuously produced by the living cells. Cellular debris adhering to the apical surface of the HR9-ECM are probably derived from dead cells that are present within the monolayer and that are not fully lysed when exposed to the Triton/NH₄OH solution.

The presence of heparan sulfate as a major glycosaminoglycan (GAG) in the ECM raised the possibility that ECM serves as a reservoir for heparin-binding growth factors that are tightly bound and stabilized by heparan sulfate in the ECM. Among these factors is bFGF, extracted from the subendothelial BCE-ECM produced *in vitro* (Vlodavsky et al., 1987) and from BM of the cornea *in vivo* (Folkman et al., 1988). bFGF was identified in a variety of endothelial and epithelial basement membranes *in vivo* (Cordon-Cardo et al., 1990; Vlodavsky et al., 1993). These and other results indicate that bFGF is an ECM component required for supporting cell proliferation, differentiation, and survival (Vlodavsky et al., 1991, 1993).

ECM-coated dishes are widely used for initiating primary cultures; for studying the control of cell adhesion, proliferation, differentiation, and survival by the local microenvironment (Vlodavsky et al., 1993); and for investigating cell migration, invasion, and metastatic behavior (Vlodavsky et al., 1983, 1992, 1995). ECM-coated dishes have been useful in restoring the normal phenotypic expression of a number of normal cell types that would otherwise have lost normal phenotypes when maintained on plastic (Gospodarowicz and Tauber, 1980; Gospodarowicz et al., 1980a,b). In the field of aging, cells maintained on ECM and exposed to serum-supplemented medium have a much longer life span in culture than cells maintained on plastic. This effect is attributed to cell survival signals that inhibit programmed cell death (Aharoni et al., 1997). ECM-coated dishes have also been useful in allowing the maintenance of a number of normal diploid cells and tumor cells under serum-free conditions, so that plasma factors involved in the control of their proliferation could be studied (Gospodarowicz and Ill, 1980; Gospodarowicz et al., 1980a,b, 1984). This property also facili-

tates the initiation of biopsy-derived epithelial cell cultures, while suppressing overgrowth by stromal fibroblasts (Biran et al., 1983). The BCE-ECM has been used in studies of nerve and glial cell differentiation (Vlodavsky et al., 1982; Lubetzki-Korn et al., 1983). The nerve cells can be either tumoral, such as the pheochromocytoma PC12 cell line, or normal sensory ganglion cells (Fujii et al., 1982; Vlodavsky et al., 1982). In either case, when cells are maintained on ECM, neurite outgrowth is initiated.

The HR9-ECM preferentially supports the proliferation and differentiation of epithelial cells. In the case of kidney tubule epithelial cells, it not only supports their growth but also promotes their differentiation into tubules that are indistinguishable from those seen in vivo (Gospodarowicz et al., 1984). HR9-ECM also allows the maintenance of kidney tubule epithelial cells under serum-free culture conditions; such cultures led to the identification of the two main plasma factors (transferrin and high-density lipoprotein) involved in supporting their growth (Gospodarowicz et al., 1984).

Critical Parameters

The goal of the described procedure is to obtain culture dishes entirely coated with a homogeneous, naturally produced, basement membrane-like matrix. The strategy is to have the main extent of cell growth in stock dishes and then seed the ECM-producing cells at a high density, allowing the ECM to be deposited over the course of two to three population doublings. The best production of ECM, in terms of thickness and homogeneity, is obtained with secondary or early-passage BCE. Cells beyond their sixth passage in culture should not be used. Also, 4% dextran T40 should be included in the growth medium in order to increase its viscosity and hence the phagocytotic/exocytotic activity of the BCE, which in turn increases the amount of ECM deposited. Unlike BCE-ECM, PF-HR9 cells produce little or no fibronectin. To enforce a firm adhesion of the HR9-ECM to the plastic substratum it is necessary to precoat the culture dishes with fibronectin. Even then, extensive washing of the HR9-ECM should be avoided, and cellular debris is often left adhering to this ECM. In contrast, no cellular debris remains associated with the BCE-ECM.

In certain experiments, denudation with Triton X-100 and NH_4OH should be avoided. For example, cell lysis should be avoided in experiments aimed to prove that bFGF is deposited

into the ECM prior to the actual denudation of the ECM, and that it is not sequestered by heparan sulfate in the ECM when the cells are lysed with Triton and NH_4OH . For this purpose, the ECM-producing cells are removed by a procedure involving little or no cell lysis. This can be best achieved by exposing the cell monolayer to 2 M urea in DMEM for 10 to 20 min at 37°C (Gospodarowicz et al., 1983). Under these conditions, the cells detach from each other, round up, and can be removed by pipetting as intact and viable cells, leaving the underlying ECM firmly bound to the tissue culture plastic (Vlodavsky et al., 1987, Gospodarowicz et al., 1983).

Anticipated Results

The described procedures enable the coating of any available tissue culture surface with intact, naturally produced ECM, similar in its molecular organization to subendothelial (BCE-ECM) and subepithelial (HR9-ECM) basement membranes in vivo. The basic approach is to allow cells to deposit their own ECM and avoid using extraction/enzymatic procedures that may alter or denature the native ECM constituent proportion and supramolecular organization.

One primary BCE culture maintained in a 6-cm dish can be subcultured into eleven 10-cm stock dishes, yielding enough cells to coat two hundred 35-mm tissue culture dishes. For PF-HR9 cells, one 10-cm stock culture provides enough cells to coat fifty 35-mm dishes.

Time Considerations

For BCE-ECM, it takes 8 to 12 days to obtain primary cultures, 8 to 10 days to get the appropriate stock cultures, and ~10 additional days for the actual deposition of ECM. A shorter time is needed for preparation of ECM deposited by the PF-HR9 teratocarcinoma-derived cell line. The HR9-ECM is obtained 5 to 6 days after seeding the cells, but unlike the BCE-ECM, it contains some cellular debris and lacks bFGF.

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Key References

Gospodarowicz et al., 1980a. See above.

See note for Vlodavsky et al. (1980).

Gospodarowicz et al., 1984. See above.

First article describing the effects of HR9-ECM on cell proliferation and differentiation.

Vlodavsky et al., 1980. See above.

This and Gospodarowicz et al. (1980a) are the first two articles describing the preparation, composition, and biological effects (induction of cell attachment, flattening, and proliferation) of BCE-ECM on normal and malignant cells.

Vlodavsky et al., 1993. See above.

Review article emphasizing the biological significance of ECM-resident bFGF and other active molecules.

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Purification of Fibronectin

UNIT 10.5

The adhesive glycoprotein fibronectin can be purified from body fluids, cultured cells, or tissues. Because of its relatively high fibronectin content, plasma is the most convenient source of this protein, which can be used to promote the adhesion, migration, and growth of virtually all cultured cells. The protocol presented in this unit for the purification of human plasma fibronectin (see Basic Protocol 1) uses immobilized gelatin (denatured collagen) to bind the protein. A pH 5.5 buffer is used to specifically elute the bound fibronectin without resorting to denaturing conditions. Because there are structural and functional differences between plasma and cell-derived fibronectins, purification protocols for the latter form are also given. Cellular fibronectin can be easily extracted from the surface of cultured cells (see Basic Protocol 2) and further purified if necessary (see Alternate Protocol 1) or harvested from cell-conditioned serum-free culture medium (see Alternate Protocol 2).

CAUTION: When working with human blood, cells, or infectious agents, appropriate biosafety practices must be followed. Even screened human plasma should be treated as potentially contaminated with infectious agents such as HIV or hepatitis viruses. Two layers of gloves, safety glasses or goggles, and suitable protective clothing should be worn at all times. Plasma should always be centrifuged in capped tubes or bottles. During thawing or warming, the plasma should be “double contained” (e.g., plastic bags of plasma should be contained in beakers before being placed in a water bath) in case of leakage. Any surfaces or skin on which plasma has been spilled or splashed should be disinfected with 10% sodium hypochlorite bleach or iodine-based disinfectant such as Wescodyne or Providone.

PURIFICATION OF PLASMA FIBRONECTIN

**BASIC
PROTOCOL 1**

This protocol describes the purification of fibronectin from surplus or outdated human plasma. It can also be used to purify fibronectin from a variety of other species, including bovine, chicken, rat, and mouse from either plasma or sera. The yield of fibronectin will be much lower from sera because the process of precipitating the fibrin clot out of plasma to produce serum can also remove as much as 60% of the fibronectin. Surplus or outdated human plasma can be most easily obtained from local blood banks, or it may be purchased commercially. This protocol as described assumes 1 liter of human plasma as the starting material and can be easily scaled up proportionally.

Materials

- 1000 ml (3 to 4 units) surplus or outdated human plasma
- Sepharose CL-4B (Amersham Pharmacia Biotech)
- Gelatin-Sepharose (Amersham Pharmacia Biotech)
- 6 M urea
- Tris-buffered saline (TBS): 0.9% (w/v) NaCl/10 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
- 0.2 M EDTA, pH 7.0
- 0.2 M PMSF (see recipe)
- ϵ -Amino-*n*-caproic acid
- Column buffers A and B (see recipes)
- Citrate elution buffer (see recipe)
- 0.5 M sodium phosphate, dibasic
- Ammonium sulfate, ultrapure (Life Technologies or Sigma)
- Dulbecco's PBS (*APPENDIX 2A*)
- CAPS/saline (optional; see recipe)

- 5-cm-diameter column, ≥ 5 cm tall, siliconized with Aquasil (Pierce)
- 5-cm-diameter column, ≥ 10 cm tall, siliconized with Aquasil

continued

**Extracellular
Matrix**

10.5.1

Contributed by Steven K. Akiyama

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Supplement 2

50-ml polycarbonate high-speed centrifuge tubes
250- or 500-ml centrifuge bottles
Dialysis tubing (12,000 to 14,000 MWCO; Spectrapor or equivalent)
High-speed refrigerated centrifuge with GS-3 and GSA rotors (Sorvall) or equivalent

Additional reagents and equipment for SDS-PAGE (UNIT 6.1)

Prepare columns

1. Pack a column (5 cm in diameter and ≥ 5 cm high) with 100 ml Sepharose CL-4B. Wash with at least 300 ml column buffer A as fast as possible by gravity flow.

This will be used as a precolumn to remove plasma components that bind nonspecifically to gelatin or Sepharose. The flow rate can be increased by using tubing connected to the bottom of the column to siphon down the column eluate more rapidly.

2. Pack a column (5 cm in diameter, 10 cm high) with 200 ml gelatin-Sepharose. Wash the gelatin-Sepharose with at least 800 ml of 6 M urea in TBS, then at least 800 ml of column buffer A.

Both of the preceding steps can be completed up to several days before the following steps.

Prepare plasma

3. Warm plasma to 37°C.

All subsequent steps are performed at room temperature, except where noted.

4. Add protease inhibitors to plasma with stirring:

25 ml 0.2 M EDTA (5 mM final)

5 ml 0.2 M PMSF (1 mM final)

6.6 g ϵ -amino-*n*-caproic acid (50 mM final).

5. Centrifuge 15 min at $10,000 \times g$, room temperature, and save the supernatant.

Use 250- to 500-ml bottles appropriate for a Sorvall GSA or GS-3 rotor or equivalent. The rotor and the centrifuge should be at room temperature prior to and during centrifugation.

Affinity purify plasma fibronectin

6. Apply the plasma to the precolumn as fast as possible, then immediately apply the flowthrough to the gelatin-Sepharose column at a flow rate of no more than ~ 500 ml/hr.

Treat the flowthrough fractions of the gelatin-Sepharose column as biohazardous waste and discard in a manner consistent with local and institutional regulations.

The precolumn removes plasma components that bind nonspecifically to the gelatin or Sepharose beads.

7. Wash the column as rapidly as possible with 800 ml column buffer B, then with 400 ml column buffer A.

8. Elute the fibronectin from the column with citrate elution buffer and collect 40-ml fractions. Check fractions for the presence of eluted fibronectin by spectrophotometry (measuring A_{280}). Pool the fibronectin-containing fractions, and immediately add 4 ml of 0.5 M dibasic sodium phosphate per fraction to neutralize the solution.

This is a possible stopping point. Neutralized fibronectin solutions can be stored up to several days at 4°C.

The yield of fibronectin can be increased by following the citrate buffer elution by a second elution with 4 M urea in TBS. The urea-eluted fibronectin will be of lower purity and usually comprises less than half the yield of the citrate-eluted fibronectin; however, it is still potentially useful, especially as starting material for the preparation of fibronectin frag-

ments. The urea should be removed immediately by dialysis before the fibronectin is concentrated by ammonium sulfate precipitation as described in step 9.

To save the gelatin-Sepharose column for reuse, wash thoroughly with TBS containing 0.1% sodium azide immediately after the urea elution and store it at 4°C. The column can be used repeatedly for many years if it is kept hydrated. Reused gelatin-Sepharose can actually provide higher yields of fibronectin than “new” gelatin-Sepharose because of irreversible occupancy of nonspecific binding sites on the beads

Concentrate fibronectin by precipitation (optional)

9. If higher concentrations of fibronectin than those eluted from the column are necessary, precipitate the fibronectin by slowly adding 0.291 g ammonium sulfate per milliliter (50% saturation) while stirring on ice until the ammonium sulfate is completely dissolved. Let the ammonium sulfate-treated fibronectin incubate on ice without stirring at least 2 hr.
10. Centrifuge 15 min at $10,000 \times g$, 4°C. Resuspend the pellet and dialyze for several days at 4°C with stirring in 3.5 liters of a suitable buffer to remove any residual ammonium sulfate. Replace the buffer each day with 3.5 liters fresh buffer.

A wide range of common buffers can be used to solubilize and dialyze the final plasma fibronectin product. It is generally easiest to use TBS or Dulbecco's PBS. It is possible to achieve fibronectin concentrations greater than ~10 mg/ml in most physiological buffers. If higher concentrations are needed, fibronectin is soluble at over 20 mg/ml in CAPS/saline.

If necessary, fibronectin can be sterilized by filtration, although yields will be low due to binding of fibronectin to the filter.

11. Determine the concentration of fibronectin.

This is most easily determined by measuring the A_{280} and using an extinction coefficient of $1.28 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$.

12. Analyze by SDS-PAGE (UNIT 6.1).

In the presence of reducing agent, plasma fibronectin appears as a doublet of 230 and 220 kDa.

13. Freeze small aliquots on dry ice and store at -80°C .

Stored at this temperature, fibronectin can remain active for 10 years or more.

If storage at -80°C is not available, the second-best option is to store fibronectin as a liquid solution on wet ice. Storage of fibronectin at -20°C should be avoided—at this temperature, fibronectin tends to form a clot that cannot be redissolved. Thaw frozen fibronectin at 37°C with minimal shaking or agitation. Avoid repeated freezing and thawing.

PURIFICATION OF FIBRONECTIN FROM CULTURED CELLS

There are two major approaches for the purification of cellular fibronectin from monolayers of cultured fibroblastic cells: (1) extracting cell surface-bound fibronectin from the cell layer and (2) purifying fibronectin that is spontaneously released from the cell monolayer and secreted into the medium (see Alternate Protocol 2). The protocols have been used to produce cellular fibronectin from fibroblasts from several species including human, chicken, rat, and mouse. Although many cell types can be used, cultured fibroblasts are usually the cells of choice. These cells are easy to culture and produce relatively large amounts of fibronectin. Fibroblasts can either be prepared from embryonic or neonatal tissue from the appropriate animal species or be purchased from commercial sources, such as the American Type Culture Collection. Yields of fibronectin can vary widely depending on cell type and culture conditions, but can be surprisingly high. For example, fibronectin can comprise up to 3% of the total protein produced by chick embryo

**BASIC
PROTOCOL 2**

**Extracellular
Matrix**

10.5.3

fibroblasts. In general, earlier-passage cells provide higher yields of fibronectin than later-passage cells.

This protocol assumes that human fibroblasts have been cultured to confluency in ten 850-cm² tissue culture roller bottles. Alternatively, 150-cm² dishes or 175-cm² flasks can be used, with volumes adjusted accordingly. Since fibronectin yields depend on cell numbers, allow the cells to grow as dense as possible yet not so dense that they detach. Many fibroblastic cells (e.g., chick embryo fibroblasts) are not truly contact inhibited and can eventually form several cell layers in culture, although they will detach from the substrate if cultured too long without passaging.

Cellular fibronectin in substantially pure form can be extracted from fibroblast monolayers with 1 to 2 M urea. If 1 M urea is used, the yield will be lower but further purification is often not necessary. Extraction with 2 M urea is often necessary to get a good yield of fibronectin, especially when human fibroblasts are used as the starting material. Users are encouraged to do small pilot extraction of 35-mm or 60-mm dishes of cells to determine the yield and purity of extracted fibronectin at different urea concentrations. Although 2 M urea extracts cell surface fibronectin from fibroblasts better than 1 M urea, the higher concentration is also more efficient at extracting other cell surface proteins. Thus, further purification of cellular fibronectin extracted with 2 M urea is usually required.

Materials

Fibroblasts grown to dense confluence in roller bottles using serum-containing medium

Hank's balanced salt solution (HBBS; *APPENDIX 2A*)

Dulbecco's modified Eagle's medium (DMEM)

100 mM glutamine

0.2 M PMSF (see recipe)

Urea, ultrapure (Life Technologies or ICN)

Ammonium sulfate, ultrapure (Life Technologies or Sigma)

0.2 M CAPS buffer, pH 11.0 (see recipe)

CAPS/saline (see recipe)

Roller bottle apparatus

Refrigerated high-speed centrifuge

50-ml polycarbonate high-speed centrifuge tubes

Dialysis tubing (12,000 to 14,000 MWCO; Spectrapor or equivalent)

1.5-cm polypropylene column, 5 cm high

12 × 75-mm polypropylene tubes

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*).

Extract cells

1. Warm 2000 ml HBSS and 1500 ml DMEM supplemented with 60 ml of 100 mM glutamine (4 mM final) and 15 ml 0.2 M PMSF (2 mM final) to 37°C.

Approximately 200 ml HBSS and 150 ml supplemented DMEM will be needed per bottle.

2. Gently rinse roller bottles four times with 50 ml warmed HBSS each time. Discard rinses.
3. Add 25 ml/bottle DMEM with 4 mM glutamine and 2 mM PMSF and incubate 1 hr at 37°C while rotating at 1 rpm to deplete the monolayer of adsorbed serum proteins.

4. Prepare 2 M urea in DMEM by dissolving 30 g ultrapure urea in DMEM supplemented with 4 mM glutamine in a total volume of 250 ml. Warm to 37°C. Just before use, add 2.5 ml of 0.2 M PMSF (2 mM final).
5. Discard the serum-free medium, then add 20 ml/bottle fresh 2 M urea in DMEM with 4 mM glutamine and 2 mM PMSF (prepared in step 4) and incubate 2 hr at 37°C while rotating at 1 rpm.

This step extracts the fibronectin from the cell surface. Usually 2 M urea is needed to extract human fibronectin, but 1 M urea can be used with some cells, for example chick embryo fibroblasts. Alternatively, 1 M urea will extract human fibronectin, albeit at low yields. However, fibronectin extracted with 1 M urea is often sufficiently pure that affinity purification is not necessary.

6. Centrifuge the extracted fibronectin in eight 50-ml polycarbonate centrifuge tubes for 15 min at 15,000 × g, 4°C. Save the supernatant, which contains the fibronectin, and discard the pellets.

Precipitate fibronectin

7. Measure the volume and slowly add 0.472 g ultrapure ammonium sulfate per milliliter of supernatant (70% saturation) with stirring on ice. After all of the ammonium sulfate is dissolved, let the solution incubate ~1 hr on ice without stirring.
8. Centrifuge the precipitated fibronectin in 50-ml polycarbonate centrifuge tubes for 15 min at 15,000 × g, 4°C. Discard the supernatant and gently but rapidly resuspend all the pellets in a total of ~10 ml of 0.2 M CAPS buffer, pH 11.0. Readjust pH to 11.0 with NaOH as needed while resuspending.

Dialyze precipitate

9. Dialyze the resuspended cellular fibronectin against 3.5 liters CAPS/saline buffer at 4°C with stirring. Replace buffer twice.

Care must be taken to seal the dialysis buffer from exposure to air as well as possible using Saran wrap or equivalent. To minimize the airspace in the beaker above the dialysis buffer, make sure the Saran wrap is touching the top of the buffer. Otherwise, carbon dioxide from the air can dissolve in the buffer, lowering its pH and precipitating the cellular fibronectin.

10. Centrifuge 20 min at 15,000 × g, 4°C, to clarify the purified fibronectin.
11. Determine the concentration of cellular fibronectin by measuring the A_{280} using an extinction coefficient of 1.28 ml·mg⁻¹·cm⁻¹ and analyze by SDS-PAGE (UNIT 6.1).

Cellular fibronectin should produce a band at ~250 kDa.

12. Rapidly freeze cellular fibronectin in small aliquots with liquid nitrogen.

To avoid partial neutralization of the CAPS buffer, do not use dry ice. Cellular fibronectin should be stored in liquid nitrogen or at -80°C. In liquid nitrogen, cellular fibronectin will remain active for several years if it is not repeatedly frozen and thawed. Cellular fibronectin should be thawed at 37°C with a minimum of shaking or agitation.

AFFINITY PURIFICATION OF EXTRACTED CELLULAR FIBRONECTIN

Cellular fibronectin extracted from cells can often be used without further treatment. However, this material can be contaminated with other cellular proteins, especially if 2 M urea is required to extract the fibronectin; in such cases further purification is necessary.

Additional Materials (also see Basic Protocol 2)

- Tris-buffered saline (TBS; APPENDIX 2A)
- Gelatin-Sepharose (Amersham Pharmacia Biotech)
- 5-ml siliconized glass or polypropylene column

ALTERNATE PROTOCOL 1

Extracellular Matrix

10.5.5

1. Extract cellular fibronectin from monolayer cells (see Basic Protocol 2, steps 1 to 6).
2. Pack 5 ml of gelatin-Sepharose into a siliconized glass or polypropylene column.
Do not use an excessively large affinity column. Recoveries of cellular fibronectin tend to be greater at higher ratios of fibronectin to gelatin-Sepharose, due a relative decrease in nonspecific binding to the beads. A 5-ml bed volume is sufficient.
3. Wash the gelatin-Sepharose with 2.5 ml of 8 M urea in TBS, then with 25 ml TBS.
4. Dilute the cellular fibronectin 1:3 with TBS to a final urea concentration of 0.5 M.
5. Apply the fibronectin to the affinity column and collect 3-ml fractions.
6. Wash the column-bound material extensively with TBS, checking the A_{280} of the effluent, until the A_{280} is <0.05 .
7. Elute with 8 M urea in TBS and collect 3-ml fractions. Save and pool the fractions containing eluted fibronectin as determined by a peak in the A_{280} .
8. Proceed with dialysis of the fractions (see Basic Protocol 2, steps 9 to 12).

**ALTERNATE
PROTOCOL 2**

**PURIFICATION OF HUMAN CELLULAR FIBRONECTIN FROM
CONDITIONED MEDIUM**

Densely cultured fibroblastic cells can release substantial amounts of cellular fibronectin into culture medium. The approach described here is to culture roller bottles of human fibroblastic cells to dense confluence in serum-containing growth medium, and then culture the cells in serum-free medium. The use of serum-free medium is required in order to avoid contamination of the cellular fibronectin with plasma fibronectin. Fibroblasts usually cannot be expected to proliferate much under serum-free culture conditions, so they must be cultured in 850-cm² roller bottles in the presence of serum until they are heavily confluent. This procedure assumes that ten such roller bottles are used as the source of fibronectin.

This protocol was initially developed as described using a serum-free medium formulation called CRCM-30 that was commercially available from Life Technologies. Unfortunately, this medium is no longer available and neither is its formulation, so the protocol is described using an alternative serum-free medium consisting of a mixture of Ham's F-12 nutrient mixture and DMEM supplemented with antibiotics, pyruvate, amino acids, insulin, selenium, transferrin, and BSA. In trial experiments, this medium did not provide as high yields as CRCM-30, but it works adequately. A wide range of serum-free media is commercially available and any formulation without serum additives should be suitable.

Additional Materials (also see Basic Protocol 2)

- Serum-free medium: e.g., 1:1 DMEM/Ham's F12; supplemented (see above)
- Gelatin-Sepharose (Amersham Pharmacia Biotech)
- BSA, crystalline (3× recrystallized; CalBiochem)
- Tris-buffered saline (TBS; APPENDIX 2A)
- Urea, ultrapure (Life Technologies or Sigma)
- CAPS/saline (see recipe)
- Ammonium sulfate
- 2.5-cm polypropylene or siliconized glass column, 5 cm high (siliconized with Aquasil, Pierce)
- 1-liter cylindrical polypropylene bottles
- 3-ml polypropylene tubes

Prepare cells and collect conditioned medium

1. Warm 2000 ml serum-free medium to 37°C.
2. Gently rinse roller-bottle fibroblast cultures four times each with 50 ml warmed serum-free medium.
3. Add 50 ml/bottle of serum-free medium and incubate at least 1 hr at 37°C while rotating at 1 rpm to deplete the monolayer of adsorbed serum proteins.

The incubation period in this step can be as long as overnight with no deleterious effects.

4. Discard the serum-free medium. Add 100 ml of fresh serum-free medium to each roller bottle and incubate 24 to 48 hr at 37°C while rotating at 1 rpm.
5. Collect the serum-free, conditioned medium from the bottles and add 10 ml of 0.2 M PMSF per 1000 ml conditioned medium (2 mM final).
6. Centrifuge the extracted fibronectin in polypropylene centrifuge tubes for 15 min at 25,000 × g, 4°C. Save the supernatant, which contains the fibronectin, and discard the pellets.

The conditioned serum-free medium from several bottles can be pooled and stored at -80°C, or used immediately in the next section.

Secreted cellular fibronectin can often be repeatedly harvested from the same roller bottles by adding more serum-free medium. Usually the cells will eventually start to detach from the roller bottles. When this happens, they can be regrown to confluence by adding serum-containing medium before repeating steps 1 to 5. As the fibroblasts age, the amount of fibronectin they secrete will decrease, so this process cannot be repeated indefinitely. When the yield of fibronectin begins to decline, the cells should be discarded.

Affinity purify secreted cellular fibronectin

7. Prepare the gelatin-Sepharose affinity column. Pack 10 ml gelatin-Sepharose per liter of conditioned medium in a 2.5-cm polypropylene or siliconized glass column.
8. Wash the gelatin-Sepharose with 30 ml of 5 mg/ml BSA in TBS. Then wash the column with 50 ml of 8 M urea in TBS followed by 50 ml TBS.

The column is prewashed with BSA in order to block nonspecific, irreversible protein binding to the gelatin-Sepharose.

9. Mix 1 ml of BSA-treated gelatin-Sepharose with 1 liter of conditioned medium and incubate for 24 hr at 4°C.

This is most easily done by mixing the beads and medium together in 1-liter cylindrical polypropylene bottles placed on a roller bottle apparatus with rotation at 3 rpm at 4°C. During this time, the fibronectin in the conditioned medium should bind to the immobilized gelatin.

Avoid mechanical stirring or excessive agitation of the gelatin-Sepharose, both of which can break up the Sepharose beads, decreasing subsequent column flow rates and the yield of fibronectin.

Recover fibronectin

10. Allow the beads to settle, decant off most of the medium, and pour the gelatin-Sepharose into a 2.5-cm diameter siliconized glass or polypropylene column.
11. Wash the column-bound material with 50 ml TBS at room temperature.
12. Elute with 8 M urea in TBS, collecting 3-ml fractions. Save and pool the fractions containing eluted fibronectin as determined by a peak in the A_{280} , until $A_{280} < 0.05$.

Concentrate fibronectin (optional)

13. If the concentration of cellular fibronectin obtained from the gelatin-Sepharose affinity column is >1 mg/ml, proceed to step 16. If a higher concentration of cellular fibronectin is needed, dialyze the eluted fibronectin against 3.5 liters CAPS/saline with stirring for at least 3 hr to remove most of the urea. Replace buffer at least twice.
14. Transfer the fibronectin to a polypropylene beaker, measure the volume, and slowly add 0.472 g ultrapure ammonium sulfate per ml (70% saturation) with stirring on ice. After all of the ammonium sulfate is dissolved, let the solution incubate at least 1 hr on ice without stirring.
15. Centrifuge the precipitated fibronectin in polypropylene centrifuge tubes for 15 min at $15,000 \times g$, 4°C . Discard the supernatant and gently but rapidly resuspend the cellular fibronectin pellets in 0.2 M CAPS buffer, pH 11.0. Readjust pH to 11.0 with NaOH if needed while resuspending.

Dialyze fibronectin

16. Dialyze the cellular fibronectin against 3.5 liters CAPS/saline buffer at 4°C with stirring. Replace buffer at least twice.

Care must be taken to seal the dialysis buffer from exposure to air as well as possible using plastic (Saran) wrap. To minimize the airspace in the beaker above the dialysis buffer, make sure the Saran wrap is touching the top of the buffer. Otherwise, carbon dioxide from the air can dissolve in the buffer and lower its pH.

17. Clarify the purified fibronectin by centrifuging 20 min at $15,000 \times g$, 4°C .
18. Determine the concentration of cellular fibronectin by measuring the A_{280} using an extinction coefficient of $1.28 \text{ ml}\cdot\text{mg}^{-1}\cdot\text{cm}^{-1}$ and analyze by SDS-PAGE (UNIT 6.1).
19. Freeze cellular fibronectin in small aliquots with liquid nitrogen.

To avoid partial neutralization of the CAPS buffer, do not use dry ice. Cellular fibronectin should be stored in liquid nitrogen or at -80°C . In liquid nitrogen, cellular fibronectin will remain active for 10 years or more if it has not been repeatedly frozen and thawed. Cellular fibronectin should be thawed at 37°C with a minimum of shaking or agitation.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

CAPS buffer, pH 11.0, 0.2 M

22.1 g CAPS (cyclohexylaminopropane sulfonic acid)
~450 ml H_2O
Adjust pH to 11.0 with 6 N NaOH
Add H_2O to 500 ml
Store protected from air up to several weeks at room temperature
After 1 to 2 months, check the pH and readjust with NaOH if necessary.

CAPS/saline

8.8 g sodium chloride
~900 ml H_2O
50 ml 0.2 M CAPS buffer, pH 11.0 (see recipe)
1 ml 1 M calcium chloride
 H_2O to 1000 ml
Store protected from air up to 1 month at room temperature
After 1 month, check the pH and readjust with NaOH if necessary.

Citrate elution buffer

11.6 g NaCl
29.4 g sodium citrate (trisodium citrate, dihydrate)
~1800 ml H₂O
2 ml 20% (w/v) sodium azide stock (see recipe)
Adjust pH to 5.5 with 6 N HCl
Add H₂O to 2000 ml
Store up to 1 month at room temperature

Composition: 0.1 M NaCl and 50 mM sodium citrate, pH 5.5.

Column buffer A

12.1 g Tris·Cl
13.1 g ε-amino-*n*-caproic acid
11.8 g sodium citrate (trisodium citrate, dihydrate)
2 ml 20% (w/v) sodium azide stock (see recipe)
H₂O to ~1900 ml
Adjust pH to 7.6 with 6 N HCl
H₂O to 2000 ml
Prepare fresh on day of experiment and use within 2 days

*Composition: 50 mM Tris·Cl, 50 mM ε-amino-*n*-caproic acid, and 20 mM sodium citrate, pH 7.6.*

Column buffer B

58.4 g sodium chloride
6.0 g Tris·Cl
6.5 g ε-amino-*n*-caproic acid
5.9 g sodium citrate (trisodium citrate, dihydrate)
~800 ml H₂O
1 ml 20% (w/v) sodium azide stock (see recipe)
Adjust pH to 7.6 with 6 N HCl
Add H₂O to 1000 ml
Prepare fresh on day of experiment and use within 2 days

Composition: 1 M NaCl, 50 mM Tris·Cl, 50 mM 6-aminohexanoic acid, and 20 mM sodium citrate, pH 7.6.

PMSF stock solution, 0.2 M

Dissolve 69.6 mg PMSF (phenylmethylsulfonyl fluoride) in 2 ml isopropyl alcohol (Sigma). Store up to several days at room temperature, or divide into aliquots and store up to several years at -20°C.

Sodium azide, 20%

Dissolve 20 g sodium azide (Sigma) in water to 100 ml. Store for several months at room temperature.

COMMENTARY

Background Information

The fibronectins have been important as substrates to promote the adhesion and culture of cells in vitro and also as model systems for investigating mechanisms of cell adhesive interactions (Hynes, 1990; Mosher, 1989; Aota and Yamada, 1995; Potts and Campbell, 1994; Akiyama et al., 1995). Fibronectin contains two distinct regions that independently promote

cell adhesion by binding integrin cell surface receptors. For most cells, adhesion is mediated by the central cell-binding domain of fibronectin through an Arg-Gly-Asp (RGD) sequence and a Pro-His-Ser-Arg-Asn (PHSRN) sequence that can interact synergistically with the RGD sequence to bind the α5β1 integrin (Pierschbacher and Ruoslahti, 1984; Yamada and Kennedy, 1984; Aota et al., 1994). A sec-

ond, independent cell-adhesive region of fibronectin that is located in the alternatively spliced IIIICS (or V) module (Kornblihtt et al., 1985) binds the $\alpha 4\beta 1$ integrin. At least two nonadjacent peptide sequences within the IIICS module contain cell adhesive activity—a Leu-Asp-Val (LDV) sequence and an Arg-Glu-Asp-Val (REDV) sequence (Humphries et al., 1987; Kormoriya et al., 1991). Unlike the central RGD and PHSRN sequences, the LDV and REDV sequences do not act in synergy but appear to have an additive effect on cell adhesion, although the REDV is much less active (~1% that of the LDV sequence).

Fibronectin is commonly found in most tissues and body fluids, but it is most easily purified from plasma and from cultured cells. The presence of cell adhesive factors in plasma or serum has been known for a long time and the existence of plasma fibronectin, in particular, has been known for about 50 years, although it has gone by a number of names, most commonly “cold insoluble globulin” or CIG (Morrison et al., 1948). Due to its relatively high content of fibronectin in soluble form, plasma is the most convenient source. Plasma-derived fibronectin is dimeric, consisting of two similar, but nonidentical, subunits, and is highly soluble under physiological conditions and over a wide range of pH values and salt concentrations.

Fibronectin has a number of ligands including heparin, collagen, and fibrin, as well as cell surface receptors. It actually binds denatured collagen (gelatin) better than it does native collagen; therefore, virtually all protocols for purifying fibronectin rely on its affinity for gelatin as described by Engvall and Ruoslahti (1977). The method in Basic Protocol 1 was first described by Miekka et al. (1982). It uses a pH 5.5 buffer to specifically elute the bound fibronectin without causing its denaturation and without eluting other gelatin-binding plasma proteins. The elution of fibronectin using a citric acid buffer provides a number of advantages over earlier procedures that use urea elution, including eliminating a second affinity purification column, preventing the denaturation of the fibronectin, and avoiding exposure of the fibronectin to urea, which can chemically modify the fibronectin or irreversibly change its conformation.

Cellular and plasma fibronectins are derived from alternatively spliced variants of the same gene (Kornblihtt et al., 1985). Cellular fibronectin contains up to three inserted sequences not present in all chains of plasma

fibronectin. The functional differences between plasma and cellular fibronectins are most striking in assays that measure morphological effects on cells, with the latter being more effective in certain adhesion and cell morphology assays (Yamada and Olden, 1978; Hashimoto-Uoshima et al., 1997; Manabe et al., 1997).

A number of cultured cell types synthesize fibronectin, including fibroblasts, endothelial cells, epithelial cells, myoblasts, and chondrocytes (Hynes, 1973; Jaffe and Mosher, 1978; Yamada and Weston, 1974; Chen et al., 1977; Dessau et al., 1978; Furcht et al., 1978; Ruoslahti et al., 1973). Cell-derived fibronectin is usually retained on the cell surface as insoluble fibers, but it can also be released into the culture medium. Virtually all cultured cells that synthesize fibronectin can be used as a source of this protein, either by direct extraction with urea or by harvesting the fibronectin released into culture medium. The abundance of cellular fibronectin on fibroblasts and the ease with which these cells are isolated and cultured made these the most convenient choice.

Critical Parameters and Troubleshooting

The preparation procedures for plasma and cellular fibronectin are relatively simple and require a minimum of equipment, all readily available. The most common problem with these procedures is low yield, which is most often caused by improper handling of the purified fibronectin. Other potential causes are poor starting material and failure of fibronectin to either bind to or elute efficiently from the affinity column.

Except where noted, all of the steps in the purification protocols for fibronectin are performed at room temperature (~22°C); it is therefore necessary to include sodium azide in all column buffers to inhibit microbial growth. The protein content of all fractions is determined by the absorbance at 280 nm (A_{280}) and should be confirmed by SDS-PAGE (UNIT 6.1) with either Coomassie blue R-250 or silver staining.

In most cases, the final products of these protocols are obtained at relatively low concentrations—usually ≤ 1 mg/ml. Ammonium sulfate precipitation followed by resolubilization of fibronectin in a small volume and dialysis is usually the best method for increasing its concentration. Because all forms of fibronectin tend to adsorb readily onto surfaces, the use of membrane-type centrifugal concentrators

(such as Centricons or Centri-preps) is not recommended. Fibronectins of all forms are also quite susceptible to proteolytic cleavage. Therefore, these protocols usually require the addition of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF; see *APPENDIX 1B*). Solid PMSF is only slightly soluble and highly unstable when added to aqueous buffers, so it must be prepared as a 200 mM stock solution in isopropanol, which is then diluted into the aqueous buffer. PMSF dissolved in isopropanol is stable for several weeks at room temperature. However, PMSF is quite unstable in aqueous solution and, therefore, must be added to buffers immediately before use. All buffers containing PMSF that are not used within 20 min should be discarded.

Improper handling of fibronectin can arise from a number of sources, many of which are unintentional. Most low yields are the result of irreversible adsorption of the fibronectin onto the surfaces of tubes and beakers, and/or precipitation or clotting of the purified fibronectin. To insure maximal recovery of fibronectin, only polypropylene or siliconized glass beakers and tubes should be used. Agitation and other physical disturbances of the fibronectin should be avoided because such rough handling can cause denaturation of fibronectin at the air-liquid interface, aggregation, and precipitation. Fibronectin can also be lost due to improper handling during freezing and storage. In fact, small amounts of fibronectin are unavoidably lost during freezing and thawing. Fibronectin is best snap-frozen in powdered dry ice (for plasma-derived protein) or liquid nitrogen and stored at -80°C . Freezing at -20°C usually results in irreversible precipitation of the fibronectin, probably because slow freezing gives the protein a chance to aggregate in the liquid phase and form a clot during the time the solution is partially liquid as it cools. Fibronectin should be thawed rapidly at 37°C with no agitation. Storing fibronectin in liquid form at or near 0°C for a week or more is preferable to repeated freezing and thawing or freezing at -20°C .

Variations in starting material are almost unavoidable. Plasma and sera, whether from human or animal sources, will show individual variations in fibronectin content, even if collected in exactly the same manner. Cultured cells can vary in their fibronectin production and secretion, and yields usually decrease substantially as cells "age." Since the preparation of fibronectin requires only 1 to 2 days, it is usually not worth the time it takes to assay a

fibronectin source prior to trying the purification protocol.

Fibronectin binds with high affinity to gelatin. Thus, low yields are rarely due to the fibronectin not binding to the affinity columns. When this does happen, it is usually because the affinity column is very old or has been stored improperly before use. The gelatin-Sepharose should always be stored near pH 7 in the presence of 0.05% sodium azide to retard microbial growth. Furthermore, although the gelatin is covalently bound to the beads, it can "bleed" off with time and repeated use, and it is susceptible to proteolysis by gelatinases in plasma. Thus, the urea prewash of the gelatin-Sepharose is a crucial step to remove gelatin fragments that can competitively inhibit fibronectin binding to the immobilized gelatin. It is also recommended that careful records be kept documenting the yield of a gelatin-Sepharose affinity column, and if a substantial drop in yields of consecutive preparations is noticed, the column should be discarded.

The most common cause of low yield is failure to elute the purified fibronectin efficiently off the gelatin-Sepharose affinity column. This is most likely due to small errors in the pH of the elution buffer. Citrate elution of fibronectin occurs because fibronectin binds poorly to gelatin at its isoelectric point. Thus, elution will not occur if the pH is either too high or too low. One way to avoid a total loss is always to elute the gelatin-Sepharose with 4 M urea following the citrate elution and collect these fractions as well. The urea-eluted fibronectin will be less pure than citrate-eluted material, but it will be useful for most cell culture purposes.

Cellular fibronectin is not very soluble at neutral pH and is best stored at pH 11. The solution can then be diluted into neutral buffers with no problems. Precautions should be taken to avoid exposing cellular fibronectin to air, and especially dry ice, for long periods of time, as carbon dioxide will eventually dissolve in the buffer, lowering its pH and precipitating the fibronectin.

Occasionally, the flow rate of the affinity column can become very slow. The most common cause is that the fibronectin has formed a "clot" at the top of the affinity column, especially if the flow rate is too slow. If this happens, use a polypropylene rod to break up the top centimeter or so of the column bed, which should increase the flow rate.

The most commonly used alternative protocol for the purification of plasma fibronectin

involves the use of two affinity columns—one of gelatin and one of heparin—as described by Hayashi and Yamada (1983). In this protocol, plasma fibronectin is first bound to a gelatin-Sepharose column and eluted with 4 M urea. This material is immediately bound to a heparin-Sepharose column and washed with 4 M urea. The urea is then washed away and the purified fibronectin is eluted with 0.3 M sodium chloride. Although this protocol takes more than 50% longer, it yields fibronectin of higher purity at the expense of having exposed the fibronectin to denaturing conditions. The extra affinity column is worth the extra effort if extremely high-purity fibronectin is required. The specific cell adhesive activities of urea-eluted and citrate-eluted fibronectins are similar.

Anticipated Results

Basic Protocol 1 should yield 100 to 300 mg of fibronectin, which is usually a more-than-adequate amount for most uses. This material is usually of very high purity, and there should be no detectable impurities when it is analyzed by gel electrophoresis. This protocol has been optimized to promote efficient use of gelatin-Sepharose, and therefore specifies a relatively high plasma to affinity support ratio. If the fibronectin is to be purified from a particularly valuable plasma or serum sample, the column, wash and elution volumes can be doubled to increase (though not double) the yield.

It is much more difficult to predict yields of cellular fibronectin since these can depend on culture conditions, passage number of the cells, and cell type as well as the efficiency of the protocol. Low-passage-number human fibroblasts in ten 850-cm² tissue culture roller bottles should typically provide between 10 and 20 mg of cellular fibronectin.

The major impurity in preparations of cellular fibronectin is the extracellular protein tenascin-C. The level of contamination can be quite high, despite the use of great care. An early paper described the separation of tenascin from preparations of chicken cell surface fibronectin (Erickson and Iglesias, 1984). The gelatin-Sepharose affinity column chromatography step should separate the cellular fibronectin from tenascin because the latter protein does not bind to gelatin (Erickson, 1989; Lightner et al., 1988).

Time Considerations

All of these protocols take ≤1 day of actual hands-on time. Basic Protocol 1 will yield purified protein 2 to 3 days after starting. Usually

the first ½ day is spent preparing buffers and packing and washing columns. The affinity purification steps are usually performed in one more day. Possible stopping points are noted in each protocol and, of course, the protocol can be stopped overnight or even over weekends at dialysis steps.

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Purification of Vitronectin

UNIT 10.6

**BASIC
PROTOCOL**

The protocol described here has been successfully used to purify vitronectin from plasma or sera of a number of species. This method takes advantage of the observation that vitronectin must be activated or “opened” by denaturation before it can bind to heparin. Plasma or serum is first passed through a heparin-Sepharose column to remove fibronectin and other heparin-binding serum components. Then urea is added to the heparin-cleared plasma (or serum) to activate the heparin-binding activity of vitronectin. The activated vitronectin then binds to heparin-Sepharose and is eluted at very high purity.

As described here, this protocol assumes the starting material consists of 400 ml of citrate-anticoagulated human plasma, which should yield 10 to 20 mg of vitronectin of high (usually >98%) purity. A similar volume of human serum, or plasma or sera from other mammals, can also be used. This protocol can be easily scaled up by using correspondingly larger columns and buffer volumes.

NOTE: Only citrate-anticoagulated, not heparin-anticoagulated, plasma or serum should be used with this protocol. Because vitronectin binds to glass, any glassware to be used in this protocol should first be siliconized (see *APPENDIX 3*). Alternatively, use plastic—polypropylene, polycarbonate, or polyethylene—equipment (polypropylene is suggested throughout but the others may be used). Polystyrene is also acceptable and is better than glass.

CAUTION: When working with human blood cells or infection agents, appropriate biosafety practices must be followed. Even screened human plasma should be treated as if it is contaminated with HIV and hepatitis viruses. Always wear two layers of gloves, safety glasses or goggles, and suitable protective clothing. Keep a 10% bleach solution, Wescodyne, or Providone on hand at all times to use as an emergency disinfection agent in case of plasma spills or splashes.

Materials

- Sepharose CL-4B (Amersham, Pharmacia Biotech)
- Heparin-Sepharose (Amersham, Pharmacia Biotech)
- Phosphate/saline/EDTA (see recipe)
- 2 M NaCl in phosphate/saline/EDTA (see recipe)
- ~400 ml (1 to 2 units) citrate-anticoagulated human plasma or serum
- 0.2 M EDTA, pH 7.0 (*APPENDIX 2A*)
- 0.2 M phenylmethanesulfonyl fluoride (PMSF; *UNIT 10.5*)
- 95% ethanol, ice cold
- Ultrapure urea
- 8 M urea in phosphate/saline/EDTA (see recipe)
- 8 M urea/2 M NaCl in phosphate/saline/EDTA (see recipe)
- 2-Mercaptoethanol
- 8 M urea/0.3 M NaCl in phosphate/saline/EDTA (see recipe)
- Dulbecco's phosphate-buffered saline (DPBS; *APPENDIX 2A*)
- 20- and 50-ml (minimum) 2.5-cm-diameter polypropylene or siliconized glass columns
- 600- and 1000-ml polypropylene beakers
- Polypropylene centrifuge tubes
- Whatman no. 2 filter paper
- 10-ml polypropylene tubes
- Quartz spectrophotometer cuvettes siliconized with Aquasil (Pierce)
- Dialysis tubing (12,000 to 14,000 MWCO)

**Extracellular
Matrix**

10.6.1

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Supplement 2

Prepare columns

1. Prepare a 2.5-cm-diameter, 8-ml Sepharose CL-4B precolumn. Wash with 24 ml phosphate/saline/EDTA.

Follow manufacturer's instructions to prepare Sepharose CL-4B and heparin-Sepharose. All column chromatography steps in this protocol are at room temperature.

2. Prepare a 2.5-cm-diameter, 20-ml heparin-Sepharose column at room temperature. Wash with 80 ml of 2 M NaCl in phosphate/saline/EDTA, then with 80 ml phosphate/saline/EDTA.

The columns can be washed up to several days in advance and kept at room temperature if 0.02% sodium azide is added to the phosphate/saline/EDTA.

3. To 400 ml citrate-anticoagulated plasma or serum, add 2.5 ml of 0.2 M EDTA and 0.5 ml of 0.2 M PMSF with rapid stirring at 4°C.
4. If serum is being used as the starting material, proceed to step 5. If plasma is used, place it in a 600-ml polypropylene beaker packed in ice and gradually add 48 ml ice-cold 95% ethanol with stirring. Continue to stir for 10 min.

This step precipitates fibrinogen in plasma.

5. Centrifuge the plasma or serum 10 min at 12,000 × g, 4°C. Discard the pellet.
6. Save the supernatant and filter through Whatman no. 2 filter paper at 4°C.

This is a possible overnight stopping point. The filtrate should be stored at 4°C.

Remove heparin-binding proteins

7. Apply the filtered plasma, preequilibrated at room temperature, to the Sepharose CL-4B precolumn to remove any plasma components that nonspecifically bind to the beads. After all the plasma has passed through the column, wash the column with 20 ml phosphate/saline/EDTA and add the wash to the plasma.
8. Apply the pooled flowthrough and wash from step 7 to the heparin-Sepharose column. After all the plasma has passed through the heparin-Sepharose column, wash the column with 50 ml phosphate/saline/EDTA and add the wash to the plasma.

At this point, the procedure can be stopped until the next day or the vitronectin can be purified immediately. If the vitronectin is not to be purified immediately, wash the heparin-Sepharose with 80 ml of 2 M NaCl in phosphate/saline/EDTA to remove heparinases or glycosidases that could degrade the beads, and store the column-treated plasma containing the vitronectin at 4°C overnight. The column can be stored at room temperature if 0.02% sodium azide is added to the 2 M NaCl.

This step removes plasma components (such as fibronectin) that bind to heparin in their native form.

Activate the vitronectin

9. Place the plasma in a 1000-ml polypropylene beaker at room temperature and add 384 g ultrapure urea to the plasma with gentle stirring. Then dilute the plasma to a final volume of 800 ml with phosphate/saline/EDTA. Let stand 1 to 2 hr at room temperature.

This step activates the vitronectin so that it can bind to heparin. The final urea concentration is 8 M.

10. While the urea-treated plasma is left to stand, wash the heparin-Sepharose column used in step 8 above with 120 ml of 8 M urea/2 M NaCl in phosphate/saline/EDTA, then with 120 ml of 8 M urea in phosphate/saline/EDTA.

Urea is unstable in aqueous solution at room temperature. Therefore, all urea-containing solutions must be prepared fresh each day and subsequently discarded if not used the same day they are prepared.

Affinity purify vitronectin

11. Apply urea-treated plasma from step 11 to the heparin-Sepharose column at a flow rate of ≤ 100 ml/hr.

This step can proceed overnight as long as precautions are taken to ensure that the column will not dry out.

If step 11 proceeds overnight, prepare fresh solutions of 8 M urea in phosphate/saline/EDTA (see recipe) and 8 M urea/0.3 M NaCl in phosphate/saline/EDTA (see recipe).

12. Wash the column with 400 ml of 8 M urea in phosphate/saline/EDTA.
13. Add 140 μ l 2-mercaptoethanol to 200 ml of 8 M urea in phosphate/saline/EDTA.
14. Wash the column with 100 ml of 8 M urea in phosphate/saline/EDTA supplemented with 2-mercaptoethanol.

If this washing step takes ≤ 1 hr, stop the column so that the total exposure of the column to 2-mercaptoethanol is at least 1 hr to ensure removal of contaminants sensitive to reduction. Longer times, up to overnight, can also be used.

If step 14 proceeds overnight, prepare fresh solutions of 8 M urea in phosphate/saline/EDTA (see recipe) and 8 M urea/0.3 M NaCl in phosphate/saline/EDTA (see recipe).

15. Wash the column with another 100 ml of 8 M urea in phosphate/saline/EDTA supplemented with 2-mercaptoethanol.

The minimal exposure time of the column to 2-mercaptoethanol should be 1 hr.

16. Wash the column with 100 ml of 8 M urea in phosphate/saline/EDTA without 2-mercaptoethanol.
17. Elute the vitronectin with 8 M urea/0.3 M NaCl in phosphate/saline/EDTA. Collect 10-ml fractions. Pool and save the fractions containing eluted vitronectin as judged by A_{280} measured in siliconized quartz cuvettes. Save fractions with $A_{280} > 0.05$.

It is recommended that the fractions containing higher concentrations ($A_{280} > 0.5$) be kept separate from those containing lower concentrations.

Dialyze fractions

18. Dialyze the fractions with higher concentrations ($A_{280} > 0.5$) at 4°C for 3 days against 3.5 liters DPBS, changing the buffer each day. Dialyze the lower-concentration fractions at 4°C for 3 days against 3.5 liters 10% DPBS (1 vol DPBS diluted with 9 vol H₂O), changing the buffer each day.
19. Centrifuge 20 min at 12,000 \times g, 4°C, to clarify the purified vitronectin. Collect the supernatant.
20. Measure the concentration of vitronectin.

The fastest way to estimate the protein concentration is to measure the A_{280} and calculate the concentration assuming an extinction coefficient of 1.36 ml/mgcm.

21. Aliquot 50 or 100 μ l vitronectin into separate polypropylene tubes and lyophilize. Label the tubes to indicate which buffer was used, the original volume, and the amount of vitronectin in each tube.

When the tubes are reconstituted with deionized water, the tubes that contained vitronectin in 10% DPBS should be reconstituted to one-tenth the original volume to yield higher concentrations of vitronectin in full-strength DPBS. Reconstituted vitronectin can be sterilized by autoclaving, if necessary.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

2 M NaCl in phosphate/saline/EDTA

29.4 g NaCl
5 ml 0.5 M sodium phosphate, pH 7.7 (see recipe)
6.3 ml 0.2 M EDTA, pH 7.0 (*APPENDIX 2A*)
0.25 ml 20% (w/v) sodium azide (*UNIT 10.5*)
Deionized H₂O to 250 ml
Store several weeks at room temperature

Phosphate/saline/EDTA

7.6 g sodium chloride (130 mM final)
20 ml 0.5 M sodium phosphate, pH 7.7 (see recipe; 10 mM final)
25 ml 0.2 M EDTA, pH 7.0 (*APPENDIX 2A*; 5 mM final)
1 ml 20% (w/v) sodium azide (0.02% final; *UNIT 10.5*)
Deionized H₂O to 1000 ml
Store several weeks at room temperature.

Sodium phosphate, pH 7.7, 0.5 M

79.7 g Na₂HPO₄·12H₂O
4.29 g NaH₂PO₄·2H₂O
Deionized H₂O to 1000 ml

8 M urea in phosphate/saline/EDTA

480 g ultrapure urea
7.6 g sodium chloride
20 ml 0.5 M sodium phosphate, pH 7.7 (see recipe)
25 ml 0.2 M EDTA, pH 7.0 (*APPENDIX 2A*)
Deionized H₂O to 1000 ml

The shelf life of this solution is <1 day.

Deionized H₂O to 500 ml

8 M urea/2 M NaCl in phosphate/saline/EDTA

60 g ultrapure urea
14.7 g NaCl
2.5 ml 0.5 M sodium phosphate, pH 7.7 (see recipe)
3.2 ml 0.2 M EDTA, pH 7.0 (*APPENDIX 2A*)
Deionized H₂O to 125 ml

The shelf life of this solution is <1 day.

8 M urea/0.3 M NaCl in phosphate/saline/EDTA

96 g ultrapure urea
3.5 g NaCl
4 ml 0.5 M sodium phosphate, pH 7.7 (see recipe)
5 ml 0.2 M EDTA, pH 7.0 (*APPENDIX 2A*)
Deionized H₂O to 200 ml

The shelf life of this solution is <1 day.

COMMENTARY

Background Information

Vitronectin, also known as serum spreading factor, S-protein, and epibolin, is found primarily in blood at concentrations ranging from 100 to 400 $\mu\text{g/ml}$ and in other biological fluids as well as tissues (Preissner, 1991). Vitronectin can appear as two species of similar size. Human vitronectin appears as distinct bands of 65 and 75 kDa under reducing conditions. The ratio of the two species can vary. Vitronectin contains an Arg-Gly-Asp sequence which, as is the case for fibronectin, functions as a cell-adhesive recognition signal for integrins. However, cellular vitronectin and fibronectin receptors are usually distinct. Cells usually adhere to fibronectin primarily through $\beta 1$ integrins while adhering to vitronectin primarily through $\beta 3$ integrins.

The protocol described here was first developed by Yatohgo et al. (1988) and has been successfully used to purify vitronectin from plasma or sera of a number of species. This protocol can generally be used across species lines. It represents a major improvement over earlier methods, which relied on the propensity of vitronectin to bind to glass and used up to four different affinity columns and required several days (Barnes and Silnutzer, 1983). The protocol described here takes advantage of the observation that vitronectin, unlike the fibronectin present in plasma, must be activated or opened with 8 M urea before it can bind to heparin. Thus, plasma components that bind specifically or nonspecifically to heparin or the Sepharose beads can be removed prior to vitronectin activation, enabling this protocol to yield vitronectin of extremely high purity.

Critical Parameters and Troubleshooting

Low yields of vitronectin can be due to analogous causes as low yields in affinity-purification of plasma fibronectin. For example, heparin-Sepharose columns tend to bleed ligand. Therefore, extensive prewashing of the affinity columns is very important. Also, care must be taken to use only citrate-anticoagulated plasma. If heparin-anticoagulated plasma is used, the heparin can competitively inhibit vitronectin binding to the affinity column, greatly reducing the yields. A recurring prob-

lem in this protocol is the slowing of column flows to distressingly low rates. Thus, if it is necessary to scale up this protocol, it is recommended that column volumes be increased by changing the column diameters, rather than heights. Columns often run well initially but eventually slow down. A faster flow rate can sometimes be reestablished by breaking up the "crust" of aggregated proteins that can form on the top of the affinity column. It may be necessary to disturb the top of the gel to a depth of 1 to 2 mm. Vitronectin was so named from its propensity to bind to glass. Therefore, only polypropylene or siliconized glass columns, beakers, and tubes should be allowed to contact the vitronectin in all steps. Using untreated glass materials in this protocol can result in much lower yields. Finally, vitronectin can be stored at -80°C without lyophilization, but it slowly loses activity over the course of a year or two.

Anticipated Results

The protocol described here should yield 10 to 20 mg of vitronectin of relatively high purity (usually $>98\%$). Purity can be checked by SDS-PAGE (UNIT 6.1). Human vitronectin usually appears as a doublet of bands at 65 and 75 kDa.

Time Considerations

This protocol requires a total of 2 to 3 days. Hands on time is between 1 and 2 days. Convenient stopping points are indicated in the protocol.

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Proteoglycan Isolation and Analysis

UNIT 10.7

This unit describes specific methods for isolation and analysis of proteoglycans and glycosaminoglycans. These molecules can be difficult to work with due to their large size, high negative charge, and tendency to aggregate and form complexes with other molecules. This requires modification of standard protocols and protocols unique to proteoglycans. Proteoglycans vary in size from small (e.g., decorin ≈ 100 kDa) to very large (e.g., aggrecan $\approx 1 \times 10^6$ kDa). Proteoglycans are present in extracellular matrices (e.g., perlecan), linked to cell surfaces by phospholipid (e.g., glypicans), as transmembrane components (e.g., syndecans), and in intracellular granules in some cells (also see UNIT 10.1). As a class of molecules, they can be isolated and purified utilizing their glycosaminoglycan chains (e.g., by ion-exchange chromatography) or their core proteins (antibody-affinity chromatography). Their glycosaminoglycan content can be further analyzed by treatment with degradative enzymes followed by size-exclusion chromatography or SDS-PAGE (UNIT 6.1), and immunoblotting (UNIT 6.2).

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety office (also see APPENDIX 1D).

NOTE: Even where chemical amounts of proteoglycan may be harvested, the addition of a trace of radiolabeled material aids in monitoring the purification process. In the absence of radiolabel, commercial assays based on the binding of 1,9-dimethylmethylene blue (a cationic dye) to sulfated glycosaminoglycans may be used to monitor isolation (e.g., Blyscan Glycosaminoglycan Assay, Biocolor; Accurate Chemical and Scientific).

ISOLATION AND PURIFICATION OF PROTEOGLYCANS

This group of protocols contains methods for isolating proteoglycans from cultured cells and for chromatographic purification.

Isolation of Proteoglycans from Cultured Cells

The following protocol is used to isolate proteoglycans from conditioned medium (soluble), as well as cell-associated proteoglycans (cell surface and intracellular) and proteoglycans in the extracellular matrix (insoluble). This is a general method applicable to all proteoglycans, as compared to Alternate Protocol 1, which is specifically designed to isolate different subcellular pools, especially those membrane-intercalated proteoglycans that act as links between the cytoskeleton and extracellular matrix. If only soluble proteoglycans are required, medium may be removed and new medium incubated with cells to increase yield.

Proteoglycans can be isolated from radiolabeled cultured cells. Since low yields are expected, particularly for some proteoglycans, the equivalent of five 10-cm-diameter culture dishes should be used for each experiment (8 ml medium/dish).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified, 37°C, 5% to 10% CO₂ incubator (depending upon medium) unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

**BASIC
PROTOCOL 1**

**Extracellular
Matrix**

10.7.1

Materials

Radiolabeled cultured cells in 10-cm tissue culture dishes (see Support Protocol)
PBS (APPENDIX 2A)
Urea, ultrapure
Solubilization buffer (see recipe) with and without Triton X-100

1. Collect supernatant from radiolabeled cultured cells by decanting into a 50-ml conical centrifuge tube. Rinse cell layer twice with 4 ml PBS each time, and add washes to the tube.
2. Centrifuge supernatant 10 min at $800 \times g$, room temperature, to remove any cellular material.
3. Add solid urea to the supernatant to a final concentration of 4 M. Store supernatant at 4°C ; do not freeze. Use for analysis as soon as possible.

All further stages with supernatant proteoglycans may be performed without detergent present in the buffers. The authors' experience, however, is that losses are decreased by the presence of 0.1% (v/v) Triton X-100.

4. Wash cell layers twice with 20 ml PBS to remove excess free radiolabel.
5. Add 2 ml solubilization buffer to the dish and scrape cells loose. Transfer suspension to a 15-ml conical tube.

Do not substitute Tween 20 for Triton, as this detergent is not as effective in solubilizing syndecans.

6. Rinse cell layer and scraper sequentially twice with 2 ml solubilization buffer and add these washes to the tube. Incubate 30 min on ice with occasional vigorous shaking.
7. Centrifuge 10 min at $800 \times g$, room temperature.

The supernatant should appear clear. If it is not, continue shaking and incubating until particulates are no longer seen. Proteoglycans (especially syndecans) aggregate readily, and may form large complexes that can be pelleted at higher g force if not solubilized.

8. Decant supernatant into a 100-ml graduated cylinder and dilute 1:5 with solubilization buffer without Triton to reduce detergent to 0.2%.
9. Store at 4°C ; do not freeze. Use for analysis as soon as possible.

ALTERNATE PROTOCOL 1

Isolation of Proteoglycan Pools

Proteoglycans can be found soluble in culture supernatant, incorporated into insoluble matrix, attached to the cell surface, or as transmembrane components, some of which may link between the cytoskeleton and extracellular matrix. Methods presented here are adapted from Woods et al. (1985).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified, 37°C , 5% to 10% CO_2 incubator (depending upon medium) unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

Additional Materials (also see Basic Protocol 1)

Serum-free culture medium
Phosphatidylinositol-specific phospholipase C (PI-PLC; Boehringer Mannheim, Molecular Probes)
Lysis buffer (see recipe) with and without detergent

Isolate conditioned medium pool

1. Wash radiolabeled cultured cell monolayers twice with 20 ml PBS and incubate 1 hour at 37°C with serum-free culture medium containing 0.1 mU PI-PLC.

This releases glycosylphosphatidylinositol (GPI)-linked proteoglycans (Schmidtchen et al., 1990).

2. Decant into a 15-ml conical centrifuge tube. Rinse cell layer twice with 2.5 ml serum-free medium without enzyme and add to tube.
3. Add solid urea to 4 M final, agitate to dissolve, and incubate on ice until used. Concentrate and purify (step 11) as soon as possible (within 24 hr).

Isolate lysate pool

4. Add 2 ml lysis buffer to the dish and incubate 15 min at 37°C.
5. Transfer lysate to a 15-ml conical tube, rinse cell layer twice with 1 ml lysis buffer, and add washes to the tube.

If desired, lysates from up to three dishes can be pooled in a single 15-ml tube, and up to twelve can be combined in a 50-ml tube.

6. Add solid urea to 4 M and stir to dissolve.
7. Dilute 1:1 with lysis buffer without detergent to dilute detergent to 0.1%. Incubate on ice until used. Concentrate and purify (step 11) as soon as possible (within 24 hr).

This is the "lysate" material, which will contain hydrophobic proteoglycans not retained due to linkage to cytoskeleton/matrix, and proteoglycans undergoing degradation or synthesis. Cell layers will lose phase density, but nuclei and stress fibers should remain.

Isolate cytoskeletal/matrix pool

8. Rinse dish three times with 20 ml PBS, 4°C.
9. Add 1 ml solubilization buffer and scrape cell remnants into the solution.
10. Dilute 1:10 with solubilization buffer without detergent to dilute detergent to 0.1%. Incubate on ice until used. Concentrate and purify (step 11) as soon as possible (within 24 hr).

This is the cytoskeletal/matrix fraction, which will contain hydrophobic proteoglycans linked to the cytoskeleton/matrix, as well as matrix proteoglycans.

Concentrate and purify pools

11. Concentrate and purify all three samples by DEAE-Sephacel chromatography (see Basic Protocol 2).

The steps above all contain protease inhibitors in the buffers because cell lysis is occurring. However, it is essential to perform anionic-exchange chromatography directly after isolation of the three pools, or proteolysis will still occur.

Radiolabeling of Proteoglycans with $^{35}\text{SO}_4$ or [^3H]Glucosamine

Because glycosaminoglycan chains (except hyaluronan) are usually highly sulfated, they can be metabolically labeled with $^{35}\text{SO}_4$. This is done by adding 40 $\mu\text{Ci/ml}$ $\text{H}_2^{35}\text{SO}_4$ (1050 to 1600 Ci/mmol; NEN Life Sciences) to the medium when seeding the cells, and allowing the cells to grow until just subconfluent (e.g., 95%). Sulfate-free medium should not be used, as it does not appear to increase incorporation of radiolabel, perhaps because sulfate becomes limiting. Streptomycin sulfate should not be used as an antibiotic in the medium, as this increases the level of unlabeled sulfate. If supernatant is not needed for analysis, it can be decanted at cell harvest and reused for labeling additional cells.

SUPPORT PROTOCOL

Extracellular Matrix

10.7.3

Glucosamine derivatives are readily incorporated into glycosaminoglycans, so [³H]glucosamine (40 to 60 Ci/mmol; Amersham) can be used to radiolabel proteoglycans and hyaluronan. This is done by adding 10 μCi/ml [³H]glucosamine at seeding and then growing cells to confluency.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified, 37°C, 5% to 10% CO₂ (depending upon medium) incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

Anion-Exchange Chromatography Purification of Proteoglycans with DEAE-Sephacel

Anion-exchange chromatography is used to purify proteoglycans from conditioned medium or cells (Basic Protocol 1) or the isolated subcellular fractions (Alternate Protocol 1). Due to the high negative charge of the glycosaminoglycan chains, proteoglycans bind with higher affinity to DEAE than do most other cellular components.

Materials

Proteoglycan samples (see Basic Protocol 1 or Alternate Protocol 1)
Diethylaminoethyl (DEAE)–Sephacel: 50:50 (v/v) slurry in PBS
PBS (*APPENDIX 2A*)
DEAE-Sephacel equilibration buffer (see recipe)
Conditioned medium from cell line of interest, incubated with cells 24 to 48 hr
DEAE low-pH buffer (see recipe)
DEAE elution buffer (see recipe)
2 × 0.5–cm minicolumns

1. Prepare DEAE-Sephacel in 2 × 0.5–cm minicolumns with ~2 to 3 ml gel bed by adding a 50:50 slurry of DEAE-Sephacel in PBS.

This is sufficient for isolation of proteoglycans from the equivalent of five 75-cm² tissue culture flasks. For larger amounts of material—e.g., from 0.5 to 10 liters of culture supernatants—use 5 to 10 ml gel volume per liter.

2. Attach inlet tubing to the top of the column. Place tubing in a PBS container and wash column with 10 column vol PBS using gravity or a pump at 16 ml/hr.

An economic system for minicolumns is to place two syringe needles (18-G) through a rubber bung. Break one needle at the metal/plastic junction and attach tubing. Place a syringe on the other needle and use this to remove air and pull liquid down the tubing into the column. For smaller volumes, a reservoir can be inserted at the column mouth.

3. Equilibrate the column with 2 column vol DEAE-Sephacel equilibration buffer.
4. Condition the column by loading 20 ml conditioned medium from the cell line being used onto the column as in step 2, using a reservoir or tubing from a suitable container.
5. Wash the column twice with 5 ml each DEAE-Sephacel equilibration buffer.
6. Wash the column twice with 5 ml each DEAE low-pH buffer.

Since this step removes most material other than proteoglycans, a less stringent protease inhibitor cocktail (i.e., lacking ε-aminocaproic acid and benzamidine-Cl) can be used in the low-pH and elution buffers.

7. Wash the column twice with 5 ml each DEAE elution buffer.

8. Reequilibrate as in step 3.
9. Load the proteoglycan sample onto the column. If a large volume is to be used, perform at 4°C.
10. Repeat washes in steps 5 and 6.
11. Elute the column with ten 1-ml aliquots of DEAE elution buffer.

Typically the proteoglycans begin to elute after 0.5 ml and are fully eluted by 10 ml. A solution of 4 M guanidinium-Cl can be used instead of high salt if the proteoglycan is to be concentrated by ethanol precipitation. If so, urea, salts, EDTA, NEM, and PMSF should be removed by washing with DEAE low-pH buffer without these ingredients.

12. Monitor elution by radiolabel if metabolically labeled, or by dye-binding assay (see unit introduction) if not. Combine fractions containing proteoglycans and store at 4°C. Do not freeze.

DEAE-Sephacel columns can be reused indefinitely. The top 1 to 2 mm of gel can be discarded before reuse if it becomes heavily contaminated (grey in color). Columns should be stored upright at 4°C in equilibration buffer containing 0.02% (w/v) sodium azide. Rinse columns with two volumes equilibration buffer prior to use. If stored long-term (6 months), recondition as in steps 4 to 8 prior to use.

Isolated proteoglycans can be dialyzed into new buffers or concentrated by precipitation and resuspended in the buffer of choice. For details, see Basic Protocol 4, steps 1a and 1b.

Isolation of Hydrophobic Proteoglycans

This protocol is used to specifically isolate membrane-intercalated proteoglycans by virtue of their hydrophobic properties. It is used after Basic Protocol 2, which isolated all proteoglycans irrespective of hydrophobicity/hydrophilicity. Hydrophobic proteoglycans from Basic Protocol 2 have Triton associated with their core proteins. In the first part of this procedure, a cholate-containing buffer is used on the DEAE-Sephacel column (from Basic Protocol 2) to exchange Triton for cholate. This weaker detergent then allows binding of the hydrophobic proteoglycans to a purifying column of octyl-Sepharose.

Additional Materials (also see Basic Protocol 2)

- DEAE-Sephacel eluate and column, *or* column loaded for elution (see Basic Protocol 2)
- DEAE low-pH buffer (see recipe) without NaCl
- Hydrophobic chromatography equilibration buffer (see recipe)
- Hydrophobic chromatography exchange buffer (see recipe)
- Hydrophobic chromatography washing buffer (see recipe)
- Hydrophobic chromatography elution buffer (see recipe)
- Octyl-Sepharose (Pharmacia): 50:50 (v/v) slurry in PBS containing 0.1% Triton X-100
- 0.1% (v/v) Triton X-100
- Octyl-Sepharose equilibration buffer (see recipe) with and without NaCl
- Octyl-Sepharose washing buffer (see recipe)
- Octyl-Sepharose elution buffer I (see recipe)
- Octyl-Sepharose elution buffer II (see recipe)
- Minicolumn

Exchange detergent

1. Dilute DEAE-Sephacel eluate 1:10 (v/v) with DEAE low-pH buffer without NaCl to give a final NaCl concentration of 0.2 M.

ALTERNATE PROTOCOL 2

2. Reequilibrate the DEAE-Sepharose column with 2 column vol hydrophobic chromatography equilibration buffer.

The column is reequilibrated to increase the pH, and thus prevent precipitation of cholate.

3. Apply diluted eluate to the reequilibrated column.

Alternately, start exchange while proteoglycans remain on DEAE-Sepharose (following step 10 of Basic Protocol 2). Rinse the column with hydrophobic chromatography equilibration buffer to increase pH, and then proceed with step 4 below.

4. Exchange proteoglycan-bound Triton with cholate by slowly passing 100 ml hydrophobic chromatography exchange buffer over the column.

This is a slow process of exchange and is best achieved using gravity rather than a pump. The column will turn a brown color due to bound detergent.

5. Wash column with 10 ml hydrophobic chromatography washing buffer.

6. Elute with 20 ml hydrophobic chromatography elution buffer, collecting 1-ml fractions.

7. Monitor fractions by counts or dye binding (see unit introduction), and combine fractions that contain proteoglycans.

The most concentrated fractions will be brown. These are typically fractions 1 to 5.

Purify hydrophobic proteoglycans

8. Prepare a minicolumn of octyl-Sepharose with a bed volume of 3 to 4 ml by adding 6 to 8 ml of 50:50 slurry in PBS containing 0.1% Triton X-100.

9. Wash with 20 ml of 0.1% Triton X-100 to remove ethanol, and then with 100 ml octyl-Sepharose equilibration buffer to remove Triton.

This column is sufficient for analysis or isolation of 10,000 to 100,000 cpm hydrophobic proteoglycans from lysate or cytoskeletal/matrix pool (Alternate Protocol 1) purified by DEAE-Sepharose (Basic Protocol 2). Individual eluate fractions or pooled fractions may be used.

10. Dilute combined fractions (step 7) to 0.6 M NaCl with octyl-Sepharose equilibration buffer without NaCl, and add to octyl-Sepharose column. Incubate overnight at 4°C.

The most efficient binding is achieved by allowing liquid above the column to drain, then adding 0.5 to 1.0 ml sample and allowing this to drain into the column before closing the column.

11. Wash column with twenty 1-ml aliquots of octyl-Sepharose washing buffer to remove cholate.

12. Wash column with twenty 1-ml aliquots of octyl-Sepharose elution buffer I to remove any material bound by ionic interactions.

13. Elute hydrophobic proteoglycans with twenty 1-ml aliquots of octyl-Sepharose elution buffer II, collecting 1-ml fractions.

During this procedure the octyl-Sepharose will swell and shrink. This is normal.

Following concentration by hydrophobic chromatography, proteoglycans may be highly aggregated. This is not reversed by boiling.

14. Use as soon as possible.

If necessary, samples can be stored at 4°C, but not for more than 2 to 3 days. Samples should not be frozen.

ANALYSIS OF PROTEOGLYCANS

Analysis of proteoglycans can be very complex. This group of protocols contains methods for analyzing the size and composition of proteoglycans isolated from cultured cells.

Analysis of Proteoglycans by Size-Exclusion Chromatography

Since proteoglycans are large and bear highly charged glycosaminoglycan chains, they often do not enter SDS-PAGE gels. One method of analysis is size-exclusion chromatography on Sepharose 4B (also see *UNIT 5.5*; Table 5.5.2). This procedure can be used for samples from Basic Protocol 2 or Alternate Protocol 2, or following immunoprecipitation (Basic Protocol 10). It is not suitable for unpurified samples (Basic Protocol 1 or Alternate Protocol 1), as high levels of unincorporated radiolabel may present a containment problem, and radiolabeled components other than proteoglycans will complicate the results obtained.

Materials

- Sepharose 4B (Pharmacia)
- SEC running buffer (see recipe)
- Proteoglycan sample (see above)
- Stock dye solution: 1 mg/ml each blue dextran (Sigma) and *N*-2,4-dinitrophenyl (DNP)-alanine (Sigma)
- 90 × 1-cm column
- Column pump

1. Prepare a 90 × 1-cm column containing a 70-ml bed volume of Sepharose 4B according to the manufacturer's instructions. Pack the column at 9 ml/hr with SEC running buffer.
2. Decrease the rate to 6 ml/hr.

This rate should be used for analysis to prevent collapse of the column.

3. Remove cap from the column and allow liquid to flow until the top of the gel is just dry. Add a maximum of 1 ml proteoglycan sample to be analyzed.

For analytical purposes 8,000 to 10,000 cpm should be used.

4. Allow sample to enter gel, then rinse the top of the gel by adding 2 ml SEC running buffer and directly removing it.

Failure to rinse will result in smearing of sample through the gel.

5. Replace cap and run column pump at 6 ml/hr. Collect 80 1-ml fractions and monitor content by radiolabel or dye-binding assay (see unit introduction).

6. Determine void volume (V_0 ; excluded volume) and total volume (V_t ; total included volume) either by coelution (200 μ l mixed dye in 800 μ l sample) of a mixture of blue dextran (high molecular weight; totally excluded) and DNP-alanine (low molecular weight; totally included), or based on comparison with a previous run containing these dyes.

V_0 is the volume at which blue dextran elutes (peak of blue color), and V_t is the volume at which DNP-alanine elutes (last fraction containing yellow color).

*See *UNIT 5.5* for details on calibration with markers from a separate run.*

7. Determine relative elution (K_{av}) using the equation $K_{av} = (V_s - V_0)/(V_t - V_0)$, where V_s is the elution volume of fractions where proteoglycans have peaks.

**BASIC
PROTOCOL 3**

**Extracellular
Matrix**

10.7.7

If HPLC equipment is available, a TSK 4000 column (TosoHaas) can be used to monitor proteoglycans. However, losses can be large if a prefilter is used, since many proteoglycans will tend to bind nonspecifically to the prefilter.

**BASIC
PROTOCOL 4**

Analysis of Glycosaminoglycan Size Following Alkaline Elimination

Free glycosaminoglycan (GAG) chains can be obtained by alkaline elimination (here) or treatment with papain (see Basic Protocol 5) to destroy the core protein. Alkaline elimination removes all protein, leaving free intact glycosaminoglycans.

Materials

Proteoglycan sample (see Alternate Protocol 2 or obtain by immunoprecipitation)
Ethanol (optional)
1 M NaOH/2 M NaBH₄
10 M acetic acid
Dialysis tubing, MWCO 12-12,000 (optional)

- 1a. *For dialysis:* Transfer proteoglycan sample to distilled water by dialyzing against three changes of 100 vol water over 24 hr at 4°C. Use dialysis tubing with a MWCO of 12-12,000.
- 1b. *For precipitation:* Precipitate proteoglycan sample with 4 vol of 80% ethanol at -20°C, wash with 80% ethanol, and air dry. Resuspend in 0 to 5 ml distilled water.
2. Add an equal volume of 1 M NaOH/2 M NaBH₄. Incubate 16 hr at 4°C.
3. Neutralize with 10 M acetic acid, checking with pH paper until pH ≈ 7.
4. Reprecipitate with ethanol, if required to reconcentrate. Solubilize in water.
5. Analyze GAG size directly by size-exclusion chromatography (see Basic Protocol 3; UNIT 5.5) or HPLC.

NOTE: If samples are not neutralized (step 3), analytical columns will be ruined.

**BASIC
PROTOCOL 5**

Analysis of Glycosaminoglycan Size Following Papain Digestion

Papain digestion degrades protein cores, leaving intact glycosaminoglycans. It requires no neutralization step.

Materials

Proteoglycan sample (see Alternate Protocol 2 or obtain by immunoprecipitation)
Papain digestion buffer (see recipe)
20 mg/ml papain (Sigma)
65°C water bath

Additional reagents and equipment for dialysis or ethanol precipitation (see Basic Protocol 4)

1. Transfer proteoglycan sample to papain digestion buffer (see Basic Protocol 4, step 1a or 1b).
2. Incubate with 2 mg/ml papain for 16 hr at 65°C.
3. Analyze GAG size directly by size-exclusion chromatography (see Basic Protocol 3; UNIT 5.5) or HPLC.

No enzyme inactivation is required.

Analysis of Glycosaminoglycan Content and Protein Core by GAG Degradation with Lyases

This analysis relies on the use of enzymes to selectively degrade glycosaminoglycans. Heparinase III will degrade heparan sulfate glycosaminoglycans. Chondroitinase ABC lyase will degrade all forms of chondroitin and dermatan sulfate glycosaminoglycans. Chondroitinase ACII lyase will degrade chondroitin but not dermatan sulfate glycosaminoglycans. Chondroitin B lyase will degrade dermatan sulfate. Each enzyme has different requirements and may not have maximum activity in the same buffer, but the heparinase III buffer listed below appears to work sufficiently well for most analyses if only core proteins are to be analyzed. Ovomuroid (Sigma) may be added at 100 µg/ml to prevent proteolysis of the protein core. These core treatments leave an intact case protein (unlike Basic Protocols 4 and 5), which can then be analyzed.

Materials

- Proteoglycan sample (see Alternate Protocol 2 or obtain by immunoprecipitation)
- Chondroitinase buffer (see recipe)
- Heparinase III buffer (see recipe)
- Chondroitin sulfate type C or B (chondroitin 6-sulfate or dermatan sulfate; Sigma)
- Chondroitinase ABC (EC 4.2.2.4), ACII (EC 4.2.2.5), or B (no EC number) (Seikagaku America)
- Heparinase III (a.k.a. heparitinase or heparitinase I; EC 4.2.2.8; Seikagaku America)
- Additional reagents and equipment for dialysis or ethanol precipitation (see Basic Protocol 4)

For treatment with chondroitinases:

- 1a. Transfer proteoglycan sample to chondroitinase buffer (see Basic Protocol 4, step 1a or 1b).

Sample volumes depend on the proteoglycan concentration in the sample and the future method of analysis. For immunoblotting, a minimum volume is needed (i.e., resuspend precipitated sample from Basic Protocol 4, step 1b, in 50 to 200 µl of appropriate buffer). For SEC or HPLC, use 0.1 to 0.5 ml (≥2000 cpm if radiolabeled).

Since chemical amounts of isolated proteoglycans are usually small (µg levels), the amounts of carrier and enzyme do not need to be varied.

If performing dual digests with chondroitinases and heparinase, use heparinase III buffer. Chondroitinase B is inhibited by phosphate.

- 2a. Add ~2 µg chondroitin sulfate type C as carrier for chondroitinase ABC or ACII, or type B for chondroitinase B.

This increases the efficiency of digestion and recovery if low amounts of material (e.g., radiolabeled preparations) are used.

- 3a. Add 0.5 to 1 mU chondroitinase ABC, ACII, or B and incubate at 37°C.

Incubation times depend on the amount of proteoglycan present. For up to ~10 µg proteoglycan, an incubation of 2 to 3 hr should suffice; where amounts are higher, adding a second aliquot of enzyme for a further 2 hr may be needed.

- 4a. Monitor glycosaminoglycan digestion by size-exclusion chromatography (see Basic Protocol 3) or HPLC (degraded material will now elute in the V_1), or by immunoblotting (see Basic Protocol 8).

For treatment with heparinase III:

- 1b. Transfer proteoglycan sample to heparinase III buffer (see Basic Protocol 4, step 1a or 1b).

Sample volumes depend on the proteoglycan concentration in the sample and the future method of analysis. For immunoblotting, a minimum volume is needed (i.e., resuspend precipitated sample from Basic Protocol 4, step 1b, in 50 to 200 μ l of appropriate buffer). For SEC or HPLC, use 0.1 to 0.5 ml (≥ 2000 cpm if radiolabeled).

Since chemical amounts of isolated proteoglycans are usually small (μ g levels), the amounts of carrier and enzyme do not need to be varied.

- 2b. Add 2 to 5 μ g heparan sulfate as carrier if low amounts of proteoglycan are present.

Do not add heparan sulfate if also digesting with chondroitinases. The products of heparan sulfate lyases are slightly inhibitory to chondroitinases. Dual digestions may, therefore, not be as efficient as separate incubations.

- 3b. Add 1 to 2 mU heparinase III and incubate at 37°C.

Time of incubation varies as indicated for chondroitinase treatment.

- 4b. Monitor glycosaminoglycan digestion by size-exclusion chromatography (see Basic Protocol 3) or HPLC (degraded material will now elute in the V_1), or by immunoblotting (see Basic Protocol 8).

BASIC PROTOCOL 7

Treatment with Nitrous Acid to Degrade Heparan Sulfate

Nitrous acid will degrade heparan sulfate glycosaminoglycans.

CAUTION: This procedure must be performed in a chemical fume hood, since nitrosamines are released.

Materials

1 N H_2SO_4
0.114 g/ml $\text{Ba}(\text{NO}_2)_2$
Proteoglycan sample (see Alternate Protocol 2 or obtain by immunoprecipitation)
10 N NaOH

1. Mix 1 ml of 1 N H_2SO_4 (0.5 mmol) with 1 ml of 0.114 g/ml $\text{Ba}(\text{NO}_2)_2$ on ice for 10 min, shaking occasionally.
2. Remove BaSO_4 precipitate by centrifuging 2 min at $500 \times g$, 4°C. Save the supernatant.

The supernatant is nitrous acid at pH 1.5. This is stable for a few hours on ice.

3. Mix sample and nitrous acid at a 1:4 (v/v) ratio and leave 2 hr on ice.

The proteoglycan sample should be unbuffered, preferably in water.

4. Neutralize with 10 N NaOH to a pH of ≈ 7 , checking with pH paper.
5. Monitor heparan sulfate digestion by size-exclusion chromatography (see Basic Protocol 3) or HPLC (degraded material will now elute in the V_1), or by immunoblotting (see Basic Protocol 8).

NOTE: *If samples are not neutralized (step 4), analytical columns will be ruined.*

Analysis of GAG Type and Core Protein

SDS-PAGE (UNIT 6.1) and immunoblotting (UNIT 6.2) using standard procedures can be used to analyze GAG type and identify the core protein. These procedures can be used for analysis of whole cells or conditioned medium without proteoglycan isolation and purification, or they can be used on purified proteoglycans. Commercial antibodies are available for detection of carbohydrate “stubs” created on protein cores by lyase treatment. Monoclonal antibodies are available (Seikagaku America) against digested heparan sulfate (Δ heparan sulfate) and digested GAGs that had contained chondroitin sulfate that was nonsulfated (Δ di-0S) or was sulfated at the 4 or 6 position on galactosamine residues (Δ di-4S or Δ di-6S). These will detect all protein cores that had these specific saccharides. Specific proteoglycans can also be detected by core protein-specific antibodies. The appearance of a discrete polypeptide by SDS-PAGE analysis, representing the core protein, with a concomitant reduction in a high-molecular-weight smear characteristic of intact proteoglycans following treatment with specific lyases, determines which type of GAG was present on the protein core.

Nitrocellulose membranes may be used for immunoblotting, but intact proteoglycans will transfer poorly. Since this method relies on resolution of deglycanated core proteins following enzyme digestions, poor transfer of intact proteoglycan may not be important. Cationic membranes can, however, be used to capture intact proteoglycans more efficiently.

Size-exclusion chromatography of radiolabeled proteoglycans can also be used to analyze the composition of proteoglycans. Intact GAGs will elute in the V_0 of a Sephadex G-50 column, whereas disaccharides released by enzyme treatment will elute at or close to the V_t . Differential treatment susceptibility indicates the type (and relative amounts) of GAG in the sample. If HPLC is available, a TSK 4000 HPLC column (TosoHaas) column may be used.

Analysis of GAG Size

This is most easily performed using HPLC, but Sephadex G-200, Sepharose CL-6B, or equivalent resins may be used if HPLC is not available.

Materials

- HPLC running buffer (see recipe)
- Proteoglycan sample, enzymatically or chemically treated (see Basic Protocols 4 and 5)
- DEAE-Sephacel column (see Basic Protocol 2)
- PD-10 gel-filtration column (Amersham Pharmacia Biotech)
- High-performance liquid chromatograph (HPLC) with TSK 4000 column (TosoHaas)

1. Isolate intact GAG chains from intact proteoglycans following treatments as above (see Basic Protocols 4 and 5), using anion-exchange chromatography on DEAE-Sephacel columns (see Basic Protocol 2).
2. Desalt samples on a PD-10 gel-filtration column according to manufacturer's instructions, using distilled water as buffer, and lyophilize.
3. Equilibrate a TSK 4000 HPLC column with HPLC running buffer.
4. Solubilize sample in HPLC running buffer. Load 100 to 500 μ l sample and elute at a flow rate of 0.5 ml/min. Collect 0.5-ml fractions.

Isolated glycosaminoglycans do not bind the prefilter.

5. Assay fractions for radioactivity or dye binding (see unit introduction).

An alternate HPLC running buffer (see recipe) containing 4 M guanidium·Cl may be used to prevent self-association of GAGs.

GAG standards of known size should be used, since globular proteins are inappropriate for calibration.

Immunoprecipitation of Proteoglycans

Although some matrix proteoglycans can be immunoprecipitated by standard methods (UNIT 7.2), there are some specific problems. First, hydrophobic proteoglycans can be a problem, especially if analysis of associated components is required (Oh et al., 1997). The type of detergent used may result in differences in co-immunoprecipitation, as seen in studies with other membrane receptors (Serru et al., 1999). Second, GAG chains may limit accessibility of antibodies against core proteins. Use of a primary and secondary antibody, followed by immobilized protein A, may improve yield. Alternatively, GAG chains may be removed enzymatically prior to immunoprecipitation. Some proteoglycans may aggregate after GAG removal, resulting in precipitation.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Alternate HPLC running buffer

382.12 g guanidine·Cl (4 M final)
6.05 g Tris base (50 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 6.0 with 1 M HCl
Add 5 ml Triton X-100 (0.5% v/v final)
Add H₂O to 1 liter
Store up to 1 month at 4°C

Chondroitinase buffer

6.05 g Tris acetate (50 mM final)
4.08 g sodium acetate (30 mM final)
0.1 g bovine serum albumin (BSA; 0.1 mg/ml final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 8.0 with 1 M acetic acid
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 1 ml Triton X-100 (for hydrophobic proteoglycans; 0.1% v/v final)
Stir slowly until Triton is dissolved
Add H₂O to 1 liter
Store up to 1 week at 4°C

DEAE elution buffer

87.66 NaCl (1.5 M final)
4.102 g sodium acetate (50 mM final)
240 g urea (4 M final)

1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 4.0 with 1 M acetic acid
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 1 ml Triton X-100 (0.1% v/v final)
Stir slowly until Triton is dissolved
Add H₂O to 1 liter
Store up to 1 week at 4°C

DEAE low-pH buffer

11.69 g NaCl (0.2 M final)
4.102 g sodium acetate (50 mM final)
240 g urea (4 M final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 4.0 with 1 M acetic acid
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 1 ml Triton X-100 (0.1% v/v final)
Stir slowly until Triton is dissolved
Add H₂O to 1 liter
Store up to 1 week at 4°C

DEAE-Sephacel equilibration buffer

11.69 g NaCl (0.2 M final)
50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
240 g urea (4 M final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
0.783 g benzamidine·Cl (5 mM final)
13.12 g ε-aminocaproic acid (0.1 M final)
7.44 g EDTA (20 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 8.0 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 1 ml Triton X-100 (0.1% v/v final)
Stir slowly until Triton is dissolved
Add H₂O to 1 liter
Store up to 1 week at 4°C

Heparinase III buffer

1.36 g sodium acetate (0.1 M final)
0.000158 g calcium acetate (0.01 mM final)
1 g ovalbumin (1% w/v final)
0.005 g leupeptin (50 μg/ml final)
0.0157 g benzamidine·Cl (1 mM final)
H₂O to 90 ml

continued

**Extracellular
Matrix**

10.7.13

Adjust pH to 7.0 with 1 M acetic acid
Add 100 μ l 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 100 μ l Triton X-100 (for hydrophobic proteoglycans; 0.1% v/v final)
Stir slowly until Triton is dissolved
Add H₂O to 100 ml
Store up to 2 days at 4°C

HPLC running buffer

13.6 g KH₂PO₄ buffer, pH 6.0 (0.1 M final)
29.2 g NaCl (0.5 M final)
2 g Zwittergent (Aldrich; 0.2% w/v final)
H₂O to 1 liter
Store up to 1 month at 4°C

Hydrophobic chromatography elution buffer

4 g sodium cholate (0.4% w/v final)
175.2 g NaCl (3 M final)
2.422 g Tris base (20 mM final)
1.25 *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H₂O to 1 liter
Store up to 1 week at 4°C

Hydrophobic chromatography equilibration buffer

5.84 g NaCl (0.1 M final)
2.422 g Tris base (20 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H₂O to 1 liter
Store up to 1 week at 4°C

Hydrophobic chromatography exchange buffer

20 g sodium cholate (2% w/v final)
2.422 g Tris base (20 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H₂O to 1 liter
Store up to 1 week at 4°C

Hydrophobic chromatography washing buffer

4 g sodium cholate (0.4% w/v final)
2.422 g Tris base (20 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H₂O to 1 liter
Store up to 1 week at 4°C

Lysis buffer

3.03 g Tris base (25 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
0.783 g benzamidinium-Cl (5 mM final)
13.12 g ε-aminocaproic acid (0.1 M final)
7.44 g EDTA (20 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 7.5 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 2 ml Triton X-100 (0.2% v/v final)
Stir slowly to dissolve Triton
Add H₂O to 1 liter
Prepare fresh just prior to use

Octyl-Sepharose elution buffer I

Prepare as for octyl-Sepharose washing buffer (see recipe), but adjust NaCl to 3 M final.

Octyl-Sepharose elution buffer II

175.2 g NaCl (3 M final)
2.422 g Tris base (20 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add 10 ml Triton X-100 (1% v/v final)
Stir slowly to dissolve Triton
Add H₂O to 1 liter
Store up to 1 week at 4°C

Octyl-Sepharose equilibration buffer

4 g sodium cholate (0.4% w/v final)
35.04 NaCl (0.6 M final)
2.422 g Tris base (20 mM final)
1.25 g *N*-ethylmaleimide (10 mM final)

continued

**Extracellular
Matrix**

10.7.15

7.44 g EDTA (20 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 20 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H₂O to 1 liter
Store up to 1 week at 4°C

Octyl-Sepharose washing buffer

5.84 g NaCl (0.1 M final)
2.422 g Tris base (20 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 7.3 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H₂O to 1 liter
Store up to 1 week at 4°C

Papain digestion buffer

11.69 g NaCl (2 M final)
0.744 g EDTA (10 mM final)
0.4102 g sodium acetate (50 mM final)
0.0175 g cysteine·Cl (0.01 M final)
H₂O to 90 ml
Adjust pH to 5.5 with 1 M acetic acid
H₂O to 100 ml
Prepare fresh just prior to use

Size-exclusion chromatography (SEC) running buffer

1 g SDS (0.1% w/v final)
20.47 g NaCl (0.35 M final)
6.06 g Tris base (50 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 8.0 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
Add H₂O to 1 liter
Store up to 2 weeks at room temperature

Solubilization buffer

10 ml Triton X-100 (1% v/v final)
240 g urea (4 M final)
3.03 g Tris base (25 mM final)
1.25 g *N*-ethylmaleimide (NEM; 10 mM final)
7.44 g EDTA (20 mM final)
0.783 g benzamidine·Cl (5 mM final)

13.12 g ϵ -aminocaproic acid (0.1 M final)
H₂O to 900 ml
Heat to dissolve
Adjust to pH 7.5 with 1 M HCl
Add 1 ml 0.2 M phenylmethylsulfonyl fluoride (PMSF) in ethanol while rapidly vortexing to prevent precipitation (0.2 mM final)
H₂O to 1 liter
Store up to 1 day at 4°C

COMMENTARY

Background Information

The analysis of proteoglycans has always been a specialized field due to the complexity of these molecules and their interactions with other molecules. Until relatively recently, it was only possible to isolate proteoglycans via the binding of their glycosaminoglycan chains to cationic matrices. Now, as core proteins are identified and characterized, antibody affinity chromatography can be used to purify individual proteoglycan species for analysis. However, analysis of glycosaminoglycans attached to the individual core proteins is still a tedious task, because the biological activity may depend on the fine structure of the glycosaminoglycan chains as well as the distinct core proteins. The glycosaminoglycan chain type can be readily identified by the use of selective enzyme degradation as detailed here. However, determining the fine structure of glycosaminoglycan chains—particularly the patterns of epimerization and sulfation of, for example, heparan sulfate chains—relies on techniques that are beyond the scope of this unit. For information on this, the reader should consult Vives et al. (1999).

Interest in proteoglycans, particularly cell-surface proteoglycans, has grown recently. This is due in part to the discoveries of the roles of cell-surface proteoglycans in signaling as co-receptors with integrins in adhesion, and with growth factors in cell cycle regulation (reviewed in Woods and Couchman, 1998; Bernfield et al., 1999; also see *UNIT 10.1*). It is often daunting for new researchers to enter the proteoglycan field, and it is hoped that these techniques will help.

Critical Parameters

The core proteins of proteoglycans are susceptible to proteolysis, but freezing often results in insoluble complexes. It is therefore important to design experiments so that isolation, purification, and analysis proceed as rapidly as possible. In addition, once the gly-

cosaminoglycans are removed from the core protein, the core proteins aggregate, especially at high concentration or if hydrophobic. Again, speed is of the essence.

Troubleshooting

Core protein aggregation, particularly of the syndecans, results in SDS-resistant multimers, which are noncovalent. This results in several bands migrating more slowly on SDS-polyacrylamide gels than expected based on molecular weight. This has now been documented in several studies but often needs to be explained in manuscripts. It is not due to disulfide bonding; it appears to be an inherent capacity of the core proteins.

The enzymes used to degrade glycosaminoglycans are susceptible to inactivation and have a limited shelf life once they are hydrated. Where they are used infrequently, enzymes can be hydrated, divided into aliquots, and freeze dried. If a negative result is seen, the particular enzyme preparation should be checked for activity under the experimental conditions used. Manufacturer's data sheets explain this procedure.

Anticipated Results

Basic Protocol 1. Yield cannot be quantified since nonincorporated radiolabel is present. After Basic Protocol 2, the supernatant fraction will yield the highest amount of labeled materials ($\sim 4 \times 10^6$ cpm), with the cell-associated materials yielding 1×10^6 cpm. These results are typical of those obtained using rat embryonic fibroblasts (confluent monolayers from five 10-cm dishes).

Alternate Protocol 1. Yield cannot be quantified since nonincorporated radiolabel is present. After Basic Protocol 2, the supernatant fraction will yield the highest amount of labeled materials ($\sim 4 \times 10^6$ cpm), with the lysate yielding somewhat less ($\sim 950,000$ cpm), and the cytoskeleton/matrix yielding the least ($\sim 150,000$ cpm). These results are typical of

those obtained using rat embryonic fibroblasts (confluent monolayers from five 10-cm dishes).

Support Protocols 1 and 2. Each cell type synthesizes its own spectrum of proteoglycans in varying amounts. For fibroblasts, total incorporation into proteoglycans can be expected to be $5\text{--}6 \times 10^6$ cpm/5 dishes.

Basic Protocol 2. Anticipated yields from this procedure are listed under Basic Protocol 1 and Alternate Protocol 1 above.

Alternate Protocol 2. Little material should be lost on reapplication of isolated proteoglycans to DEAE-Sephacel and elution. If losses do occur, the column should be reconditioned with medium before use. Yields of the different fractions will differ with cell type. Typically, using rat embryonic fibroblasts, 40% to 60% of lysate proteoglycans and 20% to 40% of cytoskeleton/matrix proteoglycan pools, respectively, elute from octyl-Sepharose with detergent buffer.

Basic Protocol 3. It is possible to resolve a 1-ml sample of proteoglycan into three to six species by size-exclusion chromatography, depending upon the cell type.

Basic Protocol 4. Alkaline elimination quantitatively separates glycosaminoglycans from the core protein.

Basic Protocol 5. Treatment of proteoglycans with papain degrades the core protein and releases free glycosaminoglycans.

Basic Protocol 6. Treatment of proteoglycans with lyases followed by immunoblotting allows identification of three to six core proteins, depending on the species produced by the cell line.

Basic Protocol 7. Treatment of proteoglycans with nitrous acid degrades heparan sulfate to low-molecular-weight products.

Basic Protocol 8. Immunoblotting allows detection of which glycosaminoglycans are bound to specific proteoglycan protein cores.

Basic Protocol 9. HPLC analysis allows determination of GAG size.

Basic Protocol 10. Specific proteoglycans and protein cores can be immunoprecipitated from cell preparations, but yield is often low.

Time Considerations

Basic Protocol 1 and Alternate Protocol 1. The whole procedure should be completed in <30 min to keep proteolysis at a minimum. Once the samples have been applied to DEAE-Sephacel and the column is washed with low-pH buffer, proteolytic risk is reduced.

Support Protocol 1. Addition of radiolabel requires 15 min, but 3 to 4 days may be needed for growth to confluency.

Basic Protocol 2. The time needed to apply the extract to the DEAE column will depend on the volume of extract. Samples should not be loaded at speeds >16 ml/hr. Washing and elution each take ~30 min. A convenient stopping point, if needed, is after the second wash (step 6).

Alternate Protocol 2. The time taken to (re)load proteoglycans onto DEAE-Sephacel will depend on the volume to be loaded. Rates >16 ml/hr should not be used. Exchange to cholate detergent is most conveniently performed overnight. Loading onto octyl-Sepharose requires overnight incubation, and washing and elution of the column requires ~3 hr.

Basic Protocol 3. Each size-exclusion chromatography run takes ~16 hr. Several columns can be run simultaneously if multichannel pumps are used.

Basic Protocol 4. Alkaline elimination requires 16 hr, with 5 to 10 min for neutralization.

Basic Protocol 5. Papain digestion requires 16 hr.

Basic Protocol 6. Incubation times depend on the amount of proteoglycan present. For up to 10 μg proteoglycan, 2 to 3 hr should suffice. Where larger amounts are present, addition of enzymes for a further 2 hr may be needed.

Basic Protocol 7. Treatment with nitrous acid requires 2.5 hr.

Basic Protocol 8. Enzyme treatment requires 2.5 hr. SDS-PAGE, transfer, and immunoblotting require 24 hr.

Basic Protocol 9. HPLC analysis requires ~4 hr, while SEC requires 16 to 18 hr.

Basic Protocol 10. Immunoprecipitation requires ~4 hr.

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Internet Resources

www.glycoforum.gr.jp

A Web site supported by Seikagaku Corp. that contains articles on glycoscience, including proteoglycans and links to other sites.

Key References

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Contributed by Anne Woods and
John R. Couchman
University of Alabama at Birmingham
Birmingham, Alabama

Matrix Metalloproteinases

UNIT 10.8

This unit describes a set of methods that are relatively unique to studies of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs, $\alpha 2M$), including cell-mediated dissolution of type I collagen fibrils (see Basic Protocol 1), direct and reverse zymography (see Basic Protocols 2 and 3), enzyme capture techniques based on $\alpha 2$ -macroglobulin ($\alpha 2M$) and TIMP-1, and -2 (see Basic Protocol 4 and Alternate Protocol), and detection and demonstration of cryptic thiol groups in MMP precursors (see Basic Protocol 5). Support Protocols are included for preparation (see Support Protocol 1) and labeling of collagen (see Support Protocol 2).

DISSOLUTION AND DEGRADATION OF COLLAGEN FIBRILS BY LIVE CELLS

BASIC
PROTOCOL 1

Comparatively few methods allow detailed analysis of how live cells orchestrate MMP and inhibitor functions in the degradation and remodeling of extracellular matrices. The methods described in this protocol were developed to study the function of matrix metalloproteinases (MMPs) in the degradation of type I collagen fibrils by live cells under controlled but readily variable conditions. In its simplest form, cells are seeded on a few-micron-thick film of reconstituted collagen fibrils, then incubated for a period of 1 to 7 days. The progressive dissolution of the film under the cell layer—in response, e.g., to changing environmental conditions, inducing agents, or inhibitors—may be monitored directly and related to the level of expression of key components of the requisite proteolytic machinery. The system is readily manipulated in a number of ways: by induction/repression of transcription of components of the signaling and effector systems; by transfection of new genes of potential importance to the process; or by selective or specific blocking strategies using antisense-, MMP-specific inhibitor-, or antibody-based approaches. The limited susceptibility of type I collagen fibrils to cleavage and dissolution by MMPs permits one to narrow the scope of the investigation to a small number of (“collagenolytic”) enzymes. This characteristic also makes it a realistic objective to dissect the entire sequence or set of reactions involved in cell-mediated dissolution of collagen fibrils, starting from the initial engagement of cell surface receptors by cytokines, growth factors, and other catabolic reagents, through the final enzymatic cleavage, dissolution, and disposal of the substrate. Important questions that may be addressed using this approach include the following:

- a. What enzymes are actually involved in the cleavage reaction itself and in the precursor activation steps?
- b. How do cells regulate the activity of the enzymes?
- c. What role is played by TIMPs in modulating, containing, and blocking the response?
- d. What is the ultimate fate of the collagen chains and peptides generated as a result of proteolysis?

Recent studies have shown that type I collagen (in solution or in reconstituted fibrillar form) may be cleaved by a larger number of enzymes than previously anticipated, including the three classical “collagenases,” MMP-1, MMP-8, and MMP-13 (Birkedal-Hansen et al., 1993; Knäuper et al., 1996). In addition, reports suggest that MMP-14 (Ohuchi et al., 1997) and TIMP-free MMP-2 may also dissolve collagen fibrils at meaningful rates under physiologic conditions (Aimes and Quigley, 1995). It is of note that although the three classical collagenases (MMP-1, MMP-8, and MMP-13) were

Extracellular
Matrix

10.8.1

Contributed by Jack Windsor, Anne Havemose Poulsen, Susan Yamada, Guy Lyons, Bente Birkedal-Hansen, William Stetler-Stevenson, and Henning Birkedal-Hansen

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discovered because of their ability to dissolve reconstituted fibrils of type I collagen, no definitive proof has yet been rendered that cleavage of collagen fibrils is indeed the exclusive or even prevailing biologic function of any of these enzymes. Admittedly, the evidence seems compelling based on a large number of *in vitro* studies.

Earlier versions of this method have been published (Birkedal-Hansen, 1987; Birkedal-Hansen et al., 1989, 1993; Lin et al., 1987). The isolation and purification techniques of type I collagen and the methods for formation of reconstituted hydrated gels of type I collagen have been described elsewhere in detail (Birkedal-Hansen, 1987). The method relies on the ability of neutral solutions of type I collagen in an appropriate concentration range (0.1 to 5 mg/ml) to form hydrated gels of reconstituted fibrils by heating to 37°C. The method also takes advantage of the observation that such loose hydrated gels may be collapsed by gentle air-drying into a thin film of uniform, densely packed, randomly oriented fibrils which remain as highly resistant to proteolysis by enzymes such as trypsin, chymotrypsin, and plasmin as hydrated gels or natural fibrils (Fig. 10.8.1). Trypsin, which is often used as a standard for testing the resistance of collagen fibrils to “unspecific” proteolytic cleavage, is unable to dissolve the collagen fibril films prepared as described. The same is true for a large number of proteinases of all four classes, and it is this unique resistance to proteolysis which renders this assay system particularly valuable as it greatly reduces the number of proteinases that are involved in the cleavage/dissolution reaction.

Several variants of the method may be used. While the authors often prefer (for ease of presentation and interpretation) to seed the cells in a small button in the middle of a much larger dish (35 mm; Fig. 10.8.1A, middle) in order to maintain medium excess, it is also possible to seed the cells over the entire collagen-coated surface, although a confluent monolayer rapidly exhausts the medium. The collagen may be used in its natural state or labeled either with radioactive or fluorescent tags to facilitate monitoring (see Support Protocol 2), retrieval, and quantification of dissolved collagen chains and fragments.

Depending on the casting conditions, collagen films may be generated with a thickness down to 1 to 2 μm , which is approximately the thickness of a single layer of well-spread cells. Most cell types seeded on this film spread within minutes to hours, although often more slowly than on plastic. Cells that express an appropriate complement of MMPs either constitutively or after exposure to cytokines and growth factors (or phorbol ester) progressively dissolve the underlying fibril coating, and, within 24 to 96 hr, clear a path to the

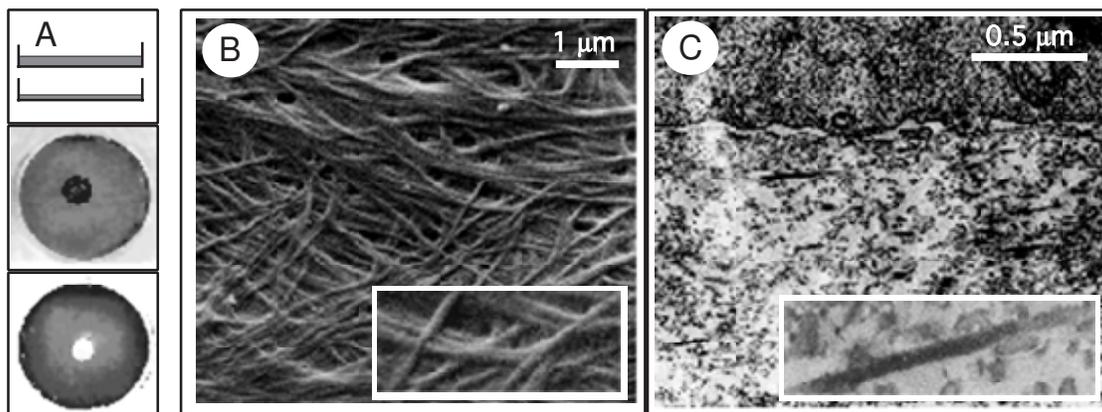


Figure 10.8.1 Reconstituted collagen fibril film. (A) Rat tail tendon type I collagen is polymerized by heat gelation. The gel is air dried and reduced in thickness to a few microns. Cells are seeded in the middle of the plate and incubated with culture medium. After incubation, the cells are removed and a clearing beneath the cell layer is exposed by staining with Coomassie blue. (B) The air-dried collagen fibril film consists of uniform, randomly oriented reconstituted fibrils (C). Detail of cell attached to the collagen fibril film.

10.8.2

plastic surface (Fig. 10.8.1A, lower; Fig. 10.8.2). Coomassie blue staining of the residual collagen fibril film after removal of the cells is usually sufficient to visualize the dissolution of the underlying film (Fig. 10.8.2).

Materials

- 3 mg/ml rat tail tendon type I collagen in 13 mM HCl (see Support Protocol 1)
- 13 mM HCl, 4°C
- Neutralizing buffer (see recipe), 4°C
- Phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ (CMF-PBS; APPENDIX 2A) supplemented with 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate
- Cells of interest (e.g., fibroblasts, keratinocytes, or tumor cells)
- DMEM (APPENDIX 2A) supplemented with 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate with and without 10% (v/v) FBS (or other medium appropriate for cell type)
- Growth factors/cytokines: e.g., IL-1β, TNF-α, TGF-α, or TPA; or phorbol ester (12-O-tetradecanoylphorbol-13-acetate, TPA, or phorbol myristate acetate, PMA)
- 1% (v/v) Triton X-100
- 0.5% (w/v) trypsin/0.53 mM EDTA (Invitrogen)
- Coomassie blue stain (see recipe)
- 6-well cell culture plates

Additional reagents and equipment for trypsinizing and counting cells (UNIT 1.1)

Prepare collagen-coated plates

1. To cast one 6-well plate, dilute 1 ml of 3 mg/ml type I collagen stock solution with 7 ml of 13 mM HCl at 4°C. Mix the collagen solution with 2 ml of cold neutralizing buffer in a precooled test tube either by gently pipetting up and down while avoiding formation of air bubbles (which will form defects in the gel) or by gently inverting the tube several times.

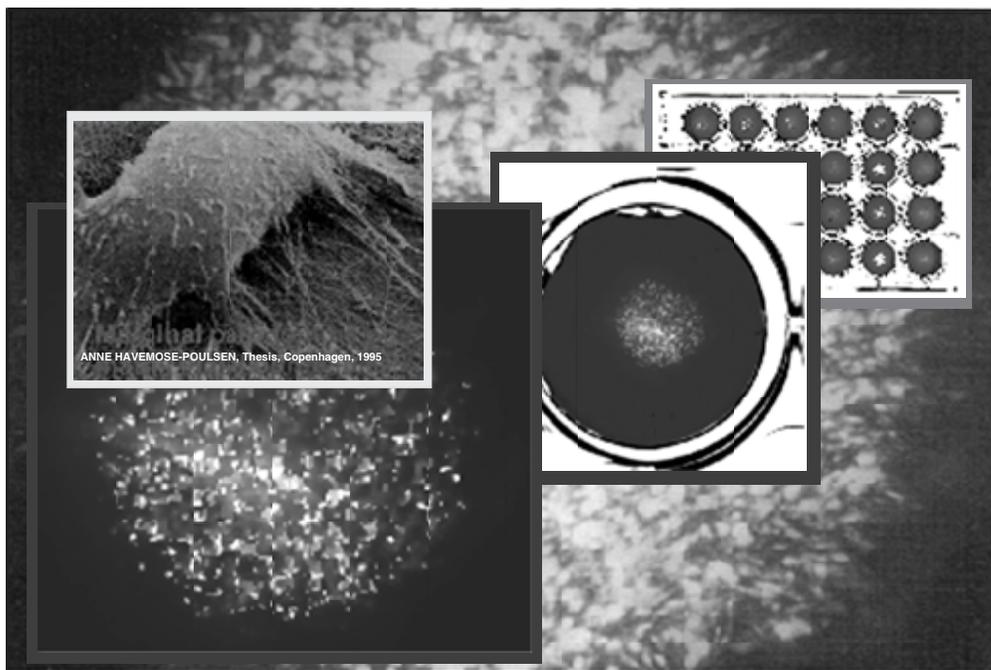


Figure 10.8.2 Dissolution of collagen fibrils by live adherent cells. Fibroblasts seeded in the center of the well dissolve the underlying collagen fibril film. Upper left panel shows scanning electron micrograph of fibroblast attached on collagen fibril film. Recreated from Havemose-Poulsen et al. (1998).

The neutralizing buffer is designed to bring the pH of the solution to 7.4 (check with pH paper). The concentration of this buffer is 0.2 M inorganic phosphate (as $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$) and 0.47 M NaCl. The final collagen concentration is 300 $\mu\text{g}/\text{ml}$ in 40 mM P_i /-0.10 M NaCl. Since pH dramatically influences the gelling properties of the collagen solution it is often advantageous to first test the efficacy of the neutralizing buffer by mixing 4 volumes of 13 mM HCl with one volume of neutralizing buffer and checking the final pH (7.4).

The final thickness of the collagen film depends on the concentration of the collagen solution. A 300 $\mu\text{g}/\text{ml}$ solution dispensed at a volume of 1.5 ml (35 mm) dish yields a film of 1.5 to 2.0 μm in thickness after drying. Higher concentrations yield thicker films. The lower concentration limit for proper gelling is around 100 $\mu\text{g}/\text{ml}$ using rat tail tendon collagen prepared as described (see Support Protocol 1) but somewhat higher (500 $\mu\text{g}/\text{ml}$) with commercial type I collagen preparations.

2. Immediately after mixing, add a 1.5-ml aliquot of neutralized collagen solution to each well of 6-well culture plate. Rotate the plate to permit the collagen to cover the entire well bottom evenly. Incubate in humidified incubator for 2 hr at 37°C. Avoid movement of gel and plate during gelling.
3. Remove plate from incubator, remove lid, and place at room temperature in an air stream (laminar flow hood) overnight (during this process the gel dries down to a thin film). Wash three times with distilled water, each time for 30 min at room temperature or 37°C, to remove salt crystals formed during the drying (check efficacy of washing step using a phase-contrast microscope). Dry again overnight in laminar flow hood and check for absence of residual salt crystals.

It is important that all salt crystals are removed by washing before the plates are used.

4. Add 2 ml CMF-PBS or DMEM supplemented with penicillin/streptomycin. Store in this solution in incubator at 37°C or in refrigerator at 4°C in closed plastic bag to prevent evaporation.

The plate can be stored in this manner for up to 2 weeks as long as evaporation is avoided.

5. Immediately before seeding cells, remove medium from wells by aspiration and wash with 2 ml distilled water for 30 min. Remove water and leave plate to air dry in hood.

Plate cells

6. Trypsinize and count cells (see UNIT 1.1), then dilute cell suspension to the appropriate concentration in DMEM/10% FBS, or in medium appropriate for the cell type being used.

Best results are obtained with 10,000 to 50,000 cells in a 25- μl aliquot, using a cell suspension of 4×10^5 to 2×10^6 cells/ml, somewhat depending on cell size. The intent is to form a coherent monolayer in a small central button (Fig. 10.8.1A, middle).

7. Deliver a 25- μl aliquot to the center of the well without touching the fragile collagen film. Fill plate volume between wells with distilled water to avoid evaporation during seeding and attachment. Place plate in plastic box on wet paper towels to avoid evaporation, and then place in incubator for 5 hr or overnight at 37°C to allow cells to attach.
8. Add to each well 2 ml DMEM/10% FBS or appropriate medium and incubate overnight at 37°C to allow cell spreading.

Some cell types can be transferred immediately to serum-free medium while others require overnight incubation in serum-supplemented medium.

Once the cells are spread, incubation may be performed either with or without serum. The result depends somewhat on cell type. Some cells tend to detach in the absence of serum

while others can be maintained for 2 to 3 days in complete absence of serum while degrading the collagen fibril matrix.

9. If the experiment is to be performed in the absence of serum, thoroughly and repeatedly wash with CMF-PBS or serum-free DMEM for 10 min at 37°C, to remove remnants of serum.

Some cells may require special media formulations, i.e., keratinocytes. Most fibroblast strains do well under serum-free conditions either in DMEM or DMEM/F12 (1:1).

Induce expression of MMPs

10. Induce cells for expression of MMPs at this stage by including in the medium cytokines such as IL-1 β (10⁻⁹M), TNF- α (10⁻⁸M), TGF- α (10⁻⁸M), or TPA (1 to 2 \times 10⁻⁷ M).

Alternatively, cytokine or TPA induction may be achieved during the last 24 hr before trypsinization and seeding. If incubated under serum-free conditions, plasminogen may be added to the medium. Some cells respond to exposure to plasminogen by greatly accelerating the rate of dissolution, while others do not. If desired, plasminogen is added from a stock solution in CMF-PBS to give a final concentration of 4 μ g/ml. Human plasminogen is either purchased from one of several commercial sources (i.e., Pharmacia Hepar or Sigma-Aldrich) or prepared as described (Deutsch and Mertz, 1970) from outdated human plasma by lysine-sepharose chromatography.

11. Incubate the plates at 37°C for 1 to 4 days (or up to 7 days) depending on the experimental design. Follow the progress of the process with a phase-contrast microscope.

To avoid evaporation it may be advantageous to fill the volume between the wells with sterile distilled water.

Stain plate and quantitate results

12. In order to visualize the dissolution of the film beneath the cell layer, remove the cells either by dissolution in 1% (v/v) Triton X-100, by 0.5% trypsin/0.53 mM EDTA (10 min, 37°C), or by a combination thereof.

Avoid use of SDS, which dissolves the collagen fibril film as well as the cells.

13. Rinse the wells with distilled water.
14. Stain with Coomassie blue stain for 5 to 15 min to visualize residual collagen film, then wash three times with distilled water.
15. Destain in distilled water for 30 min (or perform three quick washes with water) and finally allow plates to air dry.

After drying the plates, they can be stored indefinitely (Fig. 10.8.2).

In order to follow the progressive dissolution of the collagen fibril film it is advantageous to terminate sample wells on consecutive days and to contrast the dissolution after 1, 2, 3 ... days.

If desired the plates can be scanned directly into Adobe Photoshop using a scanner capable of scanning transparent originals.

16. Determine the extent or rate of dissolution of the substrate

The degree of dissolution at the conclusion of the experiment may be measured photometrically in Coomassie blue-stained plates by measuring the absorption of light in a conventional light microscope equipped with a exposure (photo)meter as described in Havemose-Poulsen et al. (1998). The relationship between amount of collagen present on

the plate and exposure time is strictly linear at least up to three times the collagen layer thickness used in this protocol.

Alternatively, if the cells are seeded evenly as a confluent monolayer over the entire collagen-coated well bottom (see below), progression may be monitored daily by removal of aliquots of medium and measuring the release of collagen chains and peptides. To this end the collagen may be labeled either with ^3H (Birkedal-Hansen, 1987; Birkedal-Hansen and Danø, 1981) or with fluorescent tags (Gherzi et al., 2001). This approach is less useful if the cells are seeded in a small 2- to 4-mm button at the center of the well, because the background release of radioactivity and fluorescent label from the entire film compromises the sensitivity of the analysis (typically only 10% to 20% of the collagen fibril film is covered by cells in this variation).

SUPPORT PROTOCOL 1

PREPARING RAT TAIL TENDON COLLAGEN TYPE I

Methods for isolation and preparation of rat tail tendon type I collagen have been described in detail elsewhere (Birkedal-Hansen, 1987; Birkedal-Hansen and Danø, 1981). Alternatively, rat, bovine or human type I collagen may be purchased from Becton Dickinson Biosciences Discovery Labware. Briefly, tendons teased from rat tails are washed with distilled water and with 0.5 M NaCl. The acid-soluble collagen fraction is then extracted in 0.5 M acetic acid, and type I collagen is purified by sequential salt precipitation at neutral to slightly alkaline pH, first with 5% NaCl, then (after redissolution in acetic acid) with 0.02 M Na_2HPO_4 .

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Tails of ~400 g rats (freshly removed or stored frozen at -80°C)
0.5 M NaCl in 50 mM Tris·Cl, pH 7.4 (see APPENDIX 2A for Tris·Cl)
5 mM, 50 mM, and 0.5 M acetic acid
NaCl (solid)
0.02 M Na_2HPO_4
13 mM HCl
Neutralizing buffer (0.2 M NaP_i)
Glass wool or cheesecloth
500-ml centrifuge bottles
High-speed centrifuge (Sorvall with SS-34 and GSA rotors, or equivalent centrifuge and rotors)
10,000 to 14,000 MWCO dialysis membrane
One large (25-liter) or several smaller (4-liter) dialysis tanks
Sterile scissors
125-ml glass Wheaton bottles
Additional reagents and equipment for dialysis (APPENDIX 3C)

Extract collagen

1. Skin 10 to 20 rat tails and place tails on ice. Break tails at joints and tease out individual collagen fibers. Wash in large volume distilled water (2 to 3 liter) for 1 hr with agitation. Change wash water three to four times.

The yield is 200 to 400 mg collagen per rat.

2. Extract overnight at 4°C with agitation in 2 liters of 0.5 M NaCl/50 mM Tris·Cl, pH 7.4. Discard extract and repeat step.

3. Discard second salt extract and wash collagen fibers extensively (over a 3-hr period with change two to three times per hr) in distilled water to remove salt.
4. Extract overnight at 4°C with slow agitation in 2 liters of 0.5 M acetic acid.
5. Remove insoluble remnants by filtration through glass wool or cheesecloth, then centrifuge in 500-ml bottles for 30 min at $11,000 \times g$ (8500 rpm in a GSA rotor), 4°C. Add solid NaCl little by little to a final concentration of 5% w/v (50 g/liter) under constant vigorous stirring. When the salt is completely dissolved, turn off stirrer, cover beaker, leave in cold room overnight and let precipitate gather at bottom of vessel.

The collagen immediately starts to precipitate upon addition of the salt.

6. Collect precipitate by centrifugation for 30 min at $11,000 \times g$, 4°C. Discard supernatant.
7. Redissolve collagen by adding 450 ml of 0.5 M acetic acid to first centrifuge bottle, transfer liquid to the second bottle, and so on, until collagen is redissolved/redispersed into ~900 to 1000 ml in 0.5 M acetic acid.
8. Stir vigorously overnight at 4°C until collagen is completely dissolved.

If not dissolved overnight, add more acetic acid and bring volume up to 1600 to 1800 ml.

Dialyze collagen solution

9. Place collagen solution, 300 to 400 ml at a time, in dialysis bags. Dialyze in tank against 25 liters of 0.5 M acetic acid, then for 3 to 4 days against 50 mM acetic acid. Change daily and mix content of bags.

See APPENDIX 3C for additional details on dialysis.

10. Dialyze against several changes of 0.02 M Na_2HPO_4 in 25-liter tank over the next 72 hr.

Precipitation should happen as fast as possible, so change solution frequently in the beginning and massage bags frequently to facilitate even distribution of reagents. The collagen precipitates as a thick white gel.

11. Harvest precipitate by centrifugation in 500-ml bottles for 30 min at $11,000 \times g$, 40°C. Redissolve collagen in 0.5 M acetic acid by vigorous stirring overnight at 4°C.
12. Dialyze 3 to 4 hr against 0.5 M acetic acid, then overnight against 50 mM acetic acid, and, finally, overnight against several changes of 5 mM acetic acid.
13. Centrifuge 1 hr at $11,000 \times g$, 4°C. Lyophilize supernatant and store in dessicator at 4°C, -20°C, or -80°C.
14. Redissolve as follows.
 - a. Weigh out no more than 150 mg collagen.
 - b. Cut into 1-cm pieces with sterile scissors.
 - c. Place collagen pieces into a 125-ml glass Wheaton bottle that has been autoclaved with a stir bar inside.
 - d. Add cold 13 mM HCl to make a 3 mg/ml solution and stir briskly at 4°C with occasional shaking for ~24 hr.

The collagen solution should be slightly opalescent.

15. Centrifuge solution for 20 min at $50,000 \times g$ (20,000 rpm in an SS-34 rotor), 4°C, to remove any insoluble material, if necessary.

Note that the solution remains somewhat opalescent even after centrifugation. This solution may be stored for months at 4°C. Freezing should be avoided.

LABELING OF COLLAGEN

Rat tail tendon type I collagen may be labeled using ^3H -acetic anhydride as described in detail in Birkedal-Hansen and Danø (1981) and Birkedal-Hansen (1987), or with fluorescent reagents. The following fluorescent labeling method was adapted from a technique devised by the Chen laboratory (G. Ghersi and W.T. Chen, unpub. observ.).

Materials

3 to 5 mg/ml rat tail tendon type I collagen originally dissolved in or dialyzed into 20 mM acetic acid (see Support Protocol 1)

DMEM

Borate buffer: 0.05 M $\text{NaB}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, pH 9.3, containing 0.04 M NaCl, sterile

2 to 3 mg/ml tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC) *or* fluorescein isothiocyanate (FITC) in DMSO

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

0.2 M acetic acid

10-cm culture dishes

1. Mix 8 ml of 3 to 5 mg/ml rat tail tendon type 1 collagen with an equal volume of DMEM, transfer to a 10-cm diameter dish, and incubate at 37°C overnight to form a 3-mm thick gel.

Rat tail tendon type I collagen may be prepared as described in this unit or purchased from Becton Dickinson Biosciences Discovery Labware; bovine skin and human placental type I collagen is also available from the same supplier.

2. Wash for 1 hr with sterile borate buffer at room temperature, with shaking.
3. Remove buffer; replace with 10 ml borate buffer plus TRITC or FITC at 2 to 3 mg/ml in a small volume of DMSO. Incubate at room temperature 15 to 20 min or until the dye diffuses through the gel. Protect from light from this point onward.
4. Wash with multiple changes of PBS for several days to remove free dye; wash salt out with water and redissolve in 0.2 M acetic acid at 4°C

Collagen is labeled in the fibrillar state so that sites important for subsequent alignment and gelling are not being blocked by the labeling procedure. Consequently, collagen labeled in this fashion readily dissolves in dilute acid and gels again upon neutralization and mild heating. Depending on the need, the fluorescently labeled collagen may be diluted up to 10-fold with unlabeled rat tail tendon collagen and still yield a strong enough signal for quantification.

GELATIN/CASEIN ZYMOGRAPHY

Zymographic methods are designed to analyze the proteolytic capacity of latent and active MMPs (Heussen and Dowdle, 1980; Birkedal-Hansen and Taylor, 1982; Birkedal-Hansen, 1987). This set of techniques is based on a number of unique properties of MMPs: (1) MMPs retain (or refold to display) catalytic activity after electrophoresis in SDS-containing buffers as long as heating and reduction are avoided (Birkedal-Hansen and Taylor, 1982); (2) brief exposure to SDS opens the “cysteine switch” (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990) so that both precursor and proteolytically truncated (“activated”) forms of the enzyme display catalytic activity; and (3) MMP catalytic activity is reversibly inhibited by SDS and readily restored when SDS is removed by washing with Triton X-100 (Birkedal-Hansen and Taylor, 1982). It is therefore possible to resolve a heterogenous group of MMPs and non-MMPs in SDS-containing gels copolymerized with a suitable substrate (gelatin, casein), remove the SDS, and develop (without distinction) the spontaneous or latent catalytic activity associated with each electrophoretic band. After appropriate incubation (to allow for proteolysis), the discrete

bands of substrate lysis are made visible by Coomassie blue staining of the gel (Fig. 10.8.3). SDS opens the “cysteine switch” but instantly inhibits the switch-open enzyme and blocks autolytic truncation normally associated with activation. The proenzyme bands therefore migrate at their expected high-molecular weight, but display proteolytic activity because the switch is unable to again “close” after removal of the SDS with Triton X-100.

Zymography using gels containing 0.1 to 1.0 mg/ml gelatin are by far the most sensitive. Gels may either be purchased (Invitrogen) or prepared as described below. Gelatin works particularly well for MMP-2 and MMP-9, whereas MMP-1, MMP-3, MMP-7, MMP-8, and MMP-10 are better identified in casein-containing gels.

Materials

Gelatin (bovine skin, Sigma-Aldrich type B6-6269) or casein (Sigma-Aldrich, technical, C-0376)

2.0 M Tris-Cl, pH 8.8 (*APPENDIX 2A*)

30/0.8 acrylamide/bisacrylamide (*UNIT 6.1*)

Glycerol

10% (w/v) SDS (*APPENDIX 2A*)

TEMED

10% (w/v) ammonium persulfate

MMP preparation of interest (for standards, use 1 to 5 ng purified MMP)

5× electrophoretic sample buffer (see recipe)

Electrophoretic running buffer (see recipe)

Gel washing buffers 1 to 4 (see recipe)

Coomassie blue stain (see recipe)

Gel destaining solution (see recipe)

50-ml centrifuge tubes

57°C water bath

Whatman no. 1 filter paper *or* 0.5- μ m syringe filter

Gel washing tray of appropriate size

Additional reagents and equipment for preparing SDS-PAGE gels according to Laemmli (*UNIT 6.1*)

NOTE: The following procedure is based on a standard 10% SDS-PAGE according to Laemmli (Laemmli, 1970; *UNIT 6.1*) using a 4% stacking gel and a pH 8.3 running buffer. It is important to avoid heating and/or reduction during sample preparation and running of the gel.

1. Weigh out appropriate amount of gelatin (for 0.1 to 1.0 mg/ml final concentration) or casein (for 1.0 mg/ml final concentration) and place in a 50-ml centrifuge tube.
2. For every 10 ml of solution to be prepared, add 4 ml of 2.0 M Tris-Cl, pH 8.8, and 6 ml water. Dissolve by heating in 57°C water bath. Filter through Whatman no. 1 filter paper or syringe filter.
3. Prepare the 10% resolving gel (also see *UNIT 6.1*) by adding the following to 10 ml filtered gelatin or casein solution (0.2 to 13 mg/ml in 0.8 M Tris-Cl, pH 8.8; see step 2):

6.6 ml 30/0.8 acrylamide/bisacrylamide

2 g glycerol

0.2 ml 10% (w/v) SDS

13.3 μ l TEMED

67 μ l 10% (w/v) ammonium persulfate

Pour gel as described in *UNIT 6.1*.

4. Prepare 4% stacking gel by combining the following (also see *UNIT 6.1*):

1 ml 30/0.8 acrylamide/bis acrylamide
0.36 ml 2 M Tris·Cl, pH 6.8
75 μ l 10% (w/v) SDS
6 ml H₂O
8 μ l TEMED
60 μ l ammonium persulfate

Pour gel as described in *UNIT 6.1*.

5. Mix 1 part MMP solution (partially or fully purified MMP, culture medium, concentrated culture medium, or other preparation containing MMP) with 4 parts of 5 \times sample buffer (final concentration, 1% SDS). Incubate at room temperature for 10 min, then load 20 to 30 μ l into each well of the 15-ml gel prepared in steps 3 and 4.

Alternatively, load 20 to 30 μ l per well of an Invitrogen minigel.

6. Run gel at 200 V for 35 to 45 min or until dye front reaches bottom of gel using electrophoretic running buffer, pH 8.3.

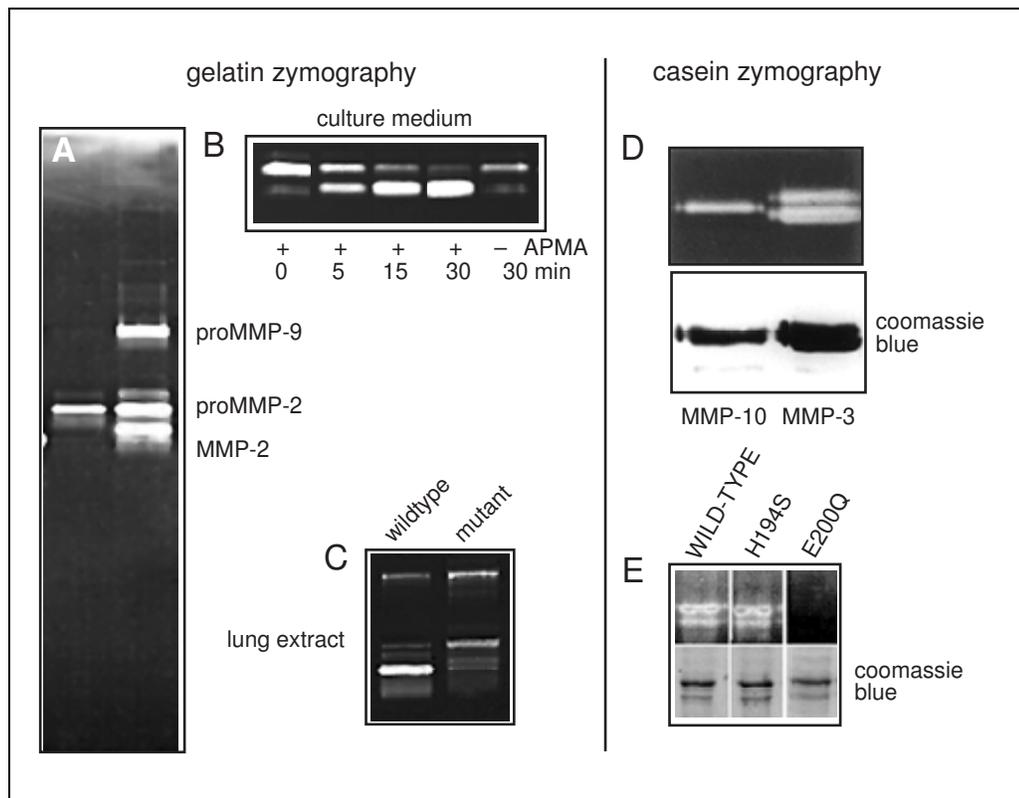


Figure 10.8.3 Zymography. **(A)** Zymography using gelatin-containing polyacrylamide gel. Culture medium containing proMMP-2 (left) or MMP-2/proMMP-2 and proMMP-9 (right). The proenzymes display catalytic activity because exposure to SDS during sample preparation opens the cysteine switch. **(B)** Detail showing conversion of proMMP-2 to MMP-2 by exposure to aminophenylmercuric acetate. From Caterina et al. (2000). **(C)** MMP-2 and MMP-9 activity in extracts of lungs of wild-type mice (left) or mice in which the TIMP-2 gene has been mutated to inactive form (modified from Caterina et al., 2000). **(D)** Zymography using casein-containing polyacrylamide gel. (pro)MMP-3 and MMP-10 cleave casein embedded in the gel (modified from Windsor et al., 1993). **(E)** Casein zymogram of mutant and wild-type MMP-1. Inactivation of catalytic activity by mutation of catalytic site glutamic acid (E) to glutamine (Q) that abolishes casein cleavage. A histidine to serine replacement outside the active site does not. Modified from Windsor et al. (1994).

7. Remove gel from electrophoretic apparatus and place in an appropriately sized container. Wash four times, 20 min each, successively, in washing buffers 1, 2, 3, and 4 at room temperature. Shake gently throughout.
8. Replace the last wash buffer with fresh washing buffer 4 and incubate 1 to 24 hr at 37°C.

A few hours of incubation is usually sufficient to reveal MMP-2 and MMP-9 by gelatin zymography. Overnight incubation is required to visualize MMP-1, MMP-3, MMP-13, MMP-7, and MMP-10 by casein zymography.

9. Stain gel with Coomassie blue stain for 30 min and destain with gel destaining solution for several hours until bands are clear.

Typical results are shown in Figure 10.8.3.

REVERSE ZYMOGRAPHY

Reverse zymography is specifically designed to identify electrophoretic bands which display MMP-inhibitory activity. The method is based on incorporation of both MMP activity and gelatin into the running gel. During the ensuing incubation, the SDS-activated MMP-2 (gelatinase A) cleaves the substrate everywhere in the gel except in and immediately around bands with inhibitory activity such as TIMPs. This method yields well resolved bands of TIMP-1, TIMP-2, TIMP-3, and TIMP-4, as well as mutant forms of these inhibitors (Fig. 10.8.4). The following protocol is developed by the Stetler-Stevenson laboratory and used in the authors' laboratory as well. Quantities are for a 15-ml gel, but can be scaled down as necessary.

Materials

- 8.7 mg/ml gelatin solution (see recipe)
- MMP-2 (Gelatinase A)
- 5× electrophoretic sample buffer (see recipe)
- 2.5% (w/v) Triton X-100
- Incubation solution (see recipe)

Additional reagents and equipment for “forward” zymography (see Basic Protocol 2)

1. Prepare separating gel (17%), copolymerizing gel with gelatin (2.5 mg/ml) and purified gelatinase A (MMP-2), by mixing the following components (also see *UNIT 6.1*):

- 4.2 ml 8.7 mg/ml gelatin solution
- 0.16 µg/ml (final concentration) gelatinase A (MMP-2)
- 8.25 ml 30/0.8 acrylamide/bisacrylamide
- 2.1 ml H₂O
- 0.29 ml 10% (w/v) SDS
- 7.3 µl TEMED
- 73 µl 10% (w/v) ammonium persulfate

Pour gel as described in *UNIT 6.1*.

Purified MMP-2 may be replaced with culture medium of cells which secrete this enzyme. The appropriate amount should be determined by trial and error.

BASIC PROTOCOL 3

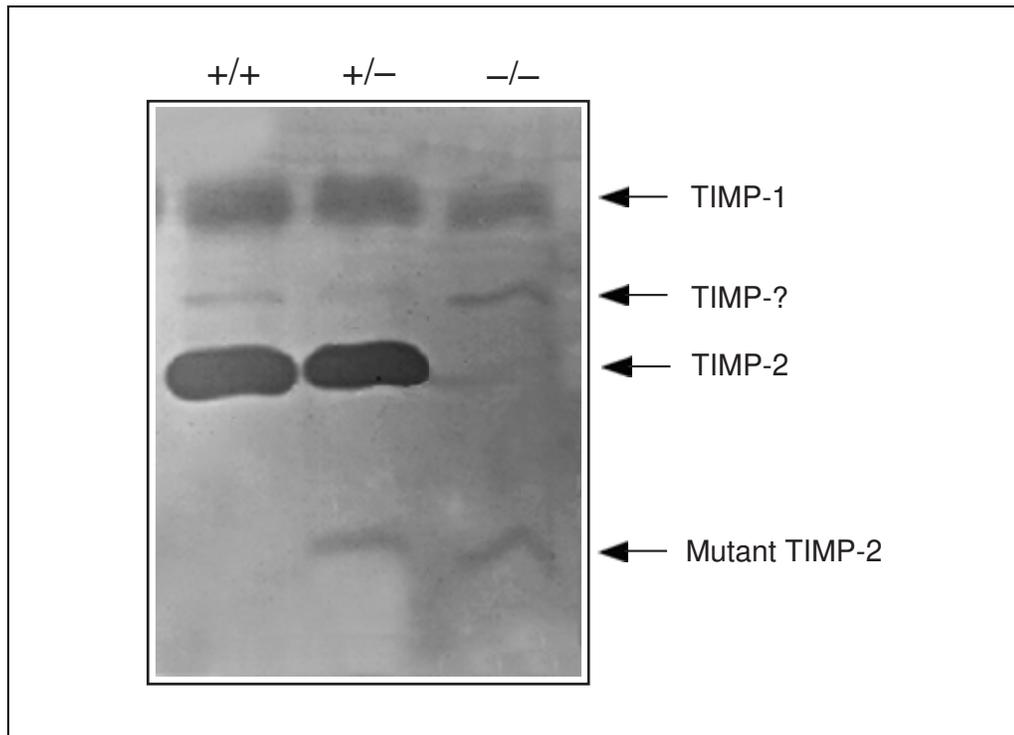


Figure 10.8.4 Reverse zymography. Inhibition of MMP-2 by TIMPs. Skin fibroblast culture medium obtained from wild-type, hemizygous, or homozygous TIMP-2-deficient mice was resolved by SDS-PAGE in a gel also containing MMP-2 and gelatin. During incubation, MMP-2 cleaves gelatin unless inhibited by electrophoretic bands of TIMPs. The TIMP-2-deficient cells still express TIMP-1 and unidentified component below TIMP-1, possibly TIMP-3 and a weakly inhibitory truncated mutant of TIMP-2. Modified from Caterina et al. (2000).

2. Prepare 5% stacking gel by combining the following (also see *UNIT 6.1*):

1.66 ml 30/0.8 acrylamide/bis acrylamide
 1.55 ml 2 M Tris·Cl, pH 6.8
 125 μ l 10% (w/v) SDS
 8.2 ml H₂O
 10 μ l TEMED
 200 μ l ammonium persulfate

Pour gel as described in *UNIT 6.1*.

3. Mix samples with 5 \times sample buffer for reverse zymography. Incubate at room temperature for ≥ 10 min, then load 20 to 30 μ l into each well of the gel.
4. Run gel at 150 V until buffer front reaches bottom of gel.
5. Remove gel and wash in three changes of 2.5% Triton X-100, for 2 hr with gentle shaking.
6. Incubate overnight at 37°C in incubation solution.
7. Stain gel with Coomassie blue stain for 20 min and destain in gel destaining solution for several hours until background is clear.

Typical results are shown in Figure 10.8.4.

α 2-MACROGLOBULIN (α 2M) CAPTURE

α 2M capture is particularly valuable because it permits assessment of the proteolytic competence and activity of single bands of MMPs in a mixture of many partially or fully processed forms. The method was originally devised (Birkedal-Hansen et al., 1976) for separation of complexes from unreacted forms by molecular sieve chromatography (Fig. 10.8.5), but it is even more valuable when combined with electrophoretic analysis. The protocol is based on the observation that α 2M forms complexes only with catalytically competent forms of MMPs. Unactivated MMP precursors or forms devoid of catalytic activity are not captured. The ensuing separation by SDS-PAGE permits easy identifica-

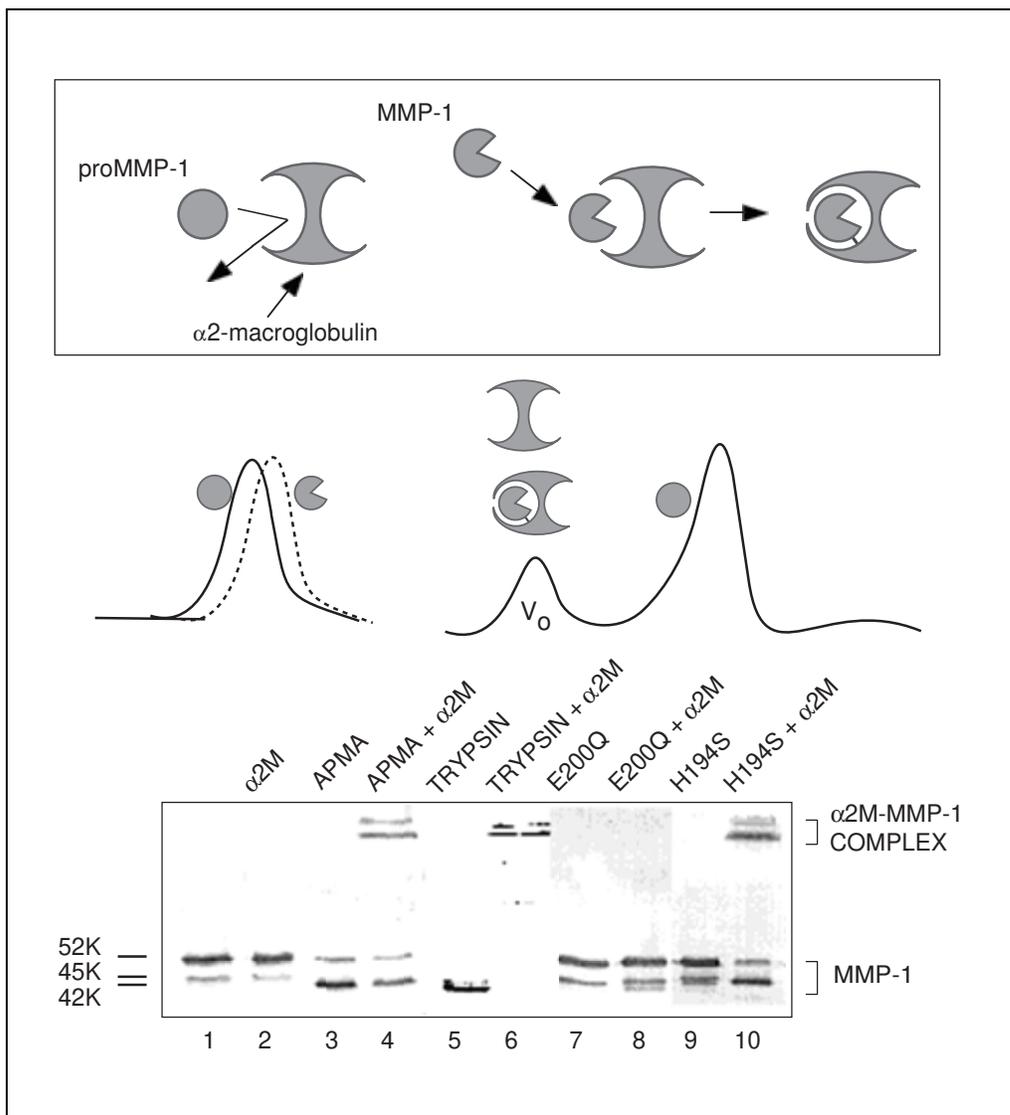


Figure 10.8.5 α 2-macroglobulin (α 2M) capture. The capture technique is based on the property that proteolytic cleavage of the α 2M bait region results in conformational and eventually covalent capture of the attacking proteinase. Because of the large disparity in molecular weight, captured and free forms of the proteinase may be separated either by molecular sieve chromatography (upper panel Birkedal-Hansen et al., 1976) or by SDS gel electrophoresis (lower panel; Windsor et al., 1994). Covalently bound proteinase is not released and is readily identified by appropriate antibody staining. Latent or inactive proteinases are not captured. The method therefore discriminates between enzyme forms with and without catalytic activity at the moment of testing. The panel shows wild-type and mutant forms of human MMP-1. Samples in lanes 3, 4, and 7 to 10 are pretreated with *p*-aminophenylmercury acetate (APMA). Samples in lanes 5, and 6 were preactivated by trypsin. Modified from Windsor et al. (1994).

tion of bands which have been captured and moved to the top of the gel because of the large molecular mass of the α 2M (Fig. 10.8.5). Bands that escape capture continue to migrate at their usual position. Complexes formed with α 2M are covalent and therefore not easily dissociated. The ability of α 2M to discriminate between latent and overtly active forms of the enzyme is a result of the α 2M inhibition mechanism. α 2M is inert until the attacking proteinase cleaves a peptide bond in the bait region. This cleavage results in rapid conformational change and liberates a thiol ester which covalently bonds to and immobilizes the attacking proteinase.

Materials

MMP solution to be tested
2 to 3 mg/ml purified α 2M in 50 mM Tris·Cl standard buffer (see recipe for buffer)
100 μ g/ml TPCK-treated trypsin (e.g., Sigma) in 50 mM Tris·Cl standard buffer (see recipe), pH 7.4
1.0 mg/ml soybean trypsin inhibitor in 50 mM Tris·Cl standard buffer (see recipe), pH 7.4
5 \times electrophoretic sample buffer (see recipe)
Antibodies to MMPs of interest
Nitrocellulose paper
Additional reagents and equipment for SDS-PAGE according to Laemmli (UNIT 6.1) immunoblotting (UNIT 6.2)

1. Mix one half of the test solution with a sufficient volume of 1.5 mg/ml α 2M to achieve a $\geq 10\times$ molar ratio of inhibitor to MMP. Incubate 15 min at room temperature.
2. To compare “activated” and “unactivated” samples, preincubate the other half of the test sample with 10 μ g/ml trypsin (added from 100 μ g/ml stock) for 10 min at room temperature, then add 100 μ g/ml soybean trypsin inhibitor (added from 1 mg/ml stock). Incubate separately with α 2M as described in step 1.

Commercial sources of α 2M are available but should always be checked for activity by titration with trypsin using a suitable substrate (Sottrup-Jensen and Birkedal-Hansen, 1989). Alternatively, the inhibitor may be prepared by standard techniques as described by Sottrup-Jensen and Birkedal-Hansen (1989) and Sottrup-Jensen et al. (1983). Activation with trypsin prior to addition of α 2M often yields more complete capture than with organomercurials—e.g., $\text{NH}_2\text{PheHgAc}$ (APMA)—which seem to gradually inactivate α 2M. Samples preincubated with organomercurials, however, still show partial capture.

3. Mix with 5 \times electrophoretic sample buffer (final concentration, 1% w/v SDS, 2.5% v/v 2-ME) without heating, resolve by SDS-PAGE using a 10% gel according to Laemmli (Laemmli, 1970; UNIT 6.1).
4. Transfer to nitrocellulose paper and stain with appropriate MMP antibody using conventional immunoblotting techniques (UNIT 6.2).

Typical results are shown in Figure 10.8.5.

TIMP CAPTURE

Complexes formed with TIMPs are not covalent, although several, but not all, withstand exposure to low concentrations of SDS, as originally observed by DeClerck et al. (1991), who first pioneered this technique. This method detects many but not all activated MMPs that bind TIMPs, including MMP-1 (collagenase-1), MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-10 (stromelysin-2), and MMP-13. Detection is most conveniently done by immunoblotting using specific antibodies to the two complex components (MMP and TIMP; Fig. 10.8.6). The method described below is the authors’ adaptation of the

ALTERNATE PROTOCOL

method of DeClerck (DeClerck et al., 1991). It is based on capture with TIMP-1, but TIMP-2 capture works just as well.

Materials

0.1 to 1.0 mg/ml TIMP-1 (Oncogene Research Products, Chemicon International; also see Bodden et al., 1994) in 50 mM Tris·Cl standard buffer (see recipe), pH 7.4

10.0 mM NH₂PheHgAc (APMA; Sigma) in Tris·Cl standard buffer (see recipe), pH 7.4

Electrophoretic sample buffer (see recipe, but use only 0.5% w/v SDS)

Antibodies to MMPs and TIMP-1 of interest (Calbiochem, Chemicon International)

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and immunoblotting (UNIT 6.2)

1. Incubate control and activated samples with 40 to 100 μg/ml TIMP-1 (added from 0.1 to 1.0 mg/ml stock) with and without 1.0 mM NH₂PheHgAc (added from 10.0 mM stock) for 90 min at 37°C.

Molecules which are activated by NH₂PheHgAc are captured almost instantly by TIMP-1.

TIMP-1 may be prepared from cultures of fibroblasts or similar cell lines that express fairly high levels of TIMP-1 activity (Bodden et al., 1994). Concentrations of this compound in the range of 0.1 to 1.0 mg/liter may be recovered from the culture medium. The purification scheme is somewhat cumbersome but greatly facilitated by use of antibody-based affinity chromatography techniques.

2. Mix with 5× electrophoretic sample buffer containing 0.5% SDS. Resolve by SDS-PAGE using a 10% gel on ice at 100 V (UNIT 6.1).

Note that the SDS concentration of the sample buffer is reduced to 0.1% (final concentration) in order to avoid dissociation of these entirely noncovalent complexes. This change is crucial to the success of the technique.

3. Transfer to nitrocellulose and stain adjacent lanes with antibodies to TIMP-1 and to MMP using standard immunoblotting techniques (UNIT 6.2).

Typical results are shown in Figure 10.8.6.

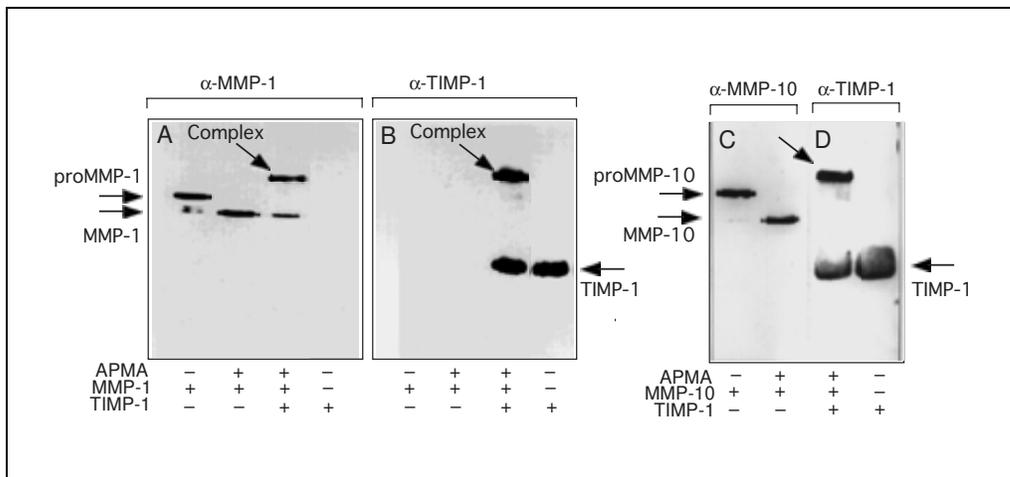


Figure 10.8.6 TIMP capture. (A, B) are identical panels stained with antibodies to either human MMP-1 or TIMP-1. Capture of activated human MMP-1 gives rise to a new band in the 70-kDa range containing both MMP-1 and TIMP-1 (arrow). (C) proMMP-10 and activated MMP-10 stained with antibody to human MMP-10. (D) Addition of TIMP-1 to activated MMP-10 results in capture of the enzyme now migrating in a complex with TIMP-1 in the M_r 70-kDa range. Modified from Windsor et al. (1993).

FLUORESCENT LABELING OF CRYPTIC CYS-RESIDUE IN MMPs

Most MMP (and ADAM) precursors contain a cryptic thiol group derived from a single, unpaired cysteine residue in the propeptide. This group is coordinately bonded directly to the active site Zn (“cysteine switch”) and in this manner plays a significant role in maintaining the catalytic latency of the proteinase precursors. The protocol below permits unmasking and detection of this cryptic thiol group (Fig. 10.8.7). The “switch” opens upon addition of SDS, which allows reaction of the liberated thiol group with a fluorescent maleimide compound (Yamamoto et al., 1977; Lyons et al., 1991).

Materials

MMP-containing samples

20 μ M fluorescent maleimide N-(7-(di-methylamino-4-methyl-3-coumarinyl) maleimide (DACM) in Tris-Cl standard buffer (see recipe for buffer; prepare from 1 mM DACM stock in DMSO or ethanol)

2-mercaptoethanol stock in electrophoretic sample buffer (see recipe for buffer): concentration appropriate to obtain 5% final concentration in reaction mixture

Fluorescent lamp

Photographic equipment

Additional reagents and equipment for SDS-PAGE (UNIT 6.1)

1. Expose companion samples of 50 to 200 μ g/ml MMP for 1 hr at room temperature to 20 μ M DACM (final concentration) either in the presence or absence of 1% (w/v) SDS.
2. Stop reaction by adding 2-mercaptoethanol (as stock solution of appropriate concentration in electrophoretic sample buffer) to a final concentration of 5% (v/v).

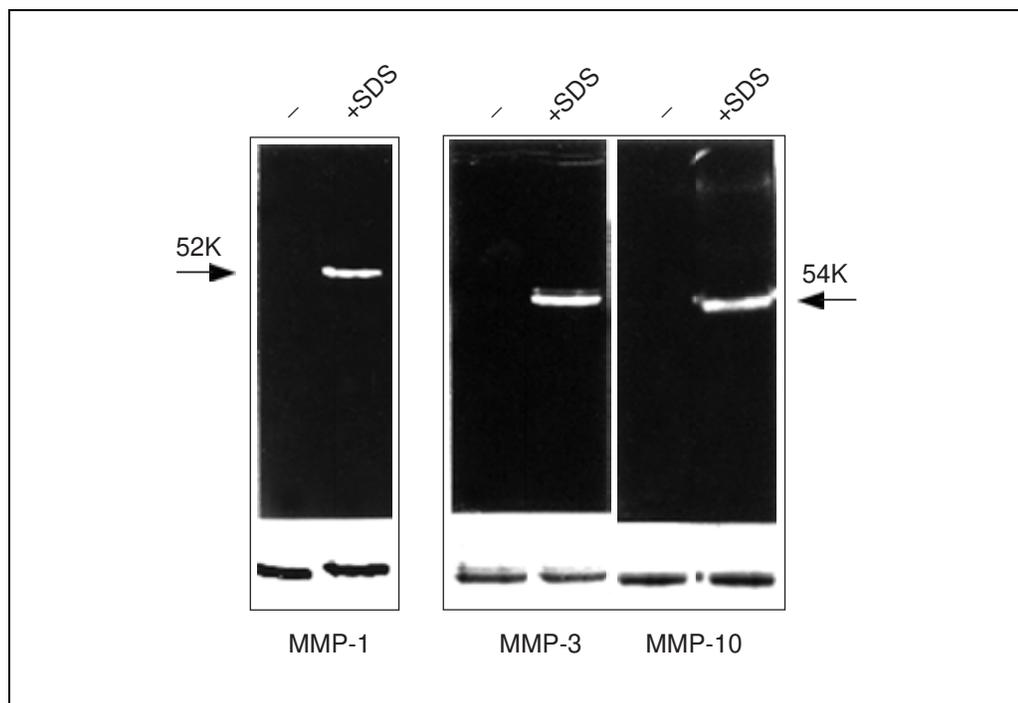


Figure 10.8.7 Fluorescent labeling of propeptide cryptic thiol residue by fluorescent maleimide. The cysteine switch is “closed” in the nascent proenzyme and therefore not reactive with a fluorescent maleimide compound (DACM). Exposure to SDS “opens” the switch and renders the cryptic thiol group reactive with the maleimide resulting in covalent modification of the proenzyme and generation of a readily detectable fluorescent band. Left panel: MMP-1. Right panel, MMP-3 and MMP-10. Lower edge of each panel shows Coomassie blue staining of the same bands. Modified from Windsor et al. (1993).

3. Resolve proteins by SDS-PAGE (UNIT 6.1).
4. Photograph under long-wavelength UV illumination.

Typical results are shown in Figure 10.8.7.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Coomassie blue stain

- 0.5% (w/v) Coomassie blue R-250
- 30% (v/v) methanol
- 10% (v/v) acetic acid
- Store up to 6 months at room temperature

Electrophoretic running buffer, pH 8.3

- 0.025 M Tris base
- 0.192 M glycine
- 0.1% (w/v) SDS
- Store up to 1 year at room temperature

Electrophoretic sample buffer, 5×

- 0.2 M Tris·Cl, pH 6.8 (APPENDIX 2A)
- 5% (w/v) SDS
- 20% (w/v) glycerol
- 0.1% (w/v) bromphenol blue
- Store up to 1 year at room temperature

This is the sample buffer used in Basic Protocol 2.

Gelatin solution, 8.7 mg/ml

Add gelatin (bovine skin, Sigma-Aldrich type B6-6269) to 1 M Tris·Cl, pH 8.8 at 8.7 mg/ml. Dissolve by heating to 57°C, then filter through Whatman no. 1 filter paper.

Gel destaining solution

- 30% (v/v) methanol
- 10% (v/v) acetic acid
- 60% (v/v) H₂O
- Store up to 1 year at room temperature

Gel washing buffers 1 to 4

Buffer 1:

- 2.5% (v/v) Triton X-100
- 3 mM NaN₃

Buffer 2:

- 2.5% (v/v) Triton X-100
- 50 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
- 3 mM NaN₃

Buffer 3:

- 2.5% (v/v) Triton X-100
- 50 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
- 3 mM NaN₃
- 5 mM CaCl₂
- 1 μM ZnCl₂

continued

**Extracellular
Matrix**

10.8.17

Buffer 4:

50 mM Tris·Cl, pH 7.5 (APPENDIX 2A)

3 mM NaN₃

5 mM CaCl₂

1 μM ZnCl₂

Buffers may be stored up to 1 year at room temperature.

Incubation solution

50 mM Tris·Cl, pH 7.4 (APPENDIX 2A)

0.2 M NaCl

5 mM CaCl₂

0.02% (w/v) Brij-35

Store up to 1 year at 4°C

Neutralizing buffer (0.2 M NaP_i)

Prepare the following stock solutions:

Solution A: 2.78 g NaH₂PO₄ in 100 ml H₂O

Solution B: 5.365 g Na₂HPO₄·7H₂O in 100 ml H₂O

Prepare working solutions as follows:

15.2 ml Solution A

64.8 ml Solution B

16.6 ml 5 M NaCl

Add 80 ml 0.1 N NaOH

Store up to 1 year at 4°C

Tris·Cl standard buffer, pH 7.4

50 mM Tris·Cl, pH 7.4 (APPENDIX 2A)

0.2 M NaCl

5 mM CaCl₂

Store up to 1 year at 4°C

COMMENTARY

Background Information

Dissolution of collagen type I

Substrate. Although collapsing the gel by air drying is advantageous for most purposes, and the resulting collagen film is more similar to the density of collagen in interstitial connective tissues (Fig. 10.8.1), it is possible to seed the cells on top of (or inside) fully hydrated gels and to monitor the process as the cells dissolve their way through the collagen gel. Electron microscopy confirms that hydrated gels are very loose, with the individual fibrils spaced far apart. The collagen content is quite low compared to the liquid phase and accounts for only 0.03% of the mass and for a similarly small volume fraction of the gel.

While use of reconstituted type I collagen fibrils as a substrate offers particular advantages because of its resistance to general proteolysis, it is possible to replace this substrate with other extracellular matrix components.

Type II collagen does not form fibrils as readily as does type I but might prove useful after additional refinement of the system. Type III collagen appears to gel adequately for this purpose and may also be used as a substrate. Films and gels of type IV collagen may also be used, as may Matrigel (predominantly composed of laminin), fibrin, and fibronectin. An important variation using fluorescently labeled fibronectin was devised by Chen and coworkers (Chen et al., 1984; Chen and Chen, 1987).

Serum. Serum contains a number of factors expected to either promote or inhibit the proteolytic dissolution of the extracellular matrix including collagen fibrils. The high concentration of α2M (2 to 3 mg/ml or 3 to 4 × 10⁻⁶ M), which effectively blocks most MMPs in test tube experiments, however, does not inhibit cell-mediated dissolution of the collagen fibril film. Serum also contains plasminogen at a concentration of ~200 μg/ml (2 × 10⁻⁶ M). Addition of even low concentrations of plasm-

nogen (4 $\mu\text{g/ml}$; 4×10^{-8} M) to serum-free cultures greatly accelerates the rate of dissolution of the collagen fibril film by human foreskin keratinocytes (or other cells) which express urokinase-type plasminogen activator (u-PA) or tissue-type plasminogen activator (t-PA). The mechanism is not quite well understood but may involve a role for plasminogen in the extracellular activation of certain proMMP precursors as an essential step in the dissolution of the substrate.

Cytokines, transcriptional activation. Addition of cytokines, growth factors, and agents such as TPA, which upregulate or induce expression of MMPs, generally accelerates dissolution of the fibril coating dramatically, but since these reagents upregulate a wide range of MMPs, it is not yet possible to determine whether a single MMP or group of MMPs is responsible for this effect.

Inhibition. That dissolution of the collagen fibril coating is mediated by metalloproteinase-dependent mechanisms is readily made evident by synthetic inhibitors. Inclusion of the Zn-chelating agent 1,10-phenanthroline completely blocks dissolution, as do synthetic MMP inhibitors such as BB94, BB2516 (British Biotech), and Galardin. A number of synthetic inhibitors currently exist; some of these may be obtained by directly contacting the pharmaceutical companies in question (British Biotech, Roche Diagnostics, Celltech). Serine proteinase inhibitors such as α 1-antitrypsin (α 1AT) and soybean trypsin inhibitor, as well as cysteine proteinase inhibitors such as E-64, have no effect on the rate of dissolution. These findings suggest that the process(es) that result in dissolution of the collagen fibrils are absolutely dependent on MMP activity.

Zymography

Gelatin zymography is a fairly straightforward yet very highly sensitive technique as long as heating and reduction are avoided during sample preparation. The method yields discrete, well-resolved, and distinct unstained bands on a blue background, which are clearly visible and easy to photograph and document with transillumination (Fig. 10.8.3). The activity may be quantified by comparison with standard curves of specific purified MMPs (Kleiner and Stetler-Stevenson, 1994), but the rate of lysis varies considerably from MMP to MMP, and the technique is primarily intended to provide qualitative information. A variation described by Lyons et al. (1991) permits moni-

toring of real-time progress of the reaction under UV-light by use of gelatin labeled by a fluorophore. Although gelatin zymography is highly sensitive, capable of detecting low picogram quantities of MMPs, the assay does not reflect the activity of these proteases present in the sample analyzed. This is because the addition of SDS to the sample prior to electrophoretic separation results in dissociation of many enzyme inhibitor complexes. Therefore, zymography represents an excellent technique for identification of MMP species present in a given sample, but overinterpretation of the results—e.g., assessment of specific activity—is a common pitfall. Casein zymograms develop more slowly, almost invariably require overnight incubation, and tend to produce less sharp bands. (Latent) proenzyme forms also show up because of the “switch”-opening effect of SDS, but these forms do not necessarily acquire full catalytic activity. “Activation” by organomercurials (0.5 to 1.0 aminophenylmercuric acetate in 50 mM Tris-HCl buffer, pH 7.5, for 20 min to 20 hr) before sample preparation often results in higher levels of proteolytic activity but also shifts the M_r of the individual bands because of autolytic cleavage and removal of the propeptide.

α 2M capture

Capture techniques permit direct assessment of the ability of various forms of MMPs to bind to natural inhibitors in a manner that resists dissolution by exposure to low concentrations of SDS. Zymographic techniques are not capable of discriminating between latent and catalytically active forms of the enzymes. That, however, can readily be achieved by α 2M capture. TIMP capture (see Alternate Protocol) on the other hand does not depend on proteolytic activity and merely requires a correctly folded, but not necessarily catalytically competent, active site (Windsor et al., 1994).

TIMP capture

The method is particularly useful for analysis of the binding capacity of mutants in TIMPs and in MMPs (Windsor et al., 1994; Caterina et al., 1997). It is important to recognize that TIMP binding is not necessarily synonymous with catalytic competence. Mutants of MMP which are correctly folded but devoid of catalytic activity, such as the E200Q mutant of MMP-1 in which the active site glutamate is replaced with glutamine (and therefore catalytically inactive) still forms complexes with

TIMP-1 fully as well as the native enzyme (Windsor et al., 1994). TIMP-1 captures both truncated and full-length forms, as long as the “switch” is open (by APMA).

Fluorescent labeling of cryptic Cys residue

The nascent closure of the “cysteine switch” by bonding of the single unpaired propeptide Cys residue to the active site Zn^{2+} converts a catalytic Zn-binding site to a structural Zn-binding site. In order to monitor the (re)opening of the switch as a preamble to zymogen activation, the authors of this unit reasoned that covalent linkage of a fluorophore to the free thiol group might render this process easily visible and potentially quantifiable. That is indeed the case. The method shows, for instance, that the phenomenon of “switch opening” can be readily visualized in the absence of propeptide cleavage by exposure either to SDS or to EDTA.

Critical Parameters and Troubleshooting

Dissolution of collagen type I

Even for the experienced operator, collagen is not an easy protein to work with. Its preparation and use require meticulous and stringent adherence to the rules and conditions that “work,” often with very little leeway for shortcuts and modifications. The most important checkpoint comes after the initial gelling. Unless there is clear and unequivocal evidence of gelling after 2 hr, efforts should be made to identify and correct the problem. Since there is no simple way to measure collagen concentration, the authors have utilized initial dry powder weight from materials stored in refrigerated dessicator jars as a guide. The concentrations mentioned in this unit refer to powder weight under these conditions. It is absolutely necessary that the solution from which the collagen is lyophilized be completely salt-free following extensive dialysis against dilute acetic acid. This problem may be avoided by purchase of commercial preparations of rat or bovine type I collagen, but it is necessary to test the gelling properties of the particular brand in question at the desired concentration and under the desired conditions. After 2 hr of gelation, the gel should be reasonably firm, i.e., it should not disintegrate upon gentle flicking of the plate. If the gel disintegrates during this test, the problem must first be solved before proceeding.

The homogeneity of the gel is also very important. This is best checked following the

first air drying and washing step by staining a newly prepared film with Coomassie blue. This will instantly reveal whether the gel is uniform and homogenous and if it contains particulate matter (which can be removed by centrifugation) or air bubbles. Both must be avoided, and the technique must be improved until each gel is completely uniform and homogenous after staining. It is also important to ascertain after the first washing of the first-time dried gel that salt-crystal deposits (formed during the initial drying phase) have been completely removed by washing. This is most easily checked using the phase-contrast microscope. The gel should look granular but uniform; any trace of crystal patterns is a certain indication of inadequate washing.

Zymography

Gelatin zymography presents few, if any, technical challenges, hence the popularity and universal application of this technique. Because of the longer incubation time required and the lesser sensitivity, casein zymograms often give less distinct and more diffuse bands. Although it has not been widely explored, it is highly likely that a large number of other substrates could be substituted for either gelatin or casein. Reverse zymography, on the other hand, is technically challenging and requires great care and skill as well as considerable practice and experience. The latter method is, however, a uniquely powerful technique to identify discrete MMP-inhibitory bands.

Inhibitor capture

While commercial preparations of $\alpha 2M$ are available the method is critically dependent on the native configuration of the inhibitor. Consequently, the authors rely only on freshly isolated inhibitor. Occasionally methods which are employed to activate MMPs, such as exposure to organomercurials, adversely affect the inhibitor and render the capture reaction partial rather than complete. In some cases trypsin activation (stopped by soy bean trypsin inhibitor) is preferable, but many mutants are highly sensitive to trypsin and rapidly degrade during activation attempts.

Fluorescent labeling of cryptic Cys residue

The method is fairly straightforward, although care must be taken to exclude any chemicals from the solutions that interfere with the Cys-maleimide reaction (e.g., heavy metals, *N*-ethylmaleimide, or iodoacetate). Photo-

graphic documentation can be tricky, but usually works well when using reflected UV light.

Anticipated Results

Dissolution of collagen type I

Use of 1- to 2- μ m films results in complete dissolution within 1 to 4 days. Initially the cells penetrate the collagen fibril coating in discrete spots, which eventually coalesce to form contiguous zones devoid of collagen fibrils (Fig. 10.8.2). Dissolution of the fibril coating is strictly limited to the area immediately beneath the cell layer and does not extend beyond the boundaries of the cell colony. A similar pattern is observed in the presence of serum or purified plasminogen.

Zymography

When performed correctly, the reverse zymography-stained gel shows discrete, well-resolved bands of TIMPs on a virtually unstained background, indicating that all of the gelatin has been degraded except in and around the TIMP bands (Fig. 10.8.4). While this method yields important information when used in qualitative or semiquantitative fashion, the read-out may be quantified as described by Kleiner and colleagues (Oliver et al., 1997).

As with direct zymography, reverse zymography is a highly sensitive technique that can detect as little as 50 to 100 pg of TIMPs in a given sample (Oliver et al., 1997). However, as with direct zymography, careful interpretation of results is essential. Again, use of SDS-containing sample buffers and electrophoretic separation of the sample results in dissociation of some protease-inhibitor complexes. Thus, the levels of TIMPs present may not accurately reflect the actual free TIMP levels present in the samples analyzed. Alternatively, as described for the TIMP capture assays, not all TIMP-MMP complexes may be dissociated by SDS, and TIMP-binding to an MMP active site does not necessarily reflect proteolytic competency of the enzyme.

α 2M capture

Incubation of native α 2M with activated proteinases that cleave the bait region result in full or partial capture of the attacking proteinase. Complete capture requires a significant molar excess of inhibitor (with the amount varying from proteinase to proteinase) which may be determined by titration in preliminary experiments. Because of the size difference, captured and uncaptured bands are readily re-

solved and identified on Western blots by staining with anti-MMP antibodies. Latent or catalytically inactive forms are not captured and remain at their usual migration position in the gel.

TIMP capture

Remarkably, most TIMP-MMP complexes survive dilute SDS solutions at room temperature and permit electrophoretic separation of free and complexed forms. The M_r difference (20 to 30 kDa) is sufficient to fully resolve the bands. As with α 2M capture, latent forms of MMPs ("switch closed") are not captured, and this method is therefore valuable in distinguishing "switch-open" and "switch-closed" forms before proteolytic excision of the propeptide during activation. The active site, however, does not have to possess catalytic activity, and inactive mutants (if correctly folded) readily form complexes with TIMPs.

Fluorescent labeling of cryptic Cys residue

Removal of Zn^{2+} with EDTA, as expected, also unmask the cryptic thiol group. Fully converted ("activated") forms of the enzyme which have lost the entire propeptide no longer react. Note, however, that the free thiol group is only a few residues upstream of the ultimate proteolytic processing site. Partially processed forms of the proenzymes therefore may still react with DACM.

Time Considerations

Analysis of the degradation of collagen gels takes ~2 days to prepare the gels and 1 to 4 days for the assay itself. It requires ~2 weeks to prepare rat tail tendon collagen type I and 3 to 4 to label the collagen with fluorophore.

Direct zymography takes 2 days to complete, while reverse zymography takes 2 days.

α 2M and TIMP capture take 1 to 2 days depending on the duration of antibody incubation in immunoblotting.

Fluorescent labeling of the cryptic Cys residue can be completed in a single day.

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Preparation of Extracellular Matrices Produced by Cultured Fibroblasts

Culturing fibroblasts on traditional two-dimensional substrates induces an artificial polarity between lower and upper surfaces of these normally nonpolar cells. Not surprisingly, fibroblast morphology and migration differ once the cells are suspended in three-dimensional collagen gels (Friedl and Brocker, 2000). However, the molecular composition of collagen gels does not mimic the natural fibroblast microenvironment. Fibroblasts secrete and organize extracellular matrix (ECM), which provides structural support for their adhesion, migration, and tissue organization, besides regulating cellular functions such as growth and survival (Buck and Horwitz, 1987; Hay, 1991; Hynes, 1999; Geiger et al., 2001). Cell-to-matrix interactions are vital for vertebrate development. Disorders in these processes have been associated with fibrosis, developmental malformations, cancer, and other diseases.

In this unit, a method is described for generating tissue culture surfaces coated with a fibroblast-derived three-dimensional ECM produced and deposited by mouse NIH-3T3 cells (see Basic Protocol). This matrix closely resembles *in vivo* mesenchymal matrices and is composed mainly of fibronectin fibrillar lattices. Utilizing *in vivo*-like three-dimensional matrices as substrates allows the acquisition of information that is physiologically relevant to cell-matrix interactions, structure, function, and signaling, and which differs from data obtained by culturing cells on conventional two-dimensional substrates *in vitro* (Cukierman et al., 2001).

These protocols were initially derived from methods described in *UNIT 10.4*. These methods were modified to obtain fibroblast-derived three-dimensional matrices and characterize cellular responses to them. The basic approach is to allow the cultured fibroblasts to produce their own three-dimensional matrix. For this purpose, fibroblasts are plated and maintained in culture in a confluent state. After 5 to 9 days, matrices are denuded of cells, and cellular remnants are removed. Such extraction results in an intact fibroblast-derived three-dimensional matrix, free of cellular debris, that remains attached to the culture surface (see Fig. 10.9.1). The fibroblast-derived three-dimensional matrices are then washed with PBS and can be stored for periods of up to 2 to 3 weeks at 4°C. In order to evaluate the quality of the fibroblast-derived three-dimensional matrix, support protocols present procedures for assessing the induction of rapid cell attachment (see Support Protocol 1) and the acquisition of *in vivo*-like spindle-shaped morphology (see Support Protocol 2) by plating new fibroblasts into the three-dimensional matrix. Additional support protocols describe how to mechanically compress the fibroblast-derived three-dimensional matrices in order to obtain two-dimensional substrate controls (see Support Protocol 3) and how to solubilize the fibroblast-derived three-dimensional matrices (see Support Protocol 4) to produce a matrix-derived protein mixture for additional two-dimensional coating controls and for subsequent biochemical analysis of the matrices (also see Commentary).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 10% CO₂ incubator unless otherwise specified.

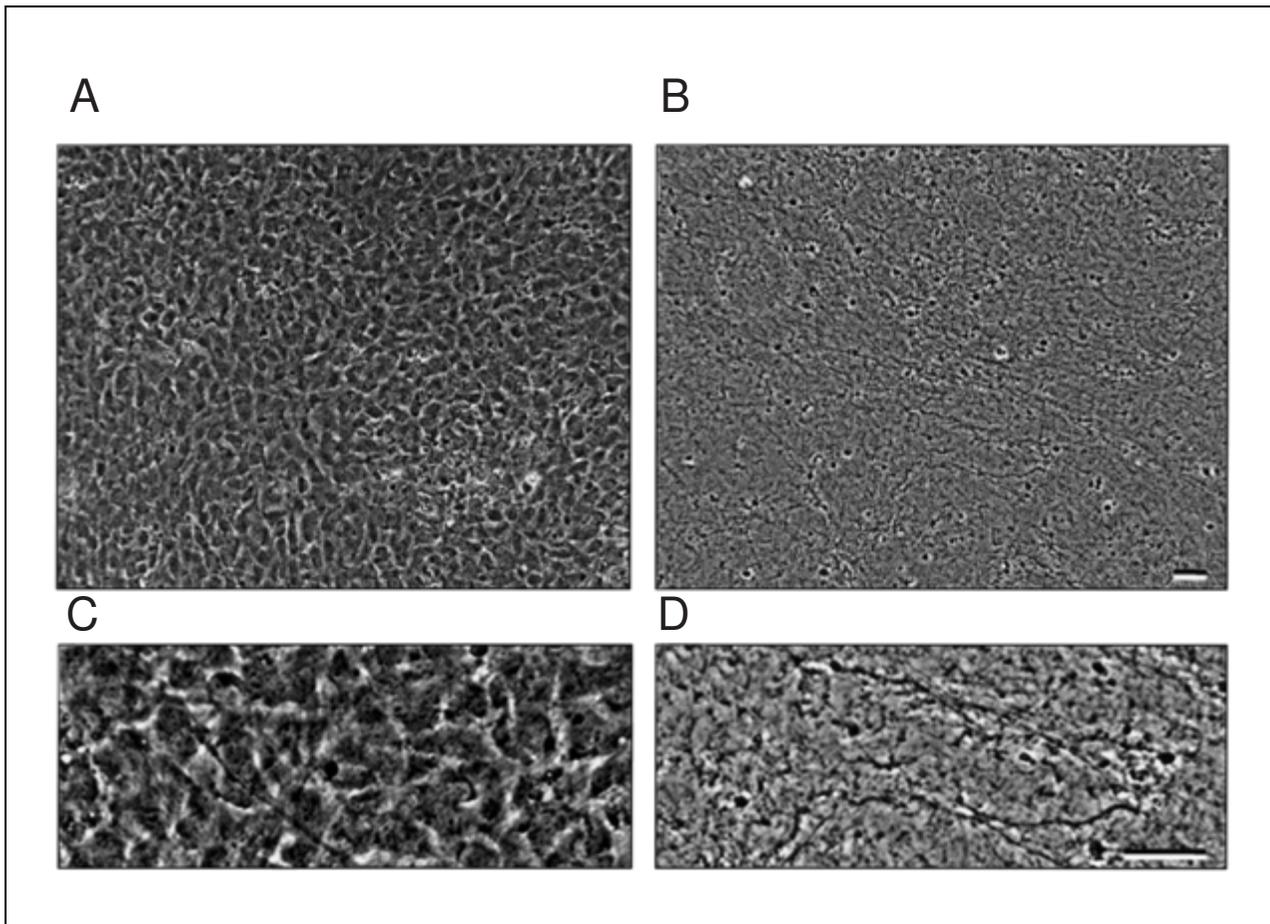


Figure 10.9.1 Fibroblast-derived three-dimensional matrices before and after extraction process. **(A)** Culture at day 5 prior to matrix extraction. **(B)** The resulting fibroblast-derived three-dimensional matrix. **(C)** Magnified inset from panel A. **(D)** Magnified inset from panel B. Bars represent 50 μm .

**BASIC
PROTOCOL**

**PREPARATION OF EXTRACELLULAR MATRICES PRODUCED BY
CULTURED FIBROBLASTS**

In this protocol, cultured fibroblasts produce their own three-dimensional matrix. The fibroblasts are plated and maintained in culture in a confluent state. After 5 to 9 days, matrices are denuded of cells, and cellular remnants are removed.

Depending on the laboratory equipment available and the anticipated uses of the fibroblast-derived three-dimensional matrices, one must select a suitable surface on which the matrices will be produced (e.g., glass-bottom dishes, coverslips, or tissue culture dishes). (1) Disposable glass-bottom dishes (MatTek) can be utilized for real-time fluorescent experiments or for quality assessment assays (e.g., cell attachment) using an inverted fluorescent microscope (see Support Protocol 1). (2) Coverslips can be used for immunofluorescence experiments in which samples are to be fixed and mounted on microscope slides (see Support Protocol 2), or for mechanical compression of the fibroblast-derived three-dimensional matrices to be used as control two-dimensional surfaces (see Support Protocol 3). (3) Regular tissue culture dishes (e.g., 35-mm diameter) can be used for *in vivo* observations with an inverted microscope, or if the matrix is to be collected and solubilized for additional characterization and/or for biochemistry analysis (see Support Protocol 4).

**Preparation of
Extracellular
Matrices
Produced by
Fibroblasts**

10.9.2

Materials

- NIH-3T3 cells growing in tissue culture (see recipe)
- Trypsin/EDTA solution (see recipe)
- Confluent medium (see recipe)
- Absolute (anhydrous) ethanol
- Phosphate-buffered saline (PBS; *APPENDIX 2A*)
- 0.2% (w/v) gelatin solution (see recipe)
- Matrix medium (see recipe)
- Extraction buffer (see recipe), 37°C
- 10 U/ml DNase I (Roche) in PBS (*APPENDIX 2A*) containing 1 mM CaCl₂ and 1 mM MgSO₄ (optional)
- PBS (*APPENDIX 2A*) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml Fungizone (additives available from Life Technologies)
- 15-cm tissue culture dishes
- Inverted phase-contrast microscope
- Small tweezers (e.g., Dumont no. 4), sterilized
- Appropriate surface for producing matrix (one of the following; see discussion above):
 - Glass-bottom no. 1.5 plates (MatTek)
 - 22-mm circular high-quality coverslips (Carolina Biological Supply) with 35-mm tissue culture dishes as containers
 - 6-well tissue culture plates *or* 35-mm dishes
- Additional reagents and equipment for tissue culture (*UNIT 1.1*)

Prepare cell cultures

1. Aspirate and discard the culture medium from a semiconfluent (80% confluent) culture of NIH-3T3 cells growing on a 15-cm culture tissue culture dish.
2. Rinse the cell layer briefly with trypsin/EDTA solution.

This rinse will remove traces of serum that contains trypsin inhibitors.
3. Add enough trypsin–EDTA solution to cover the cell layer, quickly aspirate excess liquid, and observe under an inverted microscope at room temperature until the cells have detached from the culture dish (1 to 3 min).
4. Collect the cells in 10 ml of confluent medium by adding the medium to the dish and swirling to suspend the trypsinized cells.
5. Add 2 ml of the suspended cells to a 15-cm tissue culture dish and culture for 2 to 3 days, until semiconfluent (80% confluent).

As many as five 15-cm culture dishes may be used.

Prepare surfaces for matrix deposition

6. If coverslips are to be used as the surface for fibroblast-derived three-dimensional-matrix deposition, presterilize by flaming the coverslips after dipping in absolute (anhydrous) ethanol, place in 35-mm dishes, and rinse with PBS. Use small sterilized tweezers (e.g., Dumont no. 4) to handle the coverslips.
7. Add 2 ml of 0.2% gelatin solution to the culture surfaces to be used for fibroblast-derived three-dimensional matrix deposition and incubate 1 hr at 37°C.
8. Aspirate liquid and add 2 ml PBS.

At this point, the surfaces are ready to be seeded with matrix-producing fibroblasts.

Allow matrix deposition

This protocol was developed for NIH-3T3 cells; nevertheless, other fibroblast cell lines may be used. For example, the same protocol can be followed from this point on using human or other fibroblasts.

9. Trypsinize growing cells as in steps 1 to 3.
10. Collect cells from each dish in 10 ml of matrix medium (see step 4, but substitute matrix medium for confluent medium). Count cells (*UNIT 1.1*), and dilute with matrix medium to a final concentration of 1×10^5 cells per ml.
11. Aspirate PBS from gelatin-coated dishes (see step 8).
12. Seed 2×10^5 cells (2 ml) per 35-mm dish and culture for 24 hr.
Use as many dishes as needed; each 15-cm semiconfluent dish should provide enough cells for about 100 35-mm dishes.
13. After 24 hr, carefully aspirate the medium and replace with fresh matrix medium.
14. Replace medium with freshly made matrix medium every 48 hr for a total of 5 to 9 days after step 12, until the matrix is ready to be denuded of cells (see Fig. 10.9.1A).

Extract cells from fibroblast-derived three-dimensional matrices

15. Carefully aspirate the medium and rinse gently with 2 ml PBS by touching the pipet against the dish wall rather than at the bottom of the dish where the cells are located.
16. Gently add 1 ml of prewarmed (37°C) extraction buffer.
If coverslips are being used, gently lift the coverslip with the fine-pointed (e.g., Dumont no. 4) tweezers (or a syringe needle) so that extraction buffer reaches underneath. This step will ensure that the matrix deposited on the coverslip will be separated successfully from the remainder of the matrix deposited on the bottom of the culture dish, thus facilitating subsequent handling of the coverslips without tearing the delicate matrix.
17. Observe the process of cell lysis using an inverted microscope. Incubate until no intact cells are seen (about 3 to 5 min; see Fig. 10.9.1B).
18. Dilute the cellular debris by adding 2 to 3 ml PBS.
The above dilution process should be carried out gently in order to prevent turbulence that may cause the freshly denuded matrix layer to detach from the surface.
19. As cautiously as possible (using a pipet), aspirate the diluted cellular debris, but do not completely aspirate the liquid layer.
To avoid removing the matrix layer, do not attempt to aspirate the whole volume.
20. Gently repeat steps 18 and 19.
21. *Optional:* To minimize DNA debris, incubate the matrices with 2 ml of 10 U/ml DNase 30 min at 37°C. At the end of the incubation, aspirate the enzyme solution and wash twice with PBS.
22. Cover the matrix-coated plates (or coverslips) with at least 3 ml PBS supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml Fungizone. Seal with Parafilm.

These matrices can be stored for up to 2 or 3 weeks at 4 °C. However, for signal-transduction assays in serum-free medium, see Commentary.

23. Confirm the integrity of the matrices directly before use by examining for matrix integrity using an inverted phase-contrast microscope; the matrices should be attached to the culture surface and appear similar to the example in Fig. 10.9.1B.

ASSESSING THE QUALITY OF FIBROBLAST-DERIVED THREE-DIMENSIONAL MATRICES

The quality of fibroblast-derived three-dimensional matrices can be tested by one of two assays: induction of rapid cell attachment (see Support Protocol 1) and rapid acquisition of a spindle-shape morphology (see Support Protocol 2). These assays are based on examination of fluorescently labeled cells plated on three-dimensional matrices; the prelabeling with fluorescent dye is required in order to enhance observation of cells within fibroblast-derived three-dimensional matrices.

Cell Attachment Assay

Human or mouse fibroblasts can be used to evaluate the cell adhesion-promoting activity of the fibroblast-derived three-dimensional matrices. It has been reported that these in vivo-like three-dimensional matrices are about 6-fold more effective than two-dimensional substrates in mediating cell adhesion as quantified by a 10-min cell attachment assay (Cukierman et al., 2001). Briefly, cell nuclei are prelabeled to avoid any background staining from DNA debris on the three-dimensional matrix. The live prelabeled cells are rinsed free of excess dye, trypsinized, and plated on the fibroblast-derived three-dimensional matrix to be assessed, or onto control fibronectin-coated surfaces. After 10 min, nonattached cells are washed away, and attached cells are quantified by counting labeled nuclei.

Materials

- Semiconfluent fibroblasts (human or mouse) in 15-cm dish (see Basic Protocol, steps 1 to 5)
- Confluent medium (see recipe)
- Hoechst 33342 stock solution (see recipe)
- Phosphate-buffered saline (PBS; *APPENDIX 2A*), room temperature and 4°C
- Trypsin/EDTA solution (see recipe)
- Fixing solution (see recipe)
- 15-ml conical polypropylene centrifuge tubes
- Tissue culture centrifuge with rotor suitable for 15-ml conical tubes
- End-over-end rotator
- 3 glass-bottom no. 1.5 plates containing fibroblast-derived three-dimensional matrix (see Basic Protocol)
- 3 glass-bottom no. 1.5 plates with precoated two-dimensional fibronectin (see recipe; also see Support Protocols 3 and 4)
- Inverted fluorescence microscope with appropriate camera and filter set to visualize Hoechst 33342 (see *APPENDIX 1E*)
- Image analysis software capable of counting objects (optional; e.g., MetaMorph from Universal Imaging)
- Additional reagents and equipment for tissue culture (*UNIT 1.1*)

Prepare cells

1. Aspirate and discard the culture medium from a semiconfluent (80% confluent) 15-cm culture dish containing fibroblasts (mouse or human).

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2. Add 20 ml of confluent medium containing 40 μ l of Hoechst 33342 stock solution to the cells.
3. Incubate 15 min at 37°C.
4. Rinse with PBS four times.
5. Add enough trypsin/EDTA solution to cover the cell layer, quickly aspirate excess liquid, and observe under an inverted microscope until the cells have detached from the culture dish (1 to 3 min).
6. Collect the cells in 10 ml of confluent medium by adding the confluent medium to the dish and swirling to suspend the trypsinized cells. Transfer into a 15-ml conical polypropylene centrifuge tube and count an aliquot of the cells (*UNIT 1.1*).
7. Centrifuge 5 min at 100 \times g, room temperature.
8. Discard the supernatant and gently resuspend the cells with confluent medium to a final concentration of 3.5×10^5 cells/ml.
9. Rotate cells in suspension for 20 min at 37°C on an end-over-end rotator.

Perform adhesion assay

10. Carefully place a 150- μ l drop of cell suspension onto the glass bottom part of each of the dishes coated with three-dimensional matrices or two-dimensional fibronectin controls.
11. Incubate 10 min at 37°C.
12. Remove dishes from incubator, tilt dishes slightly to dislodge the medium droplet containing unattached cells from the glass portion onto the plastic portion of the dish, then aspirate the droplet.
13. Rinse dishes by slowly adding (to the plastic portion of each dish) 3 ml PBS precooled to 4°C.
14. Aspirate the PBS carefully and add 2 ml fixing solution.
15. Incubate 20 min at room temperature.
16. Aspirate and add 2 ml PBS at room temperature.

Visualize and analyze cell attachment

17. Using an inverted fluorescence microscope, acquire five random images of the nuclei from each one of the six dishes utilizing a 10 \times or 20 \times objective and count the Hoechst-stained nuclei.

Counting of the nuclei can be done automatically utilizing commercially available image-analysis software capable of counting objects (e.g., MetaMorph from Universal Imaging Corporation). If the counting is done automatically, then images should be acquired with a 10 \times objective. However, if the nuclei are to be counted manually, then a 20 \times objective is recommended.

The mean number of cells attached to the fibroblast-derived three-dimensional matrix should be up to 6-fold higher than the number attached to the fibronectin control. This result will confirm the quality of the fibroblast-derived three-dimensional matrix (Cukierman et al., 2001).

Determination of Cell Shape

Human or mouse fibroblasts can be used to evaluate induction of spindle-shaped cell morphology promoted by good-quality *in vivo*-like three-dimensional matrix. A recent report has established that fibroblasts will acquire an *in vivo*-like spindle-shaped morphology in cell-derived three-dimensional matrices 5 hr after plating (Cukierman et al., 2001). The protocol consists of prelabeling live fibroblast membranes with a fluorescent dye and incubating the cells on fibroblast-derived three-dimensional matrices or controls for a period of 5 hr. After this period of time, the fibroblast-derived three-dimensional matrix promotes a spindle-shaped morphology resembling *in vivo* fibroblast morphology, thereby confirming the quality of the three-dimensional matrices.

Materials

- 2% (w/v) BSA, heat denatured (see recipe)
- Phosphate-buffered saline (PBS; APPENDIX 2A)
- Semiconfluent fibroblasts (human or mouse) in 15-cm dish (see Basic Protocol, steps 1 to 5)
- Trypsin/EDTA solution (see recipe)
- Confluent medium (see recipe)
- 4 $\mu\text{g/ml}$ DiI working solution (prepared in confluent medium; see recipe)
- Fixing solution (see recipe)
- Gel Mount mounting medium (Biomed)
- 3 coverslips coated with fibroblast-derived three-dimensional matrix (see Basic Protocol)
- 3 coverslips with precoated two-dimensional matrix (see recipe; also see Support Protocols 3 and 4)
- 35-mm tissue culture dishes or 6-well plates
- Small tweezers (e.g., Dumont no. 4, sterilized)
- 15-ml conical polypropylene centrifuge tubes
- End-over-end rotator
- Tissue culture centrifuge with rotor suitable for 15-ml conical tubes
- Glass microscope slides
- Fluorescent microscope equipped with digital camera
- Image analysis software capable of measuring elliptical Fourier parameters (e.g., MetaMorph from Universal Imaging)
- Additional reagents and equipment for tissue culture (UNIT 1.1)

Block nonspecific cell binding with BSA

1. Cautiously place fibroblast-derived three-dimensional matrix and two-dimensional control-coated coverslips into 35-mm tissue culture dishes (or 6-well plates) with the matrix face up. Use small sterilized tweezers (e.g., Dumont no. 4) to handle the coverslips.
2. Block nonspecific cell binding by adding 2 ml of heat-denatured 2% BSA and incubating for 1 hr at 37°C.
3. Rinse all blocked coverslips with 2 ml PBS.

At this point coverslips are ready to be seeded with the prelabeled cells.

Label cell membrane with DiI

4. Aspirate and discard the culture medium from a semiconfluent (80% confluent) 15-cm dish of fibroblasts.
5. Rinse the cell layer briefly with trypsin/EDTA solution.

This rinse will remove traces of serum that contains trypsin inhibitors.

6. Add enough trypsin–EDTA solution to cover the cell layer, quickly aspirate excess liquid, and observe under an inverted microscope at room temperature until the cells have detached from the culture dish (1 to 3 min).
7. Collect the cells in 10 ml of 4 $\mu\text{g}/\text{ml}$ DiI working solution (in confluent medium) by adding the solution to the dish and swirling to resuspend the trypsinized cells. Transfer the suspended cells into a 15-ml conical polypropylene tube.
8. Incubate the cells with the dye in suspension by rotating gently for 30 min at 37°C.
9. Centrifuge 5 min at 100 \times g, room temperature.
10. Aspirate and discard the supernatant, then gently resuspend the cells with confluent medium to a final volume of 10 ml.
11. Repeat steps 9 and 10 four additional times to remove any remaining free dye.
12. Count cells (*UNIT 1.1*) and dilute with confluent medium to a final concentration of 1×10^4 cells per ml.
13. Carefully aspirate PBS from the coverslips (see step 3).
14. Add 2 ml of the diluted cell suspension to each of the dishes containing the coverslips and incubate 5 hr at 37°C.

For fast qualitative analysis, cells can be observed and photographed at the end of 5 hr with an inverted microscope (see APPENDIX 1E for wavelength information).

15. Aspirate medium and rinse with PBS.
16. Aspirate PBS and fix for 20 min at room temperature with 1 ml of fixing solution.
17. Aspirate fixing solution and rinse with PBS.
18. Rinse with water to eliminate residual salt.
19. Carefully lift coverslip and gently discard excess liquid by touching the edge of the coverslip onto a paper towel.
20. Mount coverslips (with cells face-down) on a droplet (~20 μl) of Gel Mount placed onto a glass microscope slide.
21. Allow mounted samples to dry in the dark at room temperature for ~1 hr.

At this point samples are ready for morphometry analysis, or they can be stored overnight in the dark at 4°C.

Perform morphometry analysis

22. Acquire fluorescent digital images, slightly overexposing to visualize the contour of the cells (for wavelength information, see APPENDIX 1E).

Use a magnification that will allow visualization of the entire cell in each image. Randomly capture images of at least 12 cells per sample and a minimum of 36 cells per substrate.
23. Perform the measurements for both the length (span of the longest cord) and the breadth (caliper width) of each cell using image-analysis software.
24. Calculate the inverse axial ratio by dividing length by breadth.

The mean inverse axial ratio induced by a high-quality fibroblast-derived three-dimensional matrix should be about 3-fold greater than that induced by the two-dimensional fibronectin control (Cukierman et al., 2001).

The inverse axial ratio corresponds to the elliptical form factor (EFF) morphometric parameter found in the integrated morphometry analysis (IMA) function of MetaMorph software.

PREPARING TWO-DIMENSIONAL EXTRACELLULAR MATRIX CONTROLS

Any given cell response induced by in vivo–like fibroblast-derived three-dimensional matrices could be due to the three-dimensionality of the matrix, its molecular composition, or a combination of both. The following two support protocols provide methods for obtaining suitable two-dimensional controls with the same molecular composition as the three-dimensional matrices.

Mechanical Compression of the Fibroblast-Derived Three-Dimensional Matrix

This protocol describes how to apply pressure to the fibroblast-derived three-dimensional matrix in order to collapse the matrix to a flat substrate. Mechanical compression of the three-dimensional matrix ensures that all natural components of the three-dimensional matrix are present, with only the element of three-dimensionality lacking. Briefly, the three-dimensional sample is compressed using a known weight applied to a given area. The surface that comes into contact with the matrix is covered with a Teflon film to prevent sticking and to avoid tearing the flattened matrix as the weight is retracted.

Materials

Superglue
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Fibroblast-derived matrix on 22-mm coverslip

Ring stand equipped with a horizontal ring
Flat platform large enough to rest on the ring (see Fig. 10.9.2)
Suitable spacer smaller in width than the diameter of the ring but longer in height than the ring's depth (see Fig. 10.9.2)
12-mm round coverslips (Carolina Biological Supply)
Small tweezers (e.g., Dumont no. 4, sterilized)
Cork borer
Teflon film: protective overlay composed of, 0.001-in. FEP film on 0.008-in. vinyl film, with adhesive back (used to cover laboratory bench-tops; Cole-Parmer)
Biological hood equipped with UV light (optional)
Lifting laboratory jack (Fisher)
Weight (~158 g)
Fibroblast-derived three-dimensional matrix on 22-mm circular coverslips (see Basic Protocol)
35-mm tissue culture dishes
Inverted phase-contrast microscope

NOTE: Any other materials fulfilling the same purpose can be substituted for the hardware listed above.

Construct the weight holder for matrix compression

1. Using Superglue, glue the flat platform to the spacer in such a way that the spacer will protrude slightly beyond the bottom of the ring when the platform is placed on the ring (Fig. 10.9.2).

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PROTOCOL 3*

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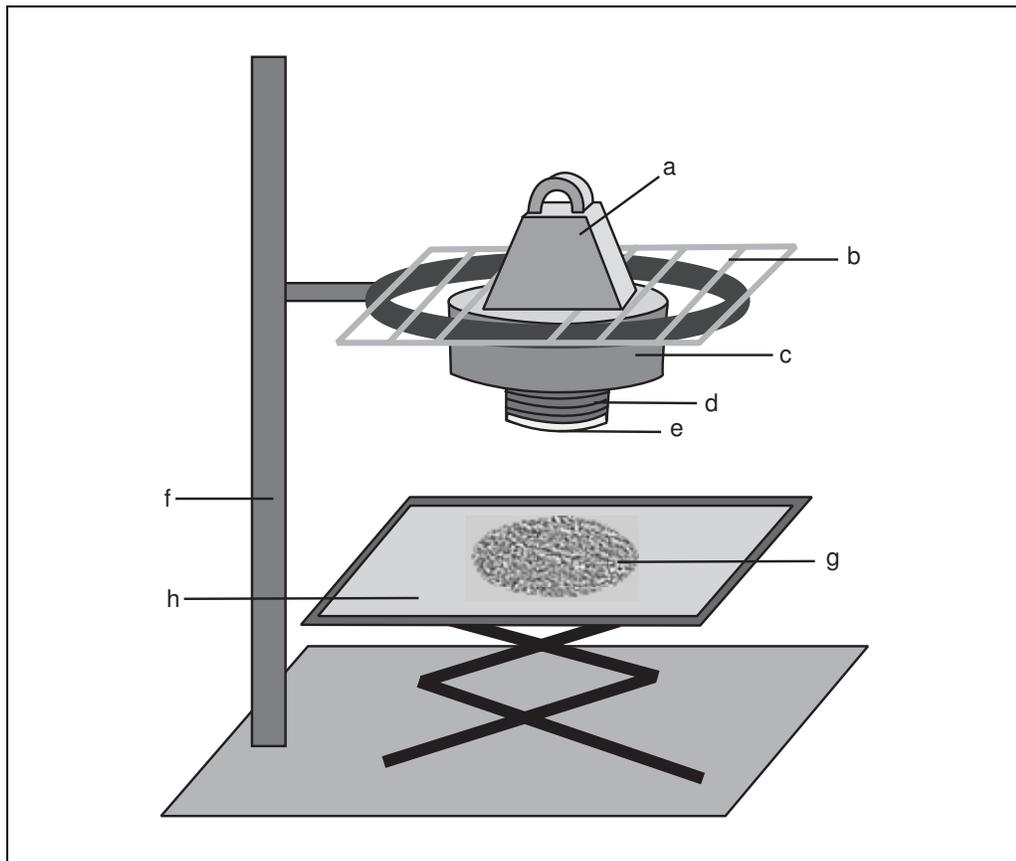


Figure 10.9.2 Diagram showing the components of the mechanical compression device. (a) weight; (b) flat platform; (c) spacer; (d) 12-mm coverslips; (e) Teflon film; (f) ring stand; (g) fibroblast-derived three-dimensional matrix to be mechanically compressed; (h) lifting laboratory jack.

2. Glue four coverslips to the end of the spacer (one on top of the other) as an extension of the spacer, and allow enough time for the Superglue to completely dry. Use small sterilized tweezers (e.g., Dumont no. 4) to handle the coverslips.

This will facilitate penetration of the coverslip portion into the matrix while avoiding contact between the matrix and the rest of the spacer, and it defines the area of compression.

3. Cut a circle (12-mm diameter) from the Teflon film with the cork borer.
4. Cover the last coverslip with the circle of Teflon film.
5. Sterilize materials by exposing them to a UV light in a biological hood for several hours with the Teflon film facing the light.

If the compressed matrices are to be in contact with cells for only short periods of time (e.g., for the 10-min cell attachment assay; see Support Protocol 1), rinsing the Teflon film with ethanol and air-drying should be sufficient to prevent contamination.

6. Place the glued platform with spacer on the ring portion of the stand with the Teflon facing down.
7. Cover the jack's flat upper surface with Parafilm and position the jack under the ring.
8. Set the weight on the platform and level the ring so that the Teflon film is situated parallel to the jack's surface (see Fig. 10.9.2).

Mechanically compress the fibroblast-derived three-dimensional matrix

9. Position the fibroblast-derived three-dimensional matrix-coated coverslip, with the matrix face-up, onto the jack directly underneath the Teflon film.
10. Slowly raise the laboratory jack until the matrix contacts the Teflon film and the platform rises above the ring.
11. Wait for 2 min.

At this point the entire weight should be resting on the matrix, compressing it at a specific weight per unit area.

12. Slowly lower the jack until the platform rests once again on the ring and the compressed matrix is separated from the Teflon film.
13. Place the coverslip with the compressed matrix into a 35-mm dish.
14. Carefully add 2 ml PBS and examine by phase-contrast microscopy to confirm continued integrity of the compressed matrix.

Solubilization of Fibroblast-Derived Three-Dimensional Matrix

This protocol describes how to solubilize fibroblast-derived three-dimensional matrix to generate a protein mixture that can be used for subsequent coating of surfaces or biochemical analysis. Briefly, the matrices are treated with a guanidine solution to denature and solubilize the matrix components, thereby producing a liquid mixture that can be stored and used for coating surfaces.

Materials

Fibroblast-derived three-dimensional matrices on 35-mm dishes (see Basic Protocol)

Solubilization reagent: 5 M guanidine containing 10 mM dithiothreitol (store indefinitely at 4°C)

Rubber policeman

End-over-end rotator

Solubilize matrix

1. Aspirate PBS from matrix-covered dishes.
2. Tip dishes ~30° with respect to the benchtop and hold in that position for 1 min to allow the excess PBS to accumulate on one side of the dish.
3. Aspirate the excess PBS carefully to avoid detaching the matrix layer.
4. Place the dishes on ice and add 300 µl of solubilization reagent.
5. Incubate on ice for 5 min.
6. Scrape the dish with the rubber policeman toward one side of the dish and pipet the mixture into a 1.5-ml microcentrifuge tube.
7. Add an additional 200 µl of solubilization reagent.
8. Rotate at 4°C for 1 hr.
9. Microcentrifuge 15 min at maximum speed, 4°C.
10. Transfer the supernatant into a fresh microcentrifuge tube and store at 4°C.

The average protein concentration is 1 to 3 mg/ml.

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10.9.11

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

BSA, heat-denatured, 2% (w/v)

Stock solution (undenatured): Dissolve 2 g bovine serum albumin (BSA) fraction V (Sigma) in 100 ml water and filter sterilize using a low-protein-binding 0.22- μ m filter. Store indefinitely at 4°C

Heat-denatured working solution: Just prior to use, heat the amount needed for 5 min at 65°C or until the solution starts to appear slightly translucent (not milky). Cool to room temperature before using for blocking procedures. Do not store the heat-denatured BSA.

Confluent medium

High-glucose Dulbecco's modified Eagle medium supplemented with:
10% (v/v) fetal bovine serum (FBS; APPENDIX 2A)
100 U/ml penicillin
100 μ g/ml streptomycin
Store up to 1 month at 4°C

Culture medium with calf serum

High-glucose Dulbecco's modified Eagle medium supplemented with:
10% (v/v) calf serum
100 U/ml penicillin
100 μ g/ml streptomycin
Store up to 1 month at 4°C

DiI stock and working solutions

Stock solution: Prepare 2.5 mg/ml DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes) in ethanol. Store up to 6 months at -20°C.

4 μ g/ml working solution: Dilute DiI stock solution to 4 μ g/ml with confluent medium (see recipe) and sterilize by filtration using a 0.22- μ m filter. Use promptly; do not store.

Extraction buffer

PBS (APPENDIX 2A) containing:
0.5% (v/v) Triton X-100
20 mM NH₄OH
Store up to 1 month at 4°C

Fibronectin-coated surfaces, precoated, two-dimensional

Prepare a 10 mg/ml solution of human plasma fibronectin (see UNIT 10.5, or purchase from Sigma) in phosphate buffered saline (PBS; APPENDIX 2A). Immediately add 1 ml of this solution per 35-mm tissue culture dish (or other surface to be coated) and incubate 1 hr at 37°C. Remove remaining fibronectin solution and rinse once with PBS.

The above procedure can be used with any desired protein for coating dishes or coverslips. If solubilized matrix mixture (see Support Protocol 4) is to be used, the coating protein concentration is 30 μ g/ml.

Fixing solution

In a 50-ml conical polypropylene conical tube, combine the following:
2 g sucrose
10 ml 16% (w/v) paraformaldehyde (EM grade; Electron Microscopy Sciences)
PBS (APPENDIX 2A) to 40 ml final
Prepare fresh before use

Gelatin solution, 0.2% (w/v)

Prepare a 0.2% (w/v) gelatin solution in PBS (*APPENDIX 2A*). Autoclave, cool, and filter through a 0.2- μ m filter. Store up to 1 year at 4°C.

Hoechst 33342 stock solution

Prepare a 2 mM solution of Hoechst 33342 (bisbenzimidazole H33342 fluorochrome, trihydrochloride; Calbiochem; mol. wt. 615.9 g) in water. Store up to 6 months at 4°C, protected from light.

Matrix medium

To confluent medium (see recipe) add L-ascorbic acid, sodium salt (Sigma) to a final concentration of 50 μ g/ml, from a freshly prepared 50 mg/ml stock solution. Sterilize by filtration with a 0.2- μ m filter.

NIH 3T3 cells, stock cultures

NIH-3T3 cells (ATCC #CRL-1658) must be routinely cultured in DMEM supplemented with 10% calf serum (see recipe for culture medium with calf serum, above) although medium using FBS is used in the protocol steps. Never allow cultured NIH-3T3 cells to become completely confluent while maintaining stock cultures. When cells reach 80% confluence (about once per week), subculture at 1:20 dilution.

Trypsin/EDTA solution

2.5 g trypsin
0.2 g tetrasodium EDTA
8 g NaCl
0.4 g KCl
1 g glucose
0.35 g NaHCO₃
0.01 g phenol red
H₂O to 1 liter
Sterilize by filtration with a 0.2- μ m filter
Store up to 3 months at -20°C

This solution is also commercially available from Life Technologies.

COMMENTARY

Background Information

Extracellular matrix (ECM) was historically regarded as a passive scaffold that stabilizes the physical structure of tissues. With time, it became evident that the ECM is much more than a simple physical scaffold. The ECM is a dynamic structure capable of inducing (and responding to) a large variety of physiological cell responses regulating the growth, migration, differentiation, survival, and tissue organization of cells (Buck and Horwitz, 1987; Hay, 1991; Hynes, 1999). Integrins are receptors for matrix molecules and can mediate these cell responses by inducing the formation of membrane-associated multimolecular complexes. These integrin-based structures (cell-matrix adhesions) mediate strong cell-substrate adhesion and transmit information in a bidirectional manner between ECM and the cytoplasm.

There are three main cell-to-matrix adhesions. The “focal adhesion” mediates firm linkage to relatively rigid substrates (Burrige and Chrzanowska-Wodnicka, 1996). Focal adhesions cooperate with “fibrillar adhesions” that generate fibrils from pliable fibronectin (Katz et al., 2000; Pankov et al., 2000). Fibroblasts require culture for several days at high cell density to generate three-dimensional matrices and evolve “three dimensional matrix adhesions.” The requirements for producing three-dimensional matrix adhesions include three dimensionality of the ECM, integrin $\alpha_5\beta_1$, fibronectin, other matrix component(s), and pliability of the matrix (Cukierman et al., 2001). The fibroblast-derived matrix provides an in vivo-like three-dimensional environment for cultured fibroblasts, thereby restoring their normally nonpolar surroundings. The fi-

broblast-derived three-dimensional matrix can be used as a suitable in vitro system to investigate in vivo-like fibroblast-to-matrix interactions, such as three-dimensional matrix–adhesion signaling.

Critical Parameters

The phenotype of cultured 3T3 fibroblasts as monitored by cell morphology is extremely important for the successful preparation of three-dimensional matrix-coated dishes. The fibroblasts should be well spread and flat under sparse culture conditions. If elongated cells are commonly observed in the cell population, recloning of the cell line may be necessary to achieve greater phenotypic homogeneity. The NIH-3T3 line obtained from ATCC (catalog no. CRL-1658) has this morphology and produces excellent matrix. The NIH-3T3 cells must be maintained routinely as subconfluent cultures in medium containing calf serum to retain the correct phenotype. However, if the matrix deposition at confluence is performed in the presence of calf serum, the resultant matrices are thicker but less stable and more likely to detach from the surface than matrices obtained after culture in fetal bovine serum. Therefore NIH-3T3 cells should be changed to medium containing fetal bovine serum prior to matrix deposition. A preadaptation of the cells in fetal bovine serum-containing medium after replating for 2 to 3 days is recommended.

The Basic Protocol could potentially be modified for other fibroblastic cell lines capable of secreting and assembling fibronectin-based matrices. In some cases, the resulting matrix may be too thick or dense to obtain efficient extraction. In such cases, more prolonged cell extraction may be needed, with extensive DNase treatment, until no cell debris is detected. The lack of contaminating cellular debris (in the case of NIH-3T3 cells) in the matrices has been confirmed by Western blotting and immunofluorescence staining for cellular proteins like actin.

Precoating surfaces with gelatin promotes fibronectin binding and results in smooth layers of relatively homogenous matrices that will not detach from the surface.

The thickness of NIH-3T3-derived three-dimensional matrices is measured using a confocal microscope without dehydration of the matrix (no mounting or fixing). The resultant thickness observed varies between 8 and 20 μm . Basic molecular characterization of the matrices revealed the presence (among other molecules) of fibronectin organized in a fibril-

lar mesh, collagen I and III, but not IV, and small traces of nonorganized laminin and perlecan.

The integrity of these three-dimensional matrices must be confirmed prior to every use. This can be accomplished by using phase-contrast microscopy and discarding any matrices that are torn or detached (see Fig. 10.8.1B). Moreover, if matrices are to be used for short-term signal transduction assays under serum-depleted conditions, freshly made matrices must be utilized. Matrices stored at 4°C (up to 2 to 3 weeks) should be used only after such assessment of integrity. Freshly prepared or stored matrices can be used to test the induction of cell responses in the presence of serum (e.g., attachment, morphology, motility, or proliferation), biochemical analysis (e.g., western blotting), and immunofluorescence staining.

Anticipated Results

The Basic Protocol is based on the ability of densely cultured fibroblasts to coat any available tissue culture surface by deposition of their natural matrix, which gradually forms a three-dimensional matrix. This intact, naturally produced ECM is similar in its molecular organization to mesenchymal fibronectin-based extracellular matrices in vivo (Cukierman et al., 2001). The basic approach is to allow cells to deposit their own ECM followed by removal of cells, while avoiding procedures that may alter or denature the native ECM constituents and supramolecular organization.

One NIH-3T3 semi-confluent (80%) cultured 15-cm dish can yield enough cells to coat 100 35-mm tissue culture dishes.

Time Considerations

The adaptation step after switching NIH-3T3 cell medium to fetal bovine serum for future matrix deposition requires 2 to 3 days. Matrix production will require between 5 and 9 days.

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Key References

Cukierman et al., 2001. See above

This is the paper upon which the procedures and materials in this unit are based. The Basic Protocol was modified from UNIT 10.4 in this manual, which was contributed by I. Vlodavsky in 1999.

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Purification and Analysis of Thrombospondin-1

UNIT 10.10

Thrombospondin-1 (TSP-1) is a trimeric matricellular protein that is expressed by many cells. It contains several different domains that allow it to participate in cell adhesion, cell migration, and cell signaling. Recently, TSP-1 has been shown to activate transforming growth factor- β (TGF- β) and to inhibit both angiogenesis and tumor growth. This unit describes two protocols: the purification of TSP-1 from platelet-rich plasma (see Basic Protocol 1) and the purification of TSP-1 proteolytic fragments (see Basic Protocol 2).

ISOLATION OF THROMBOSPONDIN-1 FROM HUMAN PLATELETS

TSP-1 is released from platelet α -granules in response to thrombin and can therefore be readily purified from the supernatant of thrombin-treated platelets. Human platelets can be obtained from the Red Cross or from hospital blood banks. Outdated pheresis units of platelet-rich plasma are a good source of TSP-1. Platelets are separated from plasma and other blood components by a series of centrifugation steps. The isolated platelets are washed repeatedly to remove plasma proteins and the washed platelets are then activated by exposure to thrombin. Next the TSP-1-containing supernatant is passed over a heparin-Sepharose column. Lower-affinity heparin-binding proteins are washed away and the TSP-1 is eluted under conditions of high salt. The TSP-1-containing fractions are pooled, precipitated, and loaded onto a 10% to 20% continuous sucrose gradient and subjected to ultracentrifugation. The gradient is divided into fractions and the protein concentrations are determined by measuring optical density. The level of purity is normally >95% as determined by SDS-PAGE (UNIT 6.1).

**BASIC
PROTOCOL 1**

Materials

Platelet-rich plasma
Baenziger A buffer (see recipe)
Baenziger B buffer (see recipe)
1 M CaCl₂ (APPENDIX 2A)
1 N NaOH (optional)
Thrombin
Diisopropyl fluorophosphate (DFP)
Heparin-Sepharose CL-6B (Amersham Pharmacia Biotech)
0.15, 0.25, 0.55, and 2.0 M heparin-Sepharose column buffers (see recipe)
Anti-vitronectin immunoaffinity column: prepare in advance according to manufacturer's instructions using an Affi-Gel Hz Immunoaffinity kit (Bio-Rad) and anti-human vitronectin antibody (e.g., GIBCO/BRL)
Ammonium sulfate
10% and 20% (w/v) sucrose gradient solutions (see recipe)
15- and 50-ml centrifuge tubes (conical bottom preferred)
Preparative centrifuge (Sorvall RC-B3 or equivalent) and rotor (H4000 or equivalent)
40-ml Oak Ridge centrifuge tubes
High-speed centrifuge (Beckman J2-MC or equivalent) and rotor (JA-20 or equivalent)
1 \times 12-cm chromatography column
Fraction collector and appropriate tubes
Spectrophotometer set at 280 nm
Gradient maker
14-ml ultracentrifuge tubes
Ultracentrifuge (Beckman LM-80 or equivalent) and rotor (SW 41Ti or equivalent)

**Extracellular
Matrix**

NOTE: Platelets are temperature sensitive and activated by untreated glass surfaces; therefore, they should be handled at room temperature in plasticware, and centrifuges and buffers should be warmed to room temperature before use.

Prepare platelets

1. Transfer platelet-rich plasma to 50-ml centrifuge tubes (conical bottom preferred) and centrifuge in a Sorvall RC-B3 preparative centrifuge 20 min at $1400 \times g$ (2800 rpm in an H4000 rotor), 20°C.

Pheresis units are preferable, but random donor units of platelet-rich plasma also work well.

2. Carefully pour off the supernatant. Gently resuspend the cell pellet in Baenziger A buffer at a ratio of 15 ml buffer per 2 ml packed cells.
3. Transfer the platelet suspension to 15-ml centrifuge tubes and centrifuge 8 min at $120 \times g$ (800 rpm in an H4000 rotor), room temperature.

Most of the platelets will remain in suspension following this centrifugation, while erythrocytes and leukocytes will pellet.

4. Leaving behind the red cell pellet, carefully transfer the platelet suspension to 50-ml centrifuge tubes (~22 ml per tube).
5. Add Baenziger A buffer to a final volume of 50 ml. Mix by inverting the tube several times and centrifuge 20 min at $1400 \times g$ (2800 rpm in an H4000 rotor), 20°C.

Wash platelet pellet

6. Carefully pour off the supernatant. Resuspend each cell pellet in 15 ml Baenziger A buffer and then add buffer to a final volume of 50 ml. Invert the tube to mix and centrifuge 20 min at $1400 \times g$ (2800 rpm in an H4000), room temperature. Repeat once.
7. Remove the supernatant and resuspend the pellet in 15 ml Baenziger B buffer. Add sufficient Baenziger B to achieve a ratio of 50 ml buffer per 2 to 3 ml packed cells. Mix the tube by inversion.
8. Add 100 μ l of 1 M CaCl_2 per 50 ml suspension.

From this point on, 2 mM calcium must be present at all times to maintain the conformational integrity of the thrombospondin molecule.

9. Check the pH of the suspension using pH paper. Adjust to pH 7.6 by adding 1 N NaOH as necessary.

Activate platelets

10. *Optional:* If the platelets are from outdated units, enhance their response to thrombin by incubating 5 min in a 37°C water bath.
11. Add 50 U thrombin per 50 ml platelet suspension and immediately mix by gentle inversion. Continue mixing 2 to 3 min at room temperature, then place on ice.

Platelet aggregation should be evident upon examination of the suspension. The platelets will form large clumps and settle to the bottom of the tube, causing the supernatant to appear somewhat clear after 2 to 3 min. Outdated platelets respond more slowly than fresh ones. Outdated units should therefore be mixed for an additional 2 to 3 min.

12. Remove the cellular debris by centrifuging the tubes 5 min at $1400 \times g$ (2800 rpm in an H4000 rotor), 4°C. Transfer supernatant to a 40-ml Oak Ridge centrifuge tube.

From this point on the TSP-1-containing supernatant must be kept on ice and all subsequent steps must be performed at 4°C.

13. Add sufficient DFP to achieve a final concentration of 1 mM (i.e., 0.181 μ l/ml).

CAUTION: DFP is a powerful serum protease inhibitor and is highly toxic. Great care should be taken in its use. DFP is volatile and should be used in a fume hood.

Isolate TSP-1 supernatant

14. Centrifuge 20 min in a Beckman J2-MC high-speed centrifuge at $34,957 \times g$ (17,000 rpm in a JA-20 rotor), 4°C.
15. Transfer the supernatant to a clean 50-ml tube. Place the sample on ice and leave overnight at 4°C.

This incubation step is necessary to allow formation of fibrin fibrils, which are then removed by centrifugation (step 17). If the supernatants are applied to the heparin-Sepharose column without performing this procedure, the fibrin fibrils will form on the top of the column and the flow rate will be decreased significantly.

Isolate TSP-1

16. Prepare and pour enough heparin-Sepharose CL-6B, according to the manufacturer's instructions, to produce a 5-ml bed volume in a 1 \times 12-cm chromatography column. Equilibrate the column with 50 ml of 0.15 M heparin-Sepharose column buffer.
17. Following the overnight incubation (step 15), centrifuge the supernatant 20 min at $1400 \times g$ (2800 rpm in an H4000 rotor), 4°C. Transfer the supernatant to a new tube.
18. Load the supernatant onto the equilibrated heparin-Sepharose column at a flow rate of ~ 3 ml/min.

The TSP-1 will be immobilized on the column following this step. If necessary, the protocol may be paused at this point; however, the column should be washed extensively with 0.15 M heparin-Sepharose column buffer before pausing. TSP-1 is stable on the column for 3 to 4 days.

19. Connect the column to a fraction collector with appropriate tubes and elute the column with 40 ml of 0.15 M heparin-Sepharose column buffer at a flow rate of ~ 3 ml/min, collecting twenty 2-ml fractions. Repeat with 0.25 M heparin-Sepharose column buffer.

Little or no TSP-1 will be present in these first two elutions.

20. Elute TSP-1 by applying 40 ml of 0.55 M heparin-Sepharose column buffer and collect in 2-ml fractions. Determine which fractions contain protein by measuring their absorbance at 280 nm. Calculate the total amount of protein in milligrams using the following formula: total protein = $OD_{280} \times 1.08 \times \text{volume}$.

After elution, >80% of total protein is TSP-1.

21. Strip the heparin-Sepharose column by applying 100 ml of 2.0 M heparin-Sepharose column buffer. Equilibrate and store the column in 0.15 M heparin-Sepharose column buffer at 4°C.

The column can be used repeatedly if treated in this manner.

22. Pool the protein-containing fractions and apply to an anti-vitronectin immunoaffinity column.

Although vitronectin is present in only trace amounts in the TSP-1-containing fraction (<1%), cells adhere strongly to vitronectin, which can pose a problem in certain applications involving purified TSP-1. It is therefore advisable to remove it. The authors are unable to detect vitronectin in the immunoaffinity flowthrough by immunoblotting.

For storage and reuse of the immunoaffinity column, refer to the manufacturer's instructions.

23. Transfer the flowthrough to a 40-ml Oak Ridge tube. Precipitate the protein by adding ammonium sulfate to 40% (w/v). Mix by inverting the tube until the solid is dissolved.
24. Centrifuge the sample 20 min at $34,957 \times g$ (17,000 rpm in a JA-20 rotor), 4°C.

The precipitated protein should form a milky-white pellet following centrifugation. It may be useful to note the orientation of the tube in the rotor to aid in locating the pellet.

25. Carefully pour off the supernatant and briefly leave the tube inverted to drain away all remaining liquid.
26. Resuspend the pellet in sufficient 0.15 M heparin-Sepharose buffer so that the protein concentration is ~1 mg/ml.

The concentration does not change significantly from the value determined in step 20.

27. Using a gradient maker, prepare 12 ml of a 10% to 20% continuous sucrose gradient in a 14-ml ultracentrifuge tube.

28. Carefully load the protein-containing sample onto the gradient, using no more than 2 mg protein on each gradient in order to achieve good resolution.

Drawing up the solution into a pipet tip and slowly discharging it by turning the volume adjustment wheel on the pipettor works well. It is important not to disturb the gradient.

See UNIT 5.3 for more information concerning sucrose gradients.

29. In a Beckman LM-80 ultracentrifuge, centrifuge the gradients 18 hr at $247,605 \times g$ (38,000 rpm in an SW 41Ti rotor), 4°C.

30. Fractionate the sucrose gradients into 0.5-ml aliquots. Read the absorbance of each fraction at 280 nm.

The concentration of TSP-1 in each fraction is determined using the equation $c = A_{280}/\epsilon l$, where ϵ is the molar extinction coefficient of TSP-1 (1.08 in $M^{-1}cm^{-1}$) and l is the pathlength of the cuvette.

TSP-1 is the major peak located in the middle third of the gradient. The peak usually appears in the eighth fraction from the bottom and continues over approximately eight fractions. There is also a minor peak located higher in the gradient that is composed mainly of β -thromboglobulin. The purified TSP-1 can be frozen directly in the sucrose gradient solution and stored 3 to 5 years at $-70^\circ C$. If necessary for specific applications, sucrose can be removed by dialysis (APPENDIX 3C).

BASIC PROTOCOL 2

ISOLATION OF PROTEOLYTIC FRAGMENTS OF TSP-1

The identification of functional sites within larger proteins can be accomplished by producing individual domains for functional studies. The proteolytic digestion of native molecules or the expression of individual domains by recombinant approaches is typically used for this purpose. While the authors were developing the procedure for the purification of TSP-1, it was observed that molecules that lacked the 25,000-Da N-terminal domain were not retained by the heparin-Sepharose column. This observation led to the early identification of the N-terminal domain as a high-affinity heparin-binding site and to the development of a procedure to purify this domain. During the development of the isolation protocol, it was also found that TSP-1 purified in the presence of calcium was distinct from that purified in the presence of EDTA. The removal of calcium from the protein renders some regions much more labile to proteolysis (Lawler and Hynes, 1986). Subsequent sequencing studies revealed that the type 3 repeats are a contiguous set of calcium-binding sites. Removal of calcium causes the type 3 repeats and the adjacent

C-terminal domain to unfold and become more labile to proteolysis. Thus, it is difficult to design a protocol that utilizes proteolysis in order to isolate the type 3 repeats and the C terminus of TSP-1. These domains are either resistant to digestion in the presence of calcium or are readily digested in its absence. In the absence of calcium, the central 70,000-Da core of the protein can be produced by chymotryptic digestion. This structure contains the intrachain disulfide bonds and hence is trimeric with a molecular weight of 210,000 Da. A procedure for preparing the N-terminal heparin-binding domain and the central core region is provided below.

Materials

TSP-1 (see Basic Protocol 1)

TBS (see recipe) containing 2 mM CaCl₂

0.5 M EDTA (*APPENDIX 2A*)

Chymotrypsin

Diisopropyl fluorophosphate (DFP)

0.8 × 3-cm column of immobilized soybean trypsin inhibitor (Pierce)

0.8 × 3-cm column of heparin-Sepharose CL-6B (Amersham Pharmacia Biotech)

0.15, 0.25, and 0.55 M heparin-Sepharose column buffers (see recipe)

Centriplus centrifugal filter device (3000-Da cutoff; Millipore)

1.3 × 30-cm column of Sephadex G-200

Fraction collector

Spectrophotometer set at 280 nm

1. Begin with 5 mg purified TSP-1 in 3 ml TBS containing 2 mM CaCl₂ on ice. Add 0.5 M EDTA to a final concentration of 5 mM.
2. Dissolve chymotrypsin in TBS to a final concentration of 1 mg/ml and add 50 μl to the sample.

This gives an enzyme-to-substrate ratio of 1:100 (w/w).

3. Digest 20 hr in a covered ice bucket in a 4°C cold room.

This approach produces a very reproducible digestion pattern.

4. Terminate the digestion by adding DFP to 1 mM (0.181 μl/ml) and incubating an additional 2 hr on ice.
5. Pass the sample over a 0.8 × 3-cm column of immobilized soybean trypsin inhibitor to remove the chymotrypsin.
6. Collect the flowthrough from the column and apply to a 0.8 × 3-cm heparin-Sepharose column connected to a fraction collector with appropriate tubes.
7. Collect 1-ml fractions while the sample is flowing onto the column and throughout the elution. Elute the column in a stepwise fashion with 20 ml (each) of 0.15, 0.25, and then 0.55 M heparin-Sepharose buffer.

The 25,000-Da fragment elutes in the 0.55 M heparin-Sepharose buffer. The protein is ~95% pure and can be used directly or concentrated further. Some applications may require dialysis to reduce the salt concentration.

The 210,000-Da fragment is in the initial flowthrough fractions. It should be dialyzed and purified on a Sephadex G-200 column as described in the following steps.

8. Pool the flowthrough fractions and concentrate to 2 ml using a Centriplus centrifugal filter device.
9. Apply this sample to a 1.3 × 30-cm Sephadex G-200 column equilibrated with TBS at 4°C.

Sephadex G-200 is fragile and care should be exercised to follow the manufacturer's directions for removing fines and for pouring and eluting the column.

10. Apply TBS and collect fifty 1-ml fractions. Measure the OD₂₈₀ of each fraction.

The 210,000-Da fragment is eluted in the molecular weight peak near the void volume of the column. This material can be concentrated using the Centriplus centrifugal filter device (step 8) and should be >95% pure.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Baenziger A buffer

0.102 M NaCl
0.0039 M K₂HPO₄
0.0039 M Na₂HPO₄
0.022 M NaH₂PO₄
0.0055 M glucose
Store up to 2 weeks at 4°C

Baenziger B buffer

0.015 M Tris·Cl, pH 7.6 (APPENDIX 2A)
0.14 M NaCl
0.005 M glucose
Store up to 2 weeks at 4°C

Heparin-Sepharose column buffers, 0.15, 0.25, 0.55, and 2.0 M

0.015 M Tris·Cl, pH 7.6 (APPENDIX 2A)
0.002 M CaCl₂
0.15, 0.25, 0.55, or 2.0 M NaCl
Store up to 3 weeks at 4°C

The molarity of the buffer refers to the concentration of the NaCl.

Sucrose gradient solutions, 10% and 20% (w/v)

0.015 M Tris·Cl, pH 7.6 (APPENDIX 2A)
0.14 M NaCl
0.002 M CaCl₂
10% or 20% (w/v) sucrose
Store up to 1 week at 4°C

TBS (Tris-buffered saline)

0.015 M Tris·Cl, pH 7.6 (APPENDIX 2A)
0.015 M NaCl
Store up to 2 weeks at 4°C

COMMENTARY

Background Information

The thrombospondins are a family of extracellular matrix proteins currently consisting of five members, thrombospondins 1 to 4 and cartilage oligomeric matrix protein (COMP). For comprehensive reviews, see Adams (2001) and Chen et al. (2000). These proteins are synthesized by many tissues with patterns of expression that are temporally and spatially

regulated. All thrombospondin family members are composed of a series of multidomain structures and have the ability to bind large numbers of calcium ions. Calcium binds to the thrombospondins through a cooperative mechanism that involves a significant conformational change in the protein. Through interactions with molecules on the cell surface and components of the extracellular matrix, the

thrombospondins play a role in cell adhesion, migration, differentiation, and proliferation.

Thrombospondin-1 (TSP-1) was the first member of the gene family to be identified and has been the most extensively characterized. TSP-1 is a large multifunctional glycoprotein with a molecular weight of 420,000 Da, and is a trimer composed of identical subunits each with a molecular weight of 142,000 Da. TSP-1 is expressed by both normal and tumor cells and has a number of domains that allow it to interact with cells and other proteins. These include (1) a heparin-binding domain that interacts with proteoglycans, integrin $\alpha 3\beta 1$, and cell-surface glycosaminoglycans (Clezardin et al., 1997; Merle et al., 1997); (2) three type 1 repeats that interact with CD36, matrix metalloproteinases, fibronectin, and heparan sulfate proteoglycans, and also activate latent TGF- β (Bornstein, 1995; Schultz-Cherry et al., 1995; Crawford et al., 1998); (3) an RGDA sequence within the last type 3 repeat, which interacts with integrin $\alpha v\beta 3$; and (4) a C-terminal cell-binding domain that contains a recognition sequence for the integrin-associated protein CD47 (Gao et al., 1996). In this unit, the authors focus on the activities of TSP-1 that involve the type 1 repeats and the interaction of TSP-1 with integrins (Fig. 10.10.1). The interaction of TSP-1 with proteoglycans is discussed in detail in a recent review by Chen et al. (2000).

TSP-1 and transforming growth factor- β

Recently, TSP-1 has been shown to activate transforming growth factor- β (TGF- β) by binding to the latency-associated protein and altering the conformation of TGF- β to make it accessible to its receptor (Schultz-Cherry et al., 1995; Crawford et al., 1998). The region of TSP-1 responsible for TGF- β activation is the amino acid sequence KRFK, which is found at the start of the second type 1 repeat (Schultz-Cherry et al., 1995; Crawford et al., 1998; Fig. 10.10.1). TGF- β is a 25-kDa homodimeric cytokine and a known tumor suppressor (Markowitz and Roberts, 1996). It is secreted in a latent complex consisting of mature TGF- β , the latency-associated protein, and sometimes an additional latent TGF- β -binding protein. The latent TGF- β -binding protein is thought to target latent TGF- β to sites in the extracellular matrix where it is sequestered until activated. Activation of TGF- β has been demonstrated in vitro by activators such as acids, plasmin, or cathepsin D (Munger et al., 1997). TSP-1 and the $\alpha v\beta 6$ integrin have been shown to activate TGF- β in vivo (Crawford et al., 1998; Munger et al., 1999). Activation of TGF- β by TSP-1 was demonstrated in vivo when TSP-1-deficient mice were injected with a peptide containing the sequence KRFK. The lungs of the injected mice became morphologically more similar to wild-type mice and active TGF- β was detected in the bronchial epithelial cells (Crawford et al., 1998). In some contexts, however, TSP-1 does not appear to be a good activator of TGF- β

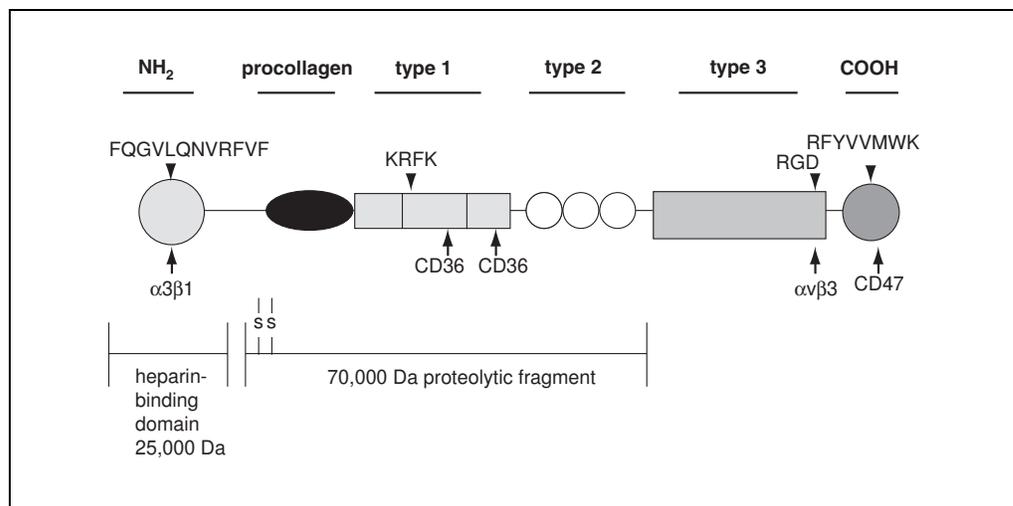


Figure 10.10.1 Representative model of TSP-1 identifying the different structural and functional domains. The binding sites for the various integrins, CD36, and CD47 are indicated below the model. Amino acid sequences that mediate receptor binding and activation of TGF- β are indicated above the model. The proteolytic fragments isolated in the protocol are shown at the bottom. The FQGVLQNVRFVF sequence is a GAG-independent cell binding site and the RFYVMWK sequence is an integrin-associated protein (CD47) binding site.

(Abdelouahed et al., 2000; Grainger and Frow, 2000). These data indicate that post-translational modification or other factors may regulate the ability of TSP-1 to activate TGF- β . Thus, co-expression of TSP-1 and TGF- β does not necessarily mean that TSP-1 will activate latent TGF- β in that tissue.

The role of TSP-1 in angiogenesis and cancer

TSP-1 has been shown to be an effective inhibitor of angiogenesis, tumor progression, and metastasis (Chen et al., 2000; Lawler, 2002). While TSP-1 levels are very low in many tumor cells, expression of TSP-1 is high in the tumor stroma (Brown et al., 1999). Overexpression of TSP-1 in MDA-MB-435 human breast carcinoma cells decreased tumorigenesis and metastasis in vivo (Weinstat-Saslow et al., 1994). Furthermore, the tumors derived from cells formed by a fusion of low-TSP-1-expressing human breast cancer cells and high-TSP-1-expressing normal breast epithelial cells were smaller in nude mice as compared to the tumors formed from the breast cancer cells alone (Zajchowski et al., 1990). Lastly, one group has shown that plasma TSP-1 secreted from primary HT1080 fibrosarcomas in nude mice inhibited growth of experimental metastases (Volpert et al., 1998). Moreover, if the implanted fibrosarcoma cells were transfected with an antisense TSP-1 construct prior to implantation, melanoma cell invasion of the lung was not inhibited.

Recently, the authors have shown that recombinant proteins comprising the second type 1 repeat of TSP-1 and containing the TGF- β activating sequence KRFK inhibited B16F10 tumor growth in mice (Miao et al., 2001). Furthermore, it was observed that treatment with a TGF- β antibody or soluble TGF- β receptor reversed this inhibition, suggesting that TSP-1 activation of TGF- β is part of the inhibitory pathway. By contrast, an effect of TGF- β was not observed with Lewis lung carcinoma because these cells have acquired mutations that have rendered them unresponsive. Vascular density was decreased in both B16F10 and Lewis lung carcinoma tumors treated with the recombinant proteins through a TGF- β -independent mechanism.

In another study, Streit et al. (1999) overexpressed full-length TSP-1 in A431 human carcinoma cells and implanted these cells in the flanks of nude mice. Decreased tumor growth and angiogenesis were observed in tumors expressing TSP-1. Recent work has demonstrated

that the KRFK sequence in the second type 1 repeat of TSP-1 is partly responsible for this growth inhibition and the decrease in tumor angiogenesis (K. Yee, unpub. observ.). In another recent study, TSP-1 null mice were crossed with *c-neu* transgenic mice to create a mouse that develops breast tumors and does not express TSP-1. These mice developed tumors that were larger and more vascular than the tumors of mice overexpressing TSP-1 (Rodríguez-Manzaneque et al., 2001). The authors also determined that the absence of TSP-1 in these tumors resulted in an increase in the amount of active matrix metalloproteinase 9 (MMP-9).

The effects of TSP-1 on endothelial cell migration and angiogenesis have been previously observed by several groups (Tolsma et al., 1993; Dawson et al., 1997; Qian et al., 1997; Iruela-Arispe et al., 1999; Jiménez et al., 2000; Nör et al., 2000). These studies demonstrate that TSP-1 is able to prevent tumor progression in several in vivo cancer models and that one of the ways TSP-1 inhibits tumor growth may be through decreasing tumor angiogenesis. In a different avenue of thinking, many groups have examined MMP-2 and MMP-9 with regards to breast cancer progression (Benaud et al., 1998; Martorana et al., 1998; Remacle et al., 1998; Rudolph-Owen et al., 1998; Lee et al., 2001). MMP-2 and -9 are gelatinases that degrade collagen types IV, V, VII, and X, as well as denatured collagen and gelatin (Dollery et al., 1995). Recently, TSP-1 has been shown to interact with MMP-2 and -9 and inhibit their activation (Bein and Simons, 2000; Rodríguez-Manzaneque et al., 2001). This interaction is mediated by the type 1 repeats of TSP-1. Therefore, one of the mechanisms through which TSP-1 inhibits both tumor progression and tumor angiogenesis may be due to its ability to inhibit MMP activation and prevent growth factor and cell mobilization.

Angiogenesis is a complex process that involves multiple cell types. TSP-1 does have possible effects on the recruitment of immune cells and on the proliferation and migration of vascular smooth muscle cells. In some assays, these effects can predominate, leading to the conclusion that TSP-1 supports angiogenesis. The preponderance of in vivo data indicates that the anti-angiogenic effects predominate in tumors.

TSP-1 and CD36

CD36 is an integral membrane glycoprotein, a member of the class B scavenger receptor

family, and is located within the caveolae of the cell membrane. It is expressed in many cells including microvascular endothelium, adipocytes, skeletal muscle, dendritic cells, and hematopoietic cells including platelets and macrophages (Febbraio et al., 2001). CD36 is also a receptor for TSP-1 and binds to the specific sequence CSVTCG in the second and third type 1 repeats of TSP-1, while TSP-1 type 1 repeats bind the CD36 LIMP-II Emp sequence homology (CLESH) region of CD36 (Crombie and Silverstein, 1998). This binding initiates a signal that involves the nonreceptor tyrosine kinases *fyn*, *lyn*, and *yes* as well as p38MAPK (Huang et al., 1991). One of the endpoints of this cascade is activation of caspase 3 and endothelial cell apoptosis (Guo et al., 1997; Jiménez et al., 2000; Nör et al., 2000). CD36 signaling is one of the mechanisms by which TSP-1 inhibits angiogenesis and tumor progression (Dawson et al., 1997; Simantov et al., 2001).

The initial work on exploring the anti-angiogenic effect of TSP-1 through CD36 utilized peptides containing the CSVTCG sequence. These peptides inhibited endothelial cell migration and angiogenesis (Iruela-Arispe et al., 1991; Tolsma et al., 1993; Dawson et al., 1999). Antibodies to CD36 also inhibited endothelial cell migration (Dawson et al., 1997) and, in CD36-null mice, TSP-1 did not inhibit angiogenesis in a cornea pocket assay (Jiménez et al., 2000). Therefore, binding of TSP-1 to CD36 on endothelial cells inhibits angiogenesis and tumor progression.

TSP-1 and integrins

Integrins are a family of cell surface receptors composed of both an α and a β subunit (Hynes, 1992). TSP-1, in both soluble and matrix-bound forms, can interact with $\beta 1$ and $\beta 3$ integrins; however, the physiological consequences of binding are dependent upon the integrin engaged, the cell type, and in some cases the involvement of accessory proteins.

TSP-1 and $\beta 1$ integrins

In breast carcinoma cells, $\alpha 3\beta 1$ is essential for chemotaxis towards TSP-1 and cell spreading on an immobilized TSP-1 matrix (Chandrasekaran et al., 1999). This interaction is mediated through binding of the integrin to residues 190 to 201 of the N-terminal region of TSP-1 (Kruttsch et al., 1999). In the presence of a $\beta 1$ -activating antibody, the adhesive properties of the carcinoma cells on TSP-1 are enhanced. This is characterized by rearrange-

ment of F actin filaments into filopodia that terminate at points that are rich in $\beta 1$ and are in contact with TSP-1. Signaling through the insulin-like growth factor-I receptor (IGF-IR) can also potentiate this adhesion. Recent evidence suggests that IGF-IR signaling activates $\alpha 3\beta 1$ by promoting association with the mitochondrial molecule heat shock protein 60 (Barazi et al., 2002).

Small-cell lung carcinoma cells also bind residues 190 to 201 of TSP-1 through $\alpha 3\beta 1$ (Guo et al., 2000). This interaction stimulates the cells to extend neurite-like processes and differentiate along a neuronal pathway. When epidermal growth factor is added to these cultures, binding to TSP-1 through this receptor also suppresses cell proliferation. This mechanism may be important for the antitumorigenic effects of TSP-1.

In response to loss of cell-cell contact, endothelial cells engage immobilized TSP-1 through $\alpha 3\beta 1$ and are stimulated to adhere to TSP-1 and proliferate (Chandrasekaran et al., 2000). This effect can be induced through disruption of cell contacts through wounding or by inhibiting vascular endothelial (VE) cadherin, indicating a role for TSP-1 in supporting repair of wounded endothelium. However, classically, TSP-1 is known for inhibiting endothelial cell proliferation and angiogenesis (Good et al., 1990). Indeed, endothelial cells exposed to a soluble TSP-1 peptide that recognizes $\alpha 3\beta 1$ have decreased proliferation and motility (Chandrasekaran et al., 2000). These opposing effects on endothelial cells suggest that tight regulation of TSP-1/ $\alpha 3\beta 1$ interaction and signaling exists. Recent studies using melanoma cells demonstrated that the ability of TSP-1 to bind $\alpha 3\beta 1$ is altered when TSP-1 is bound to fibronectin (Rodrigues et al., 2001). Conformational regulation of TSP-1 may represent one mechanism by which integrin-mediated cellular responses are controlled.

Activated T-lymphocytes can adhere to intact TSP-1 through $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins (Yabkowitz et al., 1993). This may have implications for mediating T cell activation, as stimulation of the ERK pathway by TSP-1 in these cells can be inhibited using anti- $\beta 1$ function-blocking antibodies (Wilson et al., 1999). A role for TSP-1 in modulating the inflammatory response would not be surprising since TSP-1-deficient mice suffer from inflammatory disease (Lawler et al., 1998).

TSP-1 and $\beta 3$ integrins

In platelets, it was originally discovered that $\alpha v\beta 3$ and, to a lesser extent, $\alpha IIb\beta 3$ (GPIIb/IIIa) function as adhesion receptors for TSP-1. The recognition site for these integrins is the RGD motif located in the type 3 repeats of TSP-1.

TSP-1 can influence integrin function directly and indirectly through its interaction with nonintegrin receptors. In platelets, binding of the C terminus of TSP-1 to the transmembrane receptor integrin-associated protein (IAP or CD47) leads to assembly of a TSP-1/IAP $\alpha I-Ib\beta 3$ complex on the platelet surface. This complex can further activate $\alpha IIb\beta 3$ and cause phosphorylation of focal adhesion kinase, resulting in both augmentation of platelet aggregation and attachment to fibrinogen (Chung et al., 1997). A necessity for G-protein signaling has since been added to this cascade of events (Frazier et al., 1999).

TSP-1/IAP $\alpha v\beta 3$ complexes are also important in other cell types. On vitronectin substrates, C32 human melanoma cells are stimulated to spread in response to complex formation (Gao et al., 1996). More recently, an increase in latent TGF- β activation, induced by tamoxifen treatment of breast carcinoma cells, has been shown to be dependent on localization of TSP-1 to the cell surface by this mechanism (Harpel et al., 2001).

Another example of TSP-1 affecting integrin function through cooperation with other receptors occurs in the clearance of apoptotic neutrophils. Here, TSP-1 associates with CD36 on the macrophage surface and $\alpha v\beta 3$ associates on the neutrophils where it forms a bridge, allowing the recognition of neutrophils for ingestion (Savill et al., 1992). This process can be modulated on a second exposure of macrophages to neutrophils by ligation of $\alpha v\beta 3$, $\alpha 6\beta 1$, and $\alpha 1\beta 2$ (Erwig et al., 1999).

$\alpha v\beta 3$ is also expressed on endothelial cells. In sickle cell anemia patients, both $\alpha v\beta 3$ in the endothelium (Solovey et al., 1999) and TSP-1 plasma levels are elevated. These proteins have been implicated in recurring vaso-occlusion problems in sickle cell patients caused by exaggerated adhesion of the sickle cell red blood cells (SS-RBCs) to the endothelium. Indeed, it has been demonstrated that TSP-1 enhances adhesion of SS-RBCs to cultured endothelial cells and that antibodies to $\alpha v\beta 3$ can block this event (Kaul et al., 2000). It is as yet unknown if this is a direct consequence of TSP-1/ $\alpha v\beta 3$ association.

Critical Parameters

The response of the platelets to thrombin is a critical factor contributing to the success of the purification procedure. Since platelets become less responsive during storage, the platelet-rich plasma should be processed as soon as possible after collection. Since platelets are temperature sensitive, buffers and centrifuges used in the purification procedure should be warmed to room temperature before beginning the procedure. The platelets should also be handled gently during the resuspension steps to prevent mechanical activation. Moreover, since platelets are activated by untreated glass surfaces, all transfer pipets and tubes should be plastic.

TSP-1 is susceptible to proteolysis following its secretion into the supernatant. It is important to work quickly following the activation step to minimize exposure to proteases secreted from the platelets and the thrombin used for the activation. The supernatant should be treated immediately with DFP following the debris-clearing centrifugation step in order to inactivate these proteases. The supernatant should be kept on ice at all times during the remaining purification steps.

The association of TSP-1 with calcium maintains the confirmation of the molecule. It is therefore essential that calcium be present in all solutions during and subsequent to thrombin treatment. A concentration of 2 mM is recommended.

Troubleshooting

The problem most likely to be encountered in the purification procedure is unresponsive platelets. To remedy this situation the procedure can be performed on a small scale using fresh platelets. This will provide a sense of how the aggregated platelets should appear following thrombin treatment. Another method for assaying platelet responsiveness is to perform electrophoresis on the supernatant from the thrombin-treated platelets. TSP-1 is a major component of the platelet α -granule and should appear as a prominent band running at an apparent molecular weight of 185,000 Da on discontinuous Laemmli SDS gels (UNIT 6.1). This anomalously high value for the molecular weight of the subunit is probably due to a decrease in the amount of SDS bound to the large number of negatively charged residues in the type 3 repeats.

Anticipated Results

The purification procedure should result in producing ~200 µg TSP-1 per 100 ml outdated platelet-rich plasma, which is most often >95% pure as determined by SDS-PAGE. There is evidence that some preparations of TSP-1 produced according to this method may contain trace amounts of active TGF-β bound to the TSP-1. It is possible to remove this contaminant by adjusting the pH of the sucrose gradient solutions to pH 11, as TGF-β will dissociate from TSP-1 under alkaline conditions (Murphy-Ullrich et al., 1992; Schultz-Cherry et al., 1994). The pH of the TSP-1-containing fractions should be returned to pH 7.6 immediately following centrifugation.

Whereas the protocol for purifying TSP-1 proteolytic fragments does not require many steps and is reasonably efficient, it is important to bear in mind that the N-terminal domain only represents ~18% of the total mass of the protein. Thus, if one starts with 5 mg total protein, a yield of 400 to 500 µg is appropriate. Since the 210,000-Da fragment represents about one-half of the protein, yields of 1 to 1.5 mg can be expected.

Time Considerations

The purification procedure is extended over a period of 3 days. The amount of time required to perform this procedure will depend in part on the amount of material to be processed. Approximately 3 to 4 hr should be allowed to isolate the TSP-1-containing supernatant (steps 1 to 15). Purification of TSP-1 (steps 17 to 28) will require another 3 to 4 hr. It is possible to leave the TSP-1 bound to the heparin-Sepharose column for a number of days prior to continuing the elution process.

The purification of proteolytic fragments also takes ~3 days. The limited tryptic digestion is done overnight. Elution of the heparin-Sepharose column can be done in ~1 day and the elution of the G-200 column requires another day.

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SPARC (secreted protein acidic and rich in cysteine) is a founding member of the matricellular group of proteins that have been shown to mediate interactions between cells and the extracellular matrix (ECM; Bornstein and Sage, 2002). Other proteins within this family include thrombospondins 1 and 2, osteopontin, tenascins C and X, and Cyr61. Over the last several years, a wealth of data, largely from mice with targeted disruptions of the respective genes, has emerged identifying various targets of the matricellular proteins that influence cell behavior—e.g., growth factors, cell-cycle regulatory proteins, ECM components, adhesion proteins and/or their receptors, cell survival, collagen fibrillogenesis, and immune cell function. In vivo, these effects can be translated into abnormalities in blood vessel morphogenesis and connective tissues, wound healing, bone formation, and responses to various types of injury. Therefore, study of one or more of the matricellular proteins affords insight from a somewhat unusual and underexplored perspective: the interface between the cell surface and the extracellular milieu.

SPARC belongs to a family of several genes, only one other of which, *SCI/hevin*, has been characterized beyond a limited degree (Brekken and Sage, 2000). *SPARC*-null mice exhibit many phenotypic abnormalities that follow logically from the effects of SPARC on cultured cells (i.e., de-adhesion, antiproliferation, interaction with growth factors and ECM, and regulation of collagen production). These characteristics include (1) accelerated dermal wound healing and fibrovascular invasion of sponge implants, (2) reduced foreign body response, (3) thin skin with decreased collagen, which is deposited as small-diameter fibrils, (4) excessive accumulation of adipose tissue, (5) osteopenia, and (6) cataract formation (Bornstein and Sage, 2002). Providing a mechanistic explanation for any one of these phenotypes requires experiments, largely in vitro, with active purified protein in clearly defined assays with quantitative endpoints. This unit presents several protocols for the purification of SPARC (see Basic Protocol and Alternate Protocols 1, 2, and 3), and for the measurement of its biological activity and conformation (see Support Protocols 1 and 2). Since the end product—i.e., natural SPARC or recombinant (rSPARC)—differs according to the source, guidelines for the choice of each protocol, and its advantages and limitations, have been included with the Basic Protocol (purification of SPARC from cultured cells), Alternate Protocol 1 (rSPARC from *E. coli*), Alternate Protocol 2 (rSPARC from insect cells), and Alternate Protocol 3 (SPARC from blood platelets). A method for determining endotoxin levels is presented in Support Protocol 3.

NOTE: To prevent denaturation of SPARC due to adsorption to surfaces, only polypropylene or siliconized glass should be used.

NOTE: All solutions and equipment coming into contact with live cells should be sterile and a septic technique should be used accordingly

PURIFICATION OF SPARC FROM PYS-2 CELLS

This protocol describes the purification of SPARC from cultured PYS-2 cells. This cell line, originally derived from a mouse parietal yolk sac carcinoma, has been a consistent reproducible source of biologically active SPARC for nearly two decades (Sage and Bornstein, 1995). The following procedure can be applied to most cell culture supernatants and involves essentially three steps: (1) precipitation of culture medium, (2) ion-exchange chromatography, and (3) molecular-sieve chromatography. Advantages of the PYS-2 cell line are its immortality, its high rate of growth, its copious production (secretion) of SPARC, and the presence of few other secreted products in the culture medium. It is also possible to radiolabel SPARC metabolically if desired. A commercial

BASIC PROTOCOL

Data Processing and Analysis

10.11.1

Contributed by E. Helene Sage

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Supplement 17

source of SPARC, isolated according to this protocol and of ~80% purity, is available from Sigma-Aldrich.

Materials

50% to 70% confluent PYS-2 cells (see recipe)
DMEM (serum-free; *APPENDIX 2A*)
1100 Ci/mmol (12.5 Ci/ml) [*trans*-³⁵S]methionine/cysteine (ICN; optional)
DMEM minus methionine and cysteine (optional)
0.2 M PMSF stock solution (see recipe)
N-Ethylmaleimide (NEM)
Ammonium sulfate, ultrapure
DEAE buffer, 4°C (see recipe)
NaCl
~2 × 20-cm DEAE column (see recipe)
S-200 buffer (see recipe)
Scintillation fluid (optional)
Sephacryl molecular-sieve column (see recipe)
0.05 M acetic acid

Plastic pipets
50-ml polycarbonate high-speed centrifuge tubes
Low-speed GPKR (Beckman) centrifuge with swinging bucket rotor
High-speed refrigerated centrifuge with GSA (Sorvall) or JA-17 rotors (Beckman) or equivalent
12,000- to 14,000-MWCO dialysis tubing (Spectrapor) or equivalent, prewashed with DEAE buffer
Dialysis clips (optional)
Standard gradient maker (e.g., Amersham Biosciences)
Peristaltic pump
Fraction collector
Lyophilizer
50 or 250 ml centrifuge tubes

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) with autoradiography (*UNIT 6.3*), if appropriate, and determination of protein concentration by spectroscopy (*APPENDIX 3B*)

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and surroundings. Carry out the experiments and dispose of wastes in appropriately designated area, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

Collect and precipitate tissue culture medium containing secreted SPARC

1. Replace medium in 20 to 30 dishes or flasks of PYS-2 cells (grown to 50% to 70% confluency) with 12 to 13 ml serum-free DMEM and preincubate 15 min at 37°C. Replace with fresh medium and then incubate 18 to 24 hr.

If desired, purification can be monitored by adding 500 μCi of 1100 Ci/mmol [³⁵S]methionine to one dish and processing the medium in parallel with nonlabeled medium from the other dishes. Alternatively, if radiolabeled SPARC of high specific activity is required for experimental purposes, [³⁵S]methionine/cysteine can be added to all dishes. When using label, incubate cells in serum-free DMEM lacking methionine and cysteine.

2. Collect the medium from the cell layer by gentle aspiration via plastic pipet and transfer to centrifuge tubes. Remove cellular debris by centrifuging in a clinical (i.e.,

tissue-culture) centrifuge 5 min at $1,000 \times g$, room temperature, or in GPKR centrifuge at $1000 \times g$, 4°C .

3. Pool all supernatants in a siliconized flask. Add 0.2 M PMSF drop-wise with stirring to a final concentration of 0.2 mM, and NEM to a final concentration of 10 mM. Stir on ice until medium reaches 4°C .

For 100 ml medium, add 0.1 ml PMSF stock solution and 62.5 mg NEM. Take care not to lyse cells in any of these procedures.

4. Add solid ultrapure ammonium sulfate to the medium in an amount equivalent to 50% (w/v) of the starting volume over a period of several hours. Stir 12 to 24 hr at 4°C .

For 100 ml medium, add 50 g ammonium sulfate, in very small increments (e.g., 1 to 2 g) over several hours (e.g., 3 to 5). This detail is important for maintenance of neutral pH and for efficient precipitation of protein, which consists mainly of laminin 1, type IV collagen, bovine serum albumin (BSA), and SPARC.

Do not allow the solution to foam by stirring too rapidly, as this indicates the proteins are denaturing.

5. Transfer medium to 50-ml polycarbonate high-speed centrifuge tubes and centrifuge in a high-speed refrigerated centrifuge with JA-17 rotor 30 min at $40,000 \times g$, 4°C . Discard the supernatant. Keep tubes containing pellets on ice or store up to 1 to 2 months at -70°C .
6. Thaw, if necessary, and dissolve each pellet by gentle vortexing in 2 to 5 ml DEAE buffer, 4°C . Pool these solutions and transfer to 12,000- to 14,000-MWCO dialysis tubing, prewashed with DEAE buffer and closed on one end. Rinse each centrifuge tube with 1 ml buffer and add this solution to the bag.
7. Close the open end of the dialysis bag with double knots or dialysis clips, leaving 1 to 2 in. (2.5 to 5 cm) extra space to allow for change in volume. Immerse the bag (containing ~40 ml) in a 500-ml graduated cylinder containing 500 ml DEAE buffer, 4°C . Dialyze with stirring overnight (or 4 to 6 hr), and change the dialysis buffer twice (2 to 3 hr each) for an additional 4 to 6 hr dialysis.

Wear gloves when handling dialysis tubing to minimize exposure to radioactivity as well as to protect the sample from contamination. Mix bag contents several times by inversion.

8. Remove dialysis tubing, cut tip off carefully (if knotted) or remove clips, and empty contents into one or two 50-ml centrifuge tubes. Clarify the solution by centrifuging in a JA-17 rotor 20 min at $10,000 \times g$, 4°C . If appropriate, retain 10 to 25 μl for scintillation counting and for SDS-PAGE (UNIT 6.1) with autoradiography (UNIT 6.3), as assessment of starting material.

The sample is now ready for ion-exchange chromatography.

Chromatograph on DEAE cellulose

9. Prepare gradient buffer B by adding 2.336 g NaCl to 200 ml DEAE buffer (200 mM NaCl final). Fill the front chamber of a standard gradient maker (containing a stir bar or paddle) with 200 ml DEAE buffer (gradient buffer A) and the second chamber with 200 ml gradient buffer B.

Ensure that the narrow opening between the two chambers is filled with gradient buffer A before adding gradient buffer B. An air block will inhibit flow of B into A.

10. Use a peristaltic pump to add the entire sample onto an $\sim 2 \times 20$ -cm DEAE column, and follow with one to two column volumes DEAE buffer. Discard this eluate, which contains unbound protein.
11. If phenol red (from DMEM) is seen to bind to the resin, wash the column until it is no longer visible, or until the A_{280} of the flowthrough is at baseline.

Phenol red will interfere with the monitoring of the column effluent at 280 nm.

12. Connect the gradient maker to the peristaltic pump for delivery to the column bed. Connect a fraction collector to the column and set to collect 3-ml fractions of eluate in polypropylene or siliconized glass tubes. Elute bound proteins with a linear gradient of 0% to 100% buffer B over ~300 ml.

All chromatographic procedures must be carried out at 4°C.

A less complicated alternative to the continuous gradient is the use of two stepwise elutions, the first consisting of 100 ml of 75 mM NaCl in DEAE buffer, followed by 100 ml of 175 mM NaCl in DEAE buffer. SPARC will elute in the second buffer.

13. For radiolabeled SPARC (step 2), monitor the effluent by scintillation counting 20- μ l aliquots from alternate fractions suspended in 3 ml scintillation fluid. For nonradiolabeled SPARC, monitor alternate fractions by absorbance at 280 nm.

SPARC is eluted at 150 to 175 mM NaCl. See Sage et al. (1989) for an example of the elution profile. If the location of the peak containing SPARC is in doubt, individual fractions can be analyzed by SDS-PAGE (UNIT 6.1).

14. Pool fractions containing SPARC, and dialyze the pooled sample (~20 ml) against four changes of 4 liters (each) water over 24 to 48 hr, 4°C (see steps 6 to 8).

After 24 to 48 hr, a precipitate containing SPARC, together with laminin and traces of BSA, should appear in the dialysis bag. Depending on the concentration of protein and/or the water used (pH 5.5 is optimal), precipitation may fail to occur. In this case, lyophilize the protein (step 16b), redissolve in DEAE buffer at 25% of the original volume, and repeat dialysis and precipitation (steps 14 and 15).

If the column will be reused, it should be regenerated as described (see Reagents and Solutions).

15. Decant the entire contents of the bag into a centrifuge tube and centrifuge 30 min at 48,000 \times g, 4°C. Discard the supernatant.

- 16a. *For immediate use:* Dissolve pellet in 2 ml S-200 buffer, clarify by microcentrifugation for 1 min at top speed or 10,000 \times g, and proceed to molecular-sieve chromatography (step 18).

- 16b. *For storage before chromatography:* Resuspend pellet in 2 to 4 ml water, shell-freeze by twirling the tube in dry ice/ethanol to effect freezing of the solution on the sides of the vessel, and then lyophilize. Store up to 1 to 2 months at -70°C. Before use, resuspend in 1 to 2 ml S-200 buffer, stir 4 to 6 hr at 4°C, and clarify the solution by microcentrifugation at top speed for 1 min.

Shell-freezing increases the efficiency of lyophilization and improves solubility of the protein after storage.

Pellets from several preparations can be pooled prior to molecular-sieve chromatography.

Purify SPARC by molecular-sieve chromatography

17. Remove buffer from the top of a Sephacryl molecular-sieve column and apply the sample gently onto the resin. Allow the sample (optimally 1 to 2 ml) to flow into the bed. Add 2 to 4 ml S-200 buffer to the top of the column, reconnect the buffer reservoir, and allow effluent to flow by gravity at 8 to 10 ml/hr (0.17 ml/min) by adjustment of the pressure head (i.e., the reservoir containing S-200 buffer above the column).

It is important not to disturb the column bed during sample loading, as the precision of elution can be affected.

In some cases it may be necessary to use a peristaltic pump, pulling buffer from the bottom of the column, at ~10 ml/hr. If the flow rate is too high, the column will pack too tightly and will cease to flow.

18. Collect 80 fractions of 1 to 1.5 ml each and monitor effluent by absorbance at 280 nm and/or by counting 10 to 25- μ l aliquots in 3 ml scintillation fluid.

The exact position of elution of SPARC will vary with chromatographic parameters (e.g., column size, sample size, flow rate). It is therefore advisable to monitor the column effluent and, if necessary, to check 10 to 25 μ l of each fraction by SDS-PAGE (see below). The initial peak (at V_o) contains laminin, whereas the leading shoulder of the peak corresponding to the elution position of SPARC contains most of the BSA.

19. Pool peak fractions corresponding to SPARC (approximately ten fractions, corresponding to 55 to 65 ml total column effluent). Dialyze this pool against four changes of 4 liters of 0.05 M acetic acid each, 4°C, and lyophilize.

Alternatively, the sample can be stored at -70°C in S-200 buffer without dialysis or lyophilization, or it can be dialyzed directly into another buffer as desired.

20. Determine the concentration of SPARC by absorbance at 280 nm, using the extinction coefficient (ϵ) 0.838 mg ml⁻¹ cm⁻¹ (APPENDIX 3B).

21. Analyze the purified protein by SDS-PAGE (UNIT 6.1) with autoradiography (UNIT 6.3). When heating samples at 95°C, use reducing (i.e., 50 mM DTT) and nonreducing conditions.

For detection using Coomassie blue, from 1 to 5 μ g SPARC is recommended; for detection by autoradiography, ~10⁴ cpm is recommended. A single broad band, or occasionally a doublet, should be obtained with an apparent M_r of 39,000 (with DDT) or 43,000 (without DDT), the latter co-migrating with an ovalbumin molecular weight standard. The yield of purified SPARC is ~500 μ g per 30 maxiplates (150-mm diameter) of PYS-2 cells (2 to 3 \times 10⁸ cells).

PURIFICATION OF rSPARC FROM *E. COLI*

The preceding procedure (see Basic Protocol) allows for the purification of murine SPARC from cultured (tumor) cells. Limitations of a mammalian cell culture system as a protein source are its cost, potential contamination of the product by serum and cellular proteins/proteinases, and the low yield of product. To circumvent these problems, Bassuk et al. (1996a) expressed human rSPARC with a C-terminal histidine tag in *E. coli*. A soluble (monomeric) form and an insoluble (aggregated) form of SPARC were recovered, the latter sequestered in inclusion bodies within the host. Soluble (monomeric) SPARC from *E. coli* is biologically active and can be purified in relatively large quantities with minimal contamination by endotoxin or bacterial proteins. Isolation of the soluble form is accomplished by anion-exchange, nickel-chelate affinity, and gel-filtration chromatographies. Anion-exchange chromatography on DEAE-Sepharose is used as an initial isolation step. Metal-chelate affinity chromatography provides an efficient purification of rSPARC that has been expressed with a (His)₆ sequence. Gel-filtration chromatography separates monomers of SPARC from dimers, trimers, and higher oligomers. This procedure is outlined below. It assumes that a competent strain of *E. coli*—e.g., BL21(DE3)—has been transformed with a SPARC expression plasmid—e.g., pSPARC wt (human)—with a hexahistidine (His)₆ sequence at the 3' end (Bassuk et al., 1996a) and has been propagated and frozen as a glycerol stock.

Additionally, the aggregated form can be unfolded by urea treatment, purified by nickel-chelate affinity chromatography, and renatured by gradual removal of the denaturant. After disulfide bond isomerization, the disaggregated monomers are further purified by

ALTERNATE PROTOCOL 1

Data Processing and Analysis

10.11.5

high-resolution gel-filtration chromatography (Bassuk et al., 1996b). As the disaggregation/renaturation procedure is complicated and time consuming, the reader is referred to Bassuk et al. (1996b) for this additional protocol.

Additional Materials (also see *Basic Protocol*)

- LB medium with appropriate selective reagents (*APPENDIX 2A*)
 - E. coli* strain transfected with SPARC expression vector (Bassuk et al., 1996a)
 - Inducing agent (e.g., IPTG; *APPENDIX 3A*)
 - 10 mM sodium phosphate, pH 7.0 (*APPENDIX 2A*)/10% (v/v) glycerol
 - 90 mM sodium phosphate buffer (pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF, 4°C (see recipe), with and without 0.5 M NaCl
 - DEAE-Sepharose Fast Flow anion-exchange resin (Amersham Biosciences):
 - equilibrate in 90 mM sodium phosphate buffer (pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF and allow to settle
 - 5 M NaCl (*APPENDIX 2A*)
 - 0.2 M AEBSF stock solution (see recipe)
 - Nickel/nitrilotriacetic acid (Ni-NTA) metal-chelate affinity resin (Qiagen)
 - 50 mM sodium phosphate (pH 5.3, 6.0, and 7.8)/0.5 M NaCl/10% (v/v) glycerol (see recipe)
 - 1.6 × 60–cm Superdex 70 column (see recipe)
 - 50 mM Tris-Cl (pH 8.0)/0.15 M NaCl (see recipe)
 - 1× PBS (*APPENDIX 2A*) containing 1 to 4 mM Ca²⁺ (optional)
 - French press
 - 2 × 20– and 1 × 10–cm chromatography columns
 - Flow cell coupled to a UV monitor set at 280 nm
 - Chart recorder
 - Conductivity meter (optional)
 - Disposable 10-ml gel-filtration column, sterile (optional)
- Additional reagents and equipment for transfecting SPARC expression vector (*APPENDIX 3A*) and for SDS-PAGE on minigels (*UNIT 6.1*)

Extract *E. coli*

1. Inoculate 1.3 liters LB medium containing appropriate selective reagents with a suitable *E. coli* strain transfected with SPARC expression vector using standard techniques (*APPENDIX 3A*). Grow to midexponential phase (OD₆₀₀ ~0.5) and induce with the appropriate agent.

Induction of rSPARC in midexponential phase cells is necessary for high levels of expression. The procedure and chemical(s) used depend on the E. coli strain and the vector into which SPARC cDNA is cloned. For example, IPTG was used at a final concentration of 1 mM for SPARC cloned into pET22b vector and transfected into strain BL21(DE3) (Bassuk et al., 1996a).

2. After the cells have been induced, grow an additional 1 to 4 hr.
3. Recover the cells by centrifuging 20 min at 7000 × g, room temperature. Discard the supernatant and resuspend the pellet in 20 ml of 10 mM sodium phosphate, pH 7.0, containing 10% (v/v) glycerol. Disrupt by performing two cycles in a French press at 20,000 psi.

Cells can alternatively be broken open by sonication on ice.

4. Separate soluble from insoluble material by centrifuging 30 min at 10,000 × g, 4°C. Decant soluble extract (supernatant) into a separate tube.

Soluble extracts and insoluble pellets at this stage can be stored up to 1 month at -80°C . Refer to Bassuk et al. (1996b) for details on processing pellets for aggregated SPARC.

Perform initial chromatography on DEAE-Sepharose

5. If necessary, thaw the soluble extracts on ice. Dilute to 100 ml with ice-cold 90 mM sodium phosphate buffer (pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF, 4°C .
6. Add 50 ml settled DEAE-Sepharose Fast Flow anion-exchange resin. Stir gently 12 to 18 hr at 4°C .
7. Pour slurry into a 2×20 -cm chromatography column, allow to settle, and wash with ~ 250 ml of 90 mM sodium phosphate buffer (pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF until the absorbance at 280 nm is <0.01 .
8. Assemble a linear gradient (see Basic Protocol 1, step 9) by adding 250 ml of 90 mM sodium phosphate buffer pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF to the front compartment of a gradient maker, and 250 ml of 90 mM sodium phosphate buffer (pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF with 0.5 M NaCl (7.3 g) to the other compartment.
9. At 4°C pump gradient onto the column at 3 ml/min. Collect 8-ml fractions and monitor the column eluate with a flow cell coupled to a UV monitor set at 280 nm and a chart recorder set at full scale equal to 1 OD unit.

It is advisable also to monitor the eluate by conductivity; read every fourth fraction in a conductivity meter (clean probe after each reading). rSPARC elutes at a concentration of 0.10 to 0.25 M NaCl (conductivity of 14 to 20 mmho).

10. Analyze 50- μl aliquots of fractions by SDS-PAGE on minigels (see Basic Protocol and UNIT 6.1). Pool fractions containing rSPARC, and adjust solution to 0.5 M NaCl by adding 5 M NaCl.

In the absence of post-translational modification, rSPARC migrates on an SDS-polyacrylamide gel with an apparent M_r of 34,000 to 38,000 Da after reduction. Adjustment of ionic strength can be monitored easily by conductivity measurement; the final conductivity of the pooled fractions containing SPARC should be equivalent to that of 50 mM sodium phosphate (pH 7.8)/0.5 M NaCl/10% (v/v) glycerol. At this point, fractions can be stored up to 1 month at -80°C .

Perform metal-chelate affinity chromatography

11. Add 0.2 M AEBSF stock solution to the pooled sample to a final concentration of 0.2 mM.
12. Mix sample with a slurry of Ni-NTA metal-chelate affinity resin, using 3 to 5 ml resin per liter original bacterial culture. Adjust pH to 7.8 with 1 N NaOH or 1 N HCl, and stir gently for 1 hr at 4°C .
13. Pour slurry into a chromatography column (e.g., 1×10 cm), allow to settle, and wash with ~ 60 ml of 50 mM sodium phosphate (pH 7.8)/0.5 M NaCl/10% (v/v) glycerol at a flow rate of 0.5 ml/min until the absorbance at 280 nm is <0.01 .
14. Pass 15 column volumes of 50 mM sodium phosphate (pH 6.0)/0.5 M NaCl/10% (v/v) glycerol through the column to remove nonspecifically bound proteins (i.e., until $A_{280} < 0.01$).
15. Elute rSPARC from the column with 20 ml of 50 mM sodium phosphate (pH 5.3)/0.5 M NaCl/10% (v/v) glycerol.
16. Store up to 1 month in ~ 1 -ml aliquots at -80°C .

Perform gel-filtration chromatography

17. Apply ~1 ml rSPARC solution onto a 1.6 × 60-cm Superdex 70 column and elute by gravity using 50 mM Tris·Cl (pH 8.0)/0.15 M NaCl buffer at a flow rate of 0.1 ml/min. Collect fractions of 1.25 ml each, monitored at 280 nm.

Over an elution range of 100 ml, rSPARC monomer elutes between 46 and 50 ml. This should be verified by SDS-PAGE, as should the removal of oligomers (compare migration with and without 50 mM DTT).

18. *Optional:* Perform buffer exchange as needed for experimental design using sterile disposable 10-ml gel-filtration columns. Elute in the buffer of choice (e.g., 1× PBS containing 1 to 4 mM Ca²⁺ for in vitro studies).
19. Store samples up to 3 months at –80°C.

ALTERNATE PROTOCOL 2

PURIFICATION OF rSPARC FROM INSECT (*Sf9*) CELLS

This protocol describes the purification of human rSPARC produced in a baculovirus expression system using insect (*Sf9*) cells (Bradshaw et al., 2000). Advantages of this system over those described above (see Basic Protocol and Alternate Protocol 1) are higher yield of rSPARC, production of protein in a nonbacterial system to minimize contamination by endotoxin (*E. coli*) or serum proteins (mammalian cells), and the potential for post-translational modifications of protein similar to those in mammalian cells.

This protocol assumes that the starting materials are human (or other species) SPARC cDNA (minus the signal sequence) subcloned into a baculovirus expression vector (PharMingen), *Spodoptera frugiperda* 9 (*Sf9*) insect cells cotransfected with the SPARC expression vector and linearized baculovirus, and high-titer stocks of recombinant virus generated for subsequent infections of *Sf9* cells grown in suspension in serum-free media (Invitrogen). Information on the latest versions of the *Sf9*/baculovirus expression system is readily available from the PharMingen instruction manual, *Baculovirus Expression Vector System*, and the Invitrogen manual, *Growth and Maintenance of Insect Cell Lines for Expression of Recombinant Proteins using the Baculovirus Expression System*.

Materials

- Sf9 cells (Invitrogen) infected with baculoviral SPARC expression vector, grown in serum-free *Sf*-900 II medium (Invitrogen)
- 200 mM MOPS, pH 6.5 (see recipe)
- 10 N NaOH (APPENDIX 2A)
- Q-Sepharose Fast Flow column (see recipe)
- 200 and 400 mM LiCl/20 mM MOPS, pH 6.5 (see recipe)
- 0.1 N acetic acid, 4°C: 0.6 ml glacial acetic acid in 100 ml H₂O
- Hanks' buffered saline solution (see recipe)
- 50-ml conical tube
- 0.22-µm filter bottle
- AktaPrime automated liquid chromatography system (Amersham Biosciences) or equivalent conventional model
- 10,000-NMWL Ultrafree-15 (Millipore) or Centricon Plus-80 (Amicon) centrifugal filter device
- 0.22-µm sterile syringe-driven filter

Prepare Sf9 conditioned medium for chromatography

1. Transfer Sf9 cells infected at 2 to 4×10^5 cells/ml with baculoviral SPARC expression vector, grown 4 to 5 days in serum-free Sf-900 II medium, to 50-ml conical tubes. Centrifuge 45 min at $6000 \times g$, 4°C . Transfer the supernatant to a $0.22\text{-}\mu\text{m}$ filter bottle and discard the cell pellets.

This system optimizes for the efficient secretion of recombinant protein. It is important to avoid lysis of the cells (and contamination of the medium) during this step.

2. Sterile-filter the supernatant and measure the volume. Add $\frac{1}{10}$ vol 200 mM MOPS, pH 6.5, and adjust the pH to 6.5 with 6 N NaOH.

Purify rSPARC by anion-exchange chromatography

3. Pump the sample onto a Q-Sepharose Fast Flow column at a flow rate of 5 ml/min.
4. Using either an AktaPrime automated liquid chromatography system or equivalent conventional model, assemble a continuous linear salt gradient from 200 to 400 mM. Use 200 ml of 200 mM LiCl/20 mM MOPS, pH 6.5, supplied to the buffer valve (AktaPrime System) or front chamber (conventional gradient maker) and 200 ml of 400 mM LiCl/20 mM MOPS, pH 6.5, to the buffer switch valve or rear chamber.
5. Start the gradient pumping at a rate of 5 ml/min and collect 3.5-ml fractions over 300 ml, monitoring the column effluent at 280 nm.

The fractions can also be checked by SDS-PAGE (see Basic Protocol and Alternate Protocol 1; UNIT 6.1). Human rSPARC produced by Sf9 cells migrates at $\sim 38,000$ to $40,000$ Da after reduction, and the doublet shifts to a single band of $\sim 36,000$ Da in the absence of reducing agent. The doublet is the result of heterogeneous glycosylation (Bradshaw et al., 2000).

Dialyze sample

For dialysis using acetic acid

- 6a. Pool the fractions containing rSPARC in 12,000- to 14,000-MWCO dialysis tubing and dialyze against three 1- to 2-liter changes (each) of 0.1 N acetic acid, 4°C .
- 7a. Aliquot the samples according to use. Snap-freeze on dry ice or in liquid nitrogen, lyophilize, and store at -70°C .

Acetic acid is used when the sample is to be lyophilized and concentrated.

The above procedure results in rSPARC of $\sim 80\%$ purity by SDS-PAGE. An additional purification step (entailing molecular-sieve chromatography) can be performed after the sample has been lyophilized. Follow the procedure described above (see Basic Protocol, steps 17 to 21). Significant losses of rSPARC (Sf9) should be expected with this procedure, however.

For dialysis using saline solution

- 6b. Pool the fractions containing rSPARC in 12,000- to 14,000-MWCO dialysis tubing and dialyze against three 4-liter changes (each) of $1 \times$ Hanks buffered saline solution (HBSS) containing $1 \mu\text{M}$ CaCl_2 . Concentrate in a 10,000-NMWL Ultrafree-15 or Centricon Plus-80 centrifugal filter device to 1 or 2 ml. Filter sterilize using $0.22\text{-}\mu\text{m}$ sterile syringe-driven filter.

Saline solution is used when the sample is to be used for cell culture.

- 7b. Aliquot according to use. Snap freeze on dry ice or in liquid nitrogen and store at -70°C .

Analyze SPARC

- Determine protein content by UV spectroscopy at 280 nm (*APPENDIX 3B*), using the extinction coefficient (ϵ) $0.838 \text{ mg ml}^{-1} \text{ cm}^{-1}$. Estimate the purity of SPARC by SDS-PAGE (5 μg /sample lane; *UNIT 6.1*).

The yield is 2 to 4 mg SPARC (~80% purity) per 400 ml of initial Sf9 cell culture suspension (at 2 to 4 $\times 10^6$ cells/ml).

An updated version of the Sf9/baculovirus expression system is now available from Invitrogen. Termed "InsectSelect," it is a virus-free system that relies on expression of protein from a single nonlytic, integrative plasmid transfected into Sf9 or other insect cells, and is claimed to be optimal for secreted proteins.

PURIFICATION OF SPARC/OSTEONECTIN FROM TISSUES

SPARC was originally isolated from fetal bovine mineralized bone matrix, of which it is a major noncollagenous component, and was termed osteonectin (Termine et al., 1981). Two other significant sources of SPARC are platelets (osteonectin; Kelm and Mann, 1991) and the Engelbreth-Holm-Swarm (EHS) sarcoma, a murine basement membrane-producing tumor (termed BM-40; Sasaki et al., 1999, and references therein). SPARC, osteonectin, and BM-40 are now recognized as the same protein. Many of the functional properties of SPARC were deduced from biochemical/biophysical studies of the tissue-derived protein, which can be isolated in significantly greater quantities compared to yields typically described from in vitro sources. In this protocol, purification of SPARC from human platelets is described, based on an original report by Kelm and Mann (1990). There are several advantages to using platelets as a source of SPARC: (1) human blood is a readily available source for human SPARC; (2) bovine blood is an excellent source of SPARC and requires neither screening for pathogens nor the rigorous safety procedures associated with the use of human material; and (3) denaturing conditions are not involved (the extraction of osteonectin from bone matrix or EHS tumor includes the use of EDTA and, in some cases, guanidinium-HCl; Termine et al., 1981; Kelm and Mann, 1990; Sasaki et al., 1999). It is important to note, however, that differences have been reported between bone and platelet osteonectin from the same species, notably in the specificity of collagen binding that was attributed to differences in glycosylation (Kelm and Mann, 1991). Investigators interested in tissue-specific modifications of SPARC and their functional implications are encouraged to consult the references cited above. Bovine bone and human platelet osteonectin are available commercially from Calbiochem, although their method of purification is not specified. Haematologic Technologies sells human platelet osteonectin isolated by affinity chromatography on an anti-osteonectin monoclonal antibody column, as well as bovine bone osteonectin isolated from 0.5 M EDTA extracts of demineralized bone. All commercial preparations should be tested for activity in one or more of the assays described (see Support Protocols 1 to 3).

Materials

- Platelet-rich plasma or platelet suspension, or informed, nonsmoking, aspirin-free, consenting adult blood donors
- 0.156 M citrate containing 0.1 M dextrose and 5.0 μM prostaglandin E_1 (Sigma; optional)
- 0.02 M Tris-Cl, pH 7.6/0.15 and 1.0 M NaCl (see recipe)
- Thrombin
- Sepharose 4B-AON IgG column (see recipe)
- 3.0 M NaSCN/0.02 M Tris-Cl (pH 7.6)/0.15 M NaCl (see recipe)
- 0.05 M NH_4HCO_3

19-G butterfly needles
50 or 250-ml plastic centrifuge bottles with caps
12,000 to 14,000-MWCO dialysis tubing
Lyophilizer

Additional reagents and equipment for thrombin activation of platelets (Kelm and Mann, 1990) and SDS-PAGE (UNIT 6.1; also see Basic Protocol, step 22)

Prepare activated platelet supernatant

- 1a. *For predrawn plasma:* Purchase platelet-rich plasma or platelet suspensions from a local blood bank.
- 1b. *For in-house drawn plasma:* Draw 480 ml fresh blood from informed, nonsmoking, aspirin-free, consenting adults via 19-G butterfly needles into 0.156 M citrate containing 0.1 M dextrose and 5.0 μ M prostaglandin E₁ (to prevent platelet activation). Remove red cells and leukocytes by centrifuging 30 min at 1000 \times g, room temperature.

CAUTION: Appropriate biosafety practices must be followed when working with human blood or blood products. Human blood must be screened for HIV and other infectious viruses. In addition, safety glasses, a double layer of gloves, and protective laboratory clothing should be worn at all times. Use double containment (e.g., place a tube or bag containing blood in a beaker prior to any manipulation) and ensure that all containers including centrifuge bottles are tightly capped.

2. Centrifuge platelet-rich suspension 30 min at 27,000 \times g, 4°C, to pellet the platelets. Decant supernatant.
3. Wash platelets with 200 ml of 0.02 M Tris·Cl (pH 7.6)/0.15 M NaCl, centrifuge as in step 2. Resuspend platelets in 50 ml of the same buffer. Count with a hemacytometer and suspend 4.5×10^{10} cells in 50 ml of 0.02 M Tris·Cl (pH 7.6)/0.15 M NaCl. Activate by adding 2.5 U/ml thrombin as described by Kelm and Mann (1990).
4. Transfer to plastic, capped centrifuge bottles and isolate activated platelets by centrifuging 30 min at 25,000 \times g, room temperature. Discard the pellet using appropriate containment and retain the supernatant.

CAUTION: Autoclave human products prior to disposal.

Isolate SPARC/osteonectin by immunoaffinity chromatography

5. Apply platelet supernatant to a Sepharose 4B-AON IgG column (~2 \times 20-cm). After the applied solution has permeated the resin, clamp off the column and allow the sample to remain within the column bed for 16 hr at 4°C.
6. Wash the column with 0.02 M Tris·Cl (pH 7.6)/0.15 M NaCl until the effluent shows an A₂₈₀ of 0.01.
7. Wash the column with 0.02 M Tris·Cl (pH 7.6)/1.0 M NaCl until a baseline absorbance is achieved.
8. Elute SPARC/osteonectin with 3.0 M NaSCN/0.02 M Tris·Cl (pH 7.6)/0.15 M NaCl and collect in a single tube or in fractions.
9. Transfer the effluent to 12,000 to 14,000-MWCO dialysis tubing, dialyze against two changes of 2 liters of 0.05 M NH₄HCO₃, and then lyophilize.

Peak fractions of the eluted SPARC can also be dialyzed against 0.02 M Tris·Cl (pH 7.6)/0.15 M NaCl, PBS, or DMEM (minus phenol red), as dictated by the intended use of the purified SPARC, and stored up to 1 month at -80°C. If the NH₄HCO₃ fails to lyophilize completely, redissolve the powder in water, dialyze against 0.01 N acetic acid, and repeat the lyophilization.

It is important to minimize the exposure of SPARC/osteonectin to NaSCN, and to keep all reagents at 4°C during affinity chromatography and dialysis.

10. Determine the concentration of SPARC/platelet osteonectin at A_{280} using the extinction coefficient (ϵ) $0.838 \text{ mg ml}^{-1} \text{ cm}^{-1}$.
11. Monitor the purity of SPARC by SDS-PAGE (*UNIT 6.1*; also see Basic Protocol, step 22).

Platelet SPARC/osteonectin should be >80% pure by SDS-PAGE using a Coomassie blue stain. It exhibits an apparent M_r of ~3000 greater than that of bone osteonectin purified according to the same protocol (shown to be due to differences in glycosylation), but is comparable to that reported for SPARC isolated from PYS-2 cell culture media.

Immunoaffinity chromatography typically produces somewhat low recoveries of the protein antigen, albeit in a high state of purity given the minimal steps used in the isolation protocol. The total amount of SPARC/osteonectin in human platelets (prior to affinity purification) was reported by Kelm and Mann (1990) to range from 0.65 to 2.2 $\mu\text{g}/10^8$ platelets.

ASSAYS FOR THE EVALUATION OF SPARC ACTIVITY

All proteins need to be evaluated, not only for their extent of purity, but also for their activity and conformational integrity. The latter is especially critical in the case of recombinant proteins, which are produced either in biologically “inappropriate” hosts (e.g., SPARC in a prokaryotic system) or at levels that preclude proper processing, folding, and/or editing. Moreover, the importance of post-translational modification to the functions of many proteins is poorly understood. In the case of SPARC, N-linked glycosylation (one site) appears not to be critical for activity, at least in the assays that have been used; however, rSPARC (see Alternate Protocols 1 and 2) has consistently displayed less activity (up to 50%) than SPARC purified from PYS-2 cells (see Basic Protocol; Yost et al., 1994; Bassuk et al., 1996a; Bradshaw et al., 2000). These protocols describe biological assays that test two major effects of SPARC on cultured cells: de-adhesion and inhibition of proliferation. Other assays based on biochemical measurements (e.g., circular dichroism, binding assays) are standard procedures and are discussed elsewhere (see Commentary).

SUPPORT PROTOCOL 1

Proliferation Assay

Endotoxin will inhibit cell proliferation, and endothelial (especially BAE) cells are particularly sensitive. At 10 ng endotoxin/mg SPARC, there should be <10% inhibition of [^3H]thymidine incorporation in BAE cells exposed to 60 μg SPARC/ml. To determine the effect of endotoxin on other types of cells, treat the cells with a titration of CSE and measure proliferation.

Additional Materials (also see Basic Protocol)

- Bovine aortic endothelial (BAE) cells
- DMEM/0% and 10% (w/v) FBS (*APPENDIX 2A*)
- Purified SPARC (see Basic Protocol or Alternate Protocol 1 to 3) and appropriate control buffer
- 6.71 Ci/mmol (1 mCi/ml) [methyl- ^3H]thymidine (PerkinElmer)
- 10% (w/v) trichloroacetic acid (TCA), ice cold
- 95% (v/v) ethanol
- 0.4 N NaOH
- Glacial acetic acid
- Scintillation fluid
- 24-well tissue culture plate
- 15-ml conical tube
- Radioactivity warning tape
- Additional reagents and equipment for trypsinizing cells (*UNIT 1.1*)

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and surroundings. Carry out the experiments and dispose of wastes in appropriately designated area, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

1. Starve a 100-cm dish of confluent bovine aortic endothelial (BAE) cells in serum-free DMEM for 3 to 4 days.
2. Trypsinize cells (*UNIT 1.1*), resuspend in 10 ml DMEM/10% FBS, and centrifuge briefly (i.e., 5 min at $1000 \times g$, room temperature) to pellet.
3. Rinse cells twice with 5 ml serum-free DMEM.
4. In a 24-well tissue culture plate, plate triplicate wells containing 5×10^4 cells in 500 μ l (final 1×10^5 cells/ml) of the following solutions, using the same volume for SPARC and buffer:

Serum-free DMEM (control)
DMEM/2%FBS containing 5 μ g/ml SPARC dissolved in DMEM
DMEM/2% FBS containing 20 μ g/ml SPARC dissolved in DMEM
DMEM/2% FBS containing buffer alone (control).

Other buffers compatible with cell culture may be used but not acetic acid.

5. Incubate 16 to 18 hr at 37°C.

By this time, cells will have begun to synthesize DNA (S phase).

6. Prepare label in a 15-ml conical tube by adding 20 μ l (20 μ Ci) of 6.71 Ci/mmol [methyl- 3 H]thymidine/ml DMEM. Place 55 μ l (1.1 μ Ci) of this mixture into each well. Swirl plate gently to mix. Label plate with radioactivity warning tape, and incubate 4 hr at 37°C.

CAUTION: *Perform this step in a laminar-flow hood with absorbent bench pad and radioactive waste receptacle.*

7. Wash each well twice with 500 μ l ice-cold 10% TCA, and drain completely.

CAUTION: Collect radioactive media and washes for safe disposal.

8. Wash with 500 μ l of 95% ethanol. Remove ethanol and add 500 μ l of 0.4 N NaOH per well. Incubate 30 min at room temperature with shaking.
9. Add 100 μ l glacial acetic acid to neutralize the solution.

Extremes of pH can result in precipitation of scintillation cocktail and/or quenching.

10. Place the contents of each well into a collection vial containing 3 ml scintillation fluid. Cap, mix by inversion, and measure cpm in a scintillation counter.

There should be no precipitate in the vials; check pH if this occurs and adjust to neutrality.

For rSPARC, expect >70% inhibition of [3 H]thymidine incorporation at 50 μ g SPARC/ml. For SPARC purified from PYS-2 cells, the effective dose at which 50% inhibition of [3 H]thymidine incorporation occurs (ED_{50}) is 20 μ g SPARC/ml.

De-adhesion Assay

This protocol is presented as a rapid, inexpensive, and diagnostic assay for the de-adhesive activity of SPARC on nontransformed cells in vitro. The activity is based on the diminishment of focal adhesions produced by cultured cells. These structures can be distinguished by immunofluorescence staining of vinculin in wedge-shaped structures at the periphery of the cell, which are diagnostic for focal adhesion complexes.

**SUPPORT
PROTOCOL 2**

**Data Processing
and Analysis**

10.11.13

Additional Materials (also see Basic Protocol)

One 100-mm dish of nearly-confluent bovine aortic endothelial (BAE) cells, passaged not greater than ten times, grown in DMEM/10% FBS containing appropriate antibiotics

DMEM/2% and 10% FBS (APPENDIX 2A)

Purified SPARC (see Basic Protocol and Alternate Protocols 1 to 3) and appropriate control buffer

12-well tissue culture dishes

Phase-contrast microscope (UNIT 4.1)

Additional reagents and equipment for trypsinizing cells (UNIT 1.1)

1. Trypsinize (UNIT 1.1) a 100-mm dish containing a nearly confluent monolayer of bovine aortic endothelial (BAE) cells, passaged greater than ten times, and grown in DMEM/10% FBS containing appropriate antibiotics.

The size of the dishes is optional and can be adjusted according to the availability of SPARC. Scale the volume of medium as appropriate for size of dish or well.

2. Transfer trypsinized cells to appropriate centrifuge tubes, pellet in a clinical centrifuge 5 min at $1000 \times g$, room temperature, and resuspend in an appropriate volume of DMEM/2% FBS. Plate 5 to 7.5×10^4 cells in triplicate wells of a 12-well tissue culture dish.
3. Add the following solutions to cells in triplicate, using the same volume for SPARC and buffer:

No addition
20 $\mu\text{g/ml}$ SPARC
40 $\mu\text{g/ml}$ SPARC
Appropriate control buffer.

4. Mix gently and incubate 1 hr at 37°C .
5. Check the plate carefully under a phase-contrast microscope. Examine several representative fields and count the number of cells in the following groups:
Fully spread cells (group *a*)
Partially spread cells (group *b*)
Rounded cells (group *c*).

Cells to which SPARC has not been added should be attached and beginning to spread. Cells to which SPARC has been added should be less spread (i.e., rounded). If control cells (i.e., no SPARC) have not spread, wait an additional 1 to 2 hr. There should be no toxicity or cell death.

6. Quantify the activity of SPARC according to the rounding index (RI):

$$\text{RI} = [(1 \times a) + (2 \times b) + (3 \times c)] / (a + b + c)$$

An RI = 1 represents a culture with only spread cells, whereas a culture with increasing numbers of round cells would approach the maximum, RI = 3.

A titration curve can be generated using different concentrations of SPARC. Anticipate that different types of cells will show differential sensitivity to SPARC. Cell lines (e.g., 3T3, NRK) and transformed cells typically do not respond to SPARC.

Endotoxin Assay

Endotoxin is derived from gram-negative bacteria (e.g., *E. coli*) and is a commonly encountered contaminant of buffers, columns, and glassware. In addition, soluble (monomeric) SPARC purified from *E. coli* may contain endotoxin. Endotoxin interferes with bioassays for SPARC, so it is necessary to assess samples for the presence of endotoxin.

Materials

Purified SPARC (see Basic Protocol and Alternate Protocols 1 to 3) and appropriate buffer

Limulus Amoebocyte Lysate (LAL) Pyrochrome kit (Associates of Cape Cod) for the Detection and Quantification of Gram-Negative Bacterial Endotoxin:

Pyrochrome LAL reagent

Pyrochrome Reconstitution buffer

Control Standard Endotoxin (CSE)

50% (v/v) glacial acetic acid

Nonpyrogenic 96-well tissue culture plate

Microtiter plate reader

1. Prepare SPARC titrated at 0.2, 1, and 5 $\mu\text{g}/\text{ml}$ in DMEM or HBSS. Pipet 50 μl of each into triplicate wells of a nonpyrogenic 96-well tissue culture plate, leaving 16 empty wells for standards and controls (step 5).
2. Tap vial containing Pyrochrome LAL reagent. Remove and discard stopper.
3. Add 3.2 ml Pyrochrome Reconstitution buffer to the LAL reagent. Mix gently but thoroughly. Cover with Parafilm and place for 3 to 5 min on ice. Store on ice up to 3 hr.
4. Prepare standards by adding 2.0 ml water to the vial containing the Control Standard Endotoxin (CSE) to yield 1.0 endotoxin units (EU)/ml endotoxin. Vortex and store on ice.
5. Place 200 μl water in each of five wells in a fresh 96-well plate. Make a five-step serial dilution using a ratio of 1:1 at each step, by adding 200 μl of 1.0 EU/ml endotoxin to the first well, mixing, transferring 200 μl to the next well, and repeating until the series is complete. Pipet 50 μl of each dilution into triplicate wells of the SPARC-containing plate (step 1), and include a 50- μl water-only control.

The serial dilutions above will result in final concentrations of 0.5, 0.25, 0.125, 0.0625, and 0.0313 EU/ml endotoxin, respectively.
6. Pipet 50 μl of reconstituted Pyrochrome LAL reagent (step 3) into each well, shake on a microtiter plate shaker for 30 sec, and incubate at 37°C for 30 min.
7. Stop reaction by adding 25 μl of 50% glacial acetic acid per well.
8. Measure OD₄₀₅ in a microtiter plate reader. Determine the concentration of endotoxin in the sample by comparison to the curve generated from the standards.

The expected concentration of endotoxin is <10 ng/mg SPARC, at an estimated level of 10 EU/ng endotoxin. Endotoxin levels range from 5 to 15 EU/ng. The level of endotoxin that affects cells depends on the cell type.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

AEBSF (aminoethylbenzenesulfonyl fluoride) stock solution, 0.2 M

Dissolve 4.794 g AEBSF (Calbiochem) in 100 ml water. Make fresh.

DEAE buffer

500 ml 8 M urea stock solution (see recipe)

50 ml 1 M Tris·Cl, pH 7.5 (APPENDIX 2A)

448 ml H₂O

1 ml 0.2 M PMSF stock solution (see recipe)

625 mg *N*-ethylmaleimide (NEM)

Adjust pH to 8.0 with 10 N NaOH

Chill to 4°C

Make fresh buffer for each column run

Final concentrations are 50 mM Tris·Cl, pH 8.0, 0.2 mM PMSF, 10 mM NEM, and 4 M urea.

DEAE column

Pack an ~2 × 20–cm column (e.g., Amersham Biosciences) at 4°C with a 20% slurry of DE-52 cellulose (Whatman) equilibrated in DEAE buffer (see recipe). Equilibrate with several column volumes (~70 ml each) DEAE buffer, delivered via a peristaltic pump connected from a reservoir to the bottom of the column (pumping upward ensures more efficient utilization of theoretical plates for ion exchange). The column can be stored at 4°C and reused for several months. After storage, flush the column with several volumes of fresh DEAE buffer immediately before use. To regenerate a DEAE column, pump one column volume of DEAE buffer containing 500 mM NaCl (29.2 g/liter) followed by several column volumes of DEAE buffer until the absorption and conductivity of the elution buffer is restored to baseline (see Commentary).

Columns manufactured by Amersham Biosciences work well, as they are thick walled and are equipped with high-quality fittings that can withstand the pressures delivered by a peristaltic pump.

Hanks' buffered saline solution

0.14 g/l CaCl₂ (1.26 mM final)

40 g/l KCl (5.33 mM final)

0.6 g/l potassium phosphate, monobasic (0.44 mM final)

0.1 g/l magnesium chloride, hexahydrate (0.50 mM final)

0.1 g/l magnesium sulfate, heptahydrate (0.41 mM final)

0.35 g/l sodium bicarbonate (4.00 mM final)

0.048 g/l sodium phosphate, dibasic (0.30 mM final)

Store up to 3 months at 4°C

LiCl, 200 mM/20 mM MOPS, pH 6.5

200 ml of 200 mM MOPS, pH 6.5 (see recipe)

80 ml 5 M LiCl

Add H₂O to 1700 ml

Adjust pH to 6.5 with 6 N NaOH

Add H₂O to 2000 ml

Store up to 1 year at 4°C

LiCl, 400 mM/20 mM MOPS, pH 6.5

50 ml 200 mM MOPS, pH 6.5 (see recipe)
40 ml 5 M LiCl
Add H₂O to 480 ml
Adjust pH with 6 N NaOH
Add H₂O to 500 ml
Store up to 1 year at 4 °C

LiCl, 2 M/20 mM MOPS, pH 6.5

50 ml 200 mM MOPS, pH 6.5 (see recipe)
200 ml 5 M LiCl
Add H₂O to 480 ml
Adjust pH with 6 N NaOH
Add H₂O to 500 ml
Store up to 1 year at 4 °C

Molecular-weight standards

Molecular-weight standards include a marker for the excluded (outer; V_o) and included (inner; V_i) volume of the column. For V_o , use 500 μ l of 0.1% (w/v) blue dextran in S-200 buffer (see recipe). Clarify by centrifugation before applying to the column. For V_i , use 25,000 to 100,000 cpm [³⁵S]methionine or [³H]proline (which can be detected by scintillation counting), or any small protein ($M_r < 10,000$) or peptide that is minimally hydrophobic and nonglycosylated.

MOPS, 200 mM, pH 6.5

41.86 g 3-(*N*-morpholino)propanesulfonic acid (MOPS)
Add H₂O to ~800 ml
Adjust pH to 6.5 with 6 N NaOH
Add H₂O to 1000 ml
Store up to 1 year at 4 °C

NaSCN, 3.0 M/0.02 M Tris·Cl (pH 7.6)/0.15 M NaCl

243.24 g sodium isothiocyanate (NaSCN)
20 ml 1 M 0.22- μ m-filter-sterilized Tris·Cl, pH 7.5 (APPENDIX 2A)
8.766 g NaCl
Add H₂O to ~900 ml
Adjust pH to 7.6 with 6 N NaOH
Add H₂O to 1000 ml
Store up to 1 year at 4 °C

PMSF (phenylmethylsulfonyl fluoride) stock solution, 0.2 M

Dissolve 3.48 g PMSF in 100 ml isopropanol. Store up to several years at 4 °C or room temperature.

Add this reagent to aqueous solutions drop-wise while vortexing, or it will precipitate.

PYS-2 cells, 50% to 70% confluent

Using 150-mm tissue-culture dishes or equivalent plastic flasks, grow PYS-2 cells (ATCC CRL-2745) to between 50% and 70% confluence ($7-10 \times 10^6$ cells per plate) in DMEM/10% (v/v) FBS (APPENDIX 2A).

Cells undergo >1 population doubling in 24 hr under these conditions.

Q-Sepharose Fast Flow column

Pour a 1.7 × 20-cm column of Q-Sepharose Fast Flow resin (Amersham Biosciences) equilibrated in 200 mM LiCl/20 mM MOPS, pH 6.5 (see recipe). Equilibrate by running two to three column volumes of 200 mM LiCl/20 mM MOPS, pH 6.5 (see recipe), through the resin. After use, strip the column with 100 ml of 2 M LiCl/20 mM MOPS, pH 6.5 (see recipe), and equilibrate with 60 ml of 200 mM LiCl/20 mM MOPS, pH 6.5. Store up to 1 year at room temperature.

S-200 buffer

999 ml Hanks' balanced salt solution (HBSS; see recipe) with Ca²⁺ and Mg²⁺

(Life Technologies and *APPENDIX 2A*)

1 ml 0.2 M PMSF stock solution (see recipe)

Filter sterilize with a 0.22- μ m filter

Prepare fresh for each run and keep at 4°C

Calcium is present in HBSS as 0.14 g/liter CaCl₂, and magnesium is present as 0.1 g/liter MgCl₂·6H₂O and 0.1 g/liter MgSO₄·7H₂O.

Sephacryl molecular-sieve column

At 4°C, pour an ~1 × 100-cm column (e.g., Bio-Rad) of Sephacryl S-200 (Amersham Biosciences) in a slurry of cold, sterile S-200 buffer (see recipe). Allow column bed to pack slowly but steadily, with controlled elution from the bottom port of the column at ~10 ml/hr (0.17 ml/min), to a bed height of ~95 cm. Run several column volumes (~80 ml each) of S-200 buffer through the packed bed and then calibrate using molecular-weight standards (see recipe). Store in S-200 buffer at 4°C for up to several days prior to use. For longer storage and reuse (up to several months), store in S-200 buffer containing 0.1% (w/v) sodium azide at 4°C. Periodically clean (i.e., remove sample debris from the top of the column up) and flush with fresh S-200 buffer containing 0.1% sodium azide.

Azide must be flushed out completely (monitor at 280 nm) prior to chromatography of SPARC, as azide is toxic to cells and may also interfere with the properties of SPARC.

Sepharose 4B-AON IgG column

Following manufacturer's instructions, pour anti-osteonectin (AON-5031; 20 mg) monoclonal IgG₁ antibody (Haematologic Technologies) coupled to CNBr-activated Sepharose 4B (Amersham Biosciences) into a 5- to 10-ml column, at 4°C. Equilibrate in several column volumes of 50 mM Tris·Cl (pH 8.0)/0.15 M NaCl (see recipe), by gravity flow at 0.1 to 0.5 ml/min. After use reequilibrate column in 0.02 M Tris·Cl, pH 7.6/0.15 M NaCl. Store up to 1 month in that same buffer at 4°C.

Sodium phosphate, 50 mM (pH 5.3, 6.0, or 7.8)/0.5 M NaCl/10% (v/v) glycerol

29.2 g NaCl

5.75 g sodium phosphate dibasic

1.37 g sodium phosphate monobasic

100 ml glycerol

Add H₂O to ~800 ml

Adjust pH to 5.3 or 6.0 with 6 N HCl, or to 7.8 with 6 N NaOH

Add H₂O to 1000 ml

Store up to 1 to 2 days at 4°C

Sodium phosphate, 90 mM (pH 7.8)/10% (v/v) glycerol/0.2 mM AEBSF

10.35 g sodium phosphate dibasic
2.466 g sodium phosphate monobasic
100 ml glycerol
1 ml 0.2 M AEBSF stock solution (see recipe)
Add H₂O to ~800 ml
Adjust pH to 7.8 with 6 N NaOH
Add H₂O to 1000 ml
Store up to 1 to 2 days at 4°C

Superdex 70 column

In the cold (i.e., 4°C), pour a 1.6 × 60–cm column of Superdex 70 gel-filtration resin (Amersham Biosciences) equilibrated in 50 mM Tris·Cl (pH 8.0)/0.15 M NaCl (see recipe). Calibrate using the molecular-weight standards (see recipe) blue dextran (V₀) and [³H]proline (V_i) as described.

Tris·Cl, 0.02 M (pH 7.6)/0.15 and 1.0 M NaCl

20 ml 1 M 0.22- μ m-filter-sterilized Tris·Cl, pH 7.5 (APPENDIX 2A)
8.77 or 58.44 g NaCl
Add H₂O to ~900 ml
Adjust pH to 7.6 with 1 N NaOH
Add H₂O to 1000 ml
Store up to 1 month at 4°C

Tris·Cl, 50 mM (pH 8.0)/0.15 M NaCl

8.76 g NaCl
4.44 g Tris·Cl
2.65 g Tris base
Add H₂O to ~800 ml
Adjust pH to 8.0 with 6 N NaOH
Add H₂O to 1000 ml
Store up to 1 month at 4°C

Urea stock solution, 8 M

Add 1920 g ultra pure urea (Life Technologies) in 2 liters water by dissolving ~200 g at a time. After all urea has dissolved, add water to 4 liters. Filter through Whatman no. 3 paper and store up to 1 month at 4°C.

COMMENTARY**Background Information**

The abundance of SPARC in many tissues, and its high levels of secretion by most cells in vitro, belie the difficulty of its recovery as an intact, active protein after purification. SPARC (as osteonectin) was found to be a major non-collagenous component of fetal and adult bone (Termine et al., 1981). In situ hybridization of SPARC by numerous investigators has shown that the mRNA is abundant in most fetal tissues, presumably associated with morphogenesis, growth, and angiogenesis but is somewhat limited in the corresponding adult tissues (for reviews, see Lane and Sage, 1994; Brekken and Sage, 2000; Bradshaw and Sage, 2001).

SPARC mRNA and protein are found in relatively high amounts in adult tissues that exhibit continuous turnover (gut epithelium) and remodeling (bone), and are produced in response to injury (wound healing) and certain types of pathologies (tumors, scleroderma). In more quiescent and/or established tissues, however, levels of SPARC are low. Since SPARC affects both the adhesion and proliferation of most normal cells, its association with angiogenesis and other processes requiring cell migration, differentiation, and synthesis of extracellular matrix (ECM) is not surprising.

There are several structural features of SPARC that should be considered in the context of a purification protocol.

1. SPARC is typically a secreted protein with two post-translational modifications that can be troublesome. There are fourteen cysteines, all of which are disulfide-bonded, and the folding and correct formation of disulfide bridges are not trivial in recombinant proteins produced at high levels, especially in yeast and bacteria. This has certainly been the case for SPARC (Yost et al., 1994; Bassuk et al., 1996b). Additionally, secreted SPARC contains a single complex-type carbohydrate chain (N-linked) which is not produced in nonmammalian systems. Interestingly, the carbohydrate has been shown to be variable in mammalian SPARCs—i.e., the carbohydrate from platelet SPARC is different from that from bone. In addition, cultured cells can assemble and process the oligosaccharide side-chain structures differently (Lane and Sage, 1994). It is important to remember that purification of SPARC from tissues such as bone will result in the recovery of nonsecreted SPARC that has unprocessed high-mannose-type oligosaccharide.

2. SPARC binds other proteins, including growth factors. The association of SPARC with albumin (probably through adventitious disulfide interchange) has been troublesome, but can be avoided by the use of serum-free culture (e.g., *Sf9* cells, *E. coli*, or a serum-independent mammalian cell line). Anticipate that isolation of SPARC from tissues (including platelets) can result in contamination from plasma and tissue fluid components (e.g., albumin) as well as ECM proteins to which SPARC binds (collagen types I, III, IV, V, and thrombospondin 1). Moreover, SPARC also interacts with platelet-derived growth factor (PDGF) AB and BB and vascular endothelial growth factor (VEGF) with a $K_d \cong 10^{-9}$ M. If possible, it is best to avoid these proteins when choosing a source of SPARC, as additional purification steps to remove the contaminants will invariably result in lower yields and loss of activity.

3. SPARC binds to several cations (Cu^{2+} , Fe^{2+}) and has an absolute requirement for Ca^{2+} . The disulfide-bonded EF-hand, a Ca^{2+} -binding loop at the C terminus, is reasonably stable, with a K_d for $\text{Ca}^{2+} \cong 10^{-7}$ M, and is thought to serve a structural function. The N terminus, however, contains from five to eight low-affinity ($K_d \cong 10^{-3}$ to 10^{-5} M) Ca^{2+} -binding sites (glutamic acids). Association of Ca^{2+} with this region of SPARC serves to neutralize its excessive negative charge and confers α -helicity to this domain. It is therefore critical that SPARC

is not exposed to EDTA or other chelating agents during purification, and that the protein is stored in the presence of 1 to 4 mM Ca^{2+} . One of the assays for native structure of SPARC, circular dichroism (see below), depends on α -helicity as a function of Ca^{2+} binding within this low-affinity site.

Three protocols have been discussed that maximize both the yield and the purity/native structure of either natural or rSPARC. Most cultured cells secrete reasonably high levels of SPARC into the culture medium, an environment in which SPARC is stable over several days at 37°C. Proteolytic degradation of SPARC has rarely been a problem, especially with the judicious use of protease inhibitors, as described in the protocols. Since both human and murine tumor cells can also secrete high levels of SPARC in vitro and in general are more tolerant of low serum (or, preferably, the absence of serum), they are a logical choice for the isolation of nonrecombinant SPARC, especially if they exhibit high rates of growth and secretion (see Basic Protocol).

Advantages of a recombinant protein expression system include the (theoretically) substantially higher yields of protein, as well as the potential of producing mutated versions of the protein. Both the *E. coli* and *Sf9* cell systems can achieve these goals with respect to SPARC (see Alternate Protocol 1 and 2). Additionally, SPARC from any species for which the sequence is known can be engineered by the polymerase chain reaction (APPENDIX 3F) into a suitable expression vector. Disadvantages include potential problems with folding and post-translational modification of rSPARC; however, assessment of purity and activity of the SPARC produced in both *E. coli* and *Sf9* cells has shown that these are both viable routes for the production of SPARC. Although the activity of rSPARC appears to be ~50% of that of the PYS-2-derived protein, the substantially greater yields may offset this limitation.

Any modification of the primary structure of SPARC must be considered as potentially deleterious to its conformation and/or activity. The $(\text{His})_6$ sequence, tagged onto the C terminus of SPARC to facilitate its purification by metal-affinity chromatography, could affect one or more properties of SPARC (e.g., nuclear translocation, de-adhesion) and should be controlled for in subsequent experiments. As discussed in preceding paragraphs, post-translational differences need to be considered as well—i.e., the lack of carbohydrate in *E. coli* rSPARC (see Alternate Protocol 1), and a dif-

ferent or additional type of glycosylation conferred by *Sf9* cells (see Alternate Protocol 2).

There may be situations in which the proper SPARC for study will be that isolated from a given tissue (e.g., bone). References have been included (see Alternate Protocol 3) for the extraction of SPARC from this tissue. The use of denaturants and EDTA could be problematic, although renaturation is always an option. Since both platelet and bone SPARC are available commercially (see Alternate Protocol 3), it is advisable to purchase a small amount and to test it according to the parameters required.

Critical Parameters and Troubleshooting

Many of the caveats at various stages of purification of SPARC have been detailed within each protocol. The principal problems are low recovery and poor bioactivity.

Recovery of SPARC depends on several factors, not the least important of which is the output of SPARC *in vitro*. Despite claims of immortality, transformed or tumor cells do not live forever in culture. Successive passages and cycling of cells on and off serum (or growth in the absence of serum) can affect their eventual viability. Therefore, it is important to monitor the secretion of SPARC over time (this also applies to the production of rSPARC). SPARC is produced optimally by subconfluent cells; at confluence or near-confluence, SPARC is secreted at a reduced rate, and will associate with the cell surface or ECM. Presented below is a list of other possible causes of recovery loss, as well as potential solutions; however, the reader should bear in mind that some losses are indeed unavoidable.

1. Failure of SPARC to redissolve completely in the various buffers used for purification or assay. Clarification of solutions is always recommended.
2. Precipitation of SPARC during freezing or thawing. Snap-freezing on dry ice, and quick-thawing at room temperature, are recommended.
3. Incomplete precipitation during dialysis against water, which can be checked by SDS-PAGE (UNIT 6.1) of a small aliquot of the supernatant.
4. Irreversible binding and/or denaturation of SPARC on membrane-type centrifugal concentrators (e.g., Centricons). Losses should be determined if the investigator chooses to concentrate purified SPARC in this manner. There are always new products on the market that claim to minimize this problem.

5. Degradation due to proteolysis by intrinsic proteinases or to bacterial contamination. Protease inhibitors should always be used during purification of SPARC, as described, and bacterial contamination should be minimal if sterile buffers or buffers containing sodium azide (NaN_3) are used.

6. Recovery can be compromised by the use of untreated glass vessels; only polypropylene or siliconized-glass containers should be used. Surface denaturation of SPARC occurs readily, either from adsorption to surfaces or from rapid stirring or overzealous mixing.

Denaturation of SPARC can be minimized with careful handling and attention to a few details.

1. The protein should be stored at -70° or -80°C , not at 4°C and especially not at -20°C .
2. 1 to 4 mM Ca^{2+} should be present in buffers containing SPARC.
3. Stirring of solutions should be steady but not rapid.
4. Purification of the protein should be conducted at 4°C whenever possible.
5. Only reagents (e.g., urea) of the highest purity should be used.
6. Reducing/oxidizing conditions, which can result in the scrambling of disulfide bonds, should be avoided.

Assays for SPARC bioactivity have been described elsewhere (see Support Protocols 1 to 3) and need not be repeated here. However, an important criterion for the correct folding of SPARC is the circular dichroism spectra obtained in the presence and absence of Ca^{2+} . These spectra are relatively easy to perform and interpret. Examples for SPARC purified from PYS-2 cells, *E. coli*, and *Sf9* cells have been published (Sage et al., 1989; Bassuk et al., 1996a; Bradshaw et al., 2000). The method relies on a characteristic increase of the mean residue ellipticity (θ) at 220 nm as a function of increasing concentrations of Ca^{2+} , indicative of a shift toward α -helicity. SPARC preparations that do not exhibit this transition are likely to be contaminated by other components and/or denatured.

For the use of SPARC in proliferation (i.e., [^3H]thymidine incorporation) assays, it is important to measure levels (if any) of contaminating growth factors that could affect the results. Both PDGF and VEGF bind to SPARC (see Background Information) and are anticipated to stimulate the proliferation of smooth muscle cells, fibroblasts (PDGF), and endothelial cells (VEGF). Kits based on ELISA are now available for the detection of these factors;

alternatively, detection could be accomplished by immunoblot analysis after SDS-PAGE of SPARC under reducing conditions (UNIT 6.2), although the former method allows for greater sensitivity.

Anticipated Results

Isolation from PYS-2 cells (see Basic Protocol) should yield ~500 μg per 30 maxiplates (150-mm diameter) PYS-2 cells (~ 10^7 cells/plate). The protein is of high purity (>90% by SDS-PAGE) and retains maximal biological activity. For example, an ED_{50} of 20 $\mu\text{g}/\text{ml}$ (0.6 μM) has been defined as an effective concentration for the induction of cell rounding by SPARC.

Yields of rSPARC from *E. coli* and *Sf9* cells are greater than those from PYS-2 cells (see Alternate Protocols 1 and 2), but are in large part dependent on the efficiency of the expression system (i.e., the particular expression vector, the host and its growth properties, and whether the rSPARC is secreted or retained within the cell). Using a first-generation *Sf9*/baculovirus expression system, the authors' laboratory typically recovers 2 to 4 mg human rSPARC (of ~80% purity) from an initial suspension of ~ 10^9 cells. The InsectSelect system, which eliminates the need for viral infection, is likely to be an improvement over the earlier version. rSPARC can be purified to $\geq 80\%$ and displays biological activity in cell rounding and proliferation assays.

The immunoaffinity-based chromatographic purification of SPARC from platelets will theoretically produce a highly purified protein, in reasonable yields, although the amount of SPARC in the starting material (α -granules of platelets) is low, from 0.7 to 2.2 $\mu\text{g}/10^8$ cells. One limiting factor is the availability of the monoclonal antibody used for the purification. This reagent must not only bind soluble SPARC with relatively and selectively high affinity, but must also release SPARC readily into the elution buffer without compromise of the SPARC or the antibody itself. Moreover, the antibody must function while coupled to an affinity resin. It is therefore important to ensure that a sufficient supply of the antibody is commercially available, as the column will have to be re-packed periodically with new affinity-coupled resin. An alternative is to purchase a hybridoma cell line secreting a suitable anti-SPARC IgG that can be propagated in the laboratory.

Time Considerations

The Basic Protocol and Alternate Protocols 1 to 2 each require ~1 week from the time of medium (PYS-2 and *Sf9* cells) or cell (*E. coli*) collection until the final lyophilization (or buffer exchange) step. Allow 1 to 2 days for the preparation of buffers and columns, and for the washing of columns. PYS-2 cells are usually ready for beginning the collection of medium 24 hr after plating, and medium is removed from the cells 18 to 24 hr later. Similar time frames apply to *E. coli* (grown overnight, diluted to an appropriate density in log phase, and induced) and to *Sf9* cells (grown in flasks over 3 to 4 days to generate conditioned medium containing rSPARC).

In all the protocols, convenient stopping points have been noted. There is temporal flexibility in the purification process, especially during the dialysis steps.

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CHAPTER 11

In Vitro Reconstitution

INTRODUCTION

Cell biologists employ in vitro systems as useful intermediates between the biochemistry of isolated molecules and the intricate biology of intact cells. In vitro systems can be used to study cellular functions on a number of levels. On one hand, post-cellular extracts may be used so that complicated biochemical activities are preserved but cellular structures are not. This is the case for in vitro assays of protein translation (*UNITS 11.2, 11.8, & 11.9*), cotranslational translocation of proteins into microsomes (*UNIT 11.3*), post-translational import of proteins into chloroplasts (*UNIT 11.16*), DNA replication (*UNIT 11.5*), mRNA transcription (*UNIT 11.6*), and mRNA splicing (*UNIT 11.17*). In other instances the plasma membrane of cells may be permeabilized by detergents, physical disruption, or bacterial toxins. Selective permeabilization allows the preservation of intact cellular structures. With the addition of exogenous cytosol, permeabilized cells can reconstitute aspects of membrane transport (*UNIT 11.4*), nuclear protein import (*UNIT 11.7*), peroxisomal protein import (*UNIT 11.15*), and other protein trafficking pathways. Remarkably, some in vitro systems can reconstitute not only biochemical function but also structure. This is particularly true for *Xenopus* egg extracts (*UNITS 11.10, 11.11, 11.12, & 11.13*), which can assemble either nuclei or mitotic spindles de novo from appropriate added templates, such as sperm chromatin.

Translation systems based on cellular lysates were among the earliest eukaryotic in vitro approaches developed. Over the years, they have become the basis for many widely used and versatile methods in cell biology. *UNIT 11.1* provides an overview of their origin and applications. *UNIT 11.2* gives protocols for the preparation and use of wheat germ extracts and message-dependent reticulocyte lysates. When mRNAs produced by transcription with bacteriophage RNA polymerases are introduced into wheat germ extracts or reticulocyte lysates, these systems are capable of efficient translation, thereby producing a single species of labeled protein product. Furthermore, conditions have been developed for simultaneous transcription and translation in a single reaction, making the expression of proteins from cloned DNAs extremely convenient for utilization in other assays (for instance, *UNIT 11.16*). However, reticulocyte translation systems may not always give results that are typical of other mammalian cell types, perhaps due the specialized biology of reticulocytes. This is particularly true with respect to translational initiation site selection, which is less stringent in this system. Alternate translation systems have been established using HeLa cell lysates (*UNIT 11.8*), in part to address this problem.

Needless to say, in vitro translation systems (*UNITS 11.1 & 11.2*) have been indispensable for the study of translation itself. Toward this end, methods to analyze translation complexes have been developed, monitoring the sedimentation of ribosomes, translation factors, and RNA on sucrose gradients (*UNIT 11.9*). These methods have been effective in dissecting intermediate steps in the formation of the 80S initiation complexes. They have also been utilized for examination of changes in transition initiation or elongation rates under different conditions in cell culture systems. The utility of wheat germ extracts and reticulocyte lysates can be further extended by their combination with vesicles from the rough endoplasmic reticulum, allowing cotranslational membrane translocation of secreted, luminal, and integral membrane proteins (*UNIT 11.4*). The reconstitution of mem-

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**In Vitro
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11.0.1

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brane translocation in this system permits the analysis of signal sequence peptide cleavage, protein topology after membrane insertion, and N-linked glycosylation.

Post-translational translocation of newly synthesized proteins into other membrane-bound organelles has been reconstituted for both peroxisomes (*UNIT 11.15*) and chloroplasts (*UNIT 11.16*). *UNIT 11.15* provides protocols to assay the import of biotinylated substrates into peroxisomes in semi-intact cells. The use of biotinylated substrates permits determination of the efficiency of import directly in semi-permeabilized cells or further characterization by ELISA-based methods after the isolation of cellular fractions containing the peroxisomes. *UNIT 11.16* provides methods for analysis of chloroplast protein import using pea chloroplasts, an organism that has been traditionally favored for this purpose, as well as chloroplasts from *Arabidopsis thaliana*, an organism that offers the possibility of combining genetic information with this biochemical analysis. Isolated chloroplasts from either species can take up labeled proteins that have been translated in reticulocyte lysates. Subsequently the chloroplasts are reisolated to permit determination of the extent of uptake and the suborganellar destination of the imported proteins.

Passage of proteins between the ER and Golgi has been reconstituted using either semi-intact cells or microsomal membrane preparations (*UNIT 11.3*). In order to follow the trafficking of vesicles through this pathway, the assays all utilize a temperature-sensitive mutant of a vesicular stomatitis virus glycoprotein (VSV-G ts045). At the restrictive temperature, this glycoprotein is retained in the ER because it is inappropriately folded. VSV-G ts045 protein moves synchronously out of the ER and into the Golgi when cells are shifted to the permissive temperature, in a manner that can be experimentally monitored through the maturation of its linked oligosaccharide chains. In the semi-intact cell assay, cells expressing the VSV-G ts045 protein are permeabilized at the restrictive temperature, resulting in the loss of their cytoplasmic components. VSV-G ts045 is transported from the ER to the Golgi when these permeabilized cells are incubated at the permissive temperature with exogenous cytosol. This system allows the straightforward biochemical analysis of cytosolic components, mutant proteins, and molecular manipulations upon ER-to-Golgi trafficking. Assays with microsomal membrane preparations allow the separate biochemical investigation of the budding of vesicles from the ER and their fusion to the Golgi.

Another important protein trafficking problem that has been successfully studied in semi-intact cells is the trafficking of proteins between the cytosol and the nucleus (*UNIT 11.7*). The compartmentalization of DNA within the nucleus is a distinguishing feature of eukaryotic cells. The sequestration of genetic material away from the translational machinery and cytosolic proteins serves both to define the identity of the nuclear compartment and to regulate its activity by controlling gene expression through the limited access of transcriptional regulators. Protein destined for import into nuclei encode nuclear localization sequences (NLS) that specifically target them to the nuclear compartment through a receptor-mediated process. Nuclear import has been reconstituted *in vitro* using digitonin permeabilization of cells. Treatment with digitonin preserves the integrity of the nuclear envelope, while disrupting the plasma membrane and allowing release of soluble factors required for protein import. Protein import can be reconstituted in digitonin-treated cells through the reintroduction of soluble factors from a variety of sources (reticulocyte lysates, HeLa cell lysates, and *Xenopus* oocyte cytosol), and monitored through the nuclear accumulation of fluorescently labeled protein import substrates.

Digitonin-treated cells have been an extremely powerful assay system for dissection of the biochemistry of nuclear protein import. This system is currently being extended toward a better understanding of nuclear protein import and to the regulation of protein trafficking in both directions. For analysis of RNA trafficking between the nucleus and

cytoplasm, *Xenopus* oocytes have been employed as a very powerful experimental system (UNIT 11.14). The large size of the mature oocyte makes it possible to quantitatively address questions about RNA trafficking through microinjection specifically into the cytosol or nucleus. The nucleus can be manually dissected from the cytosol at a later point and the contents of each compartment analyzed biochemically to reveal how particular molecules are translocated between these compartments. While this is actually an in vivo experimental system, it is included in this chapter on in vitro analyses because of its manipulability for analysis of nucleocytoplasmic trafficking and because it is utilized for many of the same problems addressed by other in vitro approaches covered here.

Events normally occurring within nuclei have been reconstituted in complex post-cellular fractions. DNA replication in mammalian cells has been studied using 293 cell extracts (UNIT 11.5). These extracts become competent to replicate plasmids bearing the papovavirus SV40 origin of DNA replication with the addition of deoxyribo- and ribonucleotides and a single viral protein, the large T antigen. This system has been extremely powerful for understanding mammalian DNA replication. The cellular components have been purified to near homogeneity and the succession of events that occur during the initiation of DNA replication has been well characterized, allowing detailed examination of the mechanisms whereby DNA replication is controlled. RNA polymerase II transcription has also been reconstituted in vitro, in order to identify molecularly and study biochemically the factors required for this process. UNIT 11.6 discusses protocols for the preparation and use of mammalian (HeLa cell) and *Drosophila* extracts that accurately initiate basal and activated polymerase II transcription. These systems can be used to analyze transcription from either chromatin or naked DNA templates. The capacity of these systems to faithfully recapitulate transcription in vitro permits dissection of not only the mechanisms of transcription but also the manner in which it is regulated. Finally, nuclear extracts developed for transcription have also been adapted for analysis of post-transcriptional mRNA splicing, using labeled RNAs (UNIT 11.17). This system is useful for the characterization of splicing sites within the mRNA, as well as for general identification of splicing factors. Moreover, where splicing occurs in a tissue-specific manner, nuclear extracts can be prepared from particular tissues, allowing purification and characterization of components that govern such tissue specificity.

The remarkable biology of the African clawed frog, *Xenopus laevis*, has been exploited to provide in vitro systems that can reconstitute many aspects of cellular behavior during early development, including the de novo assembly of such structures as nuclei and mitotic spindles. Extracts prepared from these eggs are able to reconstitute sperm pronuclear assembly, resulting in nuclei that are functional by a number of criteria (UNIT 11.10). These criteria include the presence of a typical double membrane nuclear envelope perforated with functional nuclear pores, the presence of an intact nuclear lamina, and the capacity of the nuclei to undergo one complete semiconservative round of DNA replication. By varying the conditions of preparation slightly, mitotic egg extracts can be produced that have the capacity to assemble intact and functional spindles from added sperm chromatin (UNIT 11.13). Moreover, by producing extracts under conditions that optimize the level of translation, it is possible to obtain extracts that will spontaneously alternate between these states, continuously alternating between interphase and mitosis (UNIT 11.11). These cycling extracts not only preserve most of the core machinery during the entry into and exit from mitosis, but they also preserve regulatory mechanisms governing such transitions. In combination, these properties have made *Xenopus* extracts an enormously powerful and useful system for study of the cell cycle. Finally, newer applications of egg extracts have been developed after it was demonstrated that they can also functionally reconstitute aspects of programmed cell death (apoptosis) within the test tube (UNIT 11.12).

Together the units in this chapter aim to cover a broad range of in vitro approaches to cell biology. Although these systems of course cannot replace experiments conducted in vivo, they allow straightforward biochemical manipulation and analysis. They therefore serve as indispensable tools in the toolbox of biologists attempting to understand how cells work.

Mary Dasso

Overview of Eukaryotic In Vitro Translation and Expression Systems

EARLY HISTORY

Today, the protocols and commercial systems for expressing gene products in vitro are well known to molecular biologists and researchers in many other fields. The present-day systems have been steadily improved in efficiency and convenience over their early predecessors. The earliest in vitro translation extracts, developed in the 1960s, were central to the breaking of the genetic code. The late 1960s and early 1970s saw the emergence of a variety of translation extracts prepared from different sources, such as Krebs II ascites cells, CHO cells, rat and mouse liver, and HeLa cells. Most of these systems faithfully translated viral and eukaryotic mRNA pools but were limited in their efficiency and by the competitive translation of endogenous mRNAs. One system that overcame these limitations was the efficient wheat germ-based expression system (Roberts and Paterson, 1973). Another system based on lysates of rabbit reticulocytes that was being developed during this period would eventually become the most widely used option. In 1968, this reticulocyte system was improved through the addition of hemin (Adamson et al., 1968; Zucker and Schulman, 1968), which allowed a steady rate of translation for 60 min. However, these early systems contained high levels of globin mRNA, which made detection and quantification of added mRNA products difficult at best. One approach to this problem was to create a reconstituted system from fractions of reticulocyte lysate from which reticulocyte mRNA had been removed (Schreier and Staehelin, 1973). Unfortunately, the reconstituted fractionated system primed with added mRNAs did not retain high activity.

IMPROVED SYSTEMS

In 1976, Pelham and Jackson described a breakthrough protocol consisting of a micrococcal nuclease treatment to destroy endogenous mRNA followed by addition of EGTA to remove the Ca^{2+} required for nuclease activity (Pelham and Jackson, 1976). This system produced the low background of fractionated systems and yet, in contrast to those systems, exhibited remarkably high activity and an exceptional response to added mRNA. In addition, the nuclease-treated reticulocyte translation system exhibited much lower nuclease

activity than the wheat germ system and could therefore generate much larger full-length gene products—up to 200 kDa. The system also retained the translational regulatory responses of the parental lysate. The system became more widely used and was continuously improved through the 1980s. Refinements included addition of exogenous calf liver tRNA to complement the limiting endogenous tRNA population strictly suited for globin expression, optimization of Mg^{2+} and K^+ ion concentrations, addition of ribonuclease inhibitors (such as RNasin) and protease inhibitors, and inclusion of sugars to maintain reducing potential (Jackson and Hunt, 1983; Jagus, 1987). Concurrently, the wheat germ system was also improved through the use of high spermidine concentrations and the addition of human placental RNase inhibitor, facilitating efficient translation of high-molecular-weight proteins (Morch et al., 1986). Many translational mechanisms were investigated and defined using these systems.

With the growing availability of cloned genes, one of the most important advancements of in vitro expression during this period was the development of a system capable of synthesizing large quantities of gene-specific mRNA (Melton et al., 1984). Genes cloned behind a phage polymerase promoter, such as the T7 or SP6 promoter, could produce mRNA transcripts through incubation of linearized DNA templates with the appropriate polymerase and reaction conditions. Capped (or uncapped) transcript mRNAs were then added to the in vitro expression systems, resulting in expression of a single gene product, rather than expression from viral genomes or mRNA pools (Krieg and Melton, 1984). In vitro translation of transcripts from cDNAs allowed the identities of many cloned genes to be verified. An added bonus of this method is that any desired mutant protein can be generated simply by altering the DNA template (Struhl, 1989).

The efficiency of this in vitro translation method was increased by maximizing the amount of RNA produced for a given amount of DNA template (Milligan et al., 1987; Gurevich et al., 1991), allowing products of the transcription reaction to be added to a cell-free translation system without prior purification of the RNA (Andrews, 1989). The greatest im-

provement in RNA yields involved uncapped transcripts, as new vectors were introduced that could create transcripts that did not need to be capped before they could be translated. The vectors pTM1 (Moss et al., 1990) and pCITE (Novagen) place the 5' untranslated leader region (UTR) of encephalomyocarditis virus (EMCV), which contains an internal ribosome entry site (IRES), upstream of the initiation codon to facilitate cap-independent translation in the reticulocyte system.

NEWER SYSTEMS AND APPROACHES

Continuous-Flow Systems

The late 1980s and the 1990s witnessed an evolution in the use of translation expression systems, from studies of translation mechanisms and simple verification of expected gene products toward the rapid and efficient production of larger quantities of functional proteins. Continuous-flow cell-free (CFCF) systems designed to work for tens of hours and produce hundreds of micrograms of synthesized protein were introduced in the late 1980s (Spirin et al., 1988). Use of the CFCF systems described by Spirin remains technically challenging, requires expensive flow cells, and has yet to demonstrate consistent results. For these reasons such systems have not been widely utilized, although they hold great potential for the production of gene products that are too toxic for production *in vivo*. Successes have been reported with CFCF systems involving simpler devices such as dialysis tubes, which have been used for prokaryotic systems (Davis et al., 1996; Kim and Choi, 1996) and a wheat germ system (K.J. Rothschild, pers. comm.). These dialysis tube-based systems are able to produce from hundreds of micrograms to >1 mg of expressed protein per milliliter of reaction. Another group has reported the use of affinity ligands to continuously remove the protein products, thereby improving translation efficiency (Marszal and Scouten, 1996).

Coupled Eukaryotic Systems

In 1992, the first eukaryotic coupled transcription/translation batch systems were introduced; these allowed direct expression from DNA containing cloned genes positioned behind T7, T3, or SP6 promoters in rabbit reticulocyte lysate or wheat germ extracts (Craig et al., 1992; Thompson et al., 1992). Transcription and translation reactions exhibit very different requirements for monovalent and divalent cat-

ions. The conditions that give optimal activity of the coupled system are closer to those needed for translation; although transcription is relatively inefficient in the coupled reaction, sufficient RNA is produced to saturate the protein synthetic capacity of the system. The fidelity and efficiency of expression in the coupled system is high. Unexpectedly, the coupling of transcription with translation often results in greater protein production (Jagus, 1993). Moreover, the ability to generate and utilize cloned DNA and the products of PCR reactions, rather than purified mRNA, has generated a multitude of new applications (Beckler, 1998—see Internet Resources).

Incorporation of Non-Natural Amino Acids

One primary advantage of *in vitro* translation is the ability to manipulate expression easily by adding exogenous compounds directly to the reaction. Incorporation of non-natural amino acids into synthesized proteins is a powerful tool for the creation of novel or mutant proteins. A technique for incorporating epsilon-amino-modified lysines into proteins synthesized in rabbit reticulocyte lysate has been described by Johnson et al. (1976). Charged tRNA complexes containing epsilon-modified lysines, such as biotinylated lysines, can be added to the translation reactions and the modified lysines will be incorporated into the synthesized protein. Biotinylated proteins can be detected nonisotopically using streptavidin-alkaline phosphatase or horseradish peroxidase conjugates (Kurzchalia et al., 1988; Hoeltke et al., 1995); these detection systems became commercially available in 1993 (Beckler and Hurst, 1993). Other groups developed alternative site-directed non-native amino-acid replacement (SNAAR) using nonsense codon suppression methods to introduce non-natural amino acids at specific sites (Noren et al., 1989). These approaches use RNA ligase to ligate *in vitro*-transcribed amber tRNA lacking the 3' terminal pCpA from the acceptor stem to a chemically acylated pCpA-X, where X can be almost anything, such as a fluorescent, spin-label, or isotopic group (Noren et al., 1990).

NEW APPLICATIONS

The PCR revolution, the genome sequencing projects, and the plethora of techniques available to look at differentially expressed genes have led many researchers to ask what a particular gene of interest codes for, what function it has, and with what other gene products

it interacts. Many basic questions centered on understanding this “genetic output” are being answered using *in vitro* expression approaches. The following applications are made possible or desirable due to the current ease with which a particular gene or gene segment can be amplified through PCR, with concurrent incorporation of a phage polymerase promoter, such as the T7 promoter (Kain et al., 1991). The PCR-generated DNA can be expressed in a eukaryotic coupled transcription/translation reaction and the activity of the translated product(s) assessed using an *in vitro* assay.

Molecular Interactions

Protein-protein interactions

One of the most popular current applications of coupled eukaryotic systems is the detection of protein-protein interactions. Often, researchers will use an *in vitro* approach to verify or confirm results obtained *in vivo*, for example using the yeast two-hybrid approach (Boyd et al., 1995; see APPENDIX 3). To define the region of protein-protein interaction, a series of constructed deletion mutants or occasionally specific point mutants are synthesized *in vitro* and compared to full-length wild-type proteins.

Fusion-tag approach. Additions to the native gene product in the form of a fusion “tag” can be extremely useful if specific antibodies are not available. [³⁵S]Methionine-labeled proteins can be synthesized in coupled reactions *in vitro* from either full-length cDNAs or deletion mutants. These radioactive proteins can be used to detect interactions with suspected protein partners that have been expressed as GST, His-tag, or epitope-tagged (UNIT 5.2) fusion proteins and purified after expression in *Escherichia coli* (Chinnaiyan et al., 1995). Fusion proteins can be bound to an affinity matrix along with the radioactive protein with which they interact (Cowell and Hurst, 1996; Sharp et al., 1997). The bound radioactive proteins are then eluted and analyzed by SDS-PAGE (UNIT 6.1) or immunoblot (western) analysis (UNIT 6.2).

Immunoprecipitation. Another approach involves detection of a radioactive fusion partner by immunoprecipitation (UNIT 7.2) using antibodies against a particular antigenic domain. For example, an influenza hemagglutinin (HA) epitope incorporated in the carboxyl terminus of an *in vitro*-expressed protein can be immunoprecipitated using anti-HA antibodies (Benedict and Clawson, 1996). Alternatively, if an antibody against one of the partners is available, detection can be accomplished by

coimmunoprecipitation (Leng et al., 1995; Sharp et al., 1997). Again, the bound radioactive proteins are eluted and analyzed by SDS-PAGE or immunoblot analysis. A variation on this analysis method utilizes *in vitro*-coupled expression of several proteins simultaneously. The relative protein expression levels can be controlled by varying the concentrations of each DNA construct. For instance, using cDNAs for chicken NF- κ B p105, NF- κ B p100, c-Rel, and v-Rel, cotranslated *in vitro*, and protein complex detection by immunoprecipitation with specific antiserum, it has been shown that one of the complexes in v-Rel-transformed spleen cells can be reconstituted *in vitro* (Sif and Gilmore, 1993).

Far western analysis. A direct detection method for identifying protein-protein interaction is the so-called “far western” analysis. In this approach, radioactive proteins are synthesized *in vitro* and then used as “probes” to detect binding to membrane-bound renatured proteins directly (Johnston et al., 1996).

Isolation of protein complexes by capture of biotinylated lysine residues. One drawback of fusion-protein techniques is the need to make the fusion protein construct. Another approach that obviates the need to develop novel constructs is to incorporate non-natural amino acids, such as biotinylated lysines, into *in vitro*-translated proteins. This approach can be taken with any gene containing lysine codons. Incorporation of non-natural amino acids does not significantly alter the efficiency of polypeptide synthesis, and usually incorporation of biotinylated lysines does not affect the function of the protein. This approach has been used to develop a novel method for capturing protein complexes that associate with biotinylated Rab5, a member of the Rab family of GTP-binding proteins (Sanford et al., 1995). The ability to bind biotinylated lysine tightly to streptavidin-linked agarose can be utilized to capture *in vitro*-synthesized biotinylated Rab5. A promising and potentially powerful modification of the biotinylated-lysine capture technique for detection and gentle purification of *in vitro*-generated polypeptides, using photocleavable (PC) biotin, has recently been described (Rothschild et al., 1997). In a recent example, the nascent PC-biotin-labeled polypeptides were captured using streptavidin-coated magnetic beads, and after a short exposure to UV, the native polypeptide was released (with 70% to 95% efficiency) with no remaining “tags” (Olejnik et al., 1995, 1998; Rothschild et al., 1997).

Protein folding, chaperonins, and luciferase. Another emerging area utilizing in vitro systems to define protein-protein interactions is the field of protein folding and chaperonin interactions. Researchers have combined the advantages of in vitro expression with the power of instantaneous reporter gene product assays. The folding of polypeptides emerging from ribosomes has been analyzed using firefly luciferase as a model protein (Frydman et al., 1994; Frydman and Hartl, 1996). The growing polypeptide interacts with a specific set of molecular chaperones, including Hsp70, the DnaJ homologue Hsp40, and the chaperonin TRiC. The ordered assembly of these components on the nascent chain forms a high-molecular-mass complex that allows the cotranslational formation of protein domains and the completion of folding once the chain is released from the ribosome.

Real-time translation/folding assays

A novel approach has been developed using a wheat germ system in which the components for the luciferase enzymatic assay have been added directly to the translation reaction and monitored continuously in real time (Kolb et al., 1994). To demonstrate that luciferase exhibits cotranslational folding, the activity of translation products produced from wild-type mRNAs was compared with that of products produced from mutant mRNAs lacking stop codons to prevent release of the polypeptide from the ribosome. Luciferase was shown to be completely folded and enzymatically fully active immediately upon release from the ribosome (Kolb et al., 1994). However, no luciferase activity was observed while full-length luciferase remained attached to the ribosome as a peptidyl-tRNA, probably because the C-terminal portion of the enzyme is masked by the ribosome and/or ribosome-associated proteins. The investigators demonstrated that the ribosome-bound enzyme acquires enzymatic activity when its C-terminus is extended by at least 26 additional amino acid residues (Makeyev et al., 1996). The results demonstrate that the acquisition of the final native conformation by a nascent protein can occur as the protein is being synthesized and that folding does not require release of the protein from the ribosome.

Macromolecular assembly and frameshifting

Many in vivo translational control mechanisms are faithfully replicated in vitro. In addition,

a variety of macromolecular complexes can be expressed and properly assembled in vitro. For example, an in vitro synthesis and assembly system for the prototypical type D retrovirus, Mason-Pfizer monkey virus (M-PMV), has been developed utilizing rabbit reticulocyte reactions expressing M-PMV-Gag precursor polyprotein precursors as the result of two ribosomal frameshift events (Sakalian et al., 1996). The frameshift efficiency in vitro is identical to that observed in vivo. These polyproteins assemble to form immature retrovirus capsids indistinguishable from those formed in the host cell cytoplasm. More importantly, this system can be utilized in combination with anti-Gag antibodies to search for potential inhibitors of retrovirus assembly.

Protein-DNA interactions

The electrophoretic mobility shift assay (EMSA) is a widely used procedure to detect DNA binding sites for proteins or protein complexes, such as transcription factors. In this technique in vitro-generated proteins with putative DNA-binding activity are combined with an oligonucleotide containing a target consensus sequence site, and changes in DNA migration due to the bound protein are detected by PAGE. Either partner can be radiolabeled (usually 5'-end-labeled [³²P]DNA is used) and with proper controls, the translation extract containing the synthesized candidate binding factor can be used directly (Lee and Chang, 1995). Many researchers investigating transcription-factor binding use coupled wheat germ rather than rabbit reticulocyte translation systems, as wheat germ extract does not contain endogenous transcription factors such as NFκB (Sif and Gilmore, 1993). If the reticulocyte system must be used, it is possible to remove endogenous DNA-binding proteins from the reticulocyte system by a rapid method using biotinylated DNA and streptavidin-conjugated magnetic beads (Ebel and Sippel, 1995).

Protein-RNA interactions

Protein-RNA interactions can also be studied with in vitro translated proteins. To demonstrate the direct interaction of the U1 snRNP-A protein with SV40 late mRNAs, [³⁵S]Met-labeled U1 snRNP-A fusion protein containing a g10 epitope tag was synthesized in vitro, mixed with ³²P-labeled in vitro transcribed RNA, and immunoprecipitated. Coprecipitating RNAs were extracted and analyzed by PAGE (Lutz and Alwine, 1994).

Characterization of molecular environments

In vitro expression systems have also been utilized extensively to understand the molecular environment of nascent polypeptides as they are translated and translocated, including the very nature of the ribosome “tunnel” the polypeptide passes through. These approaches utilize the incorporation of non-natural amino acids containing photoactivatable cross-linking groups or fluorescent groups.

Photo-cross-linking. Studies of a secretory protein utilizing the incorporation of modified Lys-tRNAs containing a photoreactive cross-linker group, with subsequent photo-cross-linking, revealed a sequential passage of the transmembrane domain through three different proteinaceous environments, and helped define the multistep process of cotranslational integration of a nascent protein into the endoplasmic reticulum membrane (Do et al., 1996).

Fluorescent quenching. Fluorescent probes incorporated into nascent secretory proteins using modified Lys-tRNAs have been used to report on the ability of various-sized quenching agents to enter the endoplasmic reticulum pore and quench fluorescence. (Hamman et al., 1997).

Membrane association. The features within the N-terminal membrane-targeting domain of the cyclic AMP phosphodiesterase RD1 that are required for membrane association have been analyzed using in vitro expression of deletion mutants of RD1-CAT chimeras. Radioactive products were generated in vitro, incubated with membranes, and then identified in either the membrane pellets or soluble supernatant fractions (Smith et al., 1996).

Characterization of transmembrane domains. The G protein-coupled receptors involved in signal transduction across the plasma membrane have been viewed as having seven transmembrane helices and a three-dimensional structure similar to that of bacteriorhodopsin. These receptors appear to insert into membranes using signal-anchor sequences followed by stop-transfer sequences. Hydrophathy plots and various experimental techniques have been used to predict the seven membrane domains, yet no one technique has allowed a direct demonstration. In vitro translation in the presence or absence of microsomal membranes was used not only to determine the presence of independent signal-anchor and stop-transfer sequences, but also to identify the amino acid sequences accounting for transmembrane folding (Bayle et al., 1997a,b). This method used constructs containing the N-terminal region of

the gastric H⁺/K⁺ ATPase or the N-terminal region of the cholecystokinin-A (CCK-A) receptor, coupled via a linker region to the last 177 amino acids of the beta subunit of the gastric H⁺/K⁺ ATPase. The latter contains five potential N-linked glycosylation sites. Translation of the mRNA encoding one, two, or more putative transmembrane domains in the absence or presence of microsomes allowed determination of signal-anchor or stop-transfer properties of the putative transmembrane domains from the molecular weight shift after SDS-PAGE. Translation of segments of the gastric H⁺/K⁺ ATPase provided evidence for only seven transmembrane segments, but coupled with other data established a ten-membrane-segment model. The CCK-A receptor showed the presence of six of the seven transmembrane segments postulated for this protein. This technique provides a useful addition to methods of determining membrane domains of integral membrane proteins, but must in general be combined with other methods to establish the number of transmembrane alpha helices.

MOLECULAR FUNCTION

Functional Genomics

Ribosomal display for cell-free protein evolution

Ribosomal display is an emerging technology for performing cell-free protein evolution in vitro as an alternative to phage display. This approach utilizes the cell-free system to transcribe a DNA library, translate the mRNA pools, and, using a variety of techniques, retain the proteins and their encoding mRNAs attached to the ribosomes. The protein-mRNA-ribosome complexes are screened for binding to a target and the retained mRNA is amplified using RT-PCR, with the resulting DNA used for another round of selection. Initially the technique utilized the prokaryotic *E. coli* coupled transcription/translation system to generate large libraries of peptides used for receptor-ligand screening (Mattheakis et al., 1994). This system was improved to allow folding of whole proteins into their native structure while on the ribosome (Hanes and Pluckthun, 1997). The first eukaryotic-based system, centered around a rabbit reticulocyte coupled transcription/translation system, used antibody-ribosome-mRNA (ARM) complexes for rapid selection to monitor the evolution of antibody combining sites (He and Taussig, 1997). ARMs carrying single-chain (VH/K) binding frag-

ments specific for progesterone have been selected using antigen-coupled magnetic beads. Selection simultaneously captured the genetic information as mRNA, making it possible to generate and amplify cDNA by single-step RT-PCR on the ribosome-bound mRNA for further manipulation. Using mutant libraries, antigen-binding ARMs were enriched by a factor of 10^4 - to 10^5 -fold in a single cycle, with further enrichment in repeated cycles. Such an approach has obvious potential for the selection of receptors or peptides from libraries.

An interesting cell-free system for performing evolution studies *in vitro* has been developed to find reaction conditions in which isothermal RNA amplification would occur simultaneously with coupled transcription and translation (Joyce, 1993). After unsuccessfully testing wheat germ extracts and coupled *E. coli* systems, investigators were able to operate the “two” reactions simultaneously using the rabbit reticulocyte coupled transcription/translation system. This system can be used to perform laboratory “evolution” by putting selective pressure on functional protein products necessary for RNA amplification.

In vitro expression cloning (IVEC)

To a considerable extent, as the era of functional genomics dawns, the current scientific focus is shifting from genomic bioinformatic analyses as such to the development of approaches to understand gene-product function. A new alternative approach to functional gene identification utilizes the manipulative ability, speed, and convenience of eukaryotic coupled transcription/translation. *In vitro* expression cloning (IVEC) consists of adding small cDNA plasmid pools (of 50 to 100 clones per pool) directly into a rabbit reticulocyte coupled transcription/translation system. The resulting protein pools can be used to identify cDNAs rapidly on the basis of almost any biochemical property for which an assay can be developed (King et al., 1997; Lustig et al., 1997). The first report of this technology detailed the screening of a total of ~500 pools of cDNAs encoding ~12,000 proteins and identification of 105 pools with potential positives. Twenty mitotically phosphorylated proteins were identified by incubating *in vitro* protein pools with interphase *Xenopus laevis* egg extracts and identifying phosphorylated protein targets from their altered SDS-PAGE migration (Stukenberg et al., 1997). Interestingly, while fifteen of the identified phosphorylated proteins share sequence similarity with proteins previously

characterized, the other five are novel. This approach has also been used to screen 100,000 cDNA clones to identify gelsolin as a substrate of caspase-3 protease, demonstrating that gelsolin is an effector of morphological changes in apoptosis (Kothakota et al., 1997). This technology appears to be an effective bridge connecting genomics and proteomics.

Molecular Characterization Studies

Enzymatic activity and mutation analysis

In vitro expression can be used to analyze synthesized proteins, whether wild-type or mutant, for enzymatic activity. While deletion mutation analysis is useful for defining protein- or DNA-binding regions, site-specific mutation analysis can be useful for understanding and modifying enzymatic activity. In one study, the hypothesis that histidine 21 of diphtheria toxin A subunit is important in ADP-ribosyltransferase activity was tested by mutagenizing codon 21 to encode all possible amino acids. The products were translated *in vitro* in a reticulocyte coupled transcription/translation system and analyzed for ADP-ribosylating activity, resulting in the determination that histidine 21 does not play a major role in diphtheria toxin catalysis (Johnson and Nicholls, 1994). *In vitro* systems offer substantial advantages over *in vivo* systems in terms of safety and expression efficiency for toxin expression.

Another group expressed a cDNA encoding a large (110-kDa) six-transmembrane protein, type IV adenylyl cyclase (ACIV), in a coupled transcription/translation system with the same specific enzymatic activity as baculoviral-produced ACIV (Warner et al., 1995). Interestingly, the *in vitro*-generated ACIV was very stable and demonstrated linear activity for 90 min at 30°C, which is much longer than impure samples (<15 min) and somewhat longer even than pure adenylyl cyclase (1 hr). Use of such a system may provide a good first screen for identifying interesting mutants or producing active chimeric constructs of topologically complex proteins like ACIV.

Similarly, generation of *in vitro*-translated aromatase in the presence of canine pancreatic microsomes gives rise to an active enzyme when supplemented with recombinant P₄₅₀ reductase (Pancharatnam et al., 1996). The *in vitro*-made enzyme has properties equivalent to those of aromatase in tissue extracts. This use of the coupled transcription/translation systems may provide a general procedure for examining structure/function relationships

among the cytochrome P₄₅₀ superfamily of enzymes without the need for transient or stable transfection studies.

Post-translational modifications

A number of post-translational activities have been extensively analyzed using *in vitro* expression systems. For example, rabbit reticulocyte lysate contains proteolytic, phosphorylation, myristylation, farnsylation, isoprenylation, and adenylation activities (Glass and Pollard, 1991; Hancock, 1995). With the addition of canine microsomal membranes, methylation, glycosylation, and signal-sequence cleavage activities can be studied (Walter and Blobel, 1983; also see *UNIT 11.4*). A reticulocyte lysate assay for studying the post-translational modifications of Ras proteins has described the analysis of a number of different modifications (Roy et al., 1997).

Proteolytic cleavage analysis

For many years, *in vitro* expression systems have been used to study viral and cellular proteolytic activity. Examples include characterization of the autoprocessing cascade of HSV-1 (Godefroy and Guenet, 1995) and of the expression of a 250-kDa polyprotein that results in detectable self-cleavage products when expressed in a coupled transcription translation system, but is incompletely processed in a standard mRNA-based translation system (Hemmer et al., 1995). Recent work has described an *in vitro* assay to activate the hepatitis C virus N2-3 protease post-translationally and methods to study the effects of several common inhibitors on the enzymatic activity (Pieroni et al., 1997).

Antisense regulation

Antisense oligonucleotides have been shown to inhibit gene expression at either the transcriptional or translational level (Curcio et al., 1997). The mechanism for translational inhibition is thought to involve activation of RNase and is currently a target of intense investigation. Attempts to rationally design effective antisense RNAs have yet to yield consistent results, giving rise to the use of an empirical approach using randomized oligonucleotide arrays to identify ideal candidate antisense oligonucleotides (Lima et al., 1997; Milner et al., 1997). An *in vitro* reticulocyte lysate coupled transcription/translation system has been established for rapid screening of antisense oligodeoxyribonucleotides to determine which are the most effective in arresting mRNA translation (Chen et al., 1997). The potential for

heteroduplex formation of an oligonucleotide has been shown to correlate closely with inhibition of translation *in vitro*.

Development of antiviral agents

The rapid reticulocyte coupled transcription/translation screening system has been applied to the identification of antisense oligodeoxynucleotides capable of inhibiting hepatic D viral replication (Chen et al., 1997). Other groups developing specific viral inhibitors have utilized *in vitro* expression to test different target regions with modified oligonucleotides (peptide nucleic acid and phosphorothioate oligonucleotides; Alt et al., 1997; Koppelhus et al., 1997). Additional studies have shown the circular 2'-deoxyribo-oligonucleotides to be potent inhibitors of luciferase expression in an *in vitro* coupled transcription/translation system (Azhayeva et al., 1997).

Ligand binding for identification of novel orphan receptors

The binding of ligands to *in vitro*-synthesized receptors can be an important aspect of identifying new receptors. For example, a search for novel "orphan" nuclear receptors and ligands cloned and characterized a novel estrogen receptor (Kuiper et al., 1996). Saturation ligand-binding and ligand-competition assays of the *in vitro*-expressed clone allowed this novel receptor to be distinguished from one previously cloned.

Characterization of antiserum

In studies utilizing antiserum, immunoprecipitation of *in vitro*-synthesized proteins can be used to demonstrate the specificity of the antiserum (Murrell et al., 1995).

Studies of Molecular Structure

Understanding of integral membrane protein function is currently limited by the difficulty of producing three-dimensional crystals. A method has been developed for probing conformational changes in proteins in which Fourier-transform infrared-difference spectroscopy is combined with amber suppression to insert a single isotopic label into a specific site within *in vitro*-synthesized polypeptides that fold into their native structure (Sonar, 1994). Unlike earlier methods using site-directed mutagenesis, this does not disrupt the protein structure. It should be applicable to a wide range of proteins, including those involved in enzyme catalysis, ion transport, and signal transduction.

Diagnostic Detection

Protein truncation test

Perhaps the fastest-growing application of coupled transcription/translation systems has been for the diagnostic detection of genetic diseases, a predominantly DNA-dominated arena. The protein truncation test (PTT), sometimes referred to as the *in vitro*-synthesized protein truncation (IVSP) assay, was first reported in 1993 as a rapid method for detecting translation-terminating mutations in the very large gene responsible for Duchenne muscular dystrophy (Roest et al., 1993) and in the familial adenomatous polyposis (APC) gene responsible for a type of hereditary colon cancer (Powell et al., 1993). In these and other diseases, such as hereditary breast cancer (Hogervorst et al., 1995), 70% to 95% of the mutations that cause disease result in a truncated gene product. In the PTT, genomic DNA or mRNA is first purified from the patient's blood or tissue. This is subjected to either RT-PCR or PCR with concurrent incorporation of a T7 promoter and an optimal translation initiation sequence surrounding the desired start codon. Often, when the source mRNA is limiting, a second nested PCR reaction is required. Large exons are amplified from genomic DNA while smaller exons are amplified together from mRNA, and the gene is segmented into overlapping amplified fragments. The amplified DNA is added directly into a coupled transcription/translation reaction, and translation-terminating mutations are detected as faster-migrating bands after SDS-PAGE analysis. The main advantages of the PTT include the ability to scan large (2- to 3-kb) DNA or RNA segments quickly and the fact that the method detects only disease-causing mutations, avoiding the fruitless evaluation of silent polymorphisms. A disadvantage has been that the interpretation of real results can be complicated by the presence of internally translated protein products and other nonspecific bands. The PTT has been improved through addition of reporter-tag sequences in the 5' primer, and by the design of overlapping segments, gel parameters, and methods to prevent nonsense-mediated mRNA decay (Rowan and Bodmer, 1997; Garvin, 1998). A recent review comparing PTT to other detection methods describes it as the first choice for mutation screening (Frayling and Rowan, 1997). Perhaps the most unusual example of the power of this method for detecting previously unknown mutations was provided by a new discovery by the Kinzler

and Vogelstein groups that uncovered a novel mechanism for producing germline mutations with resulting predisposition to colon cancer (Laken et al., 1997). IVSP screening analysis of a patient with a family history of colon cancer was found to produce truncated gene products, yet sequence analysis of the DNA revealed only a T-to-A transversion resulting in a lysine-for-isoleucine substitution. The truncation thus appeared to be an *in vitro* phenomenon caused by the transversion. However, further work revealed that this mutation creates a small hypermutable region of the gene, resulting in secondary, truncating mutations. It appears this region contains unusual secondary structure that is incorrectly processed by both the polymerases present in human cells and the phage polymerases used in the IVSP reaction. This finding has led to the development of a specific genetic screen which has demonstrated that this mutation is present in 6% of Ashkenazi Jews.

An approach similar to PTT has been applied to screening of the expression of the simian immunodeficiency virus (SIV) *nef* gene in the course of disease progression in macaque monkeys (Switzer and Heneine, 1995). SIV undergoes constant evolution, and an intact *nef* gene is important to maintain the high viral loads required for disease progression to AIDS in SIV-infected macaques. The coupled transcription/translation system has been used as a screening tool to examine the reading frames of SIV *nef* genes for the presence of premature stop codons. The screen has been further simplified by using a colony PCR step to simultaneously screen for correct recombinants and generate the T7-tagged PCR products required for analysis. Demonstrating the reliability of this technique, a 100% correlation was found between the results from coupled transcription/translation and the sequence analysis of 47 *nef* clones. This approach is much faster, cheaper, and less labor-intensive than conventional gene sequencing techniques.

Drug Screening and Pharmacology

The use of *in vitro* systems for high-throughput drug screening (HTS) is an emerging area in which there are few publications to date. However, this is not reflective of the intense activity in this area. A number of pharmaceutical companies are currently evaluating and developing *in vitro* screening protocols; material from company press releases was used for some of the descriptions below.

Screening for pharmacological agents

The *in vitro* luciferase folding/chaperonin assay described earlier has been extended to understanding the role of heat-shock factors, such as Hsp90. It is now understood that disruption of folding pathways can result in proteolytic degradation. Several groups are currently using this information to ascertain the pharmacological activities of benzoquinone ansamycins, such as geldanamycin (Schneider et al., 1996; Thulasiraman and Matts, 1996). These potentially medically important compounds were first identified as interesting because of their ability to inhibit tyrosine kinase activity. This ability appears to be due to their interaction with Hsp90, which prevents correct folding of tyrosine kinases and is followed by their proteolytic degradation. Other potentially important drugs affecting protein folding through inhibition of chaperonin function could be identified using this approach.

High-throughput screening for translation-inhibiting compounds

Viruses contain a number of different genetic elements used for promoting viral expression at the expense of host mRNA translation. Several groups are currently developing screens using *in vitro* expression of gene constructs containing a viral element—such as the 5' UTR that can harbor an internal ribosome entry site—followed by a firefly or *Renilla* luciferase gene. Chemical or antibiotic libraries can be screened for specific translation-inhibiting effects. The viral element can be placed between the firefly and *Renilla* luciferase genes, with translation of the first gene relying on normal cap-dependent initiation. Use of the two luciferase genes allows normalization of the second reporter behind the viral element. Compound efficacy can be assessed rapidly (<30 sec) by assaying light output for both the reporter and the luciferase control. As a variation on this theme, RiboGene has reported that they are developing a high-throughput screen capable of screening several hundred thousand compounds for the ability to diminish or block the required ribosomal frameshifting that occurs during translation of the HIV *gag-pol* mRNA. This screen utilizes a reporter gene in which luciferase (and light) is produced only when the frameshift occurs.

Other Applications

A highly sensitive immunoassay, known as the ultrasensitive expression immunoassay (USEI), was developed based on a “solid-

phase” coupled transcription/translation system described by Christopoulos and Chiu (1995). A DNA fragment encoding firefly luciferase is biotinylated and complexed with streptavidin. Biotinylated specific antibodies are used to quantify antigen immobilized on microtiter wells. After completion of the immunoreaction, streptavidin-DNA complex is bound to the immunocomplex. Subsequent expression of the solid phase-bound DNA by a coupled transcription/translation reaction produces luciferase. As few as 3000 molecules of DNA label can detect a minimum of 50,000 antigen molecules. The luminescence is a linear function of the number of antigen molecules in a range extending over 3 orders of magnitude. The high sensitivity achieved results from the combined amplification due to transcription/translation and the substrate turnover. The same investigators developed a similar approach for DNA detection (Chiu and Christopoulos, 1996). A denatured target DNA is hybridized to two probes, one biotinylated and one labeled with digoxigenin. The DNA label contains the firefly luciferase coding sequence downstream from a T7 RNA polymerase promoter. The target DNA (200 bp) is denatured and hybridized simultaneously with two oligonucleotide probes. One probe is immobilized in microtiter wells, via the digoxigenin/anti-digoxigenin interaction, and the other is biotinylated. After hybridization is complete, the hybrids are reacted with a streptavidin-luciferase-DNA complex. Subsequently, the solid phase-bound DNA is expressed by coupled transcription/translation. This system too is very sensitive due to the coupled transcription/translation system, and the resulting luminescence is linearly related to target DNA levels for between 5 and 5000 amol. Because the assay is performed in microtiter wells and avoids membrane hybridization and blotting, it can be adapted to automatable HTS detection.

A sensitive nonradioactive method for detecting eukaryotic nuclear extract transcription products has been developed using *in vitro* synthesis of mRNAs encoding the firefly luciferase gene. Transcriptional activity can be assessed by using vectors containing the luciferase gene (downstream of a range of different promoters) upstream of the nuclear extract transcription reactions and using the resulting transcripts to program a rabbit reticulocyte translation system. When the translation reaction products are tested using the sensitive luciferase assay, there is a linear relationship between transcriptional activity and light gener-

ated. This approach can be used either to test the transcriptional activity of the nuclear extract with regard to specific promoters or to compare promoter strengths in different extracts (Ayoubi and Van de Ven, 1995).

THE FUTURE OF IN VITRO TRANSLATION SYSTEMS

Although the process of translation—or coupled transcription/translation—is complex, the use of convenient, rapid in vitro expression systems for many different applications is simple. The emergence of the eukaryotic coupled systems, combined with the standard molecular biology techniques of PCR and RT-PCR, has produced an explosion of new applications in such diverse fields as diagnostic detection, HTS for anti-HIV drugs, screening applications for functional genomics, and ribosomal display methods for evolving novel binding partners. For rapid generation of high levels of in vitro-translated protein, continuous-flow systems currently hold more promise than concrete utility, although their practicality appears to be improving. Development of coupled transcription/translation systems from the hyperthermophilic *Archaea* may improve CFCF methodology, since these coupled systems should be more stable and longer lasting than systems derived from mesophilic organisms. The ability to manipulate both the reaction and the end product of in vitro translation systems can be expected to generate even more useful and novel tools and applications in the future.

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Internet Resources

<http://www.promega.com/promtech/tntbib.html>

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In Vitro Translation

UNIT 11.2

This unit describes the production and use of the two most widely employed eukaryotic in vitro translation systems—those derived from rabbit reticulocytes (see Basic Protocol 1) and from wheat germ (see Basic Protocol 2). These systems are useful for the production of proteins from mRNA isolated from eukaryotic cells or tissues or from mRNAs transcribed in vitro from cloned cDNAs that contain promoters for bacteriophage RNA polymerases. Also described is a time-saving variation that combines transcription and translation in a one-tube reaction (see Basic Protocol 3).

Although in vitro translation systems were developed primarily for the identification of mRNAs and the characterization of their products, the systems have been widely used for the identification of viral genes and the verification of cloned cDNAs. More recently, the systems have found a plethora of applications, including generation of protein variants that can be assayed for function in vitro (mutagenesis combined with expression-PCR), analysis of molecular interactions, characterization of molecular function, screening for mutants in disease states (protein truncation test), and high-throughput drug screening (see UNIT 11.1). The systems can be used to translate mRNAs attached to polysomes (see Alternate Protocol 1), purified from tissues or cell lines (see APPENDIX 3), or transcribed in vitro (see Support Protocols 1 and 2). Depending on the application, the systems can be used as small or large static reactions, in continuous-flow mode, and with either nonradioactive, radioactive, or modified amino acids (see Alternate Protocol 2 and Support Protocols 3 and 4).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by the local radiation safety officer (also see APPENDIX 1D).

PRODUCTION AND USE OF mRNA-DEPENDENT CELL-FREE TRANSLATION SYSTEM FROM RABBIT RETICULOCYTES

**BASIC
PROTOCOL 1**

Experimentally induced anemia results in increased accumulation of circulating reticulocytes. Treatment of rabbits with phenylhydrazine, which oxidizes red blood cell membranes, leads to premature lysis of these cells and induction of reticulocytosis. A number of variations on the phenylhydrazine protocol given here have been used successfully. However, since the translation machinery is lost as reticulocytes mature into red blood cells, it is extremely important that the treatment program be standardized to give high reticulocyte counts. It is good practice to keep rabbits for 1 week before starting to inject them to ensure a healthy starting condition. Ideally, the rabbits should weigh 3 kg. Reticulocyte count should be monitored by drawing blood from the marginal vein of the ear and examining cells stained with Brilliant Cresyl Blue. The interval between the last injection and bleeding allows the rabbits to clear the toxic products of phenylhydrazine from the circulation and increases the hematocrit and, hence, the yield of lysate.

Processing should be conducted as quickly as possible to produce a lysate of high protein-synthetic activity. If the need for lysate is high, it is possible to process five to ten rabbits at a time, since this should not increase processing time significantly. However, it is probably prudent to become familiar with the procedure with one or two rabbits before tackling ten. If processing more than one rabbit, the blood should be pooled prior to processing. If the need for lysate is low or occasional, it is probably better to purchase whichever commercially available system is most suitable. The activity of the translation systems should be tested after preparation and compared to that of previous batches or of

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Reconstitution**

commercially available systems such as those provided by Amersham, Boehringer, Novagen, or Promega. The system described below can be used to translate mRNA purified from tissues or cells (see *APPENDIX 3*) or polysomes (see Alternate Protocol 1), or from in vitro-transcribed mRNAs, which can be uncapped (see Support Protocol 1) or capped (see Support Protocol 2) depending on the application and the choice of expression vector.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Female New Zealand white rabbits, 2 to 3 kg
2.5% (w/v) phenylhydrazine (see recipe)
100 mg/ml ketamine hydrochloride
20 mg/ml xylazine hydrochloride
500 U/ml heparin in sterile water (store at -20°C)
70% ethanol
0.4 mg/ml sodium pentobarbital
Reticulocyte wash buffer I (see recipe), with and without 1 U/ml heparin (from 500 U/ml stock)
Reticulocyte wash buffer II (see recipe)
Diethylpyrocarbonate-treated water (DEPCW; *APPENDIX 2A*), sterile
100 mM CaCl_2 (*APPENDIX 2A*)
1 mM hemin hydrochloride (see recipe)
200 U/ml creatine phosphokinase (see recipe)
15,000 U/ml *Staphylococcus aureus* nuclease S7 (see recipe; prepare fresh)
200 mM EGTA (see recipe)
5 mg/ml calf liver tRNA (see recipe)
2 M KCl/10 mM magnesium chloride (K/Mg; see recipe)
1 mM amino acids minus methionine (see recipe)
 $[^{35}\text{S}]$ Methionine, translation grade (e.g., NEN Life Sciences or Amersham)
20 to 40 U/ μl RNasin ribonuclease inhibitor (Promega) or equivalent
1 M phosphocreatine (see recipe)
Translation substrate: mRNA purified from cells or tissues (see *APPENDIX 3*) *OR* uncapped or capped in vitro transcript (see Support Protocols 1 and 2)
5% and 10% trichloroacetic acid (TCA; see recipe)
60-ml disposable syringes
1-ml syringes
60-ml spring for 60-ml syringes
No. 16 and 1.5-in.-long no. 20 Huberpoint needles (Fisher)
Liquid nitrogen
Glass-fiber filters (e.g., Whatman GFC or equivalent)
Liquid scintillation counter and scintillation fluid

Prepare reticulocytes

1. Each day for 5 days, at the same time in the late afternoon, inject immature female New Zealand white rabbits (2 to 3 kg) subcutaneously in the interscapular region with 0.25 ml/kg of 2.5% phenylhydrazine hydrochloride using no. 20 Huberpoint needles.

The weight of the rabbit is only a good guide for dosage with younger rabbits, since the drug does not equilibrate with adipose tissue.

2. Allow rabbits to recover for 2 days. Monitor reticulocyte count.

3. Early in the morning of day 8, inject rabbits intramuscularly in the thigh with 15 to 20 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride using a no. 20 Huberpoint needle.
4. Coat the inside of a spring-loaded 60-ml syringe with 0.1 ml of 500 U/ml heparin and attach to a no. 16 Huberpoint needle. When the rabbit is completely unconscious, lay it on its back and spray the thorax with 70% ethanol. With the spring attached to the syringe, push the plunger in completely and insert the needle under the sternum, pointing towards the heart.

Use of the spring-loaded syringe takes most of the skill requirement out of blood collection by heart puncture. Once inside the thoracic cavity, the spring will prevent the syringe plunger from withdrawing. When the needle punctures the heart, the pressure of blood will allow the syringe to fill unassisted, a clear indication that the heart has been reached.

The combination of ketamine hydrochloride and xylazine hydrochloride anesthetizes the rabbit and relaxes the muscles while maintaining heart rate, blood pressure, and ventricular function. It is preferable to sodium pentobarbital, which decreases blood oxygen and can result in lower translation activity in the lysates prepared from isolated reticulocytes as well as death of the rabbit before bleeding is complete. Note that ketamine/xylazine anesthesia abolishes the righting reflex, but does not affect the toe-web pinch reflex.

5. Recoat inside of syringe with 0.1 ml heparin and repeat bleeding procedure in same rabbit until no more blood can be extracted.

It should be possible to withdraw between 60 and 100 ml blood from each rabbit.

6. After bleeding, euthanize each rabbit by injecting intraperitoneally with 200 mg/kg sodium pentobarbital.

Wash reticulocytes

7. Discharge blood into ~5 vol reticulocyte wash buffer I containing 1 U/ml heparin. Collect the cells by centrifugation for 10 min at $1400 \times g$, 4°C . Remove the supernatant by gentle suction and discard.

When discharging blood from the syringe into the wash buffer, remove needle first to avoid rupturing the reticulocytes.

After centrifugation the supernatant should be clear and pale straw-colored. Any redness in the supernatant suggests that the reticulocytes have not pelleted or lysis has occurred.

8. Wash twice by resuspending in ~5 to 10 vol reticulocyte wash buffer I without added heparin and centrifuging 10 min at $1400 \times g$, 4°C , discarding the supernatant each time.

The washes are to remove serum (including clotting factors) and heparin, which is inhibitory to protein synthesis. Use maximum possible rinse volumes or at least 5 times the volume of blood collected. Reticulocyte wash buffer I contains 7.5 mM MgCl_2 to stabilize the reticulocyte cell membrane, along with 1 mM glucose to prevent the reticulocytes from becoming depleted of ATP and reducing equivalents. Some published procedures call for removal of the "buffy coat," the fluffy layer of white cells that sediment on top of the heavier reticulocytes, to prevent contamination by ribonucleases contained in the white cells. In practice, however, the white cells will not lyse under the conditions used to lyse reticulocytes, so their removal is unnecessary, and it reduces the yield of reticulocytes obtained.

9. Wash once with reticulocyte wash buffer II. Centrifuge as in step 7. Remove as much supernatant as possible to avoid carryover of salts into the lysate.

Reticulocyte wash buffer II used in the final rinse is lacking the Mg^{2+} contained in reticulocyte wash buffer I. This is to prevent carryover into the reticulocyte lysate, since protein synthesis can be inhibited by Mg^{2+} at higher concentrations.

Prepare lysate

10. Transfer the cell pellet to a sterile graduated cylinder and record the volume of packed cells. Add 1.5 vol sterile DEPCW. Stir on ice for 5 min to maximize lysis.
11. Centrifuge 20 min at $20,000 \times g$, 4°C , to remove cell membranes and mitochondria. Decant the supernatant, and snap-freeze a few aliquots as “parent lysate” to compare with the mRNA-dependent lysate (MDL). Subject the rest to endonuclease treatment.

The parent lysate is useful to provide an indication of how much incorporation to expect in a cell-free translation system working at maximum efficiency/capacity. It is also useful if the MDL is not very active and it is necessary to determine whether the problem came from the reticulocyte lysate prep itself or arose during the endonuclease treatment. In addition, the parent lysate can be used to optimize each batch for K^+ , Mg^{2+} , and hemin concentrations.

12. To each 25 ml of lysate, add:
 - 250 μl 100 mM CaCl_2
 - 500 μl 1 mM hemin hydrochloride
 - 250 μl 200 U/ml creatine phosphokinase
 - 250 μl 15,000 U/ml nuclease S7.

Incubate 15 min at 20°C .

13. Add 250 μl of 200 mM EGTA to chelate the Ca^{2+} and inactivate the nuclease. Add 250 μl calf liver tRNA (5 mg/ml). Snap-freeze 500- μl aliquots and store in liquid nitrogen.

After nuclease treatment, the lysate is referred to as mRNA-dependent lysate or MDL. This preparation should produce between 30 and 40 ml MDL per rabbit. This is sufficient for 600 to 800 50- μl assays.

Calf liver tRNA is added not because endogenous tRNA is hydrolyzed by endonuclease, but because the composition of reticulocyte tRNA is related to the amino acid composition of globin and may not always support the production of full-length proteins.

Translation systems from reticulocytes retain activity for years when stored in liquid nitrogen. At -70°C , loss of activity is discernible and the lysate can only be used for 3 to 4 months. Storage at -20°C is not recommended.

14. Test each batch of MDL produced for translational activity by performing steps 15 to 20 using a suitable test RNA (mRNA purified from cells or tissues or transcribed in vitro).

A good test transcript is that produced from Promega's pGEM-luc DNA, since a functional assay measuring luciferase activity can be used. This has the advantage of not requiring a radioactive amino acid and ensures that only full-length polypeptides are scored.

Translate mRNA

15. Remove reagents from storage and place on ice. Rapidly thaw MDL by hand warming and place on ice.
16. Assemble the following reaction components in a 0.5- to 1.5-ml sterile microcentrifuge tube (for 50 μl):

- 2.5 μl K/Mg
- 2.5 μl amino acid mixture minus methionine
- 2.5 μl [^{35}S]methionine
- 0.35 μl 20 to 40 U/ μl RNasin
- 0.5 μl 1 M phosphocreatine
- 0.5 μl 200 U/ml creatine phosphokinase

continued

0.5 μ l 1 mM hemin hydrochloride
35 μ l MDL
0.5 to 2 μ l (~0.5 μ g) mRNA substrate
Nuclease-free water to 50 μ l final.

Mix by gentle vortexing and, if necessary, microcentrifuge 5 sec to return reaction mix to bottom of tube.

The reaction volume can be modified from 10 μ l to 1 ml depending on application. Capped microcentrifuge tubes should be used to avoid changes in reaction volume.

Include a control reaction without added template to assess if any translation products are arising from undegraded endogenous mRNA. Note that the composition of the reaction described above is optimal for MDL prepared as described in the preceding steps. The preparation of commercially available MDL may vary, and the manufacturer's recommendations for use should be followed.

Increased expression levels may be obtained by optimizing Mg^{2+} and K^+ concentrations for a particular mRNA species. The mRNA used may be purified from cells or tissue or transcribed in vitro from cDNAs downstream of a phage polymerase promoter. Unfractionated cytoplasmic RNA preparations contain 90% to 95% rRNA and translate very poorly.

If synthesizing a recombinant protein such as an enzyme for functional analysis, higher protein levels can be achieved by substituting 1 mM nonradioactive methionine (2.5 μ l/50- μ l reaction) for [35 S]methionine.

17. Incubate 30 min to 1 hr at 30°C.
18. Determine amount of radioactivity incorporated into TCA-precipitable material by diluting 1 to 2.5 μ l of the reaction in 1 ml ice-cold water and adding 1 ml of 10% TCA. Heat the mixture 10 min at 95°C to discharge aminoacylated tRNAs, then cool on ice.
19. Collect precipitated protein by vacuum filtration onto glass-fiber filters. Rinse with 5% TCA, and dry filters under an infrared lamp. Measure radioactivity by liquid scintillation spectroscopy.
20. Use remaining incubation mix for assay of function or analysis by SDS-PAGE (UNIT 6.1).

PRODUCTION AND USE OF mRNA-DEPENDENT CELL-FREE TRANSLATION SYSTEM FROM WHEAT GERM

In this procedure, wheat germ extract is prepared by grinding wheat germ in an extraction buffer, then centrifuging the resulting paste to remove cell debris. Endogenous amino acids and plant pigments that are inhibitory to protein synthesis are removed by gel filtration. Although the level of endogenous mRNA is low compared to that in untreated reticulocyte lysate, the response to added mRNAs is improved by treatment with calcium-dependent micrococcal nuclease with subsequent addition of sufficient EGTA to chelate the free calcium. The most important consideration in preparing wheat germ extract of high translational activity is to find a good batch of fresh commercial wheat germ low in endosperm-rich fragments. There can be considerable variability in the quality of wheat germ from one manufacturer and the activity of the resulting cell-free translation system, depending on growth conditions and conditions at harvest.

The wheat germ system can be used with all the alternative methods described for use of the reticulocyte system (see Support Protocols 1 and 2; see Alternate Protocols 1 and 2). It can also be used as a coupled transcription/translation system (see Basic Protocol 3).

**BASIC
PROTOCOL 2**

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11.2.5

Materials

Wheat germ
Wheat germ extraction buffer (see recipe; prepare fresh)
100-ml Sephadex G-25 column (Amersham Pharmacia Biotech) preequilibrated in extraction buffer
100 mM CaCl₂ (APPENDIX 2A)
1 mM hemin hydrochloride (see recipe)
200 U/ml creatine phosphokinase (see recipe)
15,000 U/ml nuclease S7 (see recipe; prepare fresh)
200 mM EGTA (see recipe)
5 mg/ml calf liver tRNA (see recipe)
50 mM ATP (see recipe for 4NTP mixture)
50 mM GTP (see recipe for 4NTP mixture)
50 mM and 1 M magnesium acetate
20 to 40 U/μl RNasin ribonuclease inhibitor (Promega) or equivalent
1 mM amino acids minus methionine (see recipe)
1 M potassium acetate
1 M phosphocreatine (see recipe)
10 mCi/ml [³⁵S]methionine (1000 Ci/mmol)
Translation substrate: mRNA purified from cells or tissues (see APPENDIX 3) or uncapped or capped in vitro transcript (see Support Protocols 1 and 2)
Diethylpyrocarbonate-treated water (DEPCW; APPENDIX 2A)
Sterile, RNase-free glass powder (see recipe)

Prepare wheat germ cell-free translation system

1. Working at 4°C with all components and apparatus prechilled, grind 5 g wheat germ with 5 g sterile glass powder and 22.5 ml wheat germ extraction buffer to give a smooth paste. Centrifuge for 10 min at 30,000 × g, 4°C.
2. Remove top 20 ml of supernatant and load onto a 100-ml Sephadex G-25 column preequilibrated in extraction buffer. Use ~100 ml of the same buffer for elution.

A low-molecular-weight inhibitor of protein synthesis and a deep yellow pigment are removed by the column.

3. As soon as all the extract has penetrated into the column, follow with 100 ml of extraction buffer. Collect 2.5-ml fractions and measure A_{260} by spectrophotometry (see APPENDIX 3). Pool the highest-absorbance fractions (which are also the most opaque fractions), usually fractions 20 to 30.
4. Let pooled fractions stand 5 min on ice, then centrifuge 10 min at 23,000 × g, 0°C. Carefully remove upper 20 ml.

A high-molecular-weight inhibitor of protein synthesis aggregates as the column fractions incubate on ice. This will disperse if temperature rises above 4° or 5°C.

5. Add the following per 20 ml of lysate:

200 μl 100 mM CaCl₂
400 μl 1 mM hemin hydrochloride
200 μl 200 U/ml creatine phosphokinase
200 μl 15,000 U/ml nuclease S7.

Incubate 15 min at 20°C.

6. Add 200 μl of 200 mM EGTA per 20 ml (to chelate Ca²⁺ and inactivate the nuclease).

7. Add the following per 20 ml of lysate:

- 200 μ l 5 mg/ml calf liver tRNA
- 625 μ l 50 mM ATP
- 125 μ l 50 mM GTP
- 37.5 μ l 1 M magnesium acetate.

Mix, then snap-freeze in 200- to 500- μ l aliquots and store in liquid nitrogen.

8. Test each batch of wheat germ extract produced for translational activity prior to use (see Basic Protocol 1, step 14).

Translate mRNA

9. Remove reagents from storage and place on ice. Rapidly thaw wheat germ extract by hand warming and place on ice.

10. Assemble the following reaction components in a 0.5- to 1.5-ml sterile microcentrifuge tube (50 μ l):

- 25 μ l wheat germ extract
- 1 μ l 20 to 40 U/ μ l RNasin
- 4 μ l amino acid mix minus methionine (or other amino acid, as appropriate)
- 3.75 μ l 1 M potassium acetate
- 1 μ l 50 mM magnesium acetate
- 2.5 μ l 10 mCi/ml [³⁵S]methionine (1000 Ci/mmol)
- 0.5 to 2 μ l mRNA substrate (to give ~0.5 to 1 μ g)
- DEPCW to 50 μ l.

Mix by gentle vortexing and, if necessary, microcentrifuge 5 sec to collect the reaction mix in the bottom of the tube.

Include a reaction without added template to measure any background incorporation from endogenous mRNA. Note that the composition of the reaction described above is optimal for wheat germ extract prepared as described above. Preparation of commercially available wheat germ extract may vary, and the manufacturer's recommendations for use should be followed.

Increased expression levels may be obtained by optimizing Mg²⁺ and K⁺ concentrations for a particular mRNA species. As with MDL, the mRNA used may be purified from cells or tissue (see APPENDIX 3) or transcribed in vitro from cDNAs downstream of a phage polymerase promoter (see Support Protocols 1 or 2).

11. Incubate 1 to 2 hr at 25°C.

12. Use 1.0 to 2.5 μ l of reaction mixture for determining radioactivity incorporated into TCA-precipitable material (see Basic Protocol 1, steps 18 and 19). Use remainder for assay of function or analyze by SDS-PAGE (UNIT 6.1).

IN VITRO PROTEIN SYNTHESIS IN COUPLED TRANSCRIPTION/TRANSLATION SYSTEMS

Transcription of cDNA clones by bacteriophage RNA polymerases may be carried out simultaneously with translation in a single reaction by adding DNA directly to the translation system along with the appropriate RNA polymerase (T3, T7, or SP6). This adaptation obviates the need for three prior reactions: (1) linearization of DNA, (2) in vitro transcription, and (3) 5' capping and methylation of mRNA. Optimization of the conditions for the combined reactions are closer to those required for in vitro translation than transcription. Under the conditions outlined, transcription is relatively inefficient, but sufficient levels of transcripts are produced to saturate the protein synthetic capacity

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PROTOCOL 3**

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11.2.7

of the system. The reaction buffer is modified to provide the NTPs and additional Mg^{2+} , with the Mg^{2+} concentration being the critical parameter.

Materials

2 M KCl/10 mM $MgCl_2$ (K/Mg; see recipe)
Amino acid mixture minus methionine (see recipe)
25× transcription/translation buffer (TX/TL buffer; see recipe)
10 mCi/ml [^{35}S]methionine (1000 Ci/mmol)
20 to 40 U/ μ l RNasin ribonuclease inhibitor (Promega) or equivalent
1 M phosphocreatine (see recipe)
200 U/ml creatine phosphokinase (see recipe)
1 mM hemin hydrochloride (see recipe)
mRNA-dependent lysate (MDL; see Basic Protocol 1, step 14, or see Basic Protocol 2, step 8)
Plasmid DNA, at ≥ 0.1 mg/ml (*APPENDIX 3*)
Appropriate bacteriophage RNA polymerase (e.g., T3, T7, or SP6)
Diethylpyrocarbonate-treated water (DEPCW; *APPENDIX 2A*)

1. Remove reagents from storage and place on ice. Rapidly thaw MDL by hand warming and place on ice.
2. Assemble the following reaction components in a 0.5- to 1.5-ml sterile microcentrifuge tube (50 μ l):
 - 2.5 μ l K/Mg
 - 2.5 μ l amino acid mixture minus methionine
 - 2.0 μ l 25× TX/TL buffer
 - 2.5 μ l 10 mCi/ml (1000 Ci/mmol) [^{35}S]methionine
 - 0.35 μ l 20 to 40 U/ μ l RNasin
 - 0.5 μ l 1 M phosphocreatine
 - 0.5 μ l 200 U/ml creatine phosphokinase
 - 1 μ l 1 mM hemin hydrochloride
 - 25 μ l MDL
 - 0.5 to 2 μ l plasmid DNA (to give ~0.5 μ g)
 - RNA polymerase to give 600 to 1000 U/ml
 - DEPCW to 50 μ l.

Mix by gentle vortexing, and if necessary microcentrifuge 5 sec to return reaction mixture to bottom of tube.

Note that the amino acid mix minus methionine and [^{35}S]methionine can be replaced by complete amino acid mix and biotinylated lysine-tRNA. Alternatively, if synthesizing a recombinant protein such as an enzyme for functional analysis, higher protein levels can be achieved by substituting 1 mM nonradioactive methionine for [^{35}S]methionine (2.5 μ l/50- μ l reaction).

Ensure that all ethanol is removed from DNA before adding to the reaction. The smaller the volume of DNA added to the reaction, the less likely it will be that a nonspecific inhibitor of protein synthesis will be introduced. Include a control incubation to which no DNA has been added. The reaction volume can be modified from 10 μ l to 1 ml depending on application. Capped microcentrifuge tubes should be used to avoid changes in reaction volume.

3. Incubate 1 hr at 30°C.
4. Analyze as for the noncoupled system (see Basic Protocol 1, steps 18 to 20).

Best results are obtained with DNA from which all contaminating RNAs are removed by a method that does not involve RNase. However, miniprep DNA prepared by a variety of methods (see APPENDIX 3) has proved to be quite adequate. Optimal production of proteins appears to be obtained with ~1 µg plasmid DNA/100 µl reaction volume, although this varies somewhat with the purification method. However, adequate levels of product may be obtained with 0.1 µg plasmid DNA/100 µl reaction. Circular plasmid DNA gives the best results although linearized versions of most plasmids can be translated. Best results will be obtained from constructs containing the EMCV 5' UTR and a 3' poly(A)⁺ sequence.

PRODUCTION OF UNCAPPED IN VITRO TRANSCRIPTS

Transcripts produced in vitro can be used in place of purified mRNA in either the reticulocyte or wheat germ translation systems. To produce high levels of uncapped mRNA, transcription reactions containing 4 mM of each nucleotide triphosphate (NTP) are used since the production of mRNA is proportional to the amount of NTP in the reaction up to this concentration (Milligan et al., 1987). Yields are also increased by the inclusion of 0.01% Triton X-100 and BSA (Milligan et al., 1987). The transcript yield is measured by the inclusion of trace amounts of radiolabeled NTP (usually [α -³²P]CTP). The percentage of [α -³²P]CTP incorporated is measured, and the total amount of CTP is calculated based on the ratio of labeled to unlabeled nucleotide. From that the amount of RNA synthesized is determined. For translation of uncapped transcripts, the efficiency of translation will be increased by using the cDNA of interest in a vector that places an internal ribosome entry site (IRES) upstream of the initiation codon to generate a mRNA that is processed via cap-independent translation. Suitable vectors for achieving this include pCITE from Novagen and the commercially unavailable pTM1 (Moss et al., 1990).

The transcripts produced by in vitro transcription can be used in place of purified mRNA in any of the basic protocols. The integrity of the RNA should be verified by agarose gel electrophoresis prior to use. Because high concentrations of RNA are produced, sufficient RNA to saturate the translation system can be added in such a small volume that prior cleanup is not necessary, as long as the free Mg²⁺ is titrated out after completion of the transcription reaction.

Materials

- Diethylpyrocarbonate-treated water (DEPCW; APPENDIX 2A), sterile
- 5× modified transcription buffer (MTB; see recipe)
- 12.5 mM 4NTP mix (see recipe)
- 1 M MgCl₂ (APPENDIX 2A)
- 250 mM DTT (see recipe)
- [α -³²P]CTP (3000 Ci/mmol) in tricine, diluted in DEPCW to 10,000 to 50,000 cpm/µl
- 20 to 40 U/µl RNasin ribonuclease inhibitor (Promega) or equivalent
- Plasmid DNA or PCR product containing appropriate phage polymerase promoter (see APPENDIX 3), linearized using a restriction enzyme that generates 5' protruding ends, at 0.2 to 0.5 mg/ml concentration
- 20 U/µl RNA polymerase (SP6, T3, or T7; Promega)
- 0.5 mg/ml yeast tRNA (carrier for TCA precipitation)
- 5% trichloroacetic acid (TCA; see recipe)
- 0.5 M EDTA (APPENDIX 2A)
- RQ1 RNase-free DNase (Promega)
- TE buffer, pH 8 (APPENDIX 2A)
- 1:1 (v/v) TE-saturated phenol/chloroform
- 24:1 (v/v) chloroform/isoamyl alcohol

SUPPORT PROTOCOL 1

In Vitro Reconstitution

11.2.9

7.5 M ammonium acetate
100% and 70% ethanol
Dry ice/ethanol bath
Glass-fiber filters (e.g., Whatman GFC or equivalent)
Liquid scintillation counter

Produce uncapped transcripts

1. Remove reagents from storage and place on ice.
2. Assemble the following reaction components, in the order listed, in a 1.5-ml sterile screw-cap microcentrifuge tube at room temperature (50 μ l total):

14.7 μ l DEPCW
10 μ l 5 \times MTB
16 μ l 12.5 mM 4NTP mix
0.8 μ l 1 M MgCl₂
2 μ l 250 mM DTT
2.5 μ l 20 to 40 U/ μ l RNasin
1 μ l 10,000 to 50,000 cpm/ μ l [α -³²P]CTP (200 to 1000 cpm/ μ l final)
1 μ l 0.2 to 0.5 mg/ml linearized DNA
2 μ l 20 U/ μ l appropriate RNA polymerase (800 U/ml final).

Mix by gentle vortexing and microcentrifuge 5 sec to collect the reaction mix in the bottom of tube.

Care should be taken in the choice of restriction enzyme for linearizing the DNA, since templates containing 3' protruding ends allow the transcription of RNA from these sites. Enzymes such as BamHI and NcoI, which produce 5' protruding ends, are good choices, whereas those such as Apa I, Sac I, and PstI that generate 3' protruding ends should be avoided.

3. Place 1- μ l spots of the mixture onto two glass-fiber filters and allow filters to dry; meanwhile, incubate remainder of reaction mixture 30 min to 1 hr at 37°C.
4. After the reaction incubation is over, pipet duplicate 1- μ l samples of the transcription mixture into 100 μ l of 0.5 mg/ml yeast carrier tRNA and add 2 ml of 5% TCA on ice. Collect precipitate on glass-fiber filters by vacuum filtration and allow to dry.
5. Determine total radioactivity (total cpm/ μ l) of pre- and postincubation samples on filters by liquid scintillation spectroscopy.

Dividing the value for the second sample by that for the first gives the percentage of [α -³²P]CTP incorporated into the transcript. These reactions give between 25% and 30% incorporation of [α -³²P]CTP, which means that 50 to 60 nmol of CTP and ~200 to 240 nmol of RNA (depending on coding sequence) are made. Based on 0.33 μ g/nmol, between 70 and 80 μ g RNA is produced in a 50- μ l reaction, giving enough RNA for ~50 50- μ l translation assays (assuming some losses during cleanup). Once this has been determined for a particular DNA preparation, scale up the reaction 10-fold to give a greater supply of transcript for use in cell-free translation reactions.

6. Add EDTA to 10 mM final (from 0.5 M stock) to chelate free Mg²⁺, and store at -20°C (good for several months).

The reaction mix can be used directly in in vitro translation reactions without further processing. When using frozen reaction mix, warm briefly to 37°C prior to direct use in translation reactions (at 0.5 to 1 μ l per 50- μ l reaction). Alternatively, the transcripts can be purified from the reaction mix as follows.

Because high concentrations of RNA are produced, sufficient RNA to saturate the translation system can be added in such a small volume that prior cleanup is not necessary, as long as the free Mg²⁺ is titrated out after completion of the transcription reaction.

Optional: Purify transcripts by phenol/chloroform extraction and ethanol precipitation

7. Add 1 U RQ1 DNase/ μg template DNA and incubate 15 min at 37°C.
8. Add 200 μl TE buffer to the reaction mixture, then extract with 250 μl of 1:1 (v/v) TE-saturated phenol/chloroform. Vortex 1 min, then microcentrifuge 5 min at maximum speed, 4°C.
9. Transfer the upper (aqueous) phase to a fresh tube and repeat extraction with 250 μl of 24:1 (v/v) chloroform/isoamyl alcohol.
10. To the final aqueous phase, add 0.5 vol of 7.5 M ammonium acetate and 2.5 vol of 100% ethanol. Place mixture in dry ice/ethanol bath for 10 min. Microcentrifuge 5 min at maximum speed, 4°C.
11. Aspirate supernatant and wash pellet with 1 ml of 70% ethanol. Dry under vacuum and resuspend in a volume of DEPCW that will give ~1 mg/ml transcript. Store at -85°C.

Dilution of the transcription reaction is only necessary to avoid large losses when dealing with small volumes. For scaled-up reactions, this dilution is not necessary.

Equivalent or higher levels of uncapped transcripts can be produced using kits from Ambion, Novagen, or Promega.

It is possible that some double-stranded RNA (dsRNA) may be produced during in vitro transcription reactions from promiscuous binding of the polymerase. This will not be removed by the usual cleanup procedures, but it can be kept to a minimum provided the DNA is not linearized with restriction enzymes that generate 3' protruding ends. If significant dsRNA is produced in the reaction, this will lead to activation of endogenous PKR, the dsRNA-activated kinase that phosphorylates and downregulates the translation factor eIF2. Higher levels of translation products may be generated by including additives that prevent PKR activation or eIF2 α phosphorylation. These include 2 to 10 mM 2-aminopurine, high levels (10 $\mu\text{g}/\text{ml}$) of dsRNA such as poly(I)·poly(C), or 100 to 200 pmol/ml of the vaccinia virus K3L gene product, the pseudosubstrate of eIF2 α .

PRODUCTION OF CAPPED IN VITRO TRANSCRIPTS

The production of capped transcripts is more expensive and more challenging than the production of uncapped transcripts and may not be necessary for the translation of many RNAs. Because of the presence of a cap analog as well as the lower concentration of transcripts generated, the reaction mix cannot be used directly in cell-free translation systems but must be purified prior to use.

Additional Materials (also see Support Protocol 1)

- 5 \times transcription buffer (TB; see recipe)
- 10 mM each ATP, CTP, UTP, and GTP (see recipe for 4NTP mixture)
- 10 mM $^7\text{m}(5')\text{Gppp}(5')\text{G}$ (cap analog; Amersham, Pharmacia Biotech)

1. Remove reagents from storage and place on ice.
2. Assemble the following reaction components, in the order listed, in a 1.5-ml sterile screw-cap microcentrifuge tube at room temperature (50 μl total):

- 13.375 μl DEPCW
- 10 μl 5 \times TB
- 5 μl each 10 mM ATP, CTP, and UTP
- 0.625 μl 10 mM GTP
- 2.5 μl 10 mM $^7\text{m}(5')\text{Gppp}(5')\text{G}$

continued

SUPPORT PROTOCOL 2

In Vitro Reconstitution

11.2.11

- 1 μ l 10,000 to 50,000 cpm/ μ l [α -³²P]CTP (200 to 1000 cpm/ μ l final)
- 2 μ l 250 mM DTT
- 2.5 μ l 20 to 40 U/ μ l RNasin
- 1 μ l 0.2 to 0.5 mg/ml linearized plasmid DNA
- 1 μ l 20 U/ μ l appropriate RNA polymerase (400 U/ml final).

Mix by gentle vortexing and microcentrifuge 5 sec to collect reaction mixture in bottom of tube.

The cap analog is present at a higher concentration than GTP, which is rate limiting for transcription. This ensures that the majority of transcripts are initiated with the cap analog. The cap analog is not used internally to any significant extent.

3. Place 1- μ l spots of the mixture onto two Whatman GFC filters and allow filters to dry; meanwhile, incubate reaction mixture 45 min at 37°C.
4. After the 45-min incubation, pipet duplicate 1- μ l samples of the reaction mixture into 100 μ l of 0.5 mg/ml yeast carrier tRNA and add 2 ml of 5% TCA on ice. Collect precipitate on glass-fiber filters by vacuum filtration and allow to dry.
5. Determine total radioactivity (cpm/ μ l) of the pre- and postincubation samples on filters by liquid scintillation spectroscopy.

Dividing the value for the second sample by that for the first gives the percent of [α -³²P]CTP incorporated into the transcript.

6. Add another 0.625 μ l of 10 mM GTP and 1 μ l of 20 U/ μ l RNA polymerase. Continue incubation for 45 min, then determine percentage of [α -³²P]CTP incorporated as in step 5.

As the reaction proceeds, the GTP concentration falls, although the concentration of m⁷(5')Gppp(5')G does not decrease significantly. The late addition of more GTP allows more synthesis to occur without affecting the use of the cap analog for initiation. These conditions allow 18% to 22% incorporation of CTP, which is equivalent to 9 to 11 nmol CTP, 36 to 44 nmol RNA, and 12 to 15 μ g RNA. Between 75% and 90% of these transcripts are capped. This gives enough RNA for ~10 50- μ l translation assays (assuming some losses during cleanup).

7. Stop reaction with RQ1 DNase and purify by phenol extraction and ethanol precipitation (see Support Protocol 1, steps 7 to 11).

ALTERNATE PROTOCOL 1

TRANSLATION OF POLYSOMAL mRNAs FROM CELLS AND TISSUES

Translation of polysome-bound mRNAs in vitro provides a means to assess in vivo recruitment of mRNAs. Polysomes or subfractions of polysomes either in a free or membrane-associated state have long been a source of mRNAs that can be translated in vitro with the aid of rabbit reticulocyte lysate or other cell-free fractions. The translation of polysomal mRNAs allows a finer assessment of changes in gene expression than can be determined by studying the changes in mRNA steady-state levels or transcriptional activity.

The method described here involves gentle lysis of the cells with the nonionic detergent Nonidet P-40 (NP-40) and isolation of polysomes by ultracentrifugation. The polysome fraction contains ribosomes, mRNA, and associated proteins, but translation is suspended in the elongation process. Translation can resume in vitro in a translation system that provides all other requirements. In vitro translation of the polysome-bound mRNA fractions in the presence of [³⁵S]methionine allows quick assessment of what mRNAs are being utilized in the cell by a variety of methods using a radioactive amino acid (e.g.,

methionine) or biotinylated lysine-tRNA. The translation products arise not just from translation runoff of polysomes, but also by reinitiation. Sufficient product can be produced to allow identification of the translation products by immunological techniques or even sequencing.

Materials

- 10× cell lysis buffer (see recipe; prepare working solution fresh before use)
- 1 M 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF)
- 8 mg/ml aprotinin
- 2 mg/ml leupeptin
- 250 mM DTT (see recipe)
- 20 to 40 U/μl RNasin ribonuclease inhibitor (Promega) or equivalent
- 0.5 M EDTA (*APPENDIX 2A*)
- 10% Nonidet P-40 (NP-40)
- Diethylpyrocarbonate-treated water (DEPCW; *APPENDIX 2A*), sterile
- 10 mg/ml cycloheximide
- Cells from which the polyribosomes are to be isolated
- PBS (*APPENDIX 2A*), ice-cold liquid *and* frozen to a slurry
- Sucrose buffer (see recipe; prepare fresh)
- HEPES/KCl buffer (see recipe)
- Beckman polyallomer thick-walled tubes
- Beckman TL100 centrifuge and TLA100.3 rotor
- Additional reagents and equipment for in vitro translation (see Basic Protocols 1, 2, and 3)

Prepare lysis buffer and cells

1. Prepare two batches of complete lysis buffer by adding the following to 100 μl of 10× cell lysis buffer on ice:

- 1 μl 1 M AEBSF
- 1 μl 8 mg/ml aprotinin
- 1 μl 2 mg/ml leupeptin
- 4 μl 250 mM DTT
- 10 μl 20 to 40 U/μl RNasin
- 2 μl 0.5 M EDTA.

Add 40 μl of 10% NP-40 to one tube. Adjust final volume in each tube to 1 ml with DEPCW. Keep tubes on ice.

2. Add 1 μl of 10 mg/ml cycloheximide per milliliter of cell medium to be used. Incubate 5 min at 37°C.

Incubation with cycloheximide prior to cell harvest piles up mRNAs in polysomes, increasing the yield of polysome-bound mRNA and increasing the translational activity. The cycloheximide is washed away during sedimentation of the polyribosomes and so does not prevent translation of the polysome-bound mRNAs in a cell-free translation system.

Lyse cells

For cells in suspension:

- 3a. To $\sim 1 \times 10^8$ cells and add an equal volume of a frozen slurry of PBS to reduce temperature rapidly. Centrifuge 7 min at $1200 \times g$, 4°C.
- 4a. Aspirate supernatant carefully. Resuspend cells in 10 ml ice-cold PBS and transfer to a sterile 15-ml conical centrifuge tube. Centrifuge as in step 3a, and discard supernatant.

- 5a. Resuspend cells in 1 ml PBS and transfer to 1.5-ml microcentrifuge tube. Microcentrifuge 10 sec at 4°C. Aspirate supernatant and discard.
- 6a. Resuspend pellet in 0.5 ml complete lysis buffer without NP-40 (from step 1); vortex very gently. Add 0.5 ml complete lysis buffer with 0.4% NP-40 to cells and mix by pipetting up and down. Incubate on ice for a maximum of 10 min to allow cells to lyse.

For cells grown in monolayers:

- 3b. Remove medium from 10 × 100-mm plates (~1 × 10⁸ cells) and place on metal blocks sitting in an ice bath.
- 4b. Rinse twice with ice-cold PBS, tilting plates after final rinse to ensure complete removal of PBS.
- 5b. Add 500 µl complete lysis buffer with NP-40. Incubate 5 min on ice to allow cells to lyse. Incubate on ice for a further 5 min.
- 6b. Scrape lysate into appropriate-sized microcentrifuge tubes.

The number of cells needed and exact lysis conditions may need to be determined for each cell type or cell line used. Since mRNAs remain in a cytoplasmic fraction, they may be contaminated with RNase, although association with polysomes seems to afford protection from degradation.

Isolate polysomes

7. Microcentrifuge 10 min at maximum speed, 4°C.
8. Layer supernatant over a 400-µl cushion of sucrose buffer in a Beckman polyallomer thick-wall tube. Centrifuge 50 min at 125,000 × g (80,000 rpm in Beckman TL100 centrifuge with TLA100.3 rotor), 4°C.

Use of a sucrose pellet traps any contaminating cell membranes that may carry ribonuclease or protease activity.

9. Aspirate supernatant and discard. Invert pellet on a laboratory wipe to remove last traces of supernatant.

The remaining polysomal pellet should be clear and slightly straw-colored.

10. Snap-freeze immediately on dry ice. Store in liquid nitrogen (good for use 1 to 2 years).

Translate polysomal bound mRNA

11. Resuspend polyribosomal pellet in 50 µl HEPES/KCl buffer.

The volume in which the polysomes are resuspended may need to be modified depending on polysome yield.

12. For a 50-µl translation reaction, combine components as described in Basic Protocol 1, step 16, or Basic Protocol 2, step 10, substituting the resuspended polysomes (1 to 4 µl) for mRNA. Adjust the final volume appropriately and incubate 1 hr at 30°C.

Alternatively, to allow capture of the newly synthesized proteins, include 1 to 2 µl biotinylated lysine-tRNA in the reaction mix. Similarly, if biotinylated lysine-tRNA is used, 1 mM methionine may be substituted for [³⁵S]methionine to give nonradioactive products. This decision should reflect anticipated use and detection method. This reaction can be scaled up (e.g., to 1 ml) to allow the isolation of sufficient biotinylated translation product for sequencing. For scaleup, optimization of the nonradioactive lysine concentration is important so that lysine is not limiting but the majority of polypeptide chains still contain biotinylated amino acids.

13. Analyze by SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3) or by immunoblotting (UNIT 6.2), as appropriate.

IN VITRO TRANSLATION WITH BIOTINYLATED AMINO ACIDS

Biotinylated lysine residues can be used for detection of proteins synthesized in cell-free translation systems, obviating the need for [³⁵S]methionine or other radioactive amino acids. These residues are provided as precharged biotinylated lysine-tRNA complexes in which the biotin moiety is linked to lysine by a spacer arm at the ε-amino group. Biotinylated lysine-tRNA is available commercially as Transcend tRNA (Promega) and the Biotin-Lysine-tRNA Set (Boehringer Mannheim).

The modified amino acid is added to the in vitro translation reactions along with the amino acid mixture, which may or may not contain unmodified lysine depending upon the amount of incorporation of biotinylated lysine desired. The protein products are subjected to SDS-PAGE (UNIT 6.1) and electroblotted for immunoblot analysis (UNIT 6.2). The proteins are detected by binding either streptavidin-alkaline phosphatase (streptavidin-AP) or streptavidin-horseradish peroxidase (streptavidin-HRP) conjugate, followed by colorimetric or chemiluminescent detection, respectively. The sensitivity is equivalent to that achieved with [³⁵S]methionine incorporation and autoradiographic detection, with the advantage that no radioisotope handling, storage, or disposal is required. Furthermore, lysine is more highly represented in proteins than methionine (average lysine content is 6.6% compared to 1.7% for methionine).

The biotin tag is stable for at least 12 months as lysine-tRNA and within synthesized proteins. In addition, the use of biotinylated lysine incorporation can open up some new applications, since the protein product can be captured with streptavidin affinity chromatography, using either immobilized streptavidin-agarose columns (see Support Protocol 3) or streptavidin-linked magnetic beads (see Support Protocol 4).

Additional Materials (also see Basic Protocols 1 and 2)

mRNA-dependent lysate (see Basic Protocol 1, step 14, or see Basic Protocol 2, step 8)

Biotinylated lysine-tRNA complex (Promega's Transcend tRNA, or Boehringer Mannheim's Biotin-Lysine-tRNA Set) at 0.5 μg/μl (12 pmol/μl)

Amino acid mix, complete (see recipe)

Streptavidin-alkaline phosphatase (streptavidin-AP) or streptavidin-horseradish peroxidase (streptavidin-HRP)

Optional: Streptavidin-linked magnetic beads (Dynal) or capture resin such as Streptavidin MagneSphere Paramagnetic Particles or Softlink Soft Release Avidin Resin (Promega), Dynabeads M-280 Streptavidin (Dynal), or Streptavidin Magnetic Particles, Streptavidin, immobilized (gel suspension; Boehringer Mannheim)

Additional reagents and equipment for production and use of reticulocyte lysate (see Basic Protocol 1) or wheat germ lysate (see Basic Protocol 2) for in vitro translation, SDS-PAGE (UNIT 6.1), and immunoblotting (UNIT 6.2)

Translate biotinylated proteins

1. Remove reagents from storage and place on ice. Rapidly thaw MDL by hand warming and place on ice.
2. Combine components for a 50-μl translation reaction (see Basic Protocol 1, step 16, or see Basic Protocol 2, step 10), substituting 2 μl of 0.5 μg/μl biotinylated lysine tRNA (1 μg; 24 pmol) for [³⁵S]methionine and complete amino acid mix for amino acids minus methionine. Adjust final volume appropriately.

Complete amino acid mix is used rather than amino acids minus lysine because lysine represents a significant percentage of amino acids; thus, since the reticulocyte contains only ~7 to 10 μM lysine, without lysine supplementation the amount of protein produced will be reduced. Alternatively, it is possible to increase the incorporation of biotinylated lysine by using amino acid mixtures minus lysine, but the total yield of protein may be less.

Biotin labeling of proteins containing few lysines can be increased by doubling the amount of biotinylated lysine-tRNA added, increasing the specific activity of the biotinylated form.

3. Incubate reaction mixture 1 hr at 30°C.

The translation reaction mix can be stored for several months at –20° to –85°C prior to analysis. Optionally, detection can be enhanced by capturing the biotinylated protein from a larger volume of translation reaction (see Support Protocols 3 and 4) prior to performing the analysis outlined in steps 4 and 5.

Reticulocyte lysate contains one minor streptavidin-binding protein that migrates at 100 kDa; some batches also contain an additional streptavidin-binding protein of ~45 kDa. Comparison with the no-template control will distinguish the endogenous streptavidin-binding protein(s) from the expressed product.

4. Analyze reaction mixture by SDS-PAGE (UNIT 6.1) and transfer to PVDF membrane (UNIT 6.2).
5. Detect with streptavidin-AP or streptavidin-HRP using either chemiluminescent or colorimetric substrates (UNIT 6.2).

SUPPORT PROTOCOL 3

CAPTURE OF BIOTINYLATED PROTEINS WITH STREPTAVIDIN-AGAROSE

After in vitro translation, the biotinylated proteins may be captured from the translation reaction using streptavidin immobilized on agarose. This technique can be used to increase the sensitivity of detection methods, and provides a quick and easy way to isolate translation products for a number of downstream applications.

Materials

Translation reaction mixture (see Alternate Protocol 2, step 3)
Softlink Soft Release Avidin Resin (Promega), washed with 5 vol TBS at 40°C
TBS (APPENDIX 2A)
2× SDS-PAGE sample buffer (see recipe)

1. Add 10 µl of translation reaction mixture to 20 µl TBS. Incubate 5 to 10 min at 30°C.
2. Add 40 µl of Softlink Soft Release Avidin Resin, prewashed with TBS, to the reaction mix and incubate for 1 hr at 4°C with periodic stirring.
3. Briefly microcentrifuge, or let stand to allow resin to settle.
4. Wash the pellet three to five times with TBS.
5. Elute the bound proteins from the resin by adding 20 to 50 µl of 2× SDS-PAGE sample buffer and heating 5 min at 95°C to release biotinylated protein from beads.

The protein is now ready for analysis (see Alternate Protocol 2, steps 4 and 5).

SUPPORT PROTOCOL 4

CAPTURE OF BIOTINYLATED PROTEINS WITH MAGNETIC BEADS

Another, rapid method of isolating biotinylated proteins from the other reticulocyte proteins in the translation mix is the use of streptavidin-linked magnetic beads. These beads consist of small metallic particles coated with streptavidin. The biotinylated protein binds the streptavidin, and the beads are removed and recovered by exposing them to a magnetic field. After elution, the protein of interest is relatively pure and may be analyzed by immunodetection, SDS-PAGE, or other methods.

Materials

Translation reaction mixture (see Alternate Protocol 2, step 3)
2× biotin/streptavidin binding buffer (2× BSBB; see recipe)
Streptavidin-linked magnetic beads (e.g., Dynabeads M-280 Streptavidin, Dynal; or MagneSphere Paramagnetic Particles, Promega)

10 mM Tris-Cl, pH 8.0 (*APPENDIX 2A*)
2× SDS-PAGE sample buffer (see recipe)
Magnetic tube holder

1. Add an equal volume of 2× BSBB to translation reaction mixture in a microcentrifuge tube. Bring the total volume up to 250 μl with 1× BSBB. Add 10 to 50 μl of streptavidin-linked magnetic beads equilibrated in BSBB.
2. Incubate with rotation 30 min at 4°C.
3. Place microcentrifuge tube in magnetic holder for 2 min to capture the magnetic beads.
4. Carefully aspirate supernatant away from magnetic beads bound to side of tube.
5. Wash magnetic beads twice with 500 μl of 1× BSBB and once with 10 mM Tris-Cl, pH 8.0, using magnetic holder to separate magnetic beads from wash buffer. Remove supernatants. Resuspend magnetic beads between washes by gentle vortexing.
6. After removal of final supernatant, add 50 μl of 2× SDS-PAGE sample buffer.
7. Incubate 5 min at 95°C to release biotinylated proteins from beads.

The protein is now ready for analysis (see Alternate Protocol 2, steps 4 and 5).

Other methods of capturing biotinylated proteins using streptavidin-coated magnetic beads are also possible; consult the section on Biomagnetic Techniques in Molecular Biology in the Dynal Technical Handbook, 2nd ed. (Dynal, 1995)

REAGENTS AND SOLUTIONS

*Use deionized, Milli-Q-purified water in all recipes and solutions except for transcription buffers and reagents for transcription reactions, which should be made with diethylpyrocarbonate-treated Milli-Q-purified water (DEPCW). Storage time for solutions kept at 4°C apply only if sterility is maintained. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.*

Amino acid mixes

<i>Amino acid:</i>	<i>Quantity:</i>
Alanine	13.35 mg
Arginine	31.5 mg
Asparagine	22.5 mg
Aspartic acid	20 mg
Cysteine	26.25 mg
Glutamine	22 mg
Glutamic acid	22 mg
Glycine	11.25 mg
Histidine	31.35 mg
Isoleucine	19.5 mg
Leucine	39 mg
Lysine	27.3 mg
Methionine	15 mg
Phenylalanine	24.75 mg
Proline	17.25 mg
Serine	15.75 mg
Threonine	17.85 mg
Tryptophan	30.6 mg
Tyrosine	27 mg
Valine	33 mg

Add water to 80 ml. Adjust pH to 7.2 with 1 N KOH, then add water to 100 ml. Filter sterilize and store in 1-ml aliquots up to 2 years at -20°C.

continued

**In Vitro
Reconstitution**

11.2.17

This yields 100 ml of a stock solution containing 3 mM leucine and valine, 1 mM methionine, and 1.5 mM each of the other 18 amino acids.

For amino acids minus valine, leucine, lysine, or methionine, prepare as described except omit the appropriate amino acid.

Biotin/streptavidin binding buffer (BSBB), 2× (10 ml)

Components:	Concentration of 2× stock:
100 μl 1 M Tris·Cl, pH 8.0	10 mM
5 ml 4 M NaCl	2 M
20 μl 500 mM EDTA	1 mM
4.88 ml DEPCW	

Prepare stock solutions with DEPCW and autoclave. Store 2× BSBB up to 6 months at 4°C.

Calf liver tRNA, 5 mg/ml

250 mg calf liver tRNA
DEPCW to 50 ml
Filter sterilize and store in 1-ml aliquots up to 2 years at -20°C

Cell lysis buffer, 10× (10 ml)

Components:	Final concentration of 10× stock:
1 ml 1 M Tris·Cl, pH 7.4	0.1 M
1 ml 1 M MgCl ₂	0.1 M
5 ml 2 M KCl	1 M
3 ml DEPCW	

Prepare stock solutions of reagents for this recipe with DEPCW and autoclave. Divide 10× buffer into 1-ml aliquots and store up to 2 years at -20°C.

Prepare complete lysis buffer from 10× cell lysis buffer stock immediately before use in the preparation of polysome-bound mRNA (see Alternate Protocol 1).

Creatine phosphokinase, 200 U/ml

500 U creatine phosphokinase
1.25 ml 100 mM KCl
20 mM HEPES-KOH (see recipe), pH 7.2
1.25 ml glycerol
Store in 500-μl aliquots up to 1 year at -20°C

Glycerol prevents the enzyme from freezing at -20°C.

DTT, 250 mM

Add 154 mg DTT to 4 ml ice-cold deaerated water. Filter sterilize and snap-freeze in 25- to 250-μl aliquots. Store up to 1 year at -20°C.

Once an aliquot is thawed for use, discard residual solution.

EGTA, 200 mM

7.6 g EGTA
H₂O to 100 ml
Adjust pH to 7.0 with 1 N NaOH
Autoclave
Store up to 6 months at 4°C

Glass powder (for production of wheat germ extract)

Suspend glass powder (Sigma) in at least 20 vol water. Add 1 ml DEPC per liter. Stir at room temperature overnight in a loosely covered container. Autoclave for 45 min. Allow glass powder to settle, remove supernatant, and dry in oven at 185°C.

Hemin hydrochloride, 1 mM

Mix:

65 mg hemin hydrochloride (Sigma)

2 ml 100 mM KOH

Sufficient 100 mM Tris·Cl, pH 7.8, to adjust pH to 7.8

Then add:

20 ml sterile H₂O

80 ml ethylene glycol

Store in light-proof bottle up to 2 years at –20°C

HEPES-KOH, pH 7.2, 1 M

238.3 g HEPES, free acid

H₂O to 800 ml

Adjust pH to 7.2 with 10 N KOH

Add water to 1 liter

Filter sterilize

Store in 1-ml aliquots up to 2 years at –20°C

HEPES/KCl buffer (10 ml)

Components:

100 µl 1 M HEPES-KOH, pH 7.2

1 ml 1 M KCl

20 µl 1 M MgCl₂

2 µl 500 mM EDTA

40 µl 250 mM DTT (see recipe)

8.84 ml DEPCW

Final concentration:

10 mM

100 mM

2 mM

0.1 mM

1 mM

Prepare all stock solutions of reagents for this recipe with DEPCW and filter sterilize. Prepare solution as described above, then store 1-ml aliquots up to 1 year at –20°C. Add 7 µl/ml RNasin RNase inhibitor (Promega) just prior to use.

Used for resuspension of polysomes.

KCl/10 mM MgCl₂ (K/Mg), 2 M

14.9 g KCl

0.214 g MgCl₂

H₂O to 100 ml

Autoclave

Store in 1-ml aliquots up to 1 year at –20°C

Modified transcription buffer (MTB), 5× (10 ml)

Components:

2 ml 1 M Tris·Cl, pH 8.1

300 µl 1 M MgCl₂

500 µl 1 M NaCl

200 µl 250 mM spermidine

250 µl 10 mg/ml BSA

200 µl 2.5% Triton X-100

6.55 ml DEPCW

Concentration of 5× stock:

200 mM

30 mM

50 mM

5 mM

250 µg/ml

0.05%

Prepare all stock solutions of reagents for this recipe with DEPCW and autoclave or filter sterilize as appropriate. Store 1-ml aliquots of 5×MTB up to 1 year at –20°C.

See Milligan et al. (1987) for discussion of this solution.

4NTP mixture, 12.5 mM

50 mM single-nucleotide stocks (ATP, CTP, GTP, and UTP): Transfer 500 mg of each to a separate sterile 50-ml tube. Add DEPCW to appropriate volume. Check pH of each stock by pipetting 1 μ l onto pH paper, and adjust by adding 1 N NaOH drop by drop until pH reaches 7.5. Filter sterilize and store in 1-ml aliquots up to 1 year at -20°C .

10 mM single-nucleotide stocks: Dilute 1/5 in DEPCW. Store in 500- μ l aliquots up to 1 year at -20°C .

12.5 mM 4NTP mix: Mix 2.5 ml each of 50 mM ATP, CTP, GTP, and UTP stocks (10 ml total). Store in 500- μ l aliquots up to 1 year at -20°C .

Nuclease S7, 15,000 U/ml

Add 1 ml water to lyophilized *Staphylococcus aureus* nuclease S7 enzyme to obtain a concentration of 15,000 U/ml. Make fresh for each use, and discard residual solution.

Used at 1/100 dilution.

Phenylhydrazine, 2.5% (w/v)

Dissolve 5 g phenylhydrazine (Sigma) in 200 ml deaerated sterile 0.85% NaCl and adjust to pH 7.0 with 1 N NaOH (the solution should be a pale straw color, not brown). Store single-use aliquots in air-tight, light-proof containers up to 3 months at -20°C .

Once an aliquot is thawed for use, discard residual solution.

Phosphocreatine, 1 M

Dissolve 0.255 g phosphocreatine in ice-cold water to 1 ml. Snap-freeze in 25- to 250- μ l aliquots and store up to 2 weeks at -20°C .

Once an aliquot is thawed for use, discard residual solution.

Reticulocyte wash buffer I

100 ml 10 \times reticulocyte wash buffer II (see recipe)

7.5 ml 1 M MgCl_2

10 ml 100 mM glucose

H_2O to 1 liter

Store up to 1 year at 4°C

Reticulocyte wash buffer II, 10 \times

38.5 g NaCl

1.875 g KCl

H_2O to 500 ml

Autoclave and store up to 1 year at 4°C

Before use, add 100 ml of 10 \times stock and 5 ml of 1 M HEPES-KOH, pH 7.2, to 900 ml sterile H_2O .

SDS-PAGE sample buffer, 2 \times (50 ml)

Components:

12.5 ml 1 M Tris-Cl, pH 6.8

3 g SDS

2.3 g DTT

5 ml 2-mercaptoethanol

500 μ l 2% bromphenol blue

20 ml glycerol

H_2O to 50 ml

Concentration of 2 \times stock:

250 mM

6%

300 mM

1.4 M

0.02%

20%

Filter and store in 1-ml aliquots up to 1 year at -20°C . Once an aliquot has been thawed for use, discard residual solution.

Sucrose buffer (for cushion; 10 ml)

<i>Components:</i>	<i>Concentration:</i>
3.42 g sucrose	1 M
1 ml 1 M KCl	100 mM
100 μ l 1 M MgCl ₂	10 mM
2 μ l 0.5 M EDTA	0.1 mM
100 μ l 1 M Tris·Cl, pH 7.4	10 mM
DEPCW to 10 ml	
Filter sterilize	
Use fresh	

Transcription buffer (TB), 5 \times (10 ml)

<i>Components:</i>	<i>Concentration of 5\times stock:</i>
2 ml 1 M Tris·Cl, pH 7.5	200 mM
300 μ l 1 M MgCl ₂	30 mM
500 μ l 1 M NaCl	50 mM
250 μ l 10 mg/ml BSA	250 μ g/ml
400 μ l 250 mM spermidine	10 mM
6.55 ml DEPCW	

Prepare all stock solutions of reagents for this recipe with DEPCW and autoclave or filter sterilize as appropriate. Store 1-ml aliquots up to 1 year at -20°C .

Transcription/translation (TX/TL) buffer, 25 \times (10 ml)

<i>Components:</i>	<i>Concentration of 25\times stock:</i>
1.4 ml 1 M HEPES-KOH (see recipe), pH 7.2	140 mM
400 μ l 1 M MgCl ₂	40 mM
200 μ l 250 mM spermidine	5 mM
8 ml 12.5 mM 4NTP mix (see recipe)	10 mM each

Prepare all stock solutions of reagents for this recipe with DEPCW and autoclave or filter sterilize as appropriate. Store 1-ml aliquots at -20°C .

Trichloroacetic acid (TCA), 5% and 10%

Weigh reagent bottle containing TCA and record weight full. Wearing rubber gloves, goggles, and plastic apron, add water and dissolve by shaking closed container. Pour solution into measuring cylinder, record weight of bottle empty, calculate amount of TCA used, and adjust volume to obtain a 10% solution. Dilute part of solution to 5% with an equal volume of water. Store both solutions in brown plastic bottles.

This method prevents burns from TCA crystals.

Wheat germ extraction buffer (50 ml)

<i>Components:</i>	<i>Final concentration:</i>
5 ml 1 M potassium acetate	0.1 M
100 μ l 1 M magnesium acetate	2 mM
1 ml 1 M HEPES-KOH, pH 7.2 (see recipe)	20 mM
8 μ l 250 mM spermidine	40 μ M
35.9 ml DEPCW	

Prepare all stock solutions of reagents for this recipe with DEPCW and autoclave or filter sterilize as appropriate. Make buffer fresh, and immediately prior to use, add 200 μ l of 250 mM DTT stock (1 mM final).

COMMENTARY

Background Information

Choice of system

The two most widely used eukaryotic in vitro translation systems are the mRNA-dependent reticulocyte lysate (Pelham and Jackson, 1976), an adaptation of an earlier mRNA-independent reticulocyte system (Adamson et al., 1968; Zucker and Schulman, 1968), and the wheat germ system developed by Roberts and Paterson (1973) with later refinements (Morch et al., 1986; Van Herwynen, 1990).

Perhaps the first question a researcher will have is “Which system should I use?” Both systems are very active and express a large range of polypeptide sizes. The most obvious advantages of the wheat germ system are its ease of preparation and its low cost. In addition, no animal use is required. With the inclusion of spermidine and RNase inhibitor in the reaction and supplementation with calf liver tRNA, an earlier drawback of the wheat germ system, its lower ability to translate high-molecular-weight proteins, has been overcome (Morch et al., 1986; Van Herwynen, 1990; Van Herwynen and Beckler, 1995). Furthermore, the wheat germ extract readily translates mRNA preparations containing contaminating double-stranded RNA or oxidized thiols, which are inhibitory in the reticulocyte system. It is also the system of choice for expressing eukaryotic transcription factors, since it does not contain endogenous counterparts (Mercurio et al., 1993).

However, the wheat germ system exhibits rather narrow ionic requirements that are very sensitive to the nature of the mRNA to be translated and can require optimization for each template used. Overall, the reticulocyte system remains the most highly used and the most widely available commercially.

The choice of system should be determined by the nature of the mRNA to be translated and the chosen assay or required function for the expressed protein. Items to consider include whether the protein will fold better in one system versus the other, whether there are endogenous enzymes available for post-translational modifications required for activity of the translation product, whether there are endogenous activities or compounds that interfere with a functional assay, and whether there are endogenous streptavidin-binding proteins that could interfere with detection of biotinylated products. Based on the above considerations, use of the reticulocyte translation system may be advantageous for stud-

ies of post-translational modification, including the use of microsomal membranes (*UNITS 11.3 & 11.4*). The reticulocyte system is also better for high-level expression using constructs containing the encephalomyocarditis 5' untranslated leader region (EMCV 5' UTR) and for nonisotopic detection using biotinylated lysine-tRNA. Conversely, the wheat germ system is preferable for studies of eukaryotic transcription factors, real-time assays monitoring low levels of luciferase, and cross-linking and fluorescent detection assays involving incorporation of non-natural amino acids. However, there are exceptions to all these generalizations.

To couple or not

The development of coupled eukaryotic transcription/translation systems had allowed direct expression from DNA containing cloned genes positioned behind T7, T3, or SP6 promoters in rabbit reticulocyte lysate or wheat germ extracts (Craig et al, 1992; Thompson et al., 1992). The availability of the coupled systems necessitates another choice—the production of mRNA prior to the translation reaction versus the use of DNA directly using the coupled system. In most cases the coupled system is more convenient, faster, and produces greater protein yields. Unless there is a problem with termination of the mRNA sequence, circular DNA can be used in the coupled system, obviating the need for linearization and subsequent clean-up of DNA. Interestingly, even though the coupled system does not use capped mRNA, several groups have reported the coupled system still produces greater protein yields than optimized expression from capped mRNA (Jagus, 1993). The eukaryotic coupled systems are much more forgiving of DNA purity than their sister prokaryotic (*E. coli*) coupled systems. Most common commercially available DNA purification systems will provide adequate DNA purity. In many cases where PCR DNA is used, the PCR reaction mix can be added directly to the coupled transcription/translation system without purification, although care should be taken not to add too much DNA (Renshaw-Gregg and Guiltinan, 1996).

On a cautionary note, when coexpressing more than one gene in a coupled transcription/translation system, care should be taken that no inhibitory antisense mRNA is generated from the use of dual opposable promoter vectors (i.e., T3/T7 or SP6/T7). If vectors using dual opposable promoters are used in such an

instance, linearization of the DNA 3' to the gene of interest will prevent the problem.

Critical Parameters

One of the most common questions of researchers expressing gene products *in vitro* is "How much protein will the system generate?" Unfortunately, there is no simple answer. A multitude of factors will affect the final protein yield, some of them outside the researcher's control. Almost all researchers will be most interested in the translation of transcripts synthesized *in vitro* from cloned cDNAs using the bacteriophage promoters such as those for T3, T7, and SP6 RNA polymerases (Melton et al., 1984; Milligan et al., 1987). There are now a number of improved commercial systems for generating high levels of mRNA from linearized DNA (e.g., Ambion, EpiCenter, Novagen, and Promega).

The highest yields of transcripts are obtained for uncapped mRNA. Production of uncapped transcripts is also cheaper, since the cap analog needed to produce capped transcripts is expensive. This makes it advantageous to use a vector that places a 5' UTR, allowing cap-independent translation, upstream of the initiation codon. Such vectors include pCITE (Novagen) and pTM1 (Moss et al., 1990), which use the internal ribosome entry site (IRES) of EMCV to promote cap-independent translation. The addition of the EMCV 5' viral IRES has been shown to increase the expression of luciferase 16-fold over a control luciferase construct without the sequence (Beckler, 1992). Interestingly, the EMCV IRES sequence does not enhance translation in the wheat germ translation system and is actually detrimental. Alternatively, high expression levels can be achieved with uncapped mRNA by optimizing salt concentrations.

To generate well-translated *in vitro* transcripts, care must be taken not only in the choice of vector but also in the design of the construct. For instance, the sequence surrounding the AUG start should be optimized to match the so-called "Kozak" consensus sequence of A/GCCAUGG to provide optimal translation initiation (Kozak, 1989). Similarly, the 5' UTR of the mRNA should not contain any significant secondary structure that may retard migration of the ribosome from the 5' cap to the initiation codon (Kozak, 1988). In general, it is safer not to include any 5' UTR sequences from the cDNA of interest since they may be inhibitory to translation. It is preferable to choose a vector that provides a 5' UTR that will ensure good translation (such as pCITE and pTM1) and to

clone in at an *NcoI* or *NdeI* restriction site that becomes the initiation codon.

However, when transcripts requiring cap-dependent translation are used, decreasing the distance of the initiation codon from the 5' cap can reduce translational efficiency (Kozak, 1991a). Conversely, inclusion of long 5' sequence that cannot form secondary structures has been shown to increase translational efficiency through the buildup of small ribosomal subunits in the 5' UTR (Kozak, 1991b). Addition of an artificial 30-nucleotide poly(A)⁺ sequence to the 3' end of an mRNA has also been shown to boost expression 7-fold (Beckler, 1992). Note that in mRNA templates containing an added 3' poly(A)⁺ tail, increasing mRNA concentration will decrease translation due to titration of poly(A)⁺ binding protein, which is involved in translation initiation.

Enhancement additives

For the rabbit reticulocyte system, a variety of factors lead to the activation of protein kinases that phosphorylate the α -subunit of initiation factor eIF2. This leads to a cessation of translation initiation. Hemin is always added to reticulocyte systems to prevent activation of the heme-sensitive eIF2 α kinase. The addition of 5'-cAMP (1 to 10 mM) may also stimulate translation by preventing eIF2 α kinase activation (Gross et al., 1988). Glucose-6-phosphate or 2-aminopurine (0.5 mM) may also stimulate translation by a variety of mechanisms (Jagus and Safer, 1981; Jackson et al., 1983; Michelson et al., 1984; Gross et al., 1988). The addition of eIF2 α homologues, such as the K3L gene product from vaccinia virus or the vaccinia virus double-stranded RNA binding protein (the E3L gene product), to the reticulocyte system can prolong the linear rate of translation, resulting in increased protein yields (reviewed by Jagus and Gray, 1994; Clemens and Elia, 1997). If the codon usage of the open reading frame contains rare codons, or is from an organism using a different genetic code (such as trypanosomes), it may be desirable to add exogenous tRNA purified from the source organism.

Optimization of ionic conditions

Optimization of Mg²⁺ and K⁺ can enhance the translation of a particular transcript (Beckler, 1992; Beckler et al., 1995). Mg²⁺ is the most critical component of the translation reaction to optimize because the Mg²⁺ optimum is very narrow: small changes in Mg²⁺ concentration can dramatically affect the efficiency and fidel-

ity of translation (Beckler, 1992). In addition, each RNA transcript exhibits a unique Mg^{2+} optimum. Capped mRNAs generally require a higher K^+ concentration than uncapped mRNAs (Kemper and Stolarsky, 1977). mRNAs containing the 5' UTR of EMCV also require higher K^+ concentrations (Jackson, 1991). The form and concentration of K^+ salts is also important for initiation at the correct site; more efficient and accurate translation is achieved using potassium chloride rather than potassium acetate (Jackson, 1991). Uncapped mRNAs produced in in vitro transcription reactions are also translated with greater fidelity using the chloride rather than the acetate (Jackson, 1991).

Anticipated Results

As stated above, there is no simple expectation of protein yield in the cell-free translation systems. The reticulocyte lysate should contain between 25 and 30 A_{260} units of ribosomes, or 250 to 300 pmol per ml. Using the MDL with added mRNA in a reaction in which the MDL constitutes 70% of the reaction volume (i.e., 175 to 200 pmol ribosomes/ml), under optimal conditions and saturating levels of globin mRNA, the yield will be up to 1 mol globin/mol ribosome/min at 37°C (Jagus, 1987). This works out to be 262.5 to 300 pmol/50- μ l reaction/30-min incubation, or 3.5 to 4.25 μ g globin. Globin mRNA is translated very efficiently. The expression level of other mRNAs will vary, depending on the integrity and purity of the mRNA and on all the characteristics of mRNA structure known to affect translatability (outlined above; see Critical Parameters).

In the coupled system, the MDL is usually used at 50% of the final reaction mix, but the yield per ribosome has the same or higher potential. However, the purity of the DNA, type of vector, and design of the construct will all have an impact. Similarly, use of enhancement additives can increase the yield by allowing translation to continue for longer.

Estimating expected yields when using biotinylated amino acids is more difficult, since in this case precharged biotinylated lysine-tRNA competes with endogenous lysine-tRNA, which is continuously recharged, so that specific activity of the biotinylated lysine-tRNA decreases throughout the incubation. With biotinylated lysine-tRNA it is difficult to determine the percent incorporation of biotinylated lysines into a translated protein. However, using 15 pmol biotinylated NBD- ^{14}C]lysine-tRNA per 25- μ l reaction, supplemented with amino acid mix minus lysine, ~50% of the biotinylated

NBD- ^{14}C]lysine was incorporated into protein (Crowley et al., 1993). This is much less yield than is seen using conventional radioisotope labeling, but in this circumstance, lysine was limiting and use of a complete amino acid mix increases both protein yield and detection (Beckler and Hurst, 1993).

Time Considerations

The time taken for in vitro translation reactions will vary depending on which system is used, the source of mRNA, the detection method, and whether a coupled system is used. Assuming that the source of mRNA consists of transcripts generated in vitro from a DNA template, then ~4 hr will be needed for synthesis and purification of the mRNA, 1 to 1.5 hr for the translation reaction, and ~7 to 12 hr for analysis by SDS-polyacrylamide minigels and fluorography, assuming a 4- to 8-hr exposure of the autoradiogram. This gives a total of ~12 to 16 hr. In comparison, use of the coupled system requires only 8 to 12 hr (Thompson et al., 1992).

If standard fluorographic detection is replaced by analysis using phosphoimaging, the time can be reduced by 2 to 6 hr. If fluorographic detection is replaced by the use of biotinylated lysine-tRNA immunodetection, the time can be cut down by 3 to 8 hr. If speed of production of translation product is a critical factor, the system of choice would be a coupled reticulocyte system purchased as a kit. If detection of the protein product is the only experimental need, the use of biotinylated lysine-tRNA with detection using avidin or streptavidin conjugated to alkaline phosphatase coupled with colorimetric detection is the fastest choice (Beckler and Hurst, 1993).

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**In Vitro
Reconstitution**

11.2.25

In Vitro Analysis of Endoplasmic-Reticulum-to-Golgi Transport in Mammalian Cells

This unit describes in vitro reconstitution of vesicular trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus in mammalian cells. The assays outlined here allow for the examination of factors that are required for and/or regulate the budding of vesicles from the ER and the subsequent fusion of these vesicles with the Golgi. The assays are based on the ability to follow the movement of a temperature-sensitive mutant of vesicular stomatitis virus–encoded glycoprotein (VSV-G ts045) as it moves out of the ER and through the Golgi. VSV-G ts045 is retained in the ER at the restrictive temperature (39.5°C), which allows for synchronized movement of this protein out of the ER upon switching to the permissive temperature (32°C). Transport is measured by following the maturation of two asparagine-linked oligosaccharide chains on VSV-G as the protein moves from the ER to the *cis*-Golgi and subsequently to the medial/*trans*-Golgi.

The first transport assay (see Basic Protocol 1) measures the movement of VSV-G in semi-intact cells (SICs). SICs are a population of cells that have lost their cytosolic contents due to mechanical perforation of the plasma membrane, but that retain their subcellular organelles. SICs prepared from normal rat kidney cells infected with VSV ts045 faithfully reconstitute vesicular transport upon addition of rat liver cytosol and ATP. The organelles in SICs are accessible to exogenous factors ranging in size from small molecules to large proteins, allowing the effects of different factors on the extent and rate of transport from the ER to the Golgi to be determined. Alternatively, the protocol can be modified to isolate and measure ER-to-*cis*-Golgi transport alone, using clone 15B CHO cells infected with the temperature-sensitive virus VSV ts045 (see Alternate Protocol).

The second transport assay uses microsomes isolated from VSV-infected mammalian cells (see Basic Protocol 2), and is similar to the SIC assay in that it involves a single incubation that reconstitutes ER-to-Golgi transport. A more detailed two-stage protocol allows for ER-derived vesicles to be formed in vitro (see Basic Protocol 3) and used in a separate second-stage reaction that measures fusion with purified Golgi membranes (see Basic Protocol 4). This microsome-based two-stage assay allows vesicle formation and the budding reaction to be studied as separate biochemical events from the targeting and fusion reactions. Furthermore the ER-derived vesicles can be purified and examined as separate entities (see Basic Protocol 3).

The unit also presents Support Protocols for preparing the necessary reagents for these assays. Preparation of microsomal membranes and Golgi membranes is described (see Support Protocols 1 and 4, respectively). VSV ts045 is propagated to generate a high-titer stock (see Support Protocol 2), which is then used as the source of virus for preparation of both SICs and microsomes. Cytosol from rat liver (see Support Protocol 3) is used in each transport assay, and is gel-filtered for use in fusion reactions of ER-derived vesicles with Golgi membranes.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

NOTE: All culture incubations should be performed in a humidified 5% CO₂ incubation unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

NOTE: VSV stocks and samples should be treated with bleach prior to disposal.

**RECONSTITUTION OF ER-TO-GOLGI TRANSPORT IN SEMI-INTACT
CELLS**

This first protocol describes the infection of normal rat kidney (NRK) cells with VSV ts045, the labeling of infected cells with ³⁵S, and the mechanical perforation of these cells by hypotonic swelling to generate labeled SICs. The labeled SICs are then used in a reaction that reconstitutes transport of VSV-G ts045 from the ER to and through the Golgi in a cytosol-, ATP-, and temperature-dependent manner. The progress of VSV-G is measured by following the acquisition of endoglycosidase H (endo H) resistance by the N-linked oligosaccharide side chains of VSV-G, which increases with time as the protein moves through the Golgi. The endo H-sensitive and endo H-resistant forms of VSV-G are separated by SDS-PAGE and quantified, and transport is expressed as the percentage of VSV-G in the endo H-resistant form.

Materials

- Normal rat kidney (NRK) cells
- Alpha minimal essential medium (α -MEM), serum-free and with 5% (v/v) FBS
(*APPENDIX 2A*)
- VSV ts045 stock ($\sim 2 \times 10^9$ pfu/ml; see Support Protocol 2)
- 1 mg/ml actinomycin D in ethanol
- Methionine-deficient labeling medium (see recipe)
- [³⁵S]Methionine (~ 11 mCi/ml, 1175 Ci/mmol; Trans³⁵S-label, ICN Biomedicals)
- 20 mM unlabeled methionine (tissue culture grade; Sigma)
- Perforation buffer (see recipe), ice cold
- Swelling buffer (see recipe), ice cold
- 1% (w/v) trypan blue
- Rat liver cytosol (see Support Protocol 3)
- 1 M HEPES acid, pH 7.4
- 0.1 M magnesium acetate
- 1 M potassium acetate
- 10 \times Ca²⁺ buffer (see recipe)
- 20 \times ATP-regenerating system (see recipe)
- 40 mM UDP-*N*-acetylglucosamine
- Endo H buffer (see recipe)
- 75 mU/ml endoglycosidase H (endo H; Boehringer Mannheim) in 0.1 M sodium acetate, pH 5.6
- 4 \times SDS sample buffer (*APPENDIX 2A*)
- Fluorographic enhancement solution: 125 mM salicylic acid (sodium salt), pH 7.0, in 30% (v/v) methanol
- Culture incubator at 32°C
- Water baths at 32°, 37°, and 39.5°C
- Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and for autoradiography and densitometry (*UNIT 6.3*)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

Infect and label NRK cells

1. Grow NRK cells to confluency on 100-mm tissue culture dishes.
2. Prepare an infection cocktail containing (per dish) 0.9 ml serum-free α -MEM, 0.1 ml of VSV ts045 stock (final $\sim 2 \times 10^8$ pfu/ml), and 5 μ l of 1 mg/ml actinomycin D (final 5 μ g/ml).

Table 11.3.1 Reaction Mix for ER-to-Golgi Transport Assay in Semi-Intact Cells

Solution	Volume in mix (μl)	Final concentration
SICs ^a	5	
Rat liver cytosol	5	3 mg/ml
1 M HEPES acid, pH 7.4	1	25 mM
0.1 M magnesium acetate	1	2.5 mM
1 M potassium acetate	2	50 mM
10× Ca ²⁺ buffer	4	1×
20× ATP-regenerating system	2	1×
40 mM UDP- <i>N</i> -acetylglucosamine	0.5	0.5 mM
Water	To 40 μl (final)	

^aAbbreviation: SICs, semi-intact cells.

- Add 1 ml infection cocktail per dish and rock continuously (or by hand at 5-min intervals) for 45 min in a 32°C culture incubator, ensuring even distribution of the infection cocktail.
- Add 5 ml α-MEM with 5% FBS to each dish and incubate at 32°C for 3 hr and 40 min.
- Move the cells to a 39.5°C water bath and wash three times with 3 ml methionine-deficient labeling medium. Incubate the cells in 3 ml methionine-deficient labeling medium 15 min at 39.5°C.
- Remove labeling medium, replace with 1.5 ml methionine-deficient labeling medium supplemented with 100 μCi [³⁵S]methionine, and incubate 10 min at 39.5°C with occasional rocking.
- Add 30 μl of 20 mM unlabeled methionine and incubate 2 min at 39.5°C.

It is essential that the temperature does not drop below 39.5°C at any point during this procedure to ensure that all of the ³⁵S-labeled VSV-G remains in the ER.

Perforate labeled cells

- Transfer cells to ice and wash three times with 5 ml ice-cold perforation buffer.
- Cover cells with 5 ml ice-cold swelling buffer and allow cells to sit on ice for 10 min.
- Aspirate swelling buffer, add 3 ml ice-cold perforation buffer, and immediately scrape the cells with a rubber policeman.
- Transfer cells to a 15-ml polypropylene tube and pellet by centrifugation at 800 × g, 3 min at 4°C.
- Wash the pellet in 3 ml ice-cold perforation buffer and recentrifuge.
- Resuspend in 200 to 300 μl ice-cold perforation buffer.
- Determine the extent of perforation by mixing 10 μl SICs with 1 μl of 1% trypan blue on a cover slip and examining by light microscopy.

The trypan blue–stained nuclei identify the perforated cells. There should be 90% to 95% perforated cells.

SICs should be used immediately (i.e., stored no more than 1 hr on ice).

Reconstitute ER-to-Golgi transport

- Prepare each reaction mix in a microcentrifuge tube as shown in Table 11.3.1. Perform reactions in duplicate along with two negative controls: complete reaction mix incubated on ice and reaction mix without cytosol, incubated at 32°C.

16. Vortex gently 3 sec and incubate 90 min in a 32°C water bath.
17. Pellet SICs by microcentrifuging 20 sec at 16,000 × *g*, 4°C.
18. Resuspend pellet in 20 μl endo H buffer.
19. Boil 5 min, microcentrifuge briefly, and cool. Add 40 μl of 75 mU/ml endo H (3 mU) in 0.1 M sodium acetate, pH 5.6.

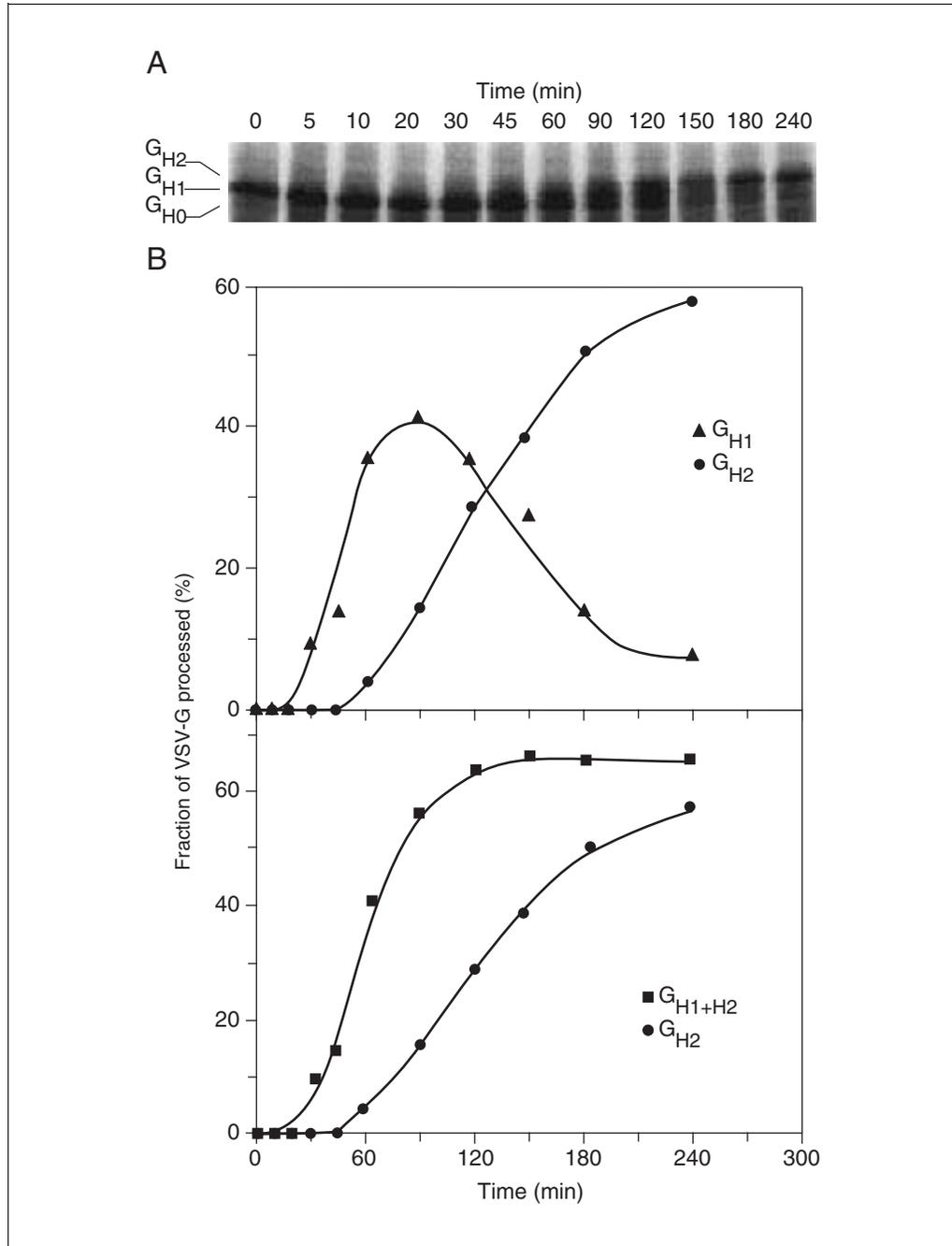


Figure 11.3.1 (A) Time course of ³⁵S-labeled VSV-G transport in semi-intact NRK cells, analyzed by endo H digestion. At the early time points, only G_{H0} is detected. G_{H1} appears as a transient intermediate and is replaced by G_{H2} as transport proceeds. These bands reflect discrete intermediates in VSV-G processing (see Background Information and Fig. 11.3.3). (B) Kinetics of VSV-G transport in semi-intact NRK cells as measured by the appearance of the G_{H1} and G_{H2} forms of VSV-G. Reprinted from Schwanger et al. (1991) with permission from the American Society for Biochemistry and Molecular Biology.

20. Incubate overnight in a 37°C water bath.
21. Stop reaction by adding 20 µl of 4× SDS sample buffer, and separate the endo H-sensitive and endo H-resistant forms of VSV-G on a large 6.75% (w/v) polyacrylamide gel (UNIT 6.1).
22. Treat the polyacrylamide gel with fluorographic enhancement solution 20 min at room temperature. Dry the gel and autoradiograph (UNIT 6.3) at –80°C.
Alternatively, expose the gel to a Phosphorimager screen.
23. Quantitate the intensities of the different forms of VSV-G by densitometry (Fig. 11.3.1; see UNIT 6.3 for densitometry).

RECONSTITUTION OF ER-TO-*cis*-GOLGI TRANSPORT IN SEMI-INTACT CELLS

ALTERNATE PROTOCOL

It is sometimes desirable to separate ER-to-*cis*-Golgi transport from ER-to-*cis*-Golgi/medial-Golgi transport. This can be accomplished in an assay using the CHO cell line Clone 15B. These cells cannot process VSV-G oligosaccharide side chains beyond the Man₅GlcNAc₂ (Man₅; endo D_s in Fig. 11.3.3) form, due to a lack of the enzyme *N*-acetylglucosaminetransferase I (GlcNAc T I). The Man₅ form is uniquely sensitive to endoglycosidase D (endo D), and therefore the acquisition of endo D sensitivity is a measure of ER-to-*cis*-Golgi transport exclusively.

Additional Materials (also see *Basic Protocol 1*)

- Clone 15B chinese hamster ovary (CHO) cells (ATCC)
- Endo D buffer (see recipe)
- 0.5 mU/µl endoglycosidase D (endo D; Boehringer Mannheim)

1. Grow Clone 15B CHO cells to confluency on 100-mm tissue culture dishes and prepare VSV-infected, ³⁵S-labeled SICs (see Basic Protocol 1, steps 2 to 14).
2. Prepare each reaction mix in a microcentrifuge tube as shown in Table 11.3.1, except omit the UDP-*N*-acetylglucosamine. Perform reactions in duplicate along with two negative controls: complete reaction mix incubated on ice and reaction mix without cytosol, incubated at 32°C.
3. Vortex gently 3 sec and incubate 90 min in a 32°C water bath.
4. Pellet SICs by microcentrifuging 20 sec at 15,000 × *g*, 4°C.
5. Resuspend pellet in 60 µl endo D buffer containing 0.5 mU endo D.
6. Incubate the reaction overnight in a 37°C water bath.
7. Analyze results (see Basic Protocol 1, steps 21 to 23; see Fig 11.3.2).

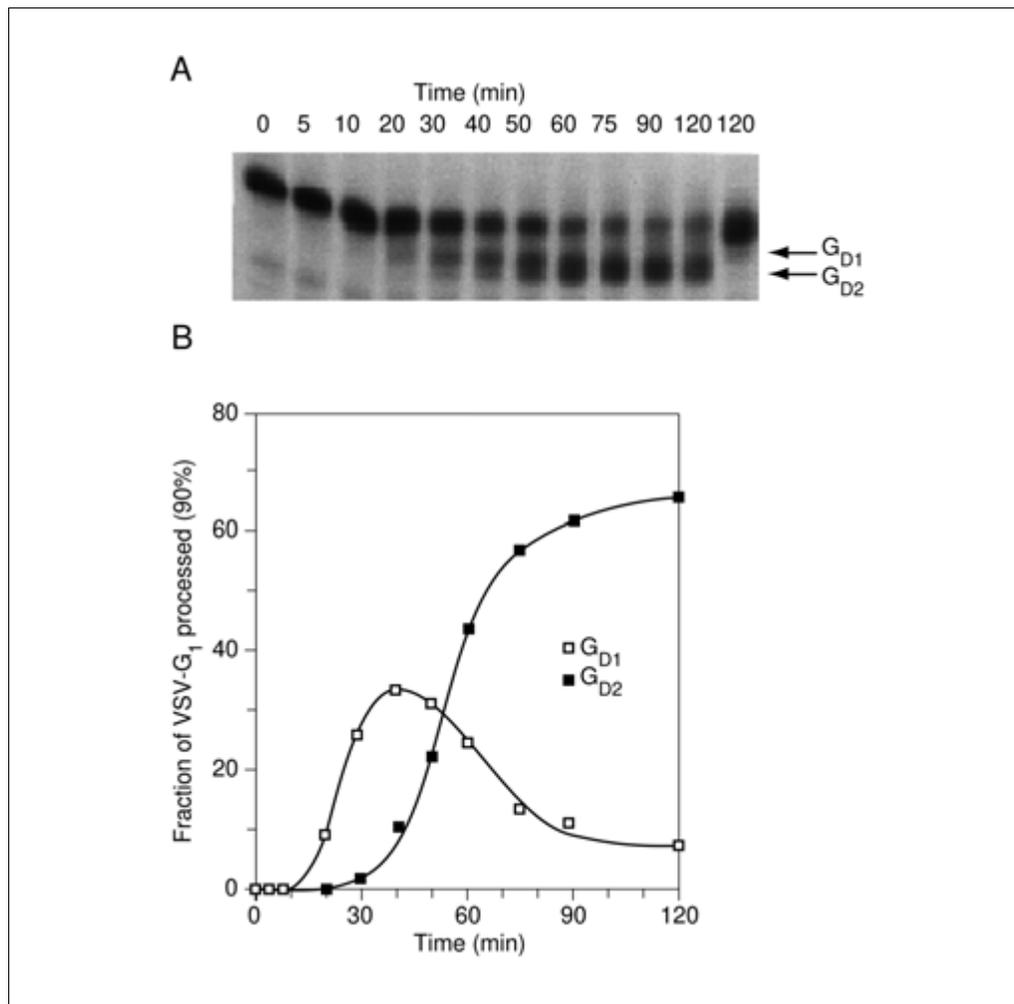


Figure 11.3.2 (A) Time course of ^{35}S -labeled VSV-G transport in semi-intact Clone 15B CHO cells, analyzed by endo D digestion. Clone 15B CHO cells are missing the enzyme GlcNAc T I, which is required for development of endo H resistance. At the early time points only G_{H0} is detected. Only G_{D2} is detected under standard SDS-PAGE conditions, although G_{D1} , which is produced when the first of the N-linked oligosaccharides is processed to the endo D-sensitive form, can sometimes be seen as an intermediate by running the dye front off the end of the gel to improve resolution. Reprinted from Schwaninger et al. (1991) with permission from the American Society for Biochemistry and Molecular Biology. (B) Kinetics of VSV-G transport as measured by the appearance of the G_{D1} and G_{D2} forms of VSV-G.

**BASIC
PROTOCOL 2**

**IN VITRO RECONSTITUTION OF ER-TO-GOLGI TRANSPORT IN
MAMMALIAN MICROSOMES**

This protocol describes a simple reaction that reconstitutes ER-to-Golgi transport in a microsomal fraction isolated from VSV-infected NRK cells. This assay is similar to the SIC assay in that it is cytosol-, ATP-, and temperature-dependent, and because transport is also measured by assessing the endo H resistance of the VSV-G oligosaccharide side chains.

Materials

- Microsomes (see Support Protocol 1)
- Rat liver cytosol (see Support Protocol 3)
- 1 M HEPES acid, pH 7.4
- 0.1 M magnesium acetate
- 1 M potassium acetate

**In Vitro Analysis
of Endoplasmic-
Reticulum-
to-Golgi
Transport in
Mammalian Cells**

11.3.6

Table 11.3.2 Reaction Mix for In Vitro ER-to-Golgi Transport Assay and for In Vitro Formation of ER-Derived Vesicles

Solution	Volume in mix (μ l)	Final concentration
Microsomes	7.5	0.5-1 mg/ml
Rat liver cytosol	7.5	4.5 mg/ml
1 M HEPES acid, pH 7.4	1	25 mM
0.1 M magnesium acetate	1	2.5 mM
1 M potassium acetate	1.4	35 mM
10 \times Ca ²⁺ buffer	4	1 \times
20 \times ATP-regenerating system	2	1 \times
2.5 M sorbitol	3	187.5 mM
40 mM UDP <i>N</i> -acetyl glucosamine	1	1 mM
Water	To 40 μ l (final)	

10 \times Ca²⁺ buffer (see recipe)

20 \times ATP-regenerating system (see recipe)

2.5 M sorbitol

40 mM UDP-*N*-acetylglucosamine

Endo H buffer (see recipe)

75 mU/ml endoglycosidase H (endo H; Boehringer Mannheim) in 0.1 M sodium acetate, pH 5.6.

4 \times SDS sample buffer (*APPENDIX 2A*)

Anti-VSV-G monoclonal antibody p5D4 (Kreis, 1986)

Horse radish peroxidase (HRP)-conjugated secondary antibody

Water baths at 32 $^{\circ}$ and 37 $^{\circ}$ C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), and densitometry (*UNIT 6.3*)

1. Prepare each reaction mix as described in Table 11.3.2 and vortex gently. Perform reactions in duplicate, using an additional complete reaction incubated on ice as a negative control.

The final concentration of potassium acetate in the assay should be 70 to 80 mM, with ~45 mM supplied by the microsomes and cytosol. The addition of other components to the assay may require altering the amount of potassium acetate added, so that the final concentration in the assay remains at 70 to 80 mM.

2. Transfer the reaction to a 32 $^{\circ}$ C water bath and incubate 60 to 90 min.
3. Stop reaction by placing the tube on ice. Collect membranes by centrifuging 10 min at 20,000 \times g, 4 $^{\circ}$ C (e.g., in a Beckman TLA 100.3 rotor). Remove the supernatant by aspiration.
4. Resuspend pellet in 20 μ l endo H buffer.
5. Boil 5 min, microcentrifuge briefly, and cool. Add 40 μ l of 75 mU/ml endo H (3 mU) in 0.1 M sodium acetate, pH 5.6.
6. Incubate in a 37 $^{\circ}$ C water bath overnight.
7. Stop reaction by adding 20 μ l of 4 \times SDS sample buffer, and separate the endo H-sensitive and endo H-resistant forms of VSV-G on a large 6.75% (w/v) polyacrylamide gel (*UNIT 6.1*).

8. Transfer the proteins to nitrocellulose at 40 V for 6.5 to 10 hr in Tris/glycine/methanol transfer buffer, and immunoblot (UNIT 6.2) using the anti-VSV-G monoclonal antibody p5D4 and an HRP-conjugated secondary, each at 1:10,000 in BLOTTO. Quantitate the intensities of the different forms of VSV-G by densitometry (Fig. 11.3.1; see UNIT 6.3 for densitometry).

In this protocol only the G_{H0} and G_{H1} forms are seen.

IN VITRO FORMATION AND ISOLATION OF ER-DERIVED VESICLES

This protocol describes an in vitro reaction that generates VSV-G-containing ER-derived vesicles from VSV-G-containing microsomes. After incubation in the presence of cytosol and ATP at 32°C, the ER-derived vesicles are separated from the microsomal membranes by differential centrifugation. The ER-derived vesicles are harvested by high-speed centrifugation, and the amount of VSV-G in the high-speed pellet is determined by SDS-PAGE (UNIT 6.1) and immunoblotting (UNIT 6.2). This gives a direct measure of the vesicle budding reaction. Timed reactions are used to examine the rate and/or extent of budding.

This protocol also describes a method for purifying the ER-derived vesicles by immunoprecipitation using an antibody against the C terminus of VSV-G. The protein constituents of the purified vesicles can then be examined by SDS-PAGE and immunoblotting. Alternatively, the ER-derived vesicles can be isolated and retained for use in a second-stage fusion reaction (see Basic Protocol 4).

Materials

- Microsomes (see Support Protocol 1)
- Rat liver cytosol (see Support Protocol 3)
- 1 M HEPES acid, pH 7.4
- 0.1 M magnesium acetate
- 1 M potassium acetate
- 10× Ca²⁺ buffer (see recipe)
- 20× ATP-regenerating system (see recipe)
- 2.5 M sorbitol
- Resuspension buffer (see recipe), ice cold
- 1× SDS sample buffer (APPENDIX 2A)
- Anti-VSV-G monoclonal antibody p5D4 (Kreis, 1986)
- Horseshoe peroxidase (HRP)-conjugated secondary antibody
- p5D4-Dynabeads: p5D4 coupled to M-500 Dynabeads (Dyna; see manufacturer's instructions)
- Immunoprecipitation buffer (see recipe)
- FBS (APPENDIX 2A)
- 100 mM EDTA (adjust to pH 8.0 with KOH)
- Transport buffer (see recipe)
- Water bath at 32°C
- Magnetic microcentrifuge tube holder
- Additional reagents and equipment for SDS-PAGE (UNIT 6.1), immunoblotting (UNIT 6.2), and densitometry (UNIT 6.3)

1. Set up a 40- to 100- μ l reaction for each desired time point as described in Table 11.3.2, except omit the UDP-*N*-acetylglucosamine. For a negative control, perform one complete reaction on ice.

To determine the rate of budding, use 40- μ l reactions with time points ranging from 0 to 60 min at 10-min intervals. To immunoprecipitate vesicles, perform three 10-min, 100- μ l

reactions. For a two-stage transport assay (see Basic Protocol 4), use one 10-min, 100- μ l reaction.

2. Incubate the reactions in a 32°C water bath, stopping each reaction at the appropriate time point by placing the tube on ice for 2 min.
3. Collect membranes by centrifugation at 135,000 \times g for 10 min, 4°C (e.g., in a Beckman TLA 100.3 rotor).
4. To resuspend pellets, add 40 to 90 μ l ice-cold resuspension buffer, resuspend by repeated pipetting, leave on ice for 10 min, and continue pipetting.

The membranes are very difficult to resuspend, but can be completely resuspended by this method.

5. Add 0.1 to 0.2 vol of a salt mix so that the final concentration of potassium acetate is 150 mM and that of magnesium acetate is 2.5 mM. Mix by pipetting and microcentrifuge immediately at 16,000 \times g for 5 min at 4°C.
6. Retain 80% of the medium-speed supernatant (MSS) and carefully aspirate the remainder away from the medium-speed pellet (MSP).

To determine the rate/extent of budding

- 7a. Centrifuge the MSS at 135,000 \times g for 10 min, 4°C (e.g., in a Beckman TLA 100.3 rotor). Aspirate the supernatant very carefully or remove it using a yellow pipet tip. Keep the high-speed pellet (HSP).
- 8a. Resuspend the MSP in 50 μ l of 1 \times SDS sample buffer and the HSP in 40 μ l of 1 \times SDS sample buffer.
- 9a. Separate the proteins by loading 10 to 20 μ l of each resuspended pellet on a 7.5% (w/v) polyacrylamide minigel (UNIT 6.1). Transfer the proteins to nitrocellulose and immunoblot for VSV-G (UNIT 6.2) using the anti-VSV-G monoclonal antibody p5D4 and an HRP-conjugated secondary, both at 1:10,000 in BLOTTO. Quantify signals by densitometry (UNIT 6.3) and determine the ratio of VSV-G in the HSP compared to total VSV-G in the HSP plus the MSP.

To purify vesicles by immunoprecipitation

- 7b. Mix 150 to 350 μ l MSS from a 10-min time point with 1×10^7 p5D4-Dynabeads in a final volume of 372 μ l immunoprecipitation buffer. Add 20 μ l FBS (final 5%) and 8 μ l of 100 mM EDTA (final 2 mM), and mix by continuous rotation at 4°C for 2 hr.

Coupling of antibodies to the beads is performed exactly as described in the manufacturer's instructions.

- 8b. Wash beads four times with 500 μ l immunoprecipitation buffer, collecting the beads each time with a magnetic tube holder.
- 9b. Solubilize vesicles on the beads into 1 \times SDS sample buffer by boiling 5 min and analyze the proteins by SDS-PAGE (UNIT 6.1).

To use vesicles in a second-stage fusion reaction

- 7c. Prepare an HSP from a 100- μ l, 10-min reaction as described in step 7a.
- 8c. Resuspend the HSP in 25 μ l transport buffer and keep on ice for stage 2 (see Basic Protocol 4).

Each 100- μ l budding reaction is sufficient for duplicate 50- μ l fusion reactions.

PREPARATION OF MICROSOMAL MEMBRANES FROM NRK CELLS

This protocol describes the preparation of microsomes from VSV-infected NRK cells. These microsomes are used in the microsome-based transport assay (see Basic Protocol 2) and as the source of ER-derived vesicles for a more detailed two-stage assay (see Basic Protocols 3 and 4).

Additional Materials (also see *Basic Protocol 1*)

PBS (*APPENDIX 2A*), ice cold
Homogenization buffer I (see recipe)
100× PIC (see recipe)
Potassium acetate buffer (see recipe)
Transport buffer (see recipe)
1-ml ball-bearing homogenizer (Balch and Rothman, 1985)
Culture incubator at 39.5°C

NOTE: The method described below is for a twelve-dish microsome preparation.

Infect and collect cells

1. Grow NRK cells to confluency on twelve 150-mm tissue culture dishes.
2. Prepare an infection cocktail containing (per dish) 4.5 ml serum-free α -MEM, 0.5 ml VSV ts045 stock (final $\sim 2 \times 10^8$ pfu/ml), and 25 μ l of 1 mg/ml actinomycin D (final 5 μ g/ml).
3. Add 5 ml per dish and rock continuously (or by hand at 5-min intervals) for 45 min in a 32°C culture incubator, ensuring even distribution of the infection cocktail.
4. Add 20 ml α -MEM with 5% FBS to each dish and incubate in a 39.5°C culture incubator (restrictive temperature) for 3 hr and 40 min.
5. Place each dish on ice. Immediately aspirate the medium and wash the cells with 10 ml ice-cold PBS.
6. Add 5 ml homogenization buffer I and scrape the cells using a rubber policeman. Transfer the cells to three 50-ml polypropylene tubes on ice, pooling cells from four dishes into each tube. Repeat the scraping procedure once.

Prepare membranes

7. Centrifuge at $720 \times g$ for 3 min at 4°C and remove the supernatant by aspiration.
8. Resuspend each cell pellet in 0.9 ml homogenization buffer I with 1× PIC. Homogenize each by three complete passes through a 1-ml ball-bearing homogenizer.
9. Pool all cell homogenates and add an equal volume of homogenization buffer I with 1× PIC. Divide the diluted homogenate into six 1-ml aliquots in 1.5-ml microcentrifuge tubes.
10. Centrifuge at $720 \times g$ for 5 min at 4°C.
11. Transfer each postnuclear supernatant to a 15-ml polypropylene tube. Add 0.5 vol potassium acetate buffer and mix.
12. Divide into 0.8- to 1-ml aliquots in 1.5-ml microcentrifuge tubes, and microcentrifuge at $12,000 \times g$ for 2 min, 4°C.
13. Remove the supernatant by aspiration and resuspend all pellets in a total volume of 1 ml transport buffer with 1× PIC. Transfer 0.5 ml into each of two microcentrifuge tubes.

14. Microcentrifuge at $12,000 \times g$ for 2 min, 4°C .
15. Resuspend the microsomal pellets in 6 to 8 vol transport buffer with $1 \times \text{PIC}$. Pool the microsomes, divide into 50- to 100- μl aliquots, freeze in liquid nitrogen, and store at -80°C .

In a typical twelve-dish preparation, 1 to 1.5 ml of resuspended microsomes (at 3 to 5 mg protein/ml) is obtained.

These microsomes can be stored for several months without the loss of transport activity.

PROPAGATION OF VSV ts045

Stocks of VSV ts045 are prepared from infected baby hamster kidney cells to provide material for these protocols.

Materials

Baby hamster kidney (BHK) cells (ATCC)
Glasgow minimal essential medium (G-MEM; Life Technologies)
Tryptose phosphate broth (TPB; Sigma)
FBS (*APPENDIX 2A*)
TD buffer (see recipe)
Vesicular stomatitis virus (VSV) ts045 stock (Indiana serotype; multiplicity of infection = 0.1; ATCC)
Culture incubator at 32°C

1. Grow BHK cells to a confluent monolayer on 100-mm tissue culture dishes in G-MEM containing 10% (v/v) TPB and 5% (v/v) FBS.
2. Wash cells with TD buffer.
3. Add 10 ml G-MEM/10% TPB containing $\sim 10^6$ plaque forming units (pfu) of VSV ts045 stock.
4. Incubate 36 to 48 hr (until cells begin to round) in a 32°C culture incubator.
5. Remove medium, which now contains the amplified virus, and centrifuge at $720 \times g$ for 10 min at 4°C .
6. Divide the supernatant into 100- μl aliquots, freeze in liquid nitrogen, and store indefinitely at -80°C .

Generally the yield of virus is $1-2 \times 10^9$ pfu/ml. The viral stock can be refrozen twice without significant loss of titer.

FUSION OF ER-DERIVED VESICLES WITH GOLGI MEMBRANES

This protocol describes an assay that measures the fusion of ER-derived vesicles with purified Golgi membranes. The vesicles are made in vitro, isolated, and incubated at 32°C with rat liver Golgi membranes in the presence of cytosol and ATP. The acquisition of endo H resistance by the VSV-G oligosaccharide side chains is measured to follow fusion and expressed as the percentage of VSV-G in the endo H-resistant form.

Materials

ER-derived vesicles (HSP; see Basic Protocol 3, step 8c)
Desalted rat liver cytosol (see Support Protocol 3)
Enriched rat liver Golgi membranes (see Support Protocol 4)
1 M HEPES acid, pH 7.4
0.1 M magnesium acetate
 $10 \times \text{Ca}^{2+}$ buffer (see recipe)

SUPPORT PROTOCOL 2

BASIC PROTOCOL 4

In Vitro Reconstitution

11.3.11

Table 11.3.3 Reaction Mix for Fusion of ER-Derived Vesicles With Golgi Membranes

Solution	Volume in mix (μl)	Final concentration
HSP ^a	10	
Desalted rat liver cytosol	8	4 mg/ml
Golgi membranes	4	1.5 mg/ml
1 M HEPES acid, pH 7.4	1.25	25 mM
0.1 M magnesium acetate	1.25	2.5 mM
10× Ca ²⁺ buffer	5	1×
20× ATP-regenerating system	2.5	1×
40 mM UDP <i>N</i> -acetylglucosamine	1.25	1 mM
2.5 M sorbitol	3.6	180 mM
Water	To 50 μl (final)	

^aAbbreviation: HSP, high-speed pellet.

20× ATP-regenerating system (see recipe)
40 mM UDP-*N*-acetylglucosamine
2.5 M sorbitol
Endo H buffer (see recipe)
75 mU/ml endoglycosidase H (endo H; Boehringer Mannheim) in 0.1 M sodium acetate, pH 5.6
4× SDS sample buffer (*APPENDIX 2A*)
Anti-VSV-G monoclonal antibody p5D4 (Kreis, 1986)
Horseradish peroxidase (HRP)-conjugated secondary antibody
Water bath at 37°C

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), and densitometry (*UNIT 6.3*)

1. Prepare each reaction mix as described in Table 11.3.3. Perform reactions in duplicate and include two negative controls: a complete reaction incubated on ice and a reaction without Golgi membranes, incubated at 32°C.

Note that desalted rat liver cytosol is used in this assay.

2. Incubate in a 32°C water bath for 75 min.
3. Stop reaction by transferring the tubes to ice.
4. Collect membranes by centrifuging at 135,000 × *g* for 10 min, 4°C (e.g., in a Beckman TLA 100.3 rotor).
5. Resuspend pellet in 20 μl endo H buffer.
6. Boil 5 min, microcentrifuge briefly, and cool. Add 40 μl of 75 mU/ml endo H (3 mU) in 0.1 M sodium acetate, pH 5.6.
7. Incubate overnight in a 37°C water bath.
8. Stop reaction by adding 20 μl of 4× SDS sample buffer, and separate the endo H-sensitive and endo H-resistant forms of VSV-G on a large 6.75% (w/v) polyacrylamide gel (*UNIT 6.1*).
9. Transfer the proteins to nitrocellulose at 40 V for 6.5 to 10 hr in Tris/glycine/methanol transfer buffer and immunoblot (*UNIT 6.2*) using the anti-VSV-G monoclonal antibody p5D4 and an HRP-conjugated secondary, both at 1:10,000 in BLOTTO. Quantify the

intensities of the different forms of VSV-G by densitometry (Fig. 11.3.1; see *UNIT 6.3* for densitometry).

In this protocol only the G_{H0} and G_{H1} forms are seen.

PREPARATION OF RAT LIVER CYTOSOL

Rat liver cytosol is required as an additive for the transport assays. The first part of this protocol describes steps used for cytosol preparation and is performed in the same manner for each type of assay. For use in the ER-vesicle-to-Golgi fusion assay (see Basic Protocol 4), it must also be desalted by gel filtration, as described in the second part of the protocol.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Male Sprague-Dawley rats
PBS (*APPENDIX 2A*), ice cold
Cytosol buffer (see recipe), ice cold
100 mM ATP
100× PIC (see recipe)
40-ml Dounce homogenizer with type A (tight-fitting) and type B (loose-fitting) glass pestles
Cheesecloth
Sephadex G-25M/PD-10 column (Pharmacia Biotech)

Prepare rat liver cytosol

1. Sacrifice two male Sprague-Dawley rats. Remove the livers and weigh them.
Two livers should have a wet weight of 15 to 20 g. The age of the rats does not seem to affect yield or performance of the preparation.
2. Wash tissue three times with 50 ml ice-cold PBS and twice with 50 ml ice-cold cytosol buffer.
3. Add 3 vol (vol/gram wet weight of liver) ice-cold cytosol buffer supplemented with 1 mM ATP and 1× PIC, and cut the tissue into small pieces on ice using scissors.
4. Transfer the diced tissue to a homogenizer in a cold room and homogenize by hand using 10 strokes with a type B pestle (loose) followed by 10 strokes with a type A pestle (tight).
5. Filter the homogenate through cheesecloth into a beaker on ice.
6. Centrifuge the filtered homogenate at $12,000 \times g$ (e.g., in a Beckman JA-20 rotor) for 10 min, 4°C.
7. Transfer the supernatant to appropriate centrifuge tubes (e.g., for a Beckman SW41 rotor) and centrifuge at $150,000 \times g$ for 90 min, 4°C.
8. Remove the overlying lipid layer by aspiration and retain the remaining supernatant (cytosol).
9. Divide the cytosol into 250- μ l aliquots, freeze in liquid nitrogen, and store up to 6 months at -80°C .

This cytosol is ready for use in Basic Protocols 1 and 2 and in the Alternate Protocol.

The normal protein concentration of the cytosol is 25 to 30 mg/ml. Two livers should provide ~150 aliquots (30 to 40 ml).

Prepare gel-filtered (desalted) cytosol

10. In a cold room, remove the caps from a Sephadex G-25M/PD-10 column and equilibrate the column with 25 ml ice-cold cytosol buffer supplemented with 1× PIC.
11. Add exactly 2.5 ml cytosol to the top of the column.
12. Allow the eluent to flow through the column until it stops, and discard the flow-through.
13. Elute the proteins with exactly 3.5 ml ice-cold cytosol buffer supplemented with 1× PIC.
14. Divide the filtered cytosol into 100- μ l aliquots, freeze in liquid nitrogen, and store up to 6 months at -80°C .

Gel-filtered cytosol is used for Basic Protocol 4.

**SUPPORT
PROTOCOL 4**

PREPARATION OF GOLGI MEMBRANES FROM RAT LIVER

Rat livers are used as a source for Golgi membranes, which are purified by sucrose-density fractionation of liver homogenate.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Male Sprague-Dawley rats
Homogenization buffer II (see recipe), ice cold
100× PIC (see recipe)
Sucrose solutions in 10 mM Tris-Cl, pH 7.4 (*APPENDIX 2A*): 0.5 M (refractive index 1.3575), 1.0 M (1.3815), 1.1 M (1.3865), 1.25 M (1.3939), and 2.35 M (1.4464)
Dilution buffer (see recipe)
Transport buffer (see recipe)
Homogenizer (Potter-Elvehjem tissue grinder, size C) and Teflon pestles with 0.026-in. (66-mm) and 0.012-in. (30-mm) clearance
Cheesecloth
Refractometer
18-G needle

Prepare postnuclear supernatant

1. Remove the livers from two sacrificed male Sprague-Dawley rats, place on adsorbent paper, and weigh them.

The two livers should have a wet weight of 15 to 20 g. The age of the rats does not seem to affect yield or performance of the preparation.
2. Place tissue in a beaker, add 5 vol (w/v) ice-cold homogenization buffer II with 1× PIC, and cut the livers into small pieces using scissors.
3. Transfer the diced tissue to a homogenizer and homogenize using a mechanical drive at a speed of 1000 rpm. Use 3 passes with a pestle of 0.026-in. clearance and 3 passes with a pestle of 0.012-in. clearance.
4. Filter the homogenate through cheesecloth into a beaker on ice. Wash the residue on the cheesecloth with 10 ml ice-cold homogenization buffer II.

5. Centrifuge the filtered homogenate at $1450 \times g$ (e.g., in a Beckman JA-20 rotor) for 10 min, 4°C .
6. Collect the supernatant.

Separate membrane fractions

7. Layer 30-ml portions of supernatant on top of 8 ml of 1.25 M sucrose solution (e.g., in Beckman SW28 tubes).
8. Centrifuge at $112,000 \times g$ (25,000 rpm in a Beckman SW28 rotor) for 90 min, 4°C .
9. Remove the top layer by aspiration and collect the membranes from the 0.5 M/1.25 M interface using a Pasteur pipet.
10. Use a refractometer to determine the sucrose concentration and adjust to 1.2 M (refractive index = 1.3915) by adding 2.35 M sucrose.
11. Place 13-ml portions of this membrane fraction at the bottom of SW28 tubes and overlay with 10 ml of 1.1 M sucrose, 10 ml of 1.0 M sucrose, and 5 ml of 0.5 M sucrose.
12. Centrifuge at $112,000 \times g$ (25,000 rpm in a Beckman SW28 rotor) for 2.5 hr, 4°C .
13. Recover the band at the 0.5 M/1.0 M interface using an 18-G needle.
14. Divide into $\sim 300\text{-}\mu\text{l}$ aliquots in 1.5-ml microcentrifuge tubes and add 4 vol dilution buffer.
15. Microcentrifuge at $16,000 \times g$, 4°C , for 10 min.
After this centrifugation the membranes smear along the side of the tube rather than forming a compact pellet.
16. Aspirate the supernatant and resuspend the membranes gently with 0.5 ml transport buffer.
17. Pool the membranes from the two tubes and repeat microcentrifugation (as in step 15).
18. Resuspend membranes in a total of 4 ml transport buffer. Divide into 75- to $100\text{-}\mu\text{l}$ aliquots, freeze with liquid nitrogen, and store up to 3 months at -80°C .

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

NOTE: The pH of all buffers is adjusted with KOH, where appropriate.

ATP-regenerating system, 20 \times

- 12 ml 46 mM ATP, pH 7.0 (20 mM final)
- 12 ml 233 mM creatine phosphate (100 mM final)
- 4 ml 700 U/ml creatine phosphate kinase (100 U/ml final)
- Divide into 100- to $200\text{-}\mu\text{l}$ aliquots
- Store up to 3 or 4 months at -80°C

Ca²⁺ buffer, 10 \times

- 20 mM HEPES acid, pH 7.4
- 50 mM EGTA
- 18 mM CaCl_2
- Store up to 6 months at 4°C

Cytosol buffer

25 mM HEPES acid, pH 7.4
125 mM potassium acetate
Store up to 6 months at 4°C

Dilution buffer

25 mM HEPES acid, pH 7.2
87.5 mM potassium acetate
1.25 mM magnesium acetate
Store up to 6 months at -20°C

Endo D buffer

50 mM sodium phosphate, pH 6.2
5 mM EDTA
0.2% (v/v) Triton X-100
Store up to 6 months at -20°C

Endo H buffer

0.1 M sodium acetate, pH 5.6
0.3% (w/v) SDS
Store up to 1 month at 4°C
Add 20% (v/v) 2-mercaptoethanol just before use

Homogenization buffer I

20 mM HEPES acid, pH 7.4
375 mM sorbitol
Store up to 6 months at -20°C

Homogenization buffer II

10 mM Tris-Cl, pH 7.4
0.5 M sucrose
5 mM EDTA
5 mM EGTA
1× PIC (see recipe)
Prepare fresh

Immunoprecipitation buffer

20 mM HEPES acid, pH 7.4
150 mM potassium acetate
0.25 M sucrose
0.1% (w/v) BSA
Prepare fresh from stock solutions stored up to 1 week at 4°C

Methionine-deficient labeling medium

Methionine-deficient labeling medium (Sigma)
1× leucine (200× solution, 79 mM; tissue grade culture; Sigma)
1× lysine (200× solution, 80 mM; tissue grade culture; Sigma)
20 mM HEPES acid, pH 7.2
Store up to 1 year at 4°C

Methionine-deficient labeling medium is minimal essential medium (MEM) with Earle's salts and L-glutamine (but without L-leucine, L-lysine, L-methionine, or sodium bicarbonate).

Perforation buffer

50 mM HEPES acid, pH 7.2
90 mM potassium acetate
Store up to 6 months at 4°C

Potassium acetate buffer

20 mM HEPES acid, pH 7.4
210 mM potassium acetate
3 mM magnesium acetate
Store up to 6 months at -20°C

Protease inhibitor cocktail (PIC), 100×

1 mg/ml leupeptin
1 mg/ml chymostatin
0.05 mg/ml pepstatin A
0.05 M phenylmethylsulfonyl fluoride (PMSF; *APPENDIX 2A*)
Prepare in water fresh before use from stock solutions stored at -20°C

Resuspension buffer

20 mM HEPES acid, pH 7.4
0.25 M sucrose
Store up to 6 months at -20°C

Swelling buffer

10 mM HEPES acid, pH 7.2
18 mM potassium acetate
Store up to 6 months at 4°C

TD buffer

138 mM NaCl
5 mM KCl
25 mM Tris base
0.4 mM Na₂HPO₄
Prepare fresh

Transport buffer

20 mM HEPES acid, pH 7.4
250 mM sorbitol
70 mM potassium acetate
1 mM magnesium acetate
Store up to 6 months at -20°C

COMMENTARY**Background Information**

The transport of proteins along the early secretory pathway is mediated by small vesicles that bud from the ER and subsequently fuse with the Golgi apparatus (Palade, 1975). A number of biochemical assays have been developed to study ER-to-Golgi transport in vitro in mammalian cells (Beckers et al., 1987; Rowe et al., 1996) and yeast (Rexach and Schekman, 1991). The assays described in this unit allow the biochemical events of ER-to-Golgi trafficking to be studied in mammalian

cells and for the role of distinct components involved in these events to be elucidated.

The assays are based on a viral glycoprotein that is transported to the cell surface in infected cells along the same pathway as endogenous plasma membrane proteins. The assays take advantage of a temperature-sensitive mutant of vesicular stomatitis virus (VSV ts045; Lafay, 1974), which encodes a glycoprotein (VSV-G ts045) that suffers from a thermoreversible folding defect. At the restrictive temperature (39.5°C), VSV-G ts045 is retained in the ER because it is misfolded. Switching to the per-

missive temperature (32°C) corrects the misfolding and triggers transport from the ER. The ability to turn on transport selectively allows the synchronized movement of VSV-G ts045 to be followed through the early secretory pathway.

The ability to retain VSV-G ts045 in the ER and control its release makes this protein an ideal reporter molecule. In theory, however, any cargo protein could be used and the assays described here could be modified to study any protein of interest. The limiting factor is the ability to distinguish cargo protein (coming from the ER during the assay) from protein that is already present in transport vesicles and the Golgi at the beginning of the assay.

The transport of VSV-G is monitored by assessing the maturation of two N-linked oligosaccharide side chains on VSV-G as the protein moves from the ER to and through the Golgi. The processing of the oligosaccharide side chains of VSV-G is a complex process and the formation of different forms of oligosaccha-

rides can be manipulated in vitro (Davidson and Balch, 1993). In the protocols described here, four different forms of VSV-G can be distinguished (Fig. 11.3.1 and Fig. 11.3.2). In the ER, VSV-G exists in a high-mannose form (Man₉GlcNAc₂; G₀; Fig. 11.3.3). In the *cis*-Golgi, α-1,2-mannosidase I (Mann I) trims G₀ to the Man₅GlcNAc₂ (G_D) form. The oligosaccharide side chains of G₀ are resistant to cleavage by the enzyme endoglycosidase D (endo D), while G_D is uniquely sensitive to endo D. Under standard SDS-PAGE conditions, only a single G_D form is detected (G_{D2}). However a second G_D form (G_{D1}) can be seen by running the dye front off the end of the gel to improve resolution (Fig. 11.3.2). G_{D1} is a transient intermediate formed when only one of the VSV-G N-linked oligosaccharide side chains is processed.

G_D is further processed by *N*-acetylglucosaminettransferase I (GlcNAc T I) and α-1,2-mannosidase II (Mann II) in the *cis* and medial compartments of the Golgi to the G_{H1} and G_{H2}

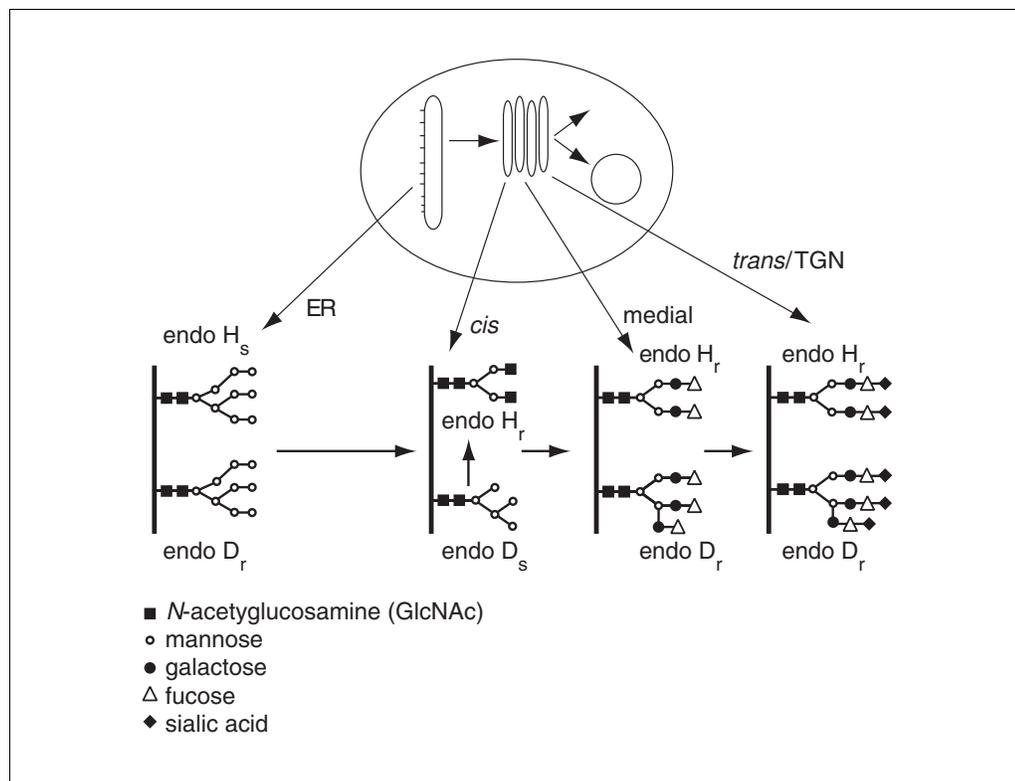


Figure 11.3.3 A schematic representation of the different oligosaccharide side chain processing forms of VSV-G. G_{H0} is the ER Man₉ form, which is endo H sensitive (endo H_s) and endo D resistant (endo D_r). G_D is formed in the *cis*-Golgi by trimming G_{H0} to the Man₅ form, which is endo D sensitive (endo D_s). This is the only intermediate in the oligosaccharide processing of VSV-G that is sensitive to endo D and cannot normally be detected in wild-type cells due to its transient formation. Further processing of G_D in the *cis*-Golgi generates G_{H1}, which is endo H resistant (endo H_r). In the medial Golgi, G_{H1} is processed to the G_{H2} form, which is also endo H resistant (endo H_r), but which migrates more slowly than G_{H1} in SDS/polyacrylamide gels. TGN, *trans*-Golgi network.

forms. G_{H1} and G_{H2} are both resistant to endoglycosidase H (endo H), while G_0 is sensitive to endo H. Under the conditions described here, G_{H1} is a transient intermediate in the pathway, and after 90 min almost all VSV-G is in the G_{H2} form. Each oligosaccharide processing form of VSV-G has a unique mobility during SDS-PAGE, allowing for a direct quantitative assay for transport (Beckers et al., 1987).

Because Clone 15B CHO cells lack the enzyme GlcNAc T I (Gottlieb et al., 1975), there is no further processing of G_D as the protein moves through the Golgi in these cells. Therefore the appearance of the G_D form is a measure of transport from the ER to the *cis*-Golgi, and the alternative semi-intact cell assay using Clone 15B CHO cells should be used when one wants to study ER-to-*cis*-Golgi trafficking exclusively.

Further processing of VSV-G that involves the addition of galactose, sialic acid, and additional GlcNAc residues (to generate a complex form of VSV-G, G_{complex}) occurs in the medial and *trans*-Golgi network (TGN), and cannot be measured using the protocols described here. However, the protocols can be modified to measure delivery of VSV-G to the TGN (Davidson and Balch, 1993).

The first protocol describes the semi-intact cell (SIC) assay, which is a relatively quick and simple way to determine the effect of any particular factor on the extent and/or rate of transport of protein from the ER to the Golgi. This assay has been used to show that GTP and ATP hydrolysis, Ca^{2+} , members of the Rab protein family, as well as general trafficking proteins such as *N*-ethylmaleimide-sensitive factor (NSF) and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are required for ER-to-Golgi transport. The use of immunodepleted cytosols, the addition of transdominant mutants, and the use of neutralizing antibodies to inhibit transport are the main approaches used to show a role for a particular protein in transport.

The SIC assay can be separated into three distinct kinetic stages (Fig. 11.3.4). The first stage, in which there is no processing of VSV-G oligosaccharide side chains, represents vesicle formation and subsequent budding and targeting of the vesicles. The second stage, in which there is a progressive increase in side chain processing, represents fusion of ER-derived vesicles with the Golgi. A plateau of processing defines the third stage, which presumably represents the maximum capacity of the SIC assay

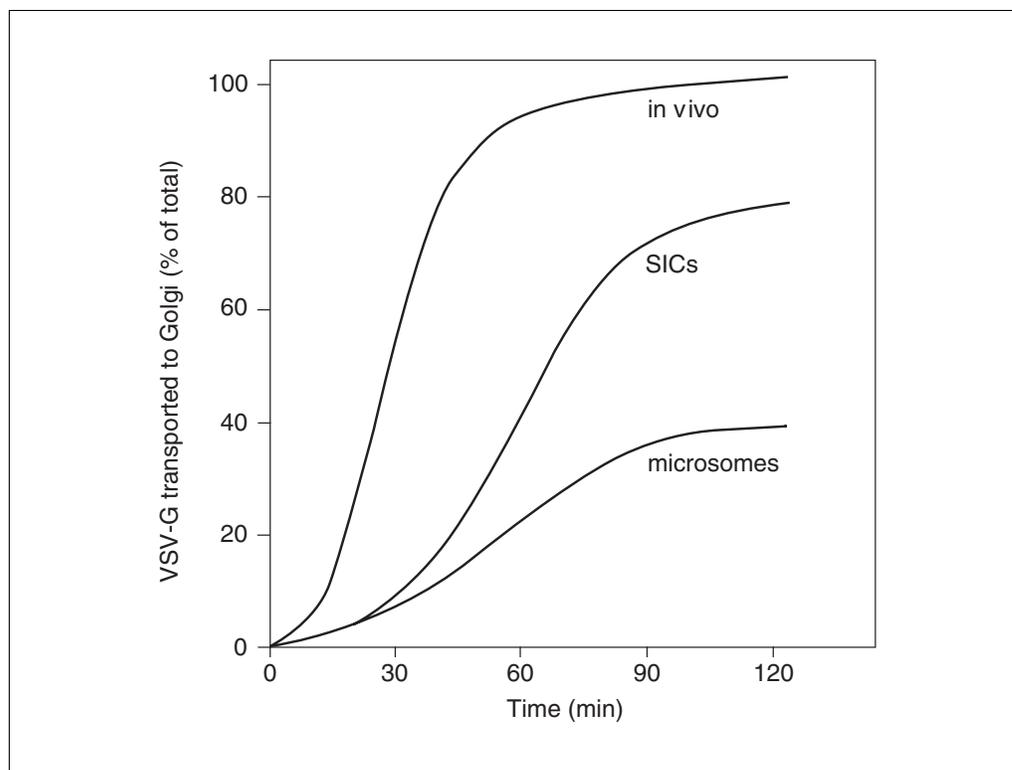


Figure 11.3.4 Comparison of the rates and extent of VSV-G transport from the ER to the Golgi apparatus measured either *in vivo*, in SIC preparations, or reconstituted using the microsome-based protocol.

to support ER-to-Golgi transport. Modifying this protocol to involve the timed addition of inhibitors during the incubation period can allow the investigator to assign a role of a particular factor to the early or late stages of transport. Furthermore, by accumulating transport intermediates with a reversible inhibitor, the requirements for further transport can be examined upon removal of the inhibitor.

The SIC assay cannot, however, accurately define a role for any factor in the separate biochemical events of ER-to-Golgi transport. The microsome-based assay described here was developed to allow for this. The protocol is a one-stage reaction in which ER-derived vesicles are formed, targeted, and allowed to fuse with Golgi membranes already present in the microsomal fraction. This assay is essentially the same as the SIC assay, except that its overall efficiency is lower (Fig. 11.3.4). It is used mainly to confirm that the microsome and cytosol preparations are competent to reconstitute ER-to-Golgi transport.

The more detailed two-stage assay allows for vesicle formation and budding, targeting of ER-derived vesicles, and fusion with the Golgi to be studied independently. This allows the investigator to accurately assign a role for any particular factor to a distinct biochemical event. Similar assays have been developed in the yeast system for studying both ER-to-Golgi transport (Rexach and Schekman, 1991) and vacuolar inheritance (Conradt et al., 1994). These assays have proved to be powerful tools in determining the order of the distinct biochemical events that are involved and the role of general trafficking proteins in these events.

The first stage of the two-stage assay involves vesicle formation *in vitro*. When used on its own, this assay allows for the factors involved in vesicle budding from the ER to be studied, as the appearance of VSV-G in the high-speed pellet (HSP) is a direct measurement of vesicle formation. The ER-derived vesicles can also be studied as separate entities by immunopurification using anti-VSV-G antibodies. This allows the protein constituents of the vesicles to be determined. The combination of the first-stage vesicle formation reaction with a separate second-stage fusion reaction allows the targeting and fusion events involved in ER-to-Golgi transport to be studied independently of vesicle formation and budding and independently of each other. The relative contributions of vesicular components, cytosolic factors, and Golgi components to the separate

biochemical events can be determined using this assay.

Critical Parameters

All assays described in this unit are based on the synchronized movement of the ts045 mutant of VSV-G from the ER. To keep VSV-G ts045 in the ER, it is essential that the temperature does not drop below 39.5°C during the preparation of SICs and microsomes.

The preparation of SICs by mechanical perforation of hypotonically swollen cells produces a yield of 90% to 95% SICs for the cell lines used routinely in the authors' laboratory. The swelling procedure can be adjusted to achieve maximum perforation of other cell lines by changing the swelling time, the composition of the swelling buffer, or the degree of adherence to the tissue culture plate. In general, cells that adhere weakly require longer swelling times, while strongly adherent cells may not even require swelling before scraping. As an alternative to scraping, perforation can be performed using nitrocellulose stripping, which tears pieces of the plasma membrane away from the surface of the cell. Transport can then be assayed in the cells that remain attached to the nitrocellulose, or the cells that remain on the plate can be harvested by gentle scraping for an assay in suspension. In all cases, the degree of perforation can be determined by assessing trypan blue staining of the nuclei of perforated cells.

The preparation of microsomes with high budding activity is dependent on the homogenization conditions. The ball-bearing homogenizer breaks the cells efficiently, giving a high yield of membranes with high budding activity. The use of other types of homogenizers is not recommended.

In the microsome-based assay, the separation of budded vesicles from the microsomes is achieved by differential centrifugation. The largest reaction volume that does not require alterations in the centrifugation conditions is 100 μ l. Larger reaction volumes may change the sedimentation properties of the membranes; therefore, the centrifugation conditions for larger reaction volumes should be characterized empirically.

Sodium ions inhibit both the SIC assay and the microsome-based assay. The pH of all buffers used in these assays is therefore adjusted with KOH, rather than NaOH. Any protein added to the assays should first be dialysed against 25 mM HEPES acid, pH 7.2, containing 125 mM potassium acetate. If it is necessary to add a component in any buffer not described

Table 11.3.4 Troubleshooting Guide

Problem	Possible cause	Solution
High percent transport in cytosol-free control in SIC assay	Poor perforation of cells	Determine percent perforation; optimize protocol for the specific cell line
	Cytosol not completely washed out of cells	Incubate cells on ice 10 min after perforation ^a
Cannot resuspend membranes completely in preparing ER-derived vesicles ^b	Concentration and/or pH of resuspension buffer are incorrect	Check concentration of sucrose using a refractometer; check pH
	Salts added before resuspension	Use salt-free resuspension buffer and add salts only after complete resuspension
No transport in SIC assay; no budding in microsome assay	Sodium ions in reaction	Adjust pH of all solutions with KOH; dialyze additives against 125 mM potassium acetate, 25 mM HEPES, pH 7.2
No fusion in second-stage fusion reaction	Sodium ions and/or sucrose in reaction	Make sure that sucrose has not been used instead of sorbitol

^aSee Basic Protocol 1, step 12.

^bSee Basic Protocol 3, step 4.

here, the effects of this buffer on the assay should be determined. See Table 11.3.4 for a brief troubleshooting guide.

Anticipated Results

The preparation of SICs from NRK, BHK, and CHO cells by scraping results in the perforation of 90% to 95% of cells. The percentage of perforation should be determined using trypan blue staining, particularly if other cell lines are to be used (see Critical Parameters).

The typical result obtained for the SIC assay is shown in Figure 11.3.1. The amount of VSV-G transported is determined by comparing the amount of the endo H-resistant form with the total amount of VSV-G in the reaction. The appearance of the different forms of VSV-G is the same after immunoblotting to measure transport in the two-stage assay, as is shown in Figure 11.3.1 for ³⁵S-labeling in the SIC assay.

In the SIC assay, 65% to 70% of VSV-G is transported after 60 to 90 min. Transport efficiency is lower in the microsome-based assay, where 35% to 40% of VSV-G is transported after 60 to 90 min (Fig. 11.3.2).

Stage 1 of the two-stage microsome assay allows for the rate of budding to be measured. Under standard conditions, VSV-G appears in the HSP after ~5 min, peaks at between 10 and 20 min, and then decreases over time. The amount of VSV-G in the HSP depends on the budding activity of the microsomes as well as on the fusion competence of the membranes in the reaction. In general, 15% to 20% of the VSV-G

is found in the HSP at the peak time point. In stage 2 of the two-stage assay, 35% to 40% of the VSV-G in the HSP appears as the endo H-resistant form after 75 to 90 min (Fig. 11.3.2).

The purification of ER-derived vesicles using immunoprecipitation allows 25% to 30% of the VSV-G in the medium-speed supernatant (MSS) to be isolated with 2.5 to 5 μg p5D4 antibody/10⁷ beads. This represents only ~5% of the total starting VSV-G. In general, therefore, the MSS from three 100-μl budding reactions (stage 1) are pooled for every immunoprecipitation reaction.

Time Considerations

The SIC assay and the microsome-based assay take 3 days from beginning the experiment to analyzing the data. The actual hands-on time for the SIC assay is 4.5 hr for infection of the cells and 3 to 3.5 hr from the preparation of SICs to the addition of endo H. The hands-on time for the microsome-based assay is considerably shorter, requiring only 2 to 2.5 hr on the first day. Digestion is done overnight (minimum 16 hr). The polyacrylamide gel (200 × 150 × 1.5 mm) takes 4 to 5 hr to run. For ³⁵S analysis, the gel is incubated in fluorographic enhancement solution for 20 min, dried for ~1 hr, and exposed to film overnight. For the microsome-based assay, the endo H-treated proteins are transferred to nitrocellulose overnight (6.5 to 10 hr total transfer time), and immunoblot analysis is done the next day.

The budding reaction (stage 1) and the immunoprecipitation of vesicles can be done in one day. Preparation of the MSS, MSP, and HSP requires ~1.5 to 2 hr. Immunoprecipitation requires 2 hr, with an additional 30 to 45 min for washing the beads. A minigel takes ~1 hr to run, and the proteins are transferred to nitrocellulose for 1 hr. Immunoblot analysis takes 3 to 4 hr.

The two-stage assay also takes a total of three days, with 1.5 to 2 hr for stage 1 and ~2 hr for stage 2, both on the first day. After the addition of endo H, the time required is the same as for the microsome assay.

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Cotranslational Translocation of Proteins into Canine Rough Microsomes

UNIT 11.4

This unit describes the isolation of small vesicular fragments of rough endoplasmic reticulum (ER), known as “rough microsomes” (RMs), and their use in cell-free protein translocation assays. By faithfully reproducing the events associated with the transport of proteins into the ER, a cell-free assay provides a tool for analyzing ER targeting signals, signal-sequence cleavage, N-linked glycosylation, and membrane-protein biogenesis. Because translocation has been shown to be a strictly cotranslational process, purified RMs are added directly to wheat germ or rabbit reticulocyte translation reactions that are programmed with appropriate mRNAs (see *UNITS 11.1 & 11.2*). A variety of post-translational treatments are then performed to facilitate interpretation of the results of the translocation reaction. Finally, the proteins in the reaction mix are resolved by SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*). Translocation or membrane insertion of the translation product is indicated by mobility shifts due to signal-sequence cleavage and glycosylation, or by cofractionation with membrane components.

This unit describes the use of RMs isolated from canine pancreas (see Support Protocol 1) in cotranslational translocation assays and optional post-translational treatments (see Basic Protocol). Also included are methods of processing and purifying RMs that optimize their compatibility with in vitro translation reactions (see Support Protocols 2 and 3).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

TRANSLOCATION INTO CANINE ROUGH MICROSOMES

In this protocol secreted, luminal, and integral membrane proteins are translocated cotranslationally into RMs that are added to rabbit reticulocyte or wheat germ translation reactions. Because many proteins contain an N-terminal, ~20- to 30-amino-acid “signal sequence” that is removed by signal peptidase as the protein is transported into the RMs, translocation can often be assessed simply by looking for a slight increase in mobility on polyacrylamide gels in the presence of RMs (see Fig. 11.4.1). Proteins that are translocated into RMs are also resistant to protease digestion. The extent of N-linked glycosylation can be assessed by looking for a retardation of mobility on polyacrylamide gels that can be reversed by the addition of glycosidases. Finally, the integration of membrane proteins can be analyzed by testing for resistance to alkaline extraction.

Although a standard cotranslational translocation reaction is performed to analyze the transport of all proteins, a variety of different post-translational treatments can be used to generate additional information about protein topology and protein modifications. Because these treatments are applicable only to specific classes of proteins and only in certain experimental situations, the investigator should assess in advance which, if any, of these optional treatments (steps 5, 6, and 7) will be appropriate. Studies by Hansen et al. (1986) and Gafvelin et al. (1997) illustrate the utility of these post-translational treatments.

**BASIC
PROTOCOL**

**In Vitro
Reconstitution**

Contributed by Harris D. Bernstein

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11.4.1

Materials

- Wheat germ *or* reticulocyte translation mix (see *UNIT 11.2*)
- 1 eq/μl RM solution (see Support Protocol 1)
- mRNA encoding a protein of interest
- mRNA encoding a control cytoplasmic protein
- Buffer C (see recipe)
- 30% trichloroacetic acid (TCA; store at 4°C), ice cold
- For optional steps:
 - 20 mg/ml proteinase K (store at –20°C)
 - Nonionic detergent: e.g., 20% Triton X-100 or Nonidet P-40 (NP-40; store at –20°C)
 - 25% sodium dodecyl sulfate (SDS; store at room temperature)
 - 1 M dithiothreitol (DTT; *APPENDIX 2A*)
 - Buffer D (see recipe)
 - Endoglycosidase H (purchased as solid or stock solution)
 - 100 mM sodium carbonate, pH 11.5, ice cold
 - Glacial acetic acid
- 1× SDS sample buffer (*APPENDIX 2A*)
- Water bath or heating block, 26° or 30°C and (for optional step) 100°C
- Tabletop ultracentrifuge and rotor (e.g., Beckman TLA 100.2 rotor; for optional step)
- Additional reagents and solutions for in vitro translation (*UNIT 11.2*) and one-dimensional SDS-PAGE (*UNIT 6.1*)

1. Determine the number and total volume of in vitro translation reactions that are required for a given experiment (*UNIT 11.2*). Keeping all reagents on ice, make a master translation mix of the components that are going to be needed in all reactions (e.g., wheat germ extract, radioactive amino acids). Pipet the appropriate amount of master mix into each reaction tube, including tubes to be used for control reactions.

Making a master mix considerably diminishes pipetting errors and reduces the number of pipetting steps needed to set up the reactions. It is a good idea to include separate control reactions lacking mRNA and lacking RMs. Because translocation of proteins into RMs is often highly efficient, small reaction volumes (e.g., 10 μl) are generally sufficient. Small-scale reactions should be performed in 0.5-ml microcentrifuge tubes. Larger reaction volumes can be used in cases where reactions are to be divided for post-translational treatments (e.g., digestion with endoglycosidase H).

2. Add 0.1 to 1 eq of RMs (0.1 to 1 μl of solution) per 10 μl of translation mix to each reaction; for RM-free control reactions, add an equivalent volume of buffer C instead. Then add appropriate amounts of mRNA (or, for RNA-free controls, buffer C). Incubate the reactions 40 to 60 min at 26°C for wheat germ reactions, or 30°C for rabbit reticulocyte translations.

Generally 10 to 50 ng of an efficiently translated mRNA per 10 μl of translation mix will give a good signal, but higher amounts can also be added.

The concentration of RMs that is added to the reaction is generally not critical, but in some cases it may be necessary to determine an optimal concentration empirically. Use one that promotes efficient translocation of a given protein and has a minimal inhibitory effect on the translation reaction. Addition of an equimolar amount of an mRNA that encodes a cytoplasmic protein (e.g., globin) to each reaction serves as a good control.

3. Stop the reactions by transferring the tubes to an ice bath.

4. If post-translational treatment(s) of the samples (e.g., protease digestion) are desired, proceed to steps 5, 6, and/or 7. Otherwise, precipitate the proteins by adding 1 vol of 30% TCA and incubating 10 min on ice; then proceed to step 8.
5. *To show that a protein or domain has been transported into the RM lumen (optional):*
 - a. Add proteinase K to 100 $\mu\text{g/ml}$ (final concentration). Incubate separate aliquots in the presence and in the absence of 0.5% to 1% nonionic detergent for 30 to 60 min at 0°C.
 - b. Stop the protease digestion by adding 2 mM PMSF (final). Incubate an additional 15 min at 0°C.
 - c. Precipitate proteins with 30% TCA (see step 4).
 - As an additional control, addition of detergent will lyse the RMs and expose a translocated protein to protease digestion. Optionally, addition of 1 mM CaCl_2 to the samples prior to protease digestion can help to stabilize the membranes and may increase the yield of protease-protected protein.*
6. *To remove N-linked carbohydrates from translocated proteins (optional):*
 - a. Add 2% SDS and 75 mM DTT (final concentrations). Boil samples for 3 min.
 - b. Dilute samples 20-fold in buffer D and add 1 $\mu\text{g/ml}$ endoglycosidase H (final). Incubate 12 to 16 hr at 37°C.
 - c. Precipitate proteins using 30% TCA (see step 4).
 - Although endoglycosidase H is most commonly used, other glycosidases that remove N-linked carbohydrates (e.g., endoglycosidase F) can be substituted.*
7. *To test for membrane integration (optional):*
 - a. Dilute translation reactions 100-fold with ice-cold 100 mM sodium carbonate, pH 11.5. Incubate 30 min on ice.
 - b. Centrifuge 30 min at $360,000 \times g_{\text{av}}$ (100,000 rpm in a Beckman TLA 100.2 tabletop ultracentrifuge rotor), 4°C.
 - c. Carefully remove the supernatant and neutralize with glacial acetic acid. Precipitate proteins with 30% TCA (see step 4).
 - d. Meanwhile, dissolve the pellet, containing ribosomes and membrane fragments, directly in 1 \times SDS sample buffer (this can be run on an SDS-polyacrylamide gel without further manipulation).
 - After the centrifugation, integral membrane proteins will be found in the pellet but soluble proteins will be found in the supernatant.*

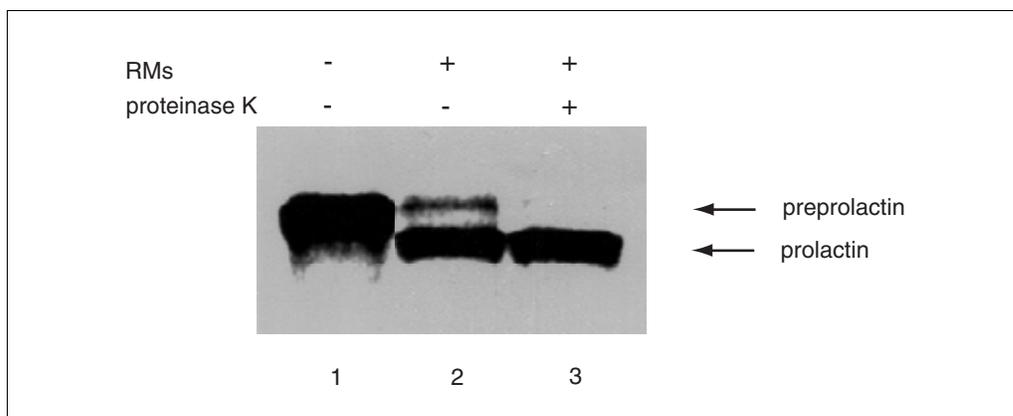


Figure 11.4.1 Translocation of preprolactin into canine pancreatic RMs. An mRNA encoding bovine preprolactin was translated in a wheat germ extract in the absence of microsomes (lane 1) or in the presence of RMs (lanes 2 and 3). After the translation was stopped, proteinase K was added to the sample shown in lane 3.

8. Pellet precipitated proteins by microcentrifuging 5 min at full speed, 4°C. Remove supernatant carefully. Boil in 1× SDS sample buffer for 3 to 5 min or until the pellet is dissolved.

If the TCA is not removed carefully, the samples may remain acidic (the bromphenol blue in the sample buffer will turn yellow). This problem can be remedied by adding small amounts of 1 M Tris base.

9. Assess protein translocation and modification by SDS-polyacrylamide gel electrophoresis (UNIT 6.1).

The gel shown in Figure 11.4.1 illustrates how protein translocation can be assessed by this method. In the absence of RMs, an mRNA encoding bovine preprolactin directs the synthesis of a 26-kDa protein (lane 1). In the presence of microsomes, efficient translocation of the preprotein results in the removal of the signal sequence and the production of the faster-migrating prolactin form (lane 2). In the presence of proteinase K, essentially all of the prolactin is protected from digestion, but residual untranslocated preprolactin is degraded (lane 3).

SUPPORT PROTOCOL 1

PREPARATION OF RMs FROM CANINE PANCREAS

This protocol describes the isolation of RMs from canine pancreas. The extremely high yield, purity, and translocation competence of RMs obtained from canine pancreas makes it an ideal source for these vesicles. In the method described here, the cells of the pancreas are first disrupted by homogenization. Then nuclei, secretory granules, plasma membranes, and mitochondria are removed to produce a “post-mitochondrial supernatant” containing Golgi and vesicular fragments of ER produced by shearing. The RMs are pelleted through a discontinuous sucrose gradient and resuspended in a buffer that is compatible with their use in protein translocation assays.

NOTE: All steps in this protocol (except dissection and centrifugation) should be performed in a cold room at 4°C. Because it is important to work as quickly as possible after dissecting the pancreas and to keep the tissue cold, all solutions should be prechilled and all equipment and glassware should be placed in the cold room in advance.

Materials

- Buffer A (see recipe), ice cold
- Dog (e.g., beagle weighing 10 to 12 kg)
- Buffer B (see recipe), ice cold
- Buffer C (see recipe), ice cold
- 1% (w/v) SDS (APPENDIX 2A), ice cold
- Liquid nitrogen
- Dissecting tools: small scissors, regular and fine forceps, scalpel (with new blade)
- Small plastic cutting board
- Single-edge razor blades (at least 5)
- Potter-Elvehjem-style tissue homogenizer (e.g., B. Braun Biotech) with 60-ml cylinder and matched Teflon plunger
- 50-ml polycarbonate or polypropylene centrifuge tubes (~28.5-mm diameter)
- Medium-speed centrifuge and rotor (Sorvall SS-34 or Beckman JA20, or equivalent)
- 25 × 89-mm (26-ml) polycarbonate ultracentrifuge bottles with tops
- 10-ml glass syringe fitted with a round-tipped pipetting needle
- Ultracentrifuge and rotor (Beckman Ti 50.2 or equivalent)
- 60-ml Dounce homogenizer fitted with type A pestle

Homogenize the pancreas

1. Cover work area with absorbent plastic-coated bench covering. Place ~100 ml ice-cold buffer A (enough to keep the tissue covered) in a 250-ml glass beaker and immerse in ice until fully chilled.
2. Sacrifice the dog, then make a large ventral incision into the abdominal cavity and isolate the pancreas. Dissect the pancreas as quickly as possible and immediately transfer it to the beaker of ice-cold buffer A.

The pancreas is an elongated, lobulated, pinkish-gray organ that runs along the small intestine and stomach. To locate it, displace the liver and find the stomach. The pancreas will reside at the junction of the stomach and descending duodenum. Grasp the pancreas and gradually detach it from other tissues. The portion of the pancreas that is attached to the duodenum (“right lobe”) is most accessible and should be detached first. Generally it is helpful to have a second person keep the pancreas exposed by holding or pushing away other organs.

If available, tissue can be obtained from dogs that are used as research subjects in other projects. Most common anesthetics, methods of sacrifice, and experimental manipulations that do not affect pancreatic function are compatible with the isolation of translocation-competent RMs. It is absolutely essential, however, to remove the pancreas as quickly as possible after sacrifice to avoid tissue necrosis and degradation of ER components that are required for protein translocation. Provided that the animal is in good health, age and sex do not appear to have a significant effect on the isolation of RMs. Because the weight of the pancreas correlates with the size of the animal, the largest quantities of RMs are obtained from large dogs.

3. Rinse the pancreas in fresh ice-cold buffer A to remove blood and other debris. Place the pancreas on a piece of Parafilm. Using a scissors and fine forceps, remove major blood vessels and adipose and connective tissues from the pancreas.

The Parafilm will help to visualize fat and connective tissue that is connected to the pancreas. It is critical to clean the pancreas carefully because extraneous tissues can interfere with effective homogenization. It is also important, however, to work rapidly to minimize necrosis of the pancreas.

4. Weigh the pancreas (keeping it in a chilled beaker) and mince finely using fresh razor blades. Change razor blades often as they become dull.

The pancreas of a 10- to 12-kg dog typically weighs 20 to 30 g.

5. Place the tissue pieces in a clean beaker and add a volume of ice-cold buffer A equal to four times the weight of the pancreas.
6. Stir the tissue pieces and buffer together to create a uniform mixture. Homogenize 60-ml aliquots of the mixture using a motor-driven Potter-Elvehjem-style instrument.

Five complete up-and-down strokes is generally sufficient to break the cells effectively. It is essential to avoid significant heating of the homogenate during this step; for this reason instruments in which the homogenizer cylinder fits into a container that can be filled with ice (e.g., the unit made by B. Braun Biotech) are recommended.

Isolate RMs

7. Pour the homogenate into a clean beaker and mix well, then transfer to 50-ml centrifuge tubes. Centrifuge 10 min at $700 \times g_{av}$ (3000 rpm in a Sorvall SS-34 or Beckman JA-20 rotor), 4°C.
8. Carefully remove the fat layer at the top of each centrifuge tube by aspiration, then pour the supernatants into a clean beaker. Mix the pooled supernatants well and transfer to clean 50-ml centrifuge tubes. Centrifuge 10 min at $8000 \times g_{av}$ (10,000 rpm in a Sorvall SS-34 or Beckman JA-20 rotor), 4°C.

9. Pour the “post-mitochondrial” supernatants into a clean beaker.

The pellets that are formed after the second centrifugation step are very soft, so pour off the supernatants carefully.

10. Pipet 16 ml of the pooled supernatants (cell extract) into 25 × 89-mm (26-ml) polycarbonate ultracentrifuge bottles.
11. Fill a 10-ml glass syringe fitted with a round-tipped pipetting needle with 8 ml ice-cold buffer B. Underlay the cell extract with this buffer. Close the bottles and centrifuge 2.5 hr at $145,000 \times g_{av}$ (40,000 rpm in a Beckmann 50.2 Ti rotor), 4°C.

In this centrifugation step the RMs should form a tight pellet. If substantial degradation of microsomal proteins and attached ribosomes occurs during the preparation, a tight pellet will not be formed; such samples should be discarded.

12. Remove the top layer carefully by aspiration and discard. Next, remove the broad cloudy band at the interface, which is enriched with Golgi. Finally, remove the buffer B cushion.
13. Add a few milliliters of ice-cold buffer C to each tube and resuspend the pelleted RMs with a rubber policeman. Transfer the resuspended pellets to a 60-ml Dounce homogenizer. Collect residual RMs by washing the tubes with a few more milliliters of buffer C.

The total volume of buffer C used to resuspend the RMs should not exceed 80% of the initial weight of the pancreas. The large off-white pellets will be resuspended as large clumps that require further dispersal.

14. Disperse the RMs in the Dounce homogenizer using five complete up-and-down strokes. Transfer the RMs to a clean 100-ml graduated cylinder.
15. Remove two 10- μ l aliquots and add each to 1 ml ice-cold 1% SDS; wipe off the the pipet tip before adding its contents to the SDS solution, because a considerable volume will stick to the outside. Pipet back and forth several times to transfer all of the RMs from inside the tip.
16. Read the absorbance of each sample at 280 nm. Using the average of the two readings, adjust the concentration of the microsomes to 50 A_{280} U/ml.

By definition this solution contains one equivalent (eq) of RMs per microliter.

17. Split the RMs into aliquots of an appropriate size and freeze in liquid nitrogen.

After adjusting the concentration of RMs as described here, the final yield should be ~1 to 1.5 ml per gram of pancreas. The RMs can be frozen and thawed at least several times without loss of activity, so a single aliquot can be used for multiple experiments.

SUPPORT PROTOCOL 2

PREPARATION OF EDTA-STRIPPED ROUGH MICROSOMES

When RMs are added to reticulocyte lysate translations, a high background of polypeptide synthesis can sometimes result from the readout of mRNAs that are attached to membrane-bound polysomes. To solve this problem, membrane-bound ribosomes can be removed by a simple EDTA treatment.

NOTE: All steps in this protocol should be performed in a cold room at 4°C. All solutions should be prechilled and all equipment and glassware should be placed in the cold room in advance.

Additional Materials (see Support Protocol 1)

Crude RM fraction (see Support Protocol 1)
Buffer C (see recipe)/50 mM EDTA, ice cold
Buffer C/0.5 M sucrose, ice cold

1. Add 1 vol buffer C/50 mM EDTA to a crude RM fraction. Incubate 15 min at 0°C.
2. Place sucrose cushion, consisting of buffer C/0.5 M sucrose, that is approximately half the volume of the RM mixture, into an ultracentrifugal tube. Carefully load the RMs over the sucrose cushion using a pipet, holding the pipet against the side of the tube to avoid disturbing the cushion. Centrifuge 1 hr at $145,000 \times g_{av}$ (40,000 rpm in a Beckmann 50.2 Ti rotor), 4°C.
3. Resuspend the pelleted membranes to half their original volume in buffer C (without EDTA or sucrose). Disperse the vesicles in a Dounce homogenizer (see Support Protocol 1, steps 13 and 14) and transfer to a graduated cylinder.
4. Adjust to the original volume with buffer C. The stripped RMs are now ready to be used or stored.

PREPARATION OF COLUMN-WASHED ROUGH MICROSOMES

Occasionally batches of RMs can inhibit *in vitro* translation reactions. This problem can be solved by separating the RMs from ribosomes and other contaminants using a gel filtration column.

NOTE: All steps in this protocol should be performed in a cold room at 4°C. All solutions should be prechilled and all equipment and glassware should be placed in the cold room in advance.

Additional Materials (also see Support Protocol 1)

Sepharose CL-2B resin (Pharmacia Biotech)
Buffer E (see recipe), ice cold
Crude RM fraction (see Support Protocol 1)

Chromatography column of volume equivalent to ten times the volume of RMs to be treated
Ultracentrifuge and rotor (Beckman 45 Ti rotor or equivalent)

1. Prepare a Sepharose CL-2B column with a column volume equivalent to ten times the volume of RMs to be treated. Equilibrate with several column volumes buffer E.
2. Load RMs onto Sepharose CL-2B column.
3. Collect fractions that represent the void volume.
4. Pool the turbid fractions and centrifuge 30 min at $85,000 \times g_{av}$ (35,000 rpm in a Beckman 45 Ti rotor), 4°C.
5. Resuspend the pelleted membranes to half their original volume in buffer C.
6. Disperse the vesicles in a Dounce homogenizer (see Support Protocol 1, steps 13 and 14) and transfer to a graduated cylinder.
7. Adjust to the original volume with buffer C. The washed RMs are now ready to be used or stored.

**SUPPORT
PROTOCOL 3**

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX. Adjust the pH of all stock solutions with acetic acid.

Buffer A

50 mM triethanolamine, pH 7.5
50 mM potassium acetate, pH 7.5
6 mM magnesium acetate
1 mM EDTA
250 mM sucrose
1 mM DTT (added immediately before use; APPENDIX 2A)
0.5 mM PMSF (added immediately before use; APPENDIX 2A)

Store solution without DTT and PMSF indefinitely at 4°C.

Preparation of one canine pancreas requires 500 ml of buffer A.

Buffer B

50 mM triethanolamine, pH 7.5
6 mM magnesium acetate
1 mM EDTA
1.3 M sucrose
1 mM DTT (added immediately before use; APPENDIX 2A)
0.5 mM PMSF (added immediately before use; APPENDIX 2A)

Store solution without DTT and PMSF indefinitely at 4°C.

Preparation of one canine pancreas requires 100 ml of buffer B.

Buffer C

50 mM triethanolamine, pH 7.5
250 mM sucrose
1 mM DTT (added immediately before use; APPENDIX 2A)

Store solution without DTT indefinitely at 4°C.

Preparation of one canine pancreas requires 100 ml of buffer C.

Buffer D

50 mM triethanolamine, pH 7.5
1.5 mM magnesium acetate
1 mM EDTA
1 mM DTT (added immediately before use; APPENDIX 2A)

Store solution without DTT indefinitely at room temperature.

Buffer E

100 mM sodium citrate, pH 5.5
0.5 mM PMSF (added immediately before use; APPENDIX 2A)

Store solution without PMSF indefinitely at room temperature.

COMMENTARY

Background Information

Pioneering studies conducted by Palade and co-workers in the 1960s demonstrated that proteins that travel through the secretory pathway in mammalian cells are synthesized on endoplasmic reticulum (ER)-bound ribosomes and are translocated across the ER membrane (Palade, 1975). The subsequent discovery that

the transport of proteins across the ER membrane, and the associated modifications, can be reproduced in vitro by adding rough microsomes (RMs) to heterologous cell-free translation reactions (Blobel and Dobberstein, 1975; Katz et al., 1977) was a remarkable breakthrough. These studies led to the development of methods for assessing protein translo-

cation (signal-sequence cleavage, glycosylation), formulation of the “signal hypothesis” (the idea that proteins contain sequence information that earmarks them for transport into the ER), and molecular dissection of the transport process itself.

Current understanding of the molecular mechanism whereby proteins are targeted to and translocated across the ER membrane has been derived largely from experiments in which canine pancreatic RMs have been added to wheat germ translation reactions. In early studies it was shown that a soluble ribonucleoprotein complex called the signal recognition particle (SRP) is required for protein transport into RMs. SRP recognizes the signal sequences of secreted proteins as they emerge from translating ribosomes (Walter and Blobel, 1981) and then targets ribosome-nascent chain complexes to the ER via an interaction with the ER-bound SRP receptor. Because SRP binds only to signal sequences that are part of a nascent polypeptide chain, protein translocation does not occur if microsomes are added post-translationally. The interaction between SRP and the SRP receptor facilitates release of the nascent chain (Gilmore et al., 1982; Meyer et al., 1982) and its insertion into a proteinaceous translocation channel comprised of the heterotrimeric Sec61p complex and an auxiliary protein called TRAM (Görlich et al., 1992; Görlich and Rapoport, 1993). Available evidence suggests that the translocation channel has properties associated with gated ion channels (Simon and Blobel, 1991; Crowley et al., 1994). During translocation, signal sequences are cleaved by the hexameric signal peptidase complex (Evans et al., 1986) and N-linked sugars are attached by the heterotrimeric oligosaccharyl transferase (Kelleher et al., 1992).

In addition to providing information about protein localization *in vivo* and insight into the mechanism of protein transport, the translocation assay described here has many other applications. Because signal-sequence processing occurs faithfully *in vitro*, N-terminal sequencing of the mature portion of a protein can be used to locate the signal-sequence cleavage site. For the large class of proteins that do not have an N-terminal signal sequence, deletion analysis can be performed to identify targeting sequences, as demonstrated, for example, by the work of Spiess and Lodish (1986). In addition, protease protection analysis following the insertion of membrane proteins can be used to study the topology of membrane proteins, which often cannot be deduced from the pri-

mary amino acid sequence (see, for example, Lipp and Dobberstein, 1986; Gafvelin et al., 1997).

Although canine pancreas is the most common source of RMs used in protein translocation studies, translocation-competent RMs have also been derived from a wide variety of other tissues including sheep pancreas (Kaderbhai et al., 1995), rat liver (Bielinska et al., 1979), ascites tumor cells (Bielinska et al., 1979), hen oviduct (Das et al., 1980), and *Drosophila* embryos (Brennan et al., 1980). Translocation-competent microsomes have also been obtained from *Saccharomyces cerevisiae* (Hansen et al., 1986), but these vesicles promote a post-translational, SRP-independent translocation reaction. Attempts to isolate translocation-competent microsomes from pig, rat, fish, and cow pancreas have not been successful (Kaderbhai et al., 1995; P. Walter, pers. comm.). At least in some of these cases it appears that the presence of high concentrations of endogenous ribonucleases destroys the integrity of the RMs. The ability to isolate translocation-competent RMs from different sources may facilitate the analysis of tissue-specific protein translocation or protein modification phenomena.

Most methods for isolating RMs are based on the observation that the attachment of ribosomes to rough ER imparts an especially high density to vesicles derived from that organelle. This high density facilitates purification of RMs on a discontinuous density gradient. In the method described here, RMs are pelleted through a 1.3 M sucrose layer whereas “smooth” microsomes, comprised mostly of Golgi and smooth ER, float at the interface between the low- and high-sucrose layers. This method works well for the isolation of RMs from specialized secretory organs such as the pancreas in which a very large percentage of the total membrane is rough ER. In a successful preparation, >90% of the high-density pellet is comprised of RMs; contamination with free ribosomes and other cellular components is minimal. Alternatively, RMs can also be purified by flotation on a very high-density (2 M) sucrose layer (Mechler and Vassalli, 1975; Behrens et al., 1996). This also yields highly purified RMs, but has the disadvantage that the vesicles may be recovered in a larger volume than desired and may require concentration by centrifugation. This method may be particularly useful in cases where pelleting the RMs would lead to unwanted contamination with free ribosomes or other cellular components.

Critical Parameters and Troubleshooting

It is essential to homogenize the pancreas effectively to obtain a good yield of RMs. Although it is neither necessary nor practical to remove all of the connective tissue and fat associated with the pancreas, excess extraneous tissue can interfere with homogenization. A good way to minimize this problem is to mince the pancreas as finely as possible before homogenization. Pieces of pancreas that prove difficult to disrupt can be removed from the homogenizer and minced again. In pelleting RMs through a sucrose cushion, the rotor and the size of the centrifuge bottles are not critical. It is important, however, to have a 2:1 to 3:1 ratio of cell extract to sucrose cushion to ensure a good separation of the large RM pellet from the smooth microsome interface.

It is very likely that pancreatic RMs obtained at the expected yield (see Support Protocol 1) will work well in translocation assays. Because the efficiency of translocation and glycosylation of different proteins can vary (see Anticipated Results), a new batch of RMs should be tested for activity using a protein that is known to be a good translocation/glycosylation substrate (e.g., preprolactin or influenza hemagglutinin). Sometimes RMs can inhibit translation or give rise to a high background of unwanted protein synthesis, but these problems can generally be eliminated by pretreating the RMs (see Support Protocols 2 and 3). Translocation assays can also be difficult to interpret if the translation reaction itself is inefficient. The optimization of translation conditions is discussed in *UNIT 11.2*.

Anticipated Results

Many but not all proteins are translocated across or inserted into canine RMs with high efficiency (50% to 100%). A few secreted proteins, in particular those that contain unusual signal sequences, are translocated very poorly under the conditions described here (Belin et al., 1996). It is possible that proteins in this class require as yet unidentified tissue-specific factors to promote their translocation.

Whereas signal-sequence cleavage is extremely effective *in vitro*, the efficiency of core glycosylation is much more variable. In some cases only a small fraction of translocated protein is glycosylated. Because glycosylation is often incomplete, several different partially modified forms of a protein that has multiple glycosylation sites may be observed on polyacrylamide gels. Post-translational treat-

ment of translocation reactions with protease (see Basic Protocol, step 5) will reduce the translational background and may facilitate the visualization of weakly glycosylated forms.

A single pancreas from a 10- to 12-kg dog should weigh 20 to 30 g and yield a high-density pellet that is >90% rough microsomes with minimal contamination from free ribosomes and other cellular components. This preparation yields sufficient rough microsomes for many thousands of small-scale assays (using 1 μ l of microsomes per reaction).

Time Considerations

The preparation of canine RMs (Support Protocol 1) can be completed in ~5 to 6 hr. The time required depends in part on the size of the pancreas; it takes longer to process a larger amount of tissue. Dissection of the pancreas, homogenization, and preparation of the post-mitochondrial supernatant requires ~1.5 to 2 hr of continuous work. Glassware and buffer C should be chilled during the subsequent 2.5-hr centrifugation step, but otherwise no work is required. Resuspension of the RMs and adjustment of the RM concentration should require <1 hr of hands-on time.

The time required to perform cotranslational translocation assays and post-translational treatments varies widely. A simple experiment involving a small number of reactions may be completed in 1.5 to 2 hr. The reactions can be assembled in <10 min. After a 40- to 60-min incubation period, an additional 20 min is required to precipitate and collect the proteins. A complex experiment with a large number of samples and different post-translational treatments may require a couple of hours of hands-on work plus incubation times (endoglycosidase H digestion usually requires an overnight incubation). The individual components of the translation and translocation reaction are very stable at 0°C, so the length of time required to assemble the reactions is not a concern. In addition, the incubation time for the translation-translocation reaction is not critical. These reactions are limited by the duration of the protein synthesis reaction, which is generally ~60 min. Shorter incubations will produce a lower signal but otherwise will give essentially the same results. After proteins are precipitated, samples may be left on ice for several hours. After the proteins are pelleted and the TCA is removed, the pellets are stable at -80°C for at least 1 week.

Both EDTA stripping of RMs (Support Protocol 2) and column washing of RMs (Support

Protocol 3) should take ~2 hr, although the time required for the latter procedure will depend to some extent on the flow rate of the column.

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Key Reference

Blobel and Dobberstein, 1975. See above.

Landmark paper containing the first clear demonstration that a presecretory protein synthesized in a cell-free translation system is transported vectorially into ER vesicles.

Contributed by Harris D. Bernstein
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In Vitro Analysis of SV40 DNA Replication

UNIT 11.5

The early studies of bacterial DNA replication provided a platform for constructing the theory of eukaryotic DNA replication. In the absence of well-defined origins of DNA replication, however, study of the eukaryotic DNA replication was almost impossible until the efficient cell-free system that supported replication from the SV40 origin of replication was developed. The system was designed to bypass the de novo replication initiation by providing SV40 origin of replication and viral initiator protein T antigen in the presence of replication-competent extract. Replication is carried out completely by cellular proteins in the presence of the viral helicase T antigen and viral origin-containing plasmid. Replication initiates at the replication origin, which is recognized by T antigen, and proceeds bilaterally in a semiconservative manner. Many cellular replication proteins have been identified through study of the SV40 replication system.

This unit outlines general methods for in vitro SV40 DNA replication and product analysis, including incorporation of nucleotides by binding to DE81 paper (see Basic Protocol) and visualization of products after alkaline denaturing gel electrophoresis (see Alternate Protocol); methods for growing a large suspension culture for the extract (see Support Protocol 1) and for preparing a replication-competent S100 cell extract from 293 cells (see Support Protocol 2); support information for the expression of T antigen in insect cells (see Support Protocol 3); and procedures for purifying T antigen from baculovirus-infected insect cells (see Support Protocol 4) and preparing the Pab419 immunoaffinity column used for the purification (see Support Protocol 5).

IN VITRO REPLICATION OF PLASMIDS BEARING SV40 ORIGIN SEQUENCES

**BASIC
PROTOCOL**

This protocol is used to study in vitro SV40 replication. The reaction consists of a mixture of T antigen and replication-competent S100 cell extract in the presence of SV40 origin-containing plasmid and [α^{32} P]ATP. Radiolabeled reaction products are analyzed by liquid scintillation counting. The reaction products can also be analyzed by agarose gel electrophoresis and autoradiography (see Alternate Protocol).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by local radiation safety officer (also see *APPENDIX 1D*).

Materials

- 5 \times replication buffer (see recipe)
- Supercoiled SV40 origin-containing DNA template
- 2 U/ μ l creatine phosphokinase in 10 mM Tris-Cl, pH 8.0/50% (w/v) glycerol
- Recombinant T antigen (see Support Protocol 4)
- [α^{32} P]dATP (800 or 6000 Ci/mmol; final volume 0.0125 ml)
- Replication-competent S100 cell extract (see Support Protocol 2)
- DE81 paper (Whatman)
- 0.5 M Na₂HPO₄
- 100% ethanol
- Scintillation cocktail

**In Vitro
Reconstitution**

11.5.1

1. Set up a reaction in a microcentrifuge tube containing:

10 μ l 5 \times replication buffer
300 ng supercoiled SV40 origin-containing DNA template
0.1 μ l 2 U/ μ l creatine phosphokinase
0.7 μ g T antigen
0.05 μ l [α -³²P]dATP (~200 to 1000 cpm/pmol)
5 to 20 μ l replication-competent S100 cell extract
H₂O to 50 μ l.

Incubate 1 hr at 37°C.

A negative control without T antigen should be included in the reaction, and the result should be treated as a baseline. Titration of the S100 is recommended to obtain optimal conditions for the reaction. Trichloroacetic acid precipitation can also be used for quantification of incorporated radioactivity.

Because the volume of reagents in a single replication reaction is often minute, it can be helpful to make a master mix before setting up the reactions. The specific activity is determined as follows (where 125 pmol is the amount of dATP present in one-tenth input):

cpm of one-tenth input from master mix for each reaction \div 125 pmol

The replication assay can also be done with purified replication proteins, protein fractions, or combinations instead of the S100 cell extract. The reaction buffer and conditions for SV40 replication remain the same.

2. Stop the reaction by placing tubes on ice.

The sample can then be analyzed for incorporation of radioactivity and by agarose gel electrophoresis and autoradiography (see Alternate Protocol).

3. Draw a grid of 1 \times 1-cm squares on a sheet of DE81 paper and label the squares accordingly.
4. Spot 5 μ l (one-tenth of output) of each reaction on the corresponding 1 \times 1-cm square of DE81 paper.

Do not allow the spots to dry.

5. Wash DE81 paper successively three times with 0.5 M Na₂HPO₄, once with double-distilled water, and once with 100% ethanol, 5 min per wash.
6. Dry paper with hair dryer or table lamp. Cut and put the squares in scintillation vials.
7. Add liquid scintillation cocktail. Count the radioactivity on squares. To determine specific activity of dATP, spot one-tenth of master mix on a square and count without washing; then calculate specific activity as described in step 1.
8. Determine the amount of dAMP incorporated in each reaction (in picomoles) using the following formula:

$[(\text{cpm of the DE81 square}) \times 10] \div \text{specific activity.}$

ALTERNATE PROTOCOL

ANALYSIS OF DNA REPLICATION REACTION PRODUCTS BY ALKALINE DENATURING AGAROSE GEL

Reaction products can also be analyzed by alkaline denaturing agarose gel electrophoresis to visualize the replicated DNA. Alternatively, one can analyze the replication products using a regular TAE or TBE agarose gel; however, the size range of nascent DNA products cannot be appreciated when using a nondenaturing gel.

Additional Materials (also see Basic Protocol)

- 3× stop solution (see recipe)
- 20 mg/ml high-quality glycogen (Life Technologies)
- 5 M NaCl (APPENDIX 2A)
- TE buffer, pH 8.0 (APPENDIX 2A)
- Agarose
- 1 N NaOH
- 0.5 M EDTA (APPENDIX 2A)
- 2× alkaline loading buffer (see recipe)
- Alkaline running buffer, made fresh (see recipe)
- 7% trichloroacetic acid (TCA)

Additional reagents and equipment for phenol/chloroform extraction and ethanol precipitation of DNA (APPENDIX 3), agarose gel electrophoresis (APPENDIX 3), and autoradiography (UNIT 6.3)

1. Perform *in vitro* replication reaction (see Basic Protocol, step 1).
2. Add $\frac{1}{3}$ vol of 3× stop solution to the replication reaction. Incubate the reaction 30 min at 37°C for protein digestion.
3. Dilute the reaction volume to 100 μ l with water. Remove the proteins by phenol/chloroform extraction.
4. Precipitate the DNA by adding 1 μ l of 20 mg/ml high-quality glycogen (as carrier) and 250 μ l of 100% ethanol.
5. Microcentrifuge 10 min at room temperature or 4°C, discard supernatant, and dissolve the DNA pellet in TE buffer.
6. Make a 1% (w/v) alkaline agarose gel: dissolve agarose in water by microwaving, cool the gel to 50°C, and add 1 N NaOH and 0.5 M EDTA to final concentrations of 30 mM and 1 mM, respectively.

Do not microwave agarose with NaOH, as this will prevent the gel from solidifying.

7. Add 1 vol of 2× alkaline loading buffer to tube and add the DNA samples. Run gel at 2 to 7 V/cm in freshly made alkaline running buffer.
8. Stop electrophoresis when xylene cyanol has migrated two-thirds of the length of the gel (12 to 14 hr). Neutralize gel in 7% TCA 30 min at room temperature.

Do not shake, as this may allow the solution to splash, contaminating the shaker with isotope.

9. Vacuum dry gel at low temperature (60°C) and autoradiograph (see UNIT 6.3).

PREPARING 293 CELL SUSPENSION CULTURE

This protocol provides guidance for expanding cells from a small-scale monolayer to a large-scale spinner culture for S100 cell extract. The cells are split to 1×10^5 cells/ml whenever the concentration reaches $0.5\text{--}1 \times 10^6$ cells/ml and are periodically checked for viability and density.

Materials

- Ten 10-cm plates of confluent 293 cell culture
- DMEM with 5% (v/v) calf serum
- Joklik's medium (JRH Bioscience) with 5% (v/v) calf serum

Additional reagents and equipment for trypsinizing monolayer cells (UNIT 1.1), counting cells, and assessing viability (UNIT 1.1).

**SUPPORT
PROTOCOL 1**

**In Vitro
Reconstitution**

11.5.3

1. Trypsinize 10-cm plates of confluent 293 cells (*UNIT 1.1*). Resuspend the trypsinized cells from ten 10-cm plates in variable amount of medium.
2. Pool cells together and determine cell count by hemacytometer.
3. Inoculate cells at a density of 1×10^5 cells/ml in suitably sized spinner flask, and maintain as suspension culture in Joklik's medium.

A good way to check if the cells are maintained as a suspension culture is to examine a drop of the culture under a light microscope: The cells should be individualized and round.

4. Monitor cells regularly (every 3 days) for viability (by Trypan blue; see *UNIT 1.1*) and density.
5. When cell density reaches 1×10^6 cells/ml, split cells by adding Joklik's medium directly to the growing cells to adjust their density to 1×10^5 cells/ml. Change to a bigger container when culture exceeds the recommended volume of the flask. Repeat this step several times, until there are enough cells for a 10-liter inoculation.
6. Split each 10-liter culture into one 8-liter and one 2-liter spinner culture. Check cell viability and density regularly (every 3 days).
7. When the cell density reaches 1×10^6 cells/ml, harvest the 8-liter culture for S100 cell extract, and split the 2-liter culture for another round of 10-liter inoculation (2 liters + 8 liters).

Maintain a 2-liter suspension culture of 293 cells to significantly shorten the time required for preparation of lab stock S100.

**SUPPORT
PROTOCOL 2**

PREPARATION OF A REPLICATION-COMPETENT S100 CELL EXTRACT

This protocol describes how to make replication-competent cytosolic cell extracts from an exponentially growing 293 suspension culture. The replication-competent S100 cell extract is used to provide replication factors in the reaction mix.

Materials

- 8 liters mid-log-phase 293 cells (see Support Protocol 1)
- PBS (*APPENDIX 2A*), 4°C
- Hypotonic lysis buffer (see recipe), 4°C
- 5 M NaCl
- Protein assay dye reagents (Bio-Rad)
- IEC PR7000 and rotor or equivalent
- IEC Clinical centrifuge or equivalent
- 40-ml Dounce homogenizer (type B pestle)
- RC5B Sorvall centrifuge with SS34 rotor
- Beckman ultracentrifuge with SW 55.1 rotor

Harvest cells

1. Harvest cells from 8 liters of mid-log-phase 293 cells at $0.5\text{--}1 \times 10^6$ cells/ml by centrifuging cells in six 1-liter centrifuge bottles 20 min at $1000 \times g$, 4°C, in an IEC PR7000 or equivalent.
2. Carefully decant supernatant, and add more 293 cell culture to the centrifuge bottles until all culture has been pelleted. Carefully decant supernatant until a small amount of medium is left.

3. Resuspend pellets with residual medium, and transfer suspension into several 50-ml centrifuge tubes.
4. Centrifuge in an IEC Clinical centrifuge or equivalent 10 min at $1000 \times g$, 4°C , and resuspend each pellet with 15 ml cold PBS. Combine resuspended pellets and repeat centrifugation until all cells fit in one tube.

This normally yields about a 10-ml cell pellet.

Lyse the cells

5. Resuspend the pellet with an equal volume (~10 ml) of freshly prepared hypotonic buffer, 4°C . Pellet the cells in the Clinical centrifuge for 10 min at $1000 \times g$, 4°C , and remove the supernatant.
6. Resuspend pellets in an equal volume of hypotonic buffer, 4°C , and incubate 10 min on ice to swell the cells.
7. Lyse cells by 30 strokes of Dounce homogenizer (with a type B pestle) on ice. Incubate the lysate 30 min on ice.

The cell lysis may be checked under a microscope.

8. Transfer the lysate into a 30-ml Corex tube, and centrifuge in an SS34 rotor of a Sorvall RC5B centrifuge 10 min at $17,000 \times g$, 4°C . Save both supernatant and pellet. Store the pellet (which contains the nuclei) at -70°C .

This pellet contains nuclear proteins, which are useful for many other purposes.

9. Measure the volume of the supernatant, and adjust the concentration to 0.1 M NaCl with 5 M NaCl. Divide the supernatant into several SW55.1 Beckman ultracentrifuge tubes, and centrifuge 1 hr at $100,000 \times g$, 4°C .

Replication factor C (RF-C) is unstable in low salt.

10. Measure protein concentration of the extracts by Bio-Rad protein assay dye reagent, and divide into desired (~500- μl) aliquots. Snap freeze the extract and store at -70°C .

This protocol usually yields 5 to 10 ml extract, with a protein concentration of 20 to 30 mg/ml. For the assay to work, the protein concentration of the extract must be ≥ 10 mg/ml.

EXPRESSION OF RECOMBINANT T ANTIGEN

Insect cells infected with recombinant T antigen baculovirus are used to express T antigen, which is then purified by immunoaffinity chromatography (see Support Protocol 4). The recombinant virus is not commercially available; however, most researchers will share cells.

Materials

Insect cells (Hi-five cells, Invitrogen)
Grace's medium with 10% fetal bovine serum (FBS)
Recombinant T antigen baculovirus stock, high titer
Serum-free medium

1. Seed ten 150-mm tissue culture plates with 2×10^7 Hi-five cells per plate.

Alternatively, if starting with fewer cells, split them into ten 150-mm plates and grow until 80% confluent.

SUPPORT PROTOCOL 3

In Vitro Reconstitution

11.5.5

**SUPPORT
PROTOCOL 4**

2. Add fresh Grace's medium to make up a final volume of 25 ml per plate before infection. Infect cells with high-titer recombinant T antigen baculovirus stock to achieve a multiplicity of infection (MOI) of 5 for optimal protein production.

The amount of medium is not important, as long as the cells are resuspended evenly.

3. Incubate cells 2 days at 27°C. Check for signs of infection (cytopathologic effect) 24 to 48 hr later.

Cytopathologic effects are usually visible by 24 hr after infection.

4. Two days after infection, harvest cells by scraping them off plates with a rubber policeman. Pellet and wash cells once with serum-free medium and store at -70°C.

The pellet can be stored 6 months to 1 year.

IMMUNOAFFINITY PURIFICATION OF T ANTIGEN

In this protocol, recombinant T antigen is purified by immunoaffinity chromatography from insect cells (Hi-five cell, Invitrogen) infected with a recombinant baculovirus containing the T antigen gene.

NOTE: All the steps in this protocol must be carried out at 4°C. Use cold buffers with proteinase inhibitor added fresh throughout.

Materials

- 1 ml Sepharose CL-4B (Amersham Pharmacia Biotech) column packed in a 3-ml syringe plugged with siliconized glass wool
- Pab419-protein A-Sepharose (Pab419-PAS) immunoaffinity column (see Support Protocol 5)
- T antigen lysis buffer (see recipe), 4°C
- Insect cell pellet containing recombinant T antigen (see Support Protocol 3)
- Wash buffer 1 (see recipe), 4°C
- Wash buffer 2 (see recipe), 4°C
- Elution buffer (see recipe), 4°C
- Protein assay dye reagent (Bio-Rad)
- Dialysis buffer (see recipe), 4°C
- 0.1 M sodium borate, pH 9.0
- 0.5 M piperazine-*N,N'*-bis(2-hydroxypropanesulfonic acid) (PIPES), pH 7.0, 4°C
- Dialysis tubing

1. Link a 1-ml Sepharose CL-4B column (for nonspecific absorption) serially to a Pab419-PAS column so the eluate from the former loads onto the latter. Equilibrate both columns with 10 ml T antigen lysis buffer.

When using the Pab419-PAS column for the first time, pre-elute it with 20 ml elution buffer to remove any noncovalently coupled antibody.

2. Resuspend cell pellet containing recombinant T antigen in 5 to 10 vol (based on pellet volume) of ice-cold T antigen lysis buffer. Let cells stand 30 min on ice to lyse, mixing occasionally.
3. Clear lysate from cellular debris by centrifuging 30 min at 8000 × g, 4°C.
4. Load the cleared lysate onto the Sepharose CL-4B column, and collect flowthrough from the Pab419-PAS column. Repeat the loading once, and collect and save the flowthrough.

Repeat the loading after disconnecting from the Sepharose CL-4B column. The second flowthrough is collected and saved in case the column condition is bad and most of the protein is still in the flowthrough.

5. Uncouple the columns, and wash the Pab419-PAS column with 100 ml wash buffer 1 followed by 50 ml wash buffer 2.
6. Elute T antigen from the Pab419-PAS column with 10 ml freshly prepared elution buffer. Collect 0.25-ml fractions in tubes containing 25 μ l of 0.5 M PIPES, pH 7.0, to neutralize each fraction immediately. Determine T antigen concentration using Bio-Rad protein assay dye reagent.

It is important to prepare the elution buffer fresh for immediate use. It is also important to add PIPES immediately to the fractions to neutralize the pH. This prevents T antigen from being inactivated owing to the high pH elution used in this protocol.

7. Pool the peak fractions, and dialyze the protein overnight at 4°C against 1 liter dialysis buffer. Divide protein into 100- μ l aliquots, and snap freeze at -70°C.
8. Regenerate the Pab419-PAS column with 10 ml elution buffer, and wash with 10 ml of 0.1 M sodium borate, pH 9.0.

The 100- μ l aliquots can be further divided into 10- μ l aliquots for the first time they are thawed to prevent repeated freeze/thaw cycles.

The Pab419-PAS column can be reused four to five times and has a shelf life of ≥ 1 year at 4°C. Make a fresh Sepharose CL-4B column each time.

PREPARATION OF Pab419 IMMUNOAFFINITY COLUMN

Purification of T antigen requires an immunoaffinity column in which mouse ascites Pab419 antibody is coupled to protein A-Sepharose (PAS). The immunoabsorbent is stabilized by cross-linking with dimethylpimelimidate. Pab419 ascites is not commercially available; however, most researchers will share the Pab419 hybridoma. A commercial laboratory can produce the ascites.

NOTE: Most of the steps in preparing an immunoaffinity column can be performed at room temperature, because both the antibody and protein A-Sepharose are fairly stable. The column is stable for up to 1 year when stored at 4°C.

Materials

Pab419 ascites
 Protein A-Sepharose (PAS; 1 ml packed volume) equilibrated in coupling buffer
 Coupling buffer (see recipe)
 Coupling buffer with 0.5% (v/v) NP-40 (4.2 ml of 10% NP-40 in 100 ml buffer)
 0.1 M sodium borate, pH 9.0
 400 mM dimethylpimelimidate (freshly prepared)
 0.2 M ethanolamine, pH 8.0
 0.2% sodium azide
 5-ml syringe plugged with silanized glass wool

1. Mix 1.6 ml Pab419 ascites with 1 ml PAS in a 15-ml tube. Bring volume to 10 ml with coupling buffer, and incubate in a rotating wheel 1 hr at room temperature.

Spinning down the particulate matter of ascites before mixing with the antibody prevents the column from clogging in later steps. Antibody sources other than ascites can be used but should be concentrated to 1 to 10 mg immunoglobulin/ml for best results.

The reaction mix can be incubated at 4°C, 4 to 5 hr or overnight, if necessary.

2. Wash off the unbound antibody with three 10-ml washes of coupling buffer followed by five 10-ml washes of coupling buffer with 0.5% NP-40.
3. Equilibrate beads with five 10-ml washes of 0.1 M sodium borate, pH 9.0. Bring up the volume to 9 ml with 0.1 M sodium borate, pH 9.0.

SUPPORT PROTOCOL 5

In Vitro Reconstitution

11.5.7

Cross-linking with dimethylpimelimidate must be performed above pH 8.3. Check the pH of the sodium borate solution before use.

4. Remove 100 μ l resuspended beads (~10 μ l packed beads) and retain separately.

This sample serves as a check for antibody conjugation before the cross-linking step, as described in step 5.

5. Add 1 ml freshly made 400 mM dimethylpimelimidate to the larger tube of beads (final concentration = 40 mM) and incubate 1 to 2 hr, room temperature, on a rotating wheel or rocker. Remove 100 μ l resuspended beads after cross-linking, but before adding ethanolamine.

Cross-linking efficiency can be determined by comparing the antibody released from the PAS beads before and after cross-linking by boiling the beads in Laemmli's buffer and conducting SDS-PAGE (UNIT 6.1). If the cross-linking took place efficiently, little antibody will be released from the beads after cross-linking compared to before cross-linking.

6. To stop the reaction, wash beads with two 15-ml washes of 0.2 M ethanolamine, pH 8.0, and then incubate 2 hr in 10 ml of 0.2 M ethanolamine, room temperature, on a rocker.
7. Transfer resuspended beads to a 5-ml syringe plugged with silanized glass wool, and wash with 20 ml of 0.1 M sodium borate, pH 9.0.

The beads can be stored stably at 4°C in 0.1 M sodium borate, pH 9.0/0.2% sodium azide for more than 1 year.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Alkaline loading buffer, 2×

60 mM NaOH
2 mM EDTA
6% (w/v) Ficoll (type 400; Amersham Pharmacia Biotech)
0.05% (w/v) bromocresol green
0.08% (w/v) xylene cyanol FF
Store at 4°C; stable \geq 1 year

Alkaline running buffer

30 mM NaOH
1 mM EDTA, pH 8.0
Prepare fresh before use

Coupling buffer

50 mM Tris·Cl, pH 8.0 (APPENDIX 2A)
150 mM NaCl
5 mM EDTA
0.1% (v/v) NP-40
Store at room temperature; stable \geq 1 year
Add 1 mM DTT and 1 mM PMSF before use

Dialysis buffer

10 mM PIPES, pH 7.0
0.1 mM EDTA
5 mM NaCl
10% glycerol
Store at room temperature; stable \geq 1 year
Add 1 mM DTT and 1 mM PMSF before use

Elution buffer

56.5 μ l triethylamine
2 ml glycerol
H₂O to 20 ml
Prepare fresh before use

Hypotonic lysis buffer

20 mM HEPES, pH 8.0
5 mM KCl
1.5 mM MgCl₂
Store at room temperature; stable \geq 1 year
Add 1 mM DTT and 1 mM PMSF before use

Replication buffer, 5 \times

150 mM HEPES, pH 7.8
2.5 mM DTT
35 mM MgCl₂
20 mM ATP
1 mM each CTP, GTP, and UTP
0.125 mM dATP
0.5 mM each dCTP and dGTP
0.6 mM TTP
200 mM creatine phosphate
Adjust pH to 7.4 to 7.8 with 10 N NaOH
Store in -20°C freezer; stable \geq 1 year

Stop solution, 3 \times

20 mM EDTA
0.3% (w/v) SDS
20 μ g pronase
Store in -20°C freezer; stable \geq 1 year

T antigen lysis buffer

50 mM Tris-Cl, pH 8.0 (*APPENDIX 2A*)
150 mM NaCl
1 mM EDTA
10% (v/v) glycerol
0.5% NP-40
Store at room temperature; stable \geq 1 year
Add 1 mM DTT and 1 mM PMSF before use

Wash buffer 1

50 mM Tris-Cl, pH 8.0 (*APPENDIX 2A*)
0.5 M LiCl
1 mM EDTA
10% (v/v) glycerol
Store at room temperature; stable \geq 1 year
Add 1 mM DTT and 1 mM PMSF before use

Wash buffer 2

10 mM PIPES, pH 7.4
5 mM NaCl
1 mM EDTA
10% glycerol
Store at room temperature; stable \geq 1 year
Add 1 mM DTT and 1 mM PMSF before use

COMMENTARY

Background Information

The SV40 replication system was established in 1984 by Li and Kelly (1984). It provides the first biochemical tool for studying eukaryotic DNA replication. By providing an SV40 replication origin in plasmid form and the initiator protein (and helicase) T antigen in the presence of a replication-competent extract, the system can replicate the plasmid. This approach bypasses difficulties posed by the absence of known cellular initiation proteins and origins of replication.

The mechanisms of SV40 replication have been extensively studied and are summarized here. T antigen initiates the replication of the SV40 origin-containing plasmid by recognizing the replication origin, which is a single 64-bp core origin DNA sequence from the SV40 virus. The DNA is unwound with the assistance of cellular replication protein A (RPA). Synthesis of a primer RNA and the first Okazaki fragment is carried out by the DNA polymerase α /primase complex after the unwinding. Both RPA and replication factor C (RF-C) interact with polymerase α /primase and stimulate lagging-strand DNA synthesis (Tsurimoto and Stillman, 1991a,b). RPA functions as a stabilizer of single-stranded DNA during the replication process. Saturating amounts of RPA, however, block polymerase α and arrest the DNA synthesis after laying down the first Okazaki fragment. Proliferating cellular nuclear antigen (PCNA), RF-C, and ATP then interact with the polymerase α /primase and form a primer recognition complex. At this stage, polymerase δ recognizes the 3' end of the first Okazaki fragment. RF-C loads PCNA onto the DNA; the latter forms a sliding clamp (Mossi et al., 1997), which interacts with polymerase δ (Lee et al., 1991) and results in the formation of a highly processive holoenzyme that efficiently replicates the leading-strand DNA. DNA polymerase α /primase, together with RPA, RF-C, and PCNA, continue the cycles of priming and DNA polymerization on the lagging strand, producing primer fragments that are extended into Okazaki fragments by DNA polymerase δ . The completion of lagging-strand DNA synthesis requires the processing of Okazaki fragments. RNase HI removes most of the RNA at the 5' ends of the Okazaki fragments, but flap endonuclease 1 (FEN-1) is required to remove the last monoribonucleotide. After dissociation of cleaved RNA, DNA ligase comes in and seals the nick

(Turchi et al., 1994). Thus the DNA replicates bidirectionally from the origin; polymerase δ continuously synthesizes the leading strand from the 3' end of the first Okazaki fragment, and the DNA polymerase α /primase complex synthesizes the Okazaki fragments along the lagging-strand template by translocating away from the replication fork in a discontinuous manner. It is believed that similar replication mechanisms exist in cellular chromosomes, except that unknown initiation proteins and origins of replication are involved.

Assays representing various stages of SV40 DNA replication are useful in studying the replication mechanism, including origin binding by T antigen (Borowiec and Hurwitz, 1988), origin DNA untwisting (Dean and Hurwitz, 1991), DNA unwinding (Dean et al., 1987; Wold et al., 1987; Dutta and Stillman, 1992; Goetz et al., 1998), RNA primer formation (Murakami et al., 1992), primer DNA formation (Bullock et al., 1991; Denis and Bullock, 1993), and interactions between various replication factors (Dornreiter et al., 1993). The details of these protocols can be found in the cited literature.

The dependence of viral initiation proteins in the SV40 system makes it difficult to study issues related to chromosomal replication initiation. The *Xenopus* in vitro replication system has some advantages over the SV40 system in this regard, because it relies totally on cellular proteins and replicates only once in the cell cycle. Yet this model is not as easily manipulated as the SV40 system. Furthermore, because both are in vitro systems that depend on cellular extracts, possible participation of insoluble cellular components such as nuclear membrane, nuclear matrix, and chromatin cannot be properly determined. Recent introduction of an in vivo mammalian replication system (Krude et al., 1997) has provided a third approach to the study of DNA replication at the biochemical level. The synthesis of knowledge acquired from all three types of in vitro reactions will probably best illuminate the biochemistry of DNA replication.

Critical Parameters

It is important to have an active cell lysate and an active T antigen for the SV40 replication assay. The cells for the replication extracts must be monitored regularly for their viability. Antifungal reagents such as amphotericin B may be needed to avoid fungal contamination in the

long-term culture. The extracts have to be made and frozen on the day of harvesting for optimal reactions. An active lysate must have a protein concentration ≥ 10 mg/ml. Repeated freezing and thawing of T antigen and S100 extract inactivates the reaction; therefore, store the proteins in small aliquots. The pH of the replication buffer is a key issue for a successful SV40 replication reaction. It is necessary to measure the pH of the replication buffer after adding nucleotides (which are usually very acidic) unless prebuffered nucleotides are used.

Troubleshooting

When incorporation of the nucleotides is poor, four possible causes must be suspected: low protein concentration of the extract, repeated freezing and thawing of the extract, inactive T antigen, and acidic replication buffer. Low yield of T antigen or inactive T antigen is usually the result of improper immunoaffinity column preparation. Check the antibody conjugation and cross-linking procedures by running an SDS-PAGE, as suggested in Support Protocol 5. Also pre-elute the column before using it the first time.

Anticipated Results

The yield of T antigen from ten 150-mm plates with a 1-ml Pab419 column is ~ 2 to 3 mg. The 8-liter 293 suspension culture usually yields 5 to 8 ml replication-competent S100 cell extract, with a concentration of 20 to 30 mg/ml. A total of 10 to 15 μ l of this extract usually gives maximal incorporation for a 50- μ l SV40 replication reaction. A total of 60 to 100 pmol nucleotide is usually incorporated in a 50- μ l reaction replication. Nucleotide incorporation decreases substantially when the conditions are not optimal.

Time Considerations

When it is started from one monolayer plate, the protocol takes ~ 3 to 4 weeks to produce enough cells for the replication competent S100 cell extract. The harvesting of cells and preparation of the extract usually takes 1 day. Insect cells infected with T antigen baculovirus are ready to be harvested 2 days after infection, and the resulting cell pellet can be stored at -70°C before purification. Preparation of the Pab419 immunoaffinity column with mouse ascites takes ~ 4 to 5 hr; most of the steps can be performed at 4°C when a longer incubation time is preferred. T antigen purification procedures take ~ 2 to 3 hr. The SV40 replication reaction takes ~ 2 hr to set up and finish. The

products can be stored at -20°C for a few days before quantification and gel analysis. Between 1 and 2 hr are required for quantification of incorporated nucleotides for 10 to 20 reactions by the DE81 paper procedure. Alkaline gel analysis of the replication products usually takes 12 to 14 hr plus time for autoradiography.

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Growth, differentiation, and development in eukaryotic organisms are largely regulated at the level of transcription. To gain an understanding of the molecular mechanisms of transcriptional regulation, it is necessary to utilize an in vitro transcription system by which the identity and function of various factors can be elucidated. The initial development of cell-free extracts from HeLa cells, capable of accurate initiation of transcription by RNA polymerase II, was an important advance in the analysis of gene regulation (Dignam et al., 1983; Manley et al., 1980). Since then, several extracts have been prepared, using diverse sources ranging from cultured mammalian (HeLa) cells (Dignam et al., 1983; Manley et al., 1980) to *Drosophila* embryos (Heiermann and Pongs, 1985; Soeller et al., 1988; Kamakaka et al., 1991), and yeast (Lue and Kornberg, 1987; Woontner and Jaehning, 1990), which mediate accurate initiation of basal and activated transcription. These extracts have been utilized to isolate and characterize the various general, as well as sequence-specific, transcription factors. The extracts have also been used to elucidate the detailed molecular mechanisms of transcription initiation in eukaryotes.

This unit describes an in vitro transcription reaction (see Basic Protocol) using either a mammalian (HeLa) cell extract (see Support Protocol 1) or a *Drosophila* embryo extract (see Support Protocol 2 or Alternate Protocol). This reaction is capable of transcribing from naked DNA or chromatin templates. Upon completion of transcription, the products are analyzed by primer extension analysis (see Support Protocol 3).

NOTE: All reagents used for these protocols should be high-quality molecular biology grade and should be RNase- and DNase-free. Likewise, all plasticware should be RNase-free, and great care should be taken to minimize contamination by RNases (*APPENDIX 2A*).

IN VITRO TRANSCRIPTION REACTIONS WITH NUCLEAR EXTRACTS

In this protocol, the nuclear extract is preincubated with a plasmid template containing an RNA polymerase II promoter to form preinitiation complexes at the promoter. Transcription is initiated by the addition of a mixture of ribonucleotide-5'-triphosphates (rNTPs) and is allowed to proceed for 30 min. After completion of the reaction, a stop solution is added. The RNA transcripts are deproteinized and precipitated. The transcripts can then be analyzed by primer extension analysis (see Support Protocol 3), S1 nuclease protection analysis of RNA, or runoff transcription assays.

NOTE: This protocol describes the conditions for 25- μ l reactions. However, this reaction can be scaled up to accommodate various experimental parameters as described below.

Materials

- Nuclear extract (see Support Protocol 1, Support Protocol 2, or Alternate Protocol)
- 10% (w/v) polyvinyl alcohol (PvOH; mol. wt. 10,000; Sigma)
- 10% (w/v) polyethylene glycol (PEG; mol. wt. 15,000 to 20,000)
- 1 M HEPES (potassium salt), pH 7.6 and 8.0, adjusted with KOH
- 200 ng/ μ l plasmid DNA template in TE buffer, pH 8.0 (*APPENDIX 2A*)
- 5 mM ribonucleotide-5'-triphosphates (rNTPs; see recipe)
- Transcription stop solution (see recipe)
- 0.3 M sodium acetate
- 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (PCIAA), equilibrated with 10 mM Tris·Cl, pH 7.5
- 75% and 100% ethanol
- 30° and 37°C water baths

BASIC PROTOCOL

In Vitro Reconstitution

11.6.1

Contributed by Rohinton T. Kamakaka and W. Lee Kraus

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Supplement 2

Prepare transcription reaction mix

1. Thaw a sufficient number of aliquots of nuclear extract for the experiment quickly in an ice-water bath. Also thaw aliquots of 10% PvOH, 10% PEG, and 5 mM rNTPs.

Keep all reagents on ice once thawed.

2. Prepare the following mix (multiplying the quantities by the number of tubes to be used in the assay):

2.5 μ l 10% PvOH
2.5 μ l 10% PEG
0.5 μ l 1 M HEPES (potassium salt), pH 8.0.

3. For each sample, combine the following reagents on ice, in the order listed, in a 1.5-ml microcentrifuge tube, mixing after each addition:

6.0 μ l H₂O
5.5 μ l PvOH/PEG/HEPES mix (from step 2)
10.0 μ l nuclear extract (or optimal amount determined empirically)
1.0 μ l 200 ng/ μ l DNA template (or optimal amount determined empirically).

The water can be added to the PvOH/PEG/HEPES mix in step 2, to decrease the amount of pipetting. Purified transcription factors or column fractions from chromatographic purifications can be added at this step in addition to the extract in place of some or all of the water, if desired.

The optimal amount of nuclear extract for each application will have to be determined empirically, but it typically ranges from 5 to 15 μ l. If <10 μ l of nuclear extract is used, the volume should be brought up with TM buffer containing 0.1 M KCl; if >10 μ l of nuclear extract is used, the volume of the water should be reduced.

The DNA template should be double-banded on CsCl gradients (see APPENDIX 3). The optimal amount of DNA template per reaction will also have to be determined empirically. It may range from 30 to 300 ng, depending on the strength of the promoter. A good starting point is 200 ng. The DNA is present in TE buffer, pH 8.0.

The reaction for each set of experimental conditions should be performed in duplicate to compensate for any pipetting errors or loss of sample. Additionally, an internal reference plasmid can be added, if desired.

4. Incubate 15 min at room temperature (~21°C) to allow the formation of preinitiation complexes.

5. Prepare the rNTP mix just before use by combining:

0.5 μ l 1 M HEPES (potassium salt), pH 7.6
2.5 μ l 5 mM rNTPs
4.5 μ l H₂O.

Perform reaction

6. Initiate transcription by adding 2.5 μ l of the rNTP mix prepared in step 5 to the incubated reaction from step 4. Incubate 30 min at 30°C.

To confirm that the RNA was synthesized by RNA polymerase II, and not some other polymerase, inhibition with α -amanitin should be performed as a control. To do this, add 1 μ l of 100 μ g/ml α -amanitin (in water; also see APPENDIX 1B) to the reaction mix to give a final concentration of 4 μ g/ml α -amanitin. RNA polymerase II is inhibited by 0.5 μ g/ml α -amanitin; RNA polymerase III is inhibited by 200 μ g/ml α -amanitin; and RNA polymerase I is resistant to α -amanitin.

7. Stop the reactions by adding 100 μ l transcription stop solution. Mix well and incubate 10 min at 37°C.

Isolate transcripts

8. Add 250 μ l of 0.3 M sodium acetate to each sample.
9. Extract each sample with 400 μ l of 25:24:1 PCIAA. Transfer the aqueous phase from each tube to a new 1.5-ml microcentrifuge tube.
10. Add 1 ml of 100% ethanol and mix well. Microcentrifuge each sample 15 min at maximum speed, room temperature, to collect the RNA in the pellet.
11. Carefully aspirate the liquid from the small white pellets and add 400 μ l of 75% ethanol to each tube. Microcentrifuge 5 min at maximum speed, room temperature.
12. Carefully aspirate all the liquid from the pellets. Dry the pellets in a rotary evaporator (e.g., a SpeedVac). Store the samples at -20°C , or proceed immediately with the primer-extension analyses (see Support Protocol 3).

PREPARATION OF A NUCLEAR EXTRACT FROM HELA CELLS

This protocol describes the preparation of a nuclear extract from HeLa cells and is based largely on the protocol described by Dignam et al. (1983). HeLa cells are incubated in a hypotonic buffer to swell them by osmotic action. The swollen cells are disrupted by Dounce homogenization, and the nuclei are collected in a pellet by centrifugation. The nuclei are then incubated in a high-salt buffer to extract the soluble proteins, including the transcription factors and RNA polymerase II, without lysing the nuclei. The nuclei are removed from the extract by centrifugation, and the extract is subjected to ammonium sulfate precipitation to concentrate the transcription factors and to remove histone H1 (a repressor of transcription). Finally, the precipitated proteins are collected by centrifugation, dissolved in buffer, dialyzed into a low-salt buffer, and used for transcription.

NOTE: This protocol describes the preparation of a HeLa nuclear extract from 12 liters of HeLa cell suspension culture (i.e., all volumes listed in the protocol below are based on an original culture volume of 12 liters). However, the preparation can be scaled up or down.

Materials

- ~ $5-8 \times 10^5$ cell/ml suspension culture of HeLa cells grown in spinner flasks
- Phosphate-buffered saline (PBS; *APPENDIX 2A*)
- Freezing buffer (see recipe)
- Glycerol
- PBS (*APPENDIX 2A*) containing 1 g/liter MgCl_2
- Hypotonic buffer (see recipe)
- High-salt buffer (see recipe)
- Powdered ammonium sulfate
- TM buffer (see recipe) with and without 0.1 M KCl
- Beckman JS-4.2 and SW-28 and Sorvall GSA and SS-34 rotors (or equivalents) and appropriate centrifuge bottles and tubes
- 40-ml Wheaton Dounce homogenizer with loose (B) pestle (or equivalent)
- Insulated magnetic stir plate
- Coffee bean grinder or mortar and pestle
- Dialysis tubing (12,000 to 14,000 MWCO)
- Conductivity meter (optional)
- Additional reagents and equipment for dialysis (*APPENDIX 3*)

SUPPORT PROTOCOL 1

In Vitro Reconstitution

11.6.3

NOTE: Perform all procedures at 4°C (i.e., on ice and in a cold room) using precooled solutions, glassware, and equipment. Perform all centrifugations at 4°C with precooled rotors. All low-speed centrifugations are done in a low-speed centrifuge (e.g., Sorvall RC-5) while high-speed centrifugations are done in an ultracentrifuge.

Isolate HeLa cell nuclei

1. Centrifuge 12 liters of HeLa cells grown under standard conditions ($\sim 5\text{--}8 \times 10^5$ cell/ml) in spinner cultures for 15 min at $1800 \times g$, 4°C, in a large, low-speed preparative rotor (e.g., Beckman JS-4.2).
2. Resuspend the cell pellets in PBS (25 to 50 ml for each liter of original culture volume), combine, and centrifuge again as in step 1 in one or two bottles.

If cells are not to be used immediately, add glycerol and freeze as in steps 3 and 4. Alternatively, the cells can be used immediately without freezing, in which case collect the cells from the spinner culture by centrifugation as in the previous steps and proceed directly to step 5.

3. Resuspend the cell pellets in 2 ml of freezing buffer for each liter of original culture volume and add glycerol to 20% (v/v), taking into account the original glycerol concentration of the freezing buffer (30% v/v), the volume contributed by the cells, and the final volume of the cell suspension.
4. Freeze the cells in aliquots in 50-ml plastic, conical tubes in liquid N₂ and store at -80° to -100°C .
5. Thaw HeLa cells (if frozen) from 12 liters of culture in an ice water bath as quickly as possible. Centrifuge 10 min at $1400 \times g$ (3000 rpm in a Sorvall GSA rotor), 4°C, add 200 ml of PBS containing 1 g/liter MgCl₂, then centrifuge again at $1400 \times g$. Aspirate the supernatant from the loose cell pellet and wash again in the same manner.

This protocol can be readily scaled up or down by a factor of two (i.e., for preparations from 6 to 24 liters of cells).

6. Aspirate the supernatant from the washed cell pellet and resuspend the cells in 60 ml hypotonic buffer. Incubate the cells on ice 15 min to allow them to swell.
7. Lyse the cells by 12 strokes in a Dounce homogenizer (with loose “B” pestle). Centrifuge the cell lysate 8 min at $3000 \times g$ (5000 rpm in a Sorvall SS-34 rotor), 4°C, to collect the nuclei in the pellet.

Cell lysis can be monitored under a microscope by staining an aliquot of cells with trypan blue (UNIT 1.1), which stains the nuclei of lysed cells. Aim for 90% cell lysis.

Work quickly after lysis of the cells to minimize the leakage of transcription factors from the nuclei.

8. Aspirate the supernatant and resuspend the isolated nuclei in a total final volume of 20 ml hypotonic buffer. Pool into one tared SS-34 tube and centrifuge 8 min at $3000 \times g$, 4°C. Aspirate the supernatant and weigh the nuclei.

A typical yield of nuclei from a 12-liter culture of HeLa cells is ~ 12 g.

Prepare a high-salt nuclear extract

9. Resuspend the nuclei in 70 ml high-salt buffer and transfer to a 200-ml glass beaker. Stir the suspension slowly in an ice-water bath on an insulated magnetic stir plate for 30 min.

10. Transfer the suspension to thick-walled tubes for a Beckman SW-28 rotor. Balance the tubes and centrifuge 60 min at $100,000 \times g$ (25,000 rpm in an SW-28 rotor), 4°C .

A swinging bucket rotor is preferable to a fixed-angle rotor for this centrifugation step. With a fixed angle-rotor, undesirable shearing of the nuclei can occur.

11. Collect the supernatants into a graduated cylinder and measure the volume (expect 70 to 75 ml).

Precipitate the extract

12. Transfer the pooled supernatants to a 200-ml glass beaker. Start stirring the suspension slowly in an ice-water bath on an insulated magnetic stir plate. Slowly, over a 15 to 20 min period, add 0.33 g powdered ammonium sulfate per ml of supernatant (final saturation 55% at 0°C). After all the ammonium sulfate has been added and has dissolved, let the mixture stir for an additional 25 to 30 min.

Pulverize the ammonium sulfate by using a coffee bean grinder or a mortar and pestle no more than 5 to 10 min before use. Powdered ammonium sulfate is hygroscopic and cannot be weighed accurately when it is wet.

13. Centrifuge the mixture 20 min $35,000 \times g$ (17,000 rpm in an SS-34 rotor), 4°C . Remove the supernatants by aspiration, trying to remove as much of the liquid as possible.

14. Dissolve the pellets in a total final volume of 5 to 6 ml TM buffer and pool into one tube.

The pellets from the ammonium sulfate precipitation are difficult to dissolve. For best results, add ~1.5 ml of TM buffer to each pellet. Then, pipet up and down gently to dislodge and resuspend the pellets. After pooling, use ~1 ml of buffer to rinse the tubes and the pipet, and add the rinse to the sample. The solution will be milky white after the pellets are dissolved.

Dialyze the extract

15. Desalt the extract by dialysis (APPENDIX 3) in 12,000 to 14,000 MWCO dialysis tubing as follows:

1 hr versus 2 liters TM buffer

1 hr versus 2 liters TM buffer containing 0.1 M KCl

2 hr versus fresh change of TM buffer (2 to 4 liters) containing 0.1 M KCl.

The dialysis is complete when the conductivity of the sample is the same as the conductivity of the dialysis buffer. If a conductivity meter is not available, it should be possible to assume complete or near-complete dialysis with this scheme.

16. Transfer the extract from the dialysis bag and centrifuge 10 min at $12,000 \times g$ (10,000 rpm in an SS-34 rotor), 4°C , to remove any insoluble material. Divide the supernatant into aliquots, freeze in liquid N_2 , and store at -80° to -100°C .

If desired, save an aliquot of the extract for determination of the protein concentration by the Coomassie blue binding assay. A convenient aliquot size for in vitro transcription experiments is 150 to 200 μl . Avoid multiple freeze-thaw cycles.

The yield is ~7 ml of HeLa extract from 12 liters of cells.

PREPARATION OF HIGH-SALT *DROSOPHILA* EXTRACTS

Two transcription extracts that are highly active for transcription in vitro can be prepared from *Drosophila* embryos (also see Alternate Protocol). Transcription extracts from *Drosophila* are particularly attractive for studies on transcriptional regulation because: (1) they are highly active and (2) many mechanisms of activation have been conserved between mammals and flies with various transcription factors being easily interchangeable in vitro. Furthermore, *Drosophila* embryos are inexpensive compared to cultured cells. Finally, *Drosophila* transcription extracts allow the use of in vitro-reconstituted chromatin as the template for transcription studies (Kamakaka et al., 1991).

This protocol is based on the method of Soeller et al. (1988) and Parker and Topol (1984). It involves the purification of nuclei from 0- to 12-hr *Drosophila* embryos, followed by extraction of nuclear proteins such as transcription factors and histones using a high-salt buffer. After removal of the cellular debris, the transcription factors are precipitated with ammonium sulfate (which, incidentally, removes nonspecific inhibitors of transcription such as histone H1). The precipitated proteins, after solubilization, are desalted by gel filtration, resulting in an active transcription extract. This extract is a rich source of both general and sequence-specific transcription factors which can be purified by further chromatography steps.

NOTE: This protocol is for use with ~100 g of embryos. The preparation, however, can be scaled up or down.

Materials

- Drosophila* cultures
- Molasses/agar plates (see recipe)
- Bleach (5.25% sodium hypochlorite) diluted 1:1 in distilled H₂O (store at room temperature)
- Embryo wash solution: 0.7% (w/v) NaCl/0.04% (v/v) Triton X-100 (store at room temperature)
- Disruption buffer (see recipe)
- Resuspension buffer (see recipe)
- 4 M ammonium sulfate, pH 7.0
- Solid ammonium sulfate
- HEMG containing 0.1 M KCl (see recipe)
- Embryo collection apparatus (construct according to Fig. 11.6.1) and paint brush
- Nylon mesh (Tetko, #3-70/43 and 3-500/49) and interlocking rings for constructing embryo collection apparatus
- Yamato LH-21 homogenizer (Thomas)
- 4 pieces of Miracloth (9 in. × 9 in.; Calbiochem)
- GSA, 45-Ti, and SS-34 rotors (or equivalents) and appropriate centrifuge bottles and tubes
- 40-ml Wheaton Dounce homogenizer with a B pestle and 15-ml homogenizer with A pestle
- Rotating mixer
- Coffee grinder
- 5 × 22-cm G-25 SF desalting column (430 ml; Pharmacia Biotech)
- Conductivity meter
- Additional reagents and equipment for growing *Drosophila* (Goldstein and Fyrberg, 1994)

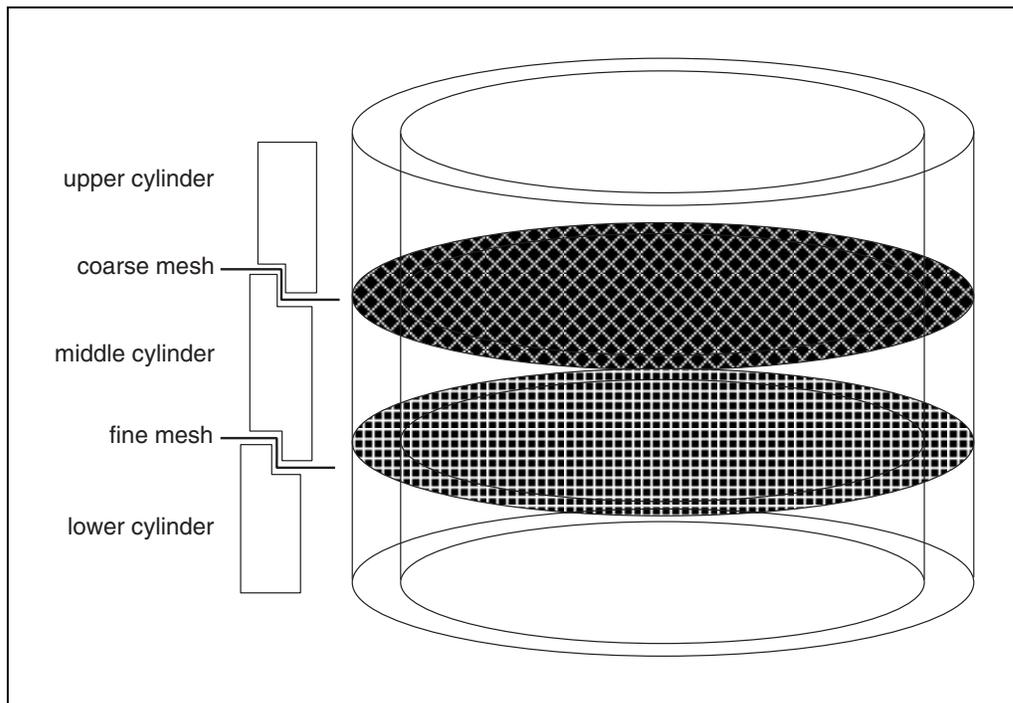


Figure 11.6.1 The embryo collection apparatus consists of three interlocking rings, which hold two nylon meshes of different porosity. The coarse mesh (Tetko 3-500/49) is placed between the upper and middle cylinders to collect any dead flies and large particulate material. The fine mesh (Tetko 3-70/43) is placed between the middle and lower cylinders to collect the embryos but allow the yeast to flow through. The dimensions of this apparatus can vary; 3-in. high, 10-in. diameter segments work well.

NOTE: Perform all procedures at 4°C (i.e., on ice and in a cold room) using precooled solutions, glassware, and equipment. Perform all centrifugations at 4°C with precooled rotors.

NOTE: The use of frozen embryos or embryos stored >3 days results in transcription extracts of a very poor quality.

Collect embryos

1. Grow wild-type flies at 25°C and 70% humidity in population cages (Goldstein and Fyrberg, 1994). Collect the embryos onto molasses/agar plates.

Embryos collected between 0 and 12 hr after fertilization will be used. The plates can be stored for up to 3 days at 4°C (Biggin and Tjian, 1988).

2. Harvest 0- to 12-hr embryos in embryo collection apparatus (Fig. 11.6.1). Wash the embryos off the molasses/agar plates onto the coarse mesh of the collection apparatus in a sink, using a paint brush and running distilled water. Once all the embryos are collected, remove the upper cylinder and the coarse mesh containing the dead flies and particulate matter.
3. Remove large foreign particles, if present, in the embryos, and wash well in the collection apparatus consisting of the middle and lower cylinders to remove the yeast.
4. Move the apparatus containing the embryos into a Nalgene tub. Pour 3 liters of 1:1 bleach/distilled water into embryo collection apparatus in the Nalgene wash tub and swirl to mix. Dechorionate the embryos by soaking 90 sec in the bleach. Lift the embryos in the bleach solution out of the tub and allow the bleach solution to drain away.

Do not soak embryos for >90 sec.

5. Quickly, rinse with 1 liter embryo wash solution by pouring embryo wash solution directly into embryo collection apparatus and swirling to mix.
6. Rinse with 1 to 3 liters water to remove excess embryo wash solution and bleach.
7. Dry the embryos to a moist cake by placing paper towels under the fine nylon mesh of the embryo collection apparatus to absorb excess water.
8. Weigh the embryos in a tared beaker.

Homogenize embryos

9. Add 3 ml disruption buffer per gram of embryos and stir with a rod to disperse the embryos evenly. Disrupt embryos with a single passage through the Yamato LH-21 homogenizer at 1000 rpm.
10. Pour the homogenate into a funnel lined with 1 layer Miracloth over a GSA bottle. After the liquid in each funnel has flowed into the GSA bottle, wash the debris remaining in the Miracloth with additional disruption buffer (2 ml per g of embryos).

The final volume of disruption buffer should be ~5 ml/g embryos.

Depending on the volume of the homogenate, between one and four funnels are used.

Once the embryos are homogenized, work quickly—proteins can leak out of the nuclei into the buffer solution.

11. Centrifuge 15 min at $10,000 \times g$ (8000 rpm in a GSA rotor), 4°C , to collect the nuclei in a pellet. Carefully decant the supernatant (the pellet will be very loose). Wipe lipids from the walls of the centrifuge bottles with Kimwipes.

Try to remove as much lipid as possible.

12. Resuspend the nuclei in 3 ml disruption buffer per gram of embryos by swirling carefully. Do not resuspend the yellow yolk, which sticks very tightly to the bottle. Transfer nuclear suspension to a Dounce homogenizer.
13. Use a 40-ml Dounce homogenizer with a B pestle to disperse the nuclei. Transfer to clean GSA bottles.
14. Centrifuge again as in step 11. Carefully decant the supernatant, and wipe the lipids from the walls of the centrifuge bottles.
15. Resuspend the nuclei in 1 ml resuspension buffer per g embryos. Use a 40-ml Dounce homogenizer with a B pestle to break up clumps of nuclei (three strokes of the Dounce are sufficient).
16. Measure the volume accurately with a 250-ml graduated cylinder and transfer to 45-Ti bottles. Add a quantity of 4 M ammonium sulfate equivalent to 1/10 vol of the liquid in 45-Ti bottles to each bottle. Mix by inversion, then rotate 20 min on a rotating mixer.

The final ammonium sulfate concentration is $0.364\text{ M} = 9\%$ saturation at 20°C .

17. Centrifuge the lysed nuclei 1 hr at $142,400 \times g$ (35,000 rpm in 45-Ti rotor), 4°C .
18. During the centrifugation, make the following preparations:
 - a. Pulverize solid ammonium sulfate crystals in a coffee grinder.
 - b. Prepare ice bath over a magnetic stirrer for ammonium sulfate precipitation.
 - c. Begin to equilibrate the G-25 SF desalting column with 2 column volumes of HEMG containing 0.1 M KCl at 400 ml/hr.

Precipitate proteins

19. Remove supernatant from the lysed nuclei with a pipet. Avoid lipid globules on the top of the tube.

It is normal for the solution to become cloudy upon standing.

20. Accurately measure the volume of the combined supernatants. Transfer the liquid to a 400-ml beaker containing a magnetic stirrer, placed in the previously prepared ice bath. Slowly (over a period of ~5 min) add 0.3 g pulverized ammonium sulfate/ml liquid. Stir for an additional 15 min.

The final ammonium sulfate concentration is 2.26 M = 56% saturation at 20°C.

21. Centrifuge ammonium sulfate precipitate 20 min at $27,000 \times g$ (15,000 rpm in an SS-34 rotor), 4°C. Decant supernatant, and drain pellets well.

The supernatant is a good source for histone H1.

22. Resuspend pellets in 0.1 ml HEMG containing 0.1 M KCl per g embryos. Use a 15-ml Dounce homogenizer with an A pestle to disperse protein clumps.
23. Centrifuge the resulting mixture 10 min at $12,000 \times g$ (10,000 rpm in an SS-34 rotor), 4°C, to remove insoluble material.

Desalt extract

24. Apply the protein extract to an equilibrated G-25 SF desalting column. Run the column at 400 ml/hr, and monitor the absorbance at 280 nm. Collect 1-min fractions (~7 ml).

In ~20 min, the protein peak will elute from the column, the protein peak will be followed by a salt peak.

25. Check the protein concentrations of the peak fractions with the Coomassie blue binding assay (APPENDIX 3), then measure the conductivity of the last protein fraction that is to be pooled.

If the conductivity of this fraction is identical to that of HEMG containing 0.1 M KCl (i.e., 20 mS/cm), then the desalting is complete. Dilute 20 ml of sample in 5 ml distilled water, and measure the conductivity of the resulting solution. 80 mS/cm corresponds to an extract of 20 mS/cm. If the conductivity does not match then use a larger column or dialyze the sample.

26. Pool the peak protein fractions, and centrifuge the solution 10 min as in step 23 to remove insoluble debris. Avoid the lipids at the top of the tubes.
27. Quick-freeze the extract in a 50-ml polypropylene screw-cap tube in liquid N₂ and store at -100°C.

Typically 100 g of embryos will yield ~40 to 50 ml of high-salt transcription extract.

PREPARATION OF THE SOLUBLE NUCLEAR FRACTION FROM ISOLATED DROSOPHILA EMBRYO NUCLEI

An alternative protocol for the preparation of *Drosophila* transcription extracts, based on the method of Kamakaka et al. (1991), has the advantages of speed, removal of nonspecific inhibitors of transcription, and allowing the preparation of extracts from very few embryos (extracts can be made from between 10 and 150 g of embryos, and quantities are simply scaled proportionately). This method involves the isolation of nuclei in a relatively low-salt buffer followed by a high-speed centrifugation of the nuclei in a small volume

**ALTERNATE
PROTOCOL**

**In Vitro
Reconstitution**

11.6.9

to maximize the concentration of transcription factors while avoiding the extraction of nonspecific inhibitors of transcription.

Additional Materials (also see Support Protocol 2)

HEMG20 containing 0.1 M KCl (see recipe)

Ultracentrifuge with SW-28 rotor

1. Prepare nuclei (see Support Protocol 2, steps 1 to 14).
2. Resuspend the nuclei in 1 ml resuspension buffer per gram of embryos. Disperse nuclei in a 40-ml Dounce homogenizer with a B pestle (three strokes of the Dounce should be sufficient).
3. Transfer the entire suspension of nuclei into one tared GSA bottle.
4. Centrifuge 10 min at $10,000 \times g$ (8000 rpm in GSA rotor), 4°C.
5. Decant the supernatant and weigh the bottle to determine the mass of the nuclei.
6. Add 0.5 ml per gram of nuclei of HEMG20 containing 0.1 M KCl.

Example: if there are 10 g nuclei, then add 5 ml HEMG20 containing 0.1 M KCl.

7. Resuspend the pellet by shaking and swirling the nuclei (do not use a homogenizer). Place the suspension on ice for 15 to 60 min.
8. Centrifuge mixture 1 hr at $100,000 \times g$ (24,000 rpm in SW-28 rotor), 4°C.

It is important to always use a swinging-bucket rotor for the preparation of the soluble nuclear fraction to reduce shearing of chromatin.

9. After centrifugation, observe the four distinct layers, which will appear from top to bottom as follows:
 - a. A thin gray-white lipid layer. Remove this with a spatula and discard.
 - b. A yellow liquid layer, which comprises ~50% of the total volume of the tube and is the soluble nuclear fraction. Remove this extract with a pipet and transfer into a 50-ml screw-cap polypropylene tube.
 - c. A gray liquid layer. Avoid the gray layer below the extract as this is enriched in transcription inhibitors and nucleases and phosphatases.
 - d. At the bottom, a solid off-white layer of nuclear material which includes DNA, scaffold etc.
10. Quick-freeze the extract (step 9, substep b, above) in 50-ml polypropylene screw cap tube in liquid N₂ and store at -100°C.

Usually, 100 g of embryos will yield 20 g of wet nuclei, which will then yield ~15 ml of the soluble nuclear fraction.

**SUPPORT
PROTOCOL 3**

**PRIMER EXTENSION ANALYSES OF IN VITRO TRANSCRIPTION
PRODUCTS**

This protocol describes primer extension analyses of in vitro transcribed RNA products. First, a short (~25 base) DNA oligonucleotide is end labeled with [γ -³²P]ATP by T4 polynucleotide kinase. Next, the radiolabeled oligomer is annealed to the RNA products from the in vitro transcription reactions, and the primers are extended with reverse transcriptase. Finally, the primer extension products are analyzed by denaturing-polyacrylamide gel electrophoresis.

Materials

~25-base DNA oligonucleotide complimentary to the RNA products from the in vitro transcription reactions
T4 polynucleotide kinase
150 $\mu\text{Ci}/\mu\text{l}$ [γ - ^{32}P]ATP (7000 Ci/mmol; ICN Biomedicals)
2.5 M ammonium acetate
10 mg/ml glycogen
100% ethanol
1 \times TE buffer, pH 8.0 (APPENDIX 2A)
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (PCIAA), equilibrated with Tris-Cl, pH 7.5
3 M sodium acetate
5 \times annealing buffer (see recipe)
RNA product (e.g., from Basic Protocol)
Primer extension mix (see recipe)
50 U/ μl Moloney murine leukemia virus (MMLV) reverse transcriptase
Formamide loading buffer (FLB; see recipe)
8% urea-polyacrylamide sequencing gel
1 \times TBE buffer (APPENDIX 2A)
37°, 58°, 70°C, and boiling water baths
Whatman 3MM filter paper

Additional reagents and equipment for primer synthesis, agarose gel electrophoresis, DNA elution, ethanol precipitation, DNA quantification, primer labeling (APPENDIX 3), and phosphorimaging or autoradiography (UNIT 6.3)

NOTE: All reagents used for this protocol should be high-quality molecular biology reagents. All procedures should be performed at room temperature unless otherwise noted. All microcentrifugations are done at maximum speed.

CAUTION. When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out experiment and dispose of waste in appropriately designated areas, following the guidelines provided by the local radiation safety officer (also see APPENDIX 1D).

Radiolabel DNA oligonucleotide

1. Design, synthesize, and purify a DNA oligonucleotide primer ~25 bases in length (APPENDIX 3).

The oligo should produce a primer-extension product 100 to 150 bases in length (from the 5' end of the annealed oligo to the transcription start site). The oligo should be gel purified on high-percentage (~10%) polyacrylamide gels run in 1 \times TBE, eluted, ethanol precipitated, dissolved in water, and quantified. Oligonucleotides with an annealing temperature of ~58°C should be designed.

2. Make a working dilution of the primer at 2.5 pmol/ μl .
3. In a 1.5-ml microcentrifuge tube, radiolabel 5 pmol (2 μl) of the primer (APPENDIX 3). Use T4 polynucleotide kinase according to the manufacturer's specifications (in a 20- μl reaction) in the presence of 2 μl of 150 $\mu\text{Ci}/\mu\text{l}$ [γ - ^{32}P]ATP. Incubate the reaction at 37°C for 1 hr.

CAUTION: Great care should be taken, and appropriate shielding used, when labeling with [γ - ^{32}P]ATP. All bench surfaces, pipettors, tube racks, hands, and forearms should be carefully monitored with a Geiger counter (see APPENDIX 1D).

Precipitate DNA

4. Add 100 μl of 2.5 M ammonium acetate. Heat the reaction to 70°C for 15 min to inactivate the T4 polynucleotide kinase.
5. Allow the tube to cool to room temperature, then add 2 μl of 10 mg/ml glycogen and 375 μl of 100% ethanol to precipitate the radiolabeled primer. Mix by vortexing.

When vortexing to mix the radioactive solutions or to dissolve precipitated primer in this protocol, use careful, gentle vortexing, avoiding contact of the liquids with the inside of the tube lid. This will minimize leakage of radioactive material from the tube and reduce contamination of the microcentrifuge and the investigator's hands.

6. Microcentrifuge the ethanol precipitates 15 min. Carefully remove the ethanol supernatant from the pellet with a pipet and discard in an appropriate waste container. Add 100 μl TE buffer, pH 8.0, and dissolve the pellet by vortexing.
7. Add 100 μl PCIAA and mix by vortexing. Microcentrifuge the tube 5 min to separate the organic and aqueous phases. Remove the upper (aqueous) phase and transfer to a new 1.5-ml microcentrifuge tube.
8. Add 10 μl of 3 M sodium acetate and 300 μl of 100% ethanol to precipitate the radiolabeled primer. Mix by vortexing, and microcentrifuge 15 min.
9. Carefully remove and discard the ethanol supernatant. Dry the pellet 10 min at room temperature.

Do not dry the pellets under vacuum or they will be very difficult to dissolve.

10. Dissolve the pellet in 150 μl TE buffer, pH 8.0 (to a final concentration of 0.033 pmol/ μl), by vortexing. Store the radiolabeled primer at -20°C.

Primers labeled in this manner may be stored under these conditions for 2 to 3 weeks pending primer-extension analyses.

Perform primer extension

11. Thaw an aliquot of radiolabeled primer complementary to the in vitro transcription RNA products. Make a mix containing 10 μl of 1 \times annealing buffer (2 μl of 5 \times annealing buffer and 8 μl TE, pH 8.0) and 0.25 μl of labeled primer (~8 fmol) for each sample to be analyzed in the assay.
12. Add 10 μl of this primer extension annealing mix to each tube of RNA product. Dissolve the RNA by vortexing.
13. Heat the samples to 70°C for 2 min, then incubate 40 min at 58°C to anneal the primer to the RNA product/template.

The optimal annealing conditions will vary depending on the length and G+C content of the primer.

14. Microcentrifuge the samples quickly to collect condensation from the insides of the lids to the bottoms of the tubes, and allow the samples to cool to room temperature.
15. Add 40 μl primer extension mix and 0.5 μl MMLV reverse transcriptase combined just before use and mix by gentle vortexing. Incubate 40 min at 37°C.
16. Add 225 μl of 100% ethanol. Mix the samples by gentle vortexing and microcentrifuge 15 min.
17. Remove the ethanol supernatant and dry the pellets in a rotary evaporator (e.g., SpeedVac). Dissolve the pellets in 6 μl FLB. Store samples at -20°C or proceed immediately with denaturing polyacrylamide gel electrophoresis.

Avoid storage of the primer extension products for more than 2 or 3 days before gel analysis.

Analyze the products

18. Pour a standard 8% urea-polyacrylamide sequencing gel (*APPENDIX 3*). Boil the primer extension samples 4 min in a water bath. Run the samples on the gel in 1× TBE until the bromphenol blue tracking dye is 3 to 5 cm from the bottom of the gel.

A 25-base primer will run in the vicinity of the bromphenol blue tracking dye on an 8% sequencing gel.

19. Dry the gel on a piece of Whatman 3MM paper. Subject the dried gel to phosphorimaging analysis or autoradiography (*UNIT 6.3*).

If the aim of the experiment is to quantify the amount of primer-extension product, fixing the gel in 10% acetic acid for 15 min is recommended. Prior to fixation of the gel, cut the gel to remove the free primer. Fixation of a gel with the free primer band results in the diffusion of the primer during fixation which increases the background.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.*

Annealing buffer, 5×

- 10 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
 - 1 mM EDTA
 - 1.25 M KCl
- Store up to 6 months at -20°C

Disruption buffer

- 15 mM HEPES (potassium salt), pH 7.6
 - 10 mM KCl
 - 5 mM MgCl_2
 - 0.1 mM EDTA
 - 0.5 mM EGTA
 - 350 mM sucrose
- Store up to 1 month at 4°C
- Just before use add:*
- 1 mM DTT
 - 1 mM sodium metabisulfite
 - 0.2 mM PMSF (also see *APPENDIX 1B*)
 - 1 mM benzamidine

Formamide loading buffer (FLB)

- 80% formamide
 - 10 mM EDTA
 - 1 mg/ml xylene cyanol
- Store up to 6 months at -20°C

Freezing buffer

- 50 mM Tris·Cl, pH 7.9
 - 30% glycerol
 - 1 mM EDTA
 - 0.5 mM DTT
- Prepare fresh at 4°C

HEMG containing 0.1 M KCl

- 25 mM HEPES (potassium salt), pH 7.6
- 100 mM KCl

continued

**In Vitro
Reconstitution**

11.6.13

12.5 mM MgCl₂
0.1 mM EDTA
10% (v/v) glycerol
Store up to 1 month at 4°C
Just before use add:
1.5 mM DTT
1 mM sodium metabisulfite
0.1 mM PMSF (also see *APPENDIX 1B*)
1 mM benzamidine

HEMG20 containing 0.1 M KCl

25 mM HEPES (potassium salt), pH 7.6
100 mM KCl
12.5 mM MgCl₂
0.1 mM EDTA
20% (v/v) glycerol
Store up to 1 month at 4°C
Just before use add:
1.5 mM DTT
1 mM sodium metabisulfite
0.1 mM PMSF (also see *APPENDIX 1B*)
1 mM benzamidine

It is also possible to use 0.4 M potassium glutamate monohydrate (Sigma), instead of 0.1 M KCl, for the preparation of “nuclei juice.”

High-salt buffer

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
10% (w/v) sucrose
0.42 M KCl
5 mM MgCl₂
0.1 mM EDTA
20% (v/v) glycerol
Store up to 1 month at 4°C
Just before use add:
2 mM DTT
0.2 mM PMSF (also see *APPENDIX 1B*)
1 mM benzamidine

The 0.42 M KCl in the high-salt buffer can be substituted with 0.42 M NaCl, if desired.

Hypotonic buffer

10 mM Tris·Cl, pH 7.9 (*APPENDIX 2A*)
10 mM KCl
0.1 mM EDTA
0.1 mM EGTA
Store up to 1 month at 4°C
Just before use add:
2 mM DTT
0.2 mM PMSF (also see *APPENDIX 1B*)
1 mM benzamidine
2 µg/ml aprotinin
2 µg/ml leupeptin
0.75 mM spermidine
0.15 mM spermine
2 µg/ml pepstatin

Molasses/agar plates

100 ml water
14.6 ml molasses
3.6 g fine-ground agar

Autoclave, pour into styrofoam meat trays, and let set. Just before use, spread the top with thick yeast paste (prepared by mixing Red Star yeast with water to a consistency that can be easily spread but is not so thin that the flies get stuck, approximately that of canned tomato paste). Store up to 1 week at 4°C.

Primer extension mix (1 ml)

Evaporate 62.5 µl of 2 mg/ml actinomycin D in ethanol to dryness. Add to this 886 µl water followed by 62.5 µl of 1 M Tris·Cl, pH 8.3. Vortex to dissolve actinomycin D. Add 12.5 µl of 100 mM MnCl₂ (do not use a 1 M stock). Add 3.5 µl of each 10 mM dNTP (14 µl total). Vortex again. Finally, add 25 µl of 0.5 M DTT.

Resuspension buffer

15 mM HEPES (potassium salt), pH 7.6
110 mM KCl
5 mM MgCl₂
0.1 mM EDTA
Store up to 1 month at 4°C

Just before use add:

2 mM DTT
1 mM sodium metabisulfite
0.2 mM PMSF (also see *APPENDIX 1B*)
1 mM benzamidine

Ribonucleotide-5'-triphosphates (rNTPs), 5 mM

Dilute 100 mM stocks of each rNTP (Amersham Pharmacia Biotech) in buffer (the authors use 20 mM sodium phosphate buffer, pH 7.5, but others—including potassium phosphate, Tris, and HEPES—would work) such that each nucleotide is at a final concentration of 5 mM. Store up to 1 year at -70°C

TM buffer

50 mM Tris·Cl, pH 7.9 (*APPENDIX 2A*)
0.1 M KCl (omit where specified)
12.5 mM MgCl₂
1 mM EDTA
10% (v/v) glycerol
Store up to 1 month at 4°C

Just before use add:

1 mM DTT
0.1 mM PMSF (also see *APPENDIX 1B*)
1 mM benzamidine
1 mM sodium metabisulfite

Transcription stop solution

20 mM EDTA, pH 8.0
0.2 M NaCl
1% (w/v) SDS
0.25 µg/µl glycogen
Store up to 1 month at room temperature

COMMENTARY

Background Information

In general, transcription extracts are prepared from whole cells or nuclei by the extraction of factors using high salt concentrations—e.g., 0.42 M NaCl or 0.36 M ammonium sulfate (Dignam et al., 1983; Manley et al., 1980). These extracts are a rich source of general transcription factors, as well as some sequence-specific factors, and they are capable of accurate initiation of transcription from eukaryotic promoters mediated by RNA polymerase II. Results from several laboratories have demonstrated that addition of sequence-specific factors during the transcription reaction results in activated transcription in a binding site-dependent manner. Typically, most of these high-salt extracts utilize between 2% and 5% of the DNA template in a single round of transcription, and most systems allow two to three rounds of transcription. Unfortunately, these high-salt extracts are also a rich source of transcription repressors such as the histones (Croston et al., 1991) and other nonspecific DNA-binding proteins which inhibit elevated amounts of transcription *in vitro*. Removal of these inhibitors is important in order to dissect the exact mechanisms of transcription activation by sequence-specific factors (see, e.g., Croston et al., 1991).

Use of low-salt nuclear extracts avoids the extraction of certain transcription repressors such as the histones. The low-salt extract is therefore very efficient in template usage, and, depending on the promoter, it is possible to use up to 20% of the template per round of transcription with the possibility of multiple rounds of transcription (Kamakaka et al., 1991). The absence of histones in the extract makes it particularly useful in the transcriptional analysis of reconstituted chromatin templates (Kamakaka et al., 1993). Since the soluble nuclear fraction is prepared with low-salt buffers, it can be used directly in *in vitro* reactions without desalting.

Critical Parameters

The protocol for preparation of transcription extracts involves the isolation of nuclei followed by extraction of the factors using either high-salt buffers or high-speed centrifugation.

The most critical consideration after nuclear isolation speed is that all subsequent steps be performed at 4°C as rapidly as possible.

During the high-speed centrifugation, the authors have noticed that use of a fixed-angle rotor generates extracts of very poor quality;

hence it is recommended that only a swinging-bucket rotor be used.

In the authors' experience, dilute extracts are inefficient for *in vitro* transcription reactions.

The use of sterile, high-grade reagents and clean glassware is critical.

High-salt extracts, in general, are more efficient in extracting both general and sequence-specific factors from nuclei. However the method is more tedious (taking 7 to 10 hr) and it is impractical to generate extracts from very small quantities of material. The soluble nuclear fraction does not suffer from these problems.

Anticipated Results

Extracts with a protein concentration of between 10 and 30 mg/ml are expected, and these extracts are usually competent for transcription.

All of these extracts should be initially tested for transcription competence using well characterized eukaryotic promoters and sequence-specific transcription activators. The adenovirus major late promoter should be used to test the HeLa extract while the Kruppel promoter is recommended for the *Drosophila* extract. Gal4-VP16 is a potent transcription activator that can be used with promoter templates containing binding sites for Gal4 with the HeLa and *Drosophila* extracts. In studies carried out in the authors' laboratory RNA synthesis has been monitored by primer extension analysis of the RNA (see Kamakaka et al., 1991). Generation of a primer extension product of a specific size is an indication of a functional extract.

Extraction of 12 liters of HeLa cells should provide 7 ml of active extract. Extraction of 100 g of *Drosophila* embryos should provide 40 to 50 ml high-salt extract or 15 ml soluble nuclear extract.

Time Considerations

The *in vitro* transcription reaction takes 1 to 2 hr and analysis by primer extension another 2 to 3 hr. Preparation of the HeLa extract requires 11 hr (~6 hr to make the extract from frozen cells with an additional 4 to 5 hr for dialysis and freezing). Preparation of the *Drosophila* embryo high salt extract requires 8 to 9 hr and the soluble nuclear extract requires 4 to 5 hr.

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Nuclear Import in Digitonin-Permeabilized Cells

UNIT 11.7

The development of the nuclear import assay in permeabilized cells in 1990 was a windfall for the examination of the mechanism of nuclear import. This assay (see Basic Protocol) has been used to detect and examine the factors required for import of nuclear substrates and has lately been adapted for the study of nuclear export as well. This unit will primarily deal with how to analyze the nuclear import of a substrate in an in vitro system. These experiments generally have two goals: first, to demonstrate that a given protein is imported into the nucleus and second, to define the particular sequence (termed the nuclear localization sequence or NLS) in the protein which is responsible for that localization. The nuclear import assay as described here uses *Xenopus* ovarian cytosol as a source of import factors to support import of fluorescent substrate into the nuclei of cells in which the plasma membrane has been permeabilized with digitonin (see Basic Protocol). The presence or absence of import substrate within the nuclei is subsequently detected by fluorescence microscopy.

More challenging than the nuclear import assay itself is generating the reagents necessary to perform the assay. This includes generation of the fluorescent nuclear import substrate and production of cytosol to support nuclear import of that substrate. Production of *Xenopus* ovarian cytosol is described here (see Support Protocol 1). It is a simple, dependable, and inexpensive procedure which does not require a relatively large tissue culture capability. Many laboratories instead use HeLa cytosol or reticulocyte lysate (UNIT 11.2) as a source of import factors.

Two protocols are given for production of fluorescent nuclear import substrate controls. The first protocol (see Support Protocol 2) describes how to make TRITC-BSA-NLS, which consists of bovine serum albumin (BSA) that is made fluorescent by coupling to rhodamine (for ease of detection) and then chemically coupled to NLS peptides to confer nuclear localization. A more recent version of import substrate (developed by Dr. Manfred Lohka; see Support Protocol 3) is a fusion protein composed of green fluorescent protein (GFP), glutathione *S*-transferase (GST), and an NLS. The recombinant import substrates have the advantage of containing a specific number of NLSs (one) and fluorophores (one) per molecule. This is not true of chemically conjugated import substrates which, due to the method of their production, are a heterogeneous population. However, the recombinant substrate has not been used as extensively and is therefore not as thoroughly characterized as the chemical conjugates.

Finally, the method of detection of imported substrate must be adapted to the protein in question. There are three common alternatives. First, if the protein has been cloned, it can be fused to GFP and its import assayed directly, assuming the total molecular weight of the substrate is greater than ~50 kDa. Second, the protein can be made fluorescent by direct conjugation to a fluorophore. Third, the protein can be detected by indirect immunofluorescence microscopy (see UNIT 4.3).

NUCLEAR IMPORT ASSAY IN ATTACHED HELA CELLS

The following protocol describes a nuclear import assay using a known NLS-containing substrate. The HeLa cell plasma membrane is permeabilized with digitonin, and soluble cellular components are washed away. These soluble components include proteins and nucleotides required for nuclear import to occur. The components are therefore added back to the permeabilized cells in the form of *Xenopus* ovarian cytosol, ATP, and GTP.

**BASIC
PROTOCOL**

**In Vitro
Reconstitution**

11.7.1

Contributed by Mary Shannon Moore and Eric D. Schwoebel

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Supplement 5

There are two choices of positive control substrate (see Support Protocols 2 and 3), both of which have the same basic properties: (1) the substrate is fluorescent to aid in detection and, (2) the substrate contains an NLS directing import into the nuclei. This substrate is mixed with the cytosol and nucleotide, and added to the cells. The import reaction is allowed to continue for 15 min, and is stopped by addition of cold buffer and fixation.

Materials

70% ethanol
30,000 cell/ml suspension of HeLa cells (ATCC CCL-2) in complete DMEM/10% FBS
DMEM/10% FBS (see recipe)
1× transport buffer (TB; see recipe), ice cold
Digitonin working solution (see recipe), ice cold
Import reaction mixture (see recipe)
Paraformaldehyde working solution (see recipe), ice cold
p-phenylenediamine mounting medium (see recipe)
Clear nail polish

12-mm circular glass coverslips (Fisher) sterilized by autoclaving
24-well tissue culture plates
Whatman 3 mm filter paper
Fine-tipped forceps with large radius curved shanks (e.g., Dumont #7, Fine Science Tools)
Fluorescence microscope

NOTE: All solutions and equipment coming into contact with tissue culture cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All tissue culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

Prepare HeLa cultures

1. Place 12-mm coverslips in wells of 24-well plate using forceps that have been sterilized by immersion in 70% ethanol and air dried. Add 1 ml of HeLa cells at 30,000 cells/ml in complete DMEM/10% FBS. Return plate to incubator and incubate 16 hr.

The number of cells plated may have to be optimized to give ~50% confluence after 16 hr.

Permeabilize cells

2. Place plate on ice for 5 min. Aspirate medium and gently add 1 ml ice-cold TB.

If using a repeating pipettor, designate one syringe for each solution (TB, digitonin, and fixative). Be gentle when adding solutions to the coverslips. Pipet slowly and do not spray directly onto the cells, but rather down the side of the well. From this point on, sterility is not required.

3. Aspirate TB and add 1 ml ice-cold digitonin solution. Incubate 5 min on ice.

Digitonin preferentially solubilized cholesterol from membranes, which leaves large holes through which soluble material passes. Because the plasma membrane contains higher concentrations of cholesterol than the nuclear envelope, it is selectively permeabilized while the nuclear envelope is not.

4. Aspirate the digitonin solution and add 1 ml of ice-cold TB. Continue to hold plates on ice up to 30 min.

Perform import reaction

5. Tape Parafilm onto the benchtop, ensuring that it is flat on the surface, and mark with numbers in the same pattern as the plate (i.e., in rows of six). Place 40- μ l drops of the import reaction mixture on the Parafilm so as to correspond to each well. Using forceps, remove coverslips from wells and wick off excess TB by touching the edge of the coverslip to filter paper. Gently invert each coverslip (cell-side-down) on its designated drop of import mixture.

The coverslip will float on top of the drop of import mix.

Controls for nuclear import include reaction mixes prepared with probes containing positive and negative NLS sequences (see Critical Parameters).

6. Cover the reactions with an opaque tray to prevent fading, and incubate 15 min at room temperature.
7. Gently pipet 250 μ l of ice-cold TB underneath the coverslip to stop the import reaction and to float the coverslip up from the Parafilm. Gently remove coverslip and place back in the TB (cell-side-up) in the 24-well plate on ice.

Fix cells

8. Aspirate TB and add 1 ml of fresh ice-cold TB to each coverslip. Aspirate TB and add 1 ml of ice-cold 3% paraformaldehyde. Cover to protect from light, and incubate on ice for 15 min.
9. Remove coverslip, wick off excess fixative on filter paper, and gently invert on a small drop of mounting medium (cell-side-down). Allow top of coverslip to dry for 5 min before sealing the edges of the coverslips with clear nail polish.

Assess cells for nuclear import

10. Observe by fluorescence microscopy using 63 \times or 100 \times oil objective.

The simplest way to record the results is photographically. To ensure that different panels of a given figure are comparable, the different samples should be photographed with the same exposure time and printed identically. The relative differences in fluorescence intensity between different samples is generally very reproducible. However, the overall fluorescence intensity of the assay does show some inter-assay variation from day to day. Therefore all the relevant samples for a given figure should be done in a single experiment. If desired, the prints from these photographs can be scanned and quantitated. However, if quantitation is to be used on a regular basis, it is strongly recommended that the investigator access or purchase a quantitation program and a system (such as a CCD camera or confocal microscope; UNIT 4.5) specifically designed for that purpose.

PREPARATION OF XENOPUS OVARIAN CYTOSOL

Cytosol from *Xenopus* ovaries is used to provide the cytosolic factors necessary for protein import.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

- Eight female *Xenopus* frogs
- PBS (see recipe), ice-cold
- Protease inhibitor tablets (Boehringer Mannheim Complete, EDTA-free)
- 1 \times homogenization buffer (see recipe), ice cold
- 1 \times transport buffer (TB; see recipe), ice cold

SUPPORT PROTOCOL 1

In Vitro Reconstitution

11.7.3

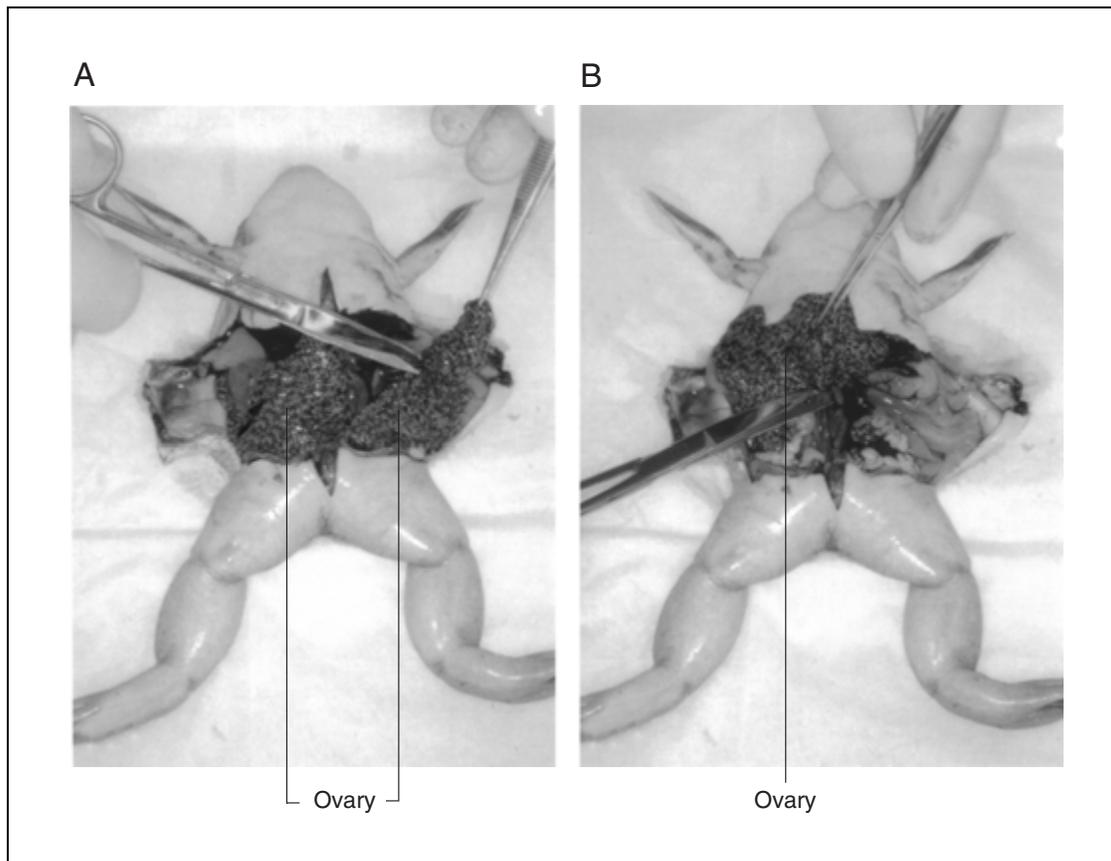


Figure 11.7.1 Frog ovary dissection. These panels show the exposed abdominal cavity of the female *Xenopus*. **(A)** Upon opening the abdominal cavity, the ovary is by far the most conspicuous organ. **(B)** To remove, gently lift the ovary with forceps and clip attachment points as near to the ovarian tissue as possible.

Carbon dioxide tank with attached tubing
 Polytron homogenizer, PRO300D (PRO Scientific)
 250-ml plastic centrifuge bottles
 Sorvall centrifuge with GSA centrifuge rotor (or equivalent)
 Cheesecloth
 Beckman ultracentrifuge with Ti 70.1 rotor (or equivalent), and appropriate ultracentrifuge tubes
 Plastic tubing, thin
 20-ml syringe
 Dialysis tubing (4000 to 6000 MWCO)
 Centriplus-10 concentrators (Amicon)
 Additional reagents and equipment for determining protein concentration
 (APPENDIX 3B)

Dissect ovaries

1. Place frogs and water into a covered bucket with two pipet-sized holes drilled in the top for gas exchange. Sacrifice frogs by bubbling carbon dioxide through the frog water for 20 min under a fume hood. Transfer dead frogs into ice to keep cold.

Approximately 350 mg of cytosol can be prepared from eight frogs.

2. Remove ovaries (Fig. 11.7.1) and place in a beaker (kept on ice) containing 1 liter of cold PBS.

Try to avoid harvesting connective tissue.

3. After all of the ovaries are collected, wash them several times with a total of 4 liters of ice-cold PBS. Let the ovaries settle between washes and decant the supernatant.
4. Place the ovaries in a large graduated cylinder containing cold PBS to measure the wet volume of the ovaries.
5. Using a large pair of forceps, remove ovaries from beaker and briefly blot on paper towel to remove excess liquid. Transfer to a plastic beaker.

Prepare homogenate

6. Add an equal volume of cold homogenization buffer containing dissolved protease tablets (1 tablet per 50 ml buffer).
7. Homogenize with a Polytron homogenizer using several short bursts (5 to 10 sec each).

This tissue is very soft and homogenizes easily. Use a relatively low setting on the homogenizer. Do not operate at high enough speeds to foam the slurry.
8. Transfer homogenate to 250-ml plastic centrifuge bottles and centrifuge 20 min in a GSA rotor at $16,000 \times g$, 4°C .
9. Decant the supernatant, avoiding the floating fat layer as much as possible. Filter supernatant through several layers of cheesecloth. Discard pellet and fat layer.
10. Transfer the supernatant to ultracentrifuge tubes using homogenization buffer to top off the last tube. Centrifuge 2 hr in a Ti 70.1 rotor at $150,000 \times g$, 4°C .

Collect clarified cytosol

11. Attach thin tubing to a 20-ml syringe. Insert tubing approximately one-half of the distance into the tube, between the floating fat layer and the loose yellow precipitate near the bottom. Draw off this clear middle layer.

It is better to leave a little cytosol behind than to draw up contaminants at this step.

12. Transfer this cytosol to dialysis tubing (4000 to 6000 MWCO). Dialyze into a total of 6 liters of TB at 4°C . Change buffer three times with the third change followed by overnight dialysis.

Additional insoluble material will form during dialysis, making the cytosol appear cloudy.

13. Transfer dialysate to ultracentrifuge tubes and centrifuge 1 hr in a Ti 70.1 rotor at $100,000 \times g$, 4°C .
14. Perform a Bio-Rad protein assay on the resulting cytosol (APPENDIX 3B). Concentrate the cytosol to ~ 20 mg protein/ml using Centriplus-10 concentrators.

The protein concentration at the end of the cytosol preparation is usually 4 to 6 mg/ml. Typically, the optimal final concentration required to support nuclear import is ~ 10 mg/ml. Because additional constituents will be added during the import assay, it is convenient to concentrate the cytosol to ~ 20 mg/ml. A concentration curve must be performed on each batch to determine the optimal concentration required to support nuclear import.

15. Divide the concentrated cytosol into aliquots, snap freeze, and store at -80°C up to 6 to 8 months.

A 550- μl 10 mg/ml aliquot would be enough cytosol for 24 cover slips.

PRODUCTION OF FLUORESCENT IMPORT SUBSTRATE: TRITC-BSA-NLS

A nuclear localization substrate is prepared by coupling BSA to an NLS and conjugating the resulting protein to TRITC.

Materials

Sephadex G-25 and G-50 (Pharmacia Biotech)
0.1 M sodium phosphate, pH 7.0 and 8.0 (*APPENDIX 2A*), ice cold
Bovine serum albumin, fatty-acid free (Boehringer Mannheim)
0.1 M sodium carbonate, pH 9.0/50 mM NaCl (store indefinitely at room temperature)
Tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC; Molecular Probes)
DMSO
1.5 M hydroxylamine-HCl, pH 8.5 (see recipe)
Sulfo-SMCC (Pierce)
NLS or mutant NLS peptide (~1 mg)
20 mM HEPES (potassium salt), pH 7.3/110 mM potassium acetate (prepare fresh)
1× transport buffer (TB; see recipe)
Disposable 20-ml columns (Bio-Rad Laboratories, Cat # 732-1010)
Rocking platform
Centricon-30 concentrators (Amicon)
Additional reagents and equipment for dialysis (*APPENDIX 3C*), determining protein concentration (*APPENDIX 3B*), and SDS-PAGE (*UNIT 6.1*)

Prepare columns

1. Using disposable 20-ml columns, prepare three 15-ml bed volume desalting columns containing the following media.

Sephadex G-25 equilibrated with 0.1 M sodium phosphate, pH 7.0

Sephadex G-25 equilibrated with 0.1 M sodium phosphate, pH 8.0

Sephadex G-50 equilibrated with 0.1 M sodium phosphate, pH 7.0

Equilibrate each column at 4°C with at least 75 ml of the appropriate cold phosphate buffer.

Label BSA with TRITC

2. Dissolve 10 mg BSA in 1 ml of 0.1 M sodium carbonate, pH 9.0/50 mM NaCl. Save a 20- μ l aliquot of this solution at 4°C as standard for SDS-PAGE analysis of conjugated products.

This protocol will yield ~8 mg of TRITC-BSA-NLS import substrate.

If more (or less) import substrate is required, scale entire procedure including BSA, TRITC, sulfo-SMCC, and peptide proportionally.

3. Dissolve 0.4 mg TRITC in 40 μ l DMSO.
4. Add 12.5 μ l of the TRITC/DMSO solution to dissolved BSA, 2.5 μ l at a time, mixing after each addition. After all of the TRITC is added, wrap the tube in foil and incubate on a rocking platform 1 hr at room temperature.

TRITC and its subsequent conjugates are sensitive to fading by ambient light, and prolonged exposure will result in noticeably dimmer import substrate. Protect from light whenever possible from this point onward.

5. Add 100 μ l of 1.5 M hydroxylamine-HCl, pH 8.5, to the TRITC-BSA, wrap in foil, and mix for an additional 1 hr on the rocking platform at room temperature.

Hydroxylamine terminates the conjugation reaction and removes unstable conjugates from the BSA.

6. Microcentrifuge 10 min at $15,000 \times g$, 4°C , and save the supernatant.

Some insoluble complexes may form during steps 9 and 10.

Purify TRITC-BSA

7. Load the TRITC-BSA onto the 15-ml Sephadex G-25 column equilibrated with 0.1 M sodium phosphate, pH 8.0 (see step 1). Elute with 0.1 M sodium phosphate, pH 8.0, collecting 1-ml fractions. Pool the fractions containing the first red peak.

The separation of the TRITC-BSA (faster) and unconjugated TRITC (slower) should be obvious.

8. Concentrate eluate to ~ 0.5 ml in a Centricon-30. Remove a $10\text{-}\mu\text{l}$ aliquot and save at 4°C for SDS-PAGE analysis.

Note that if the separation of TRITC from TRITC-BSA on the Sephadex G-25 was effective, the filtrate from the Centricon should be colorless.

Activate TRITC-BSA

9. Dissolve 2 mg of sulfo-SMCC in 200 μl water (23 mM final). Add 166 μl of sulfo-SMCC to the TRITC-BSA. Wrap in foil and incubate 30 min at 37°C . Vortex lightly every ~ 5 min.

10. Microcentrifuge 10 min at $15,000 \times g$, 4°C , and collect the supernatant.

11. Load sample onto the 15-ml Sephadex G-25 column equilibrated with 0.1 M sodium phosphate, pH 7.0 (see step 1). Elute with 0.1 M sodium phosphate, pH 7.0, collecting 1-ml fractions. Pool the fractions containing the red eluate, which is the sulfo-SMCC activated TRITC-BSA. Remove 20 μl and save for SDS-PAGE analysis.

This column will separate unbound sulfo-SMCC (slower and colorless) from that bound to the TRITC-BSA (faster and red).

Conjugate NHS peptide to TRITC-BSA

12. Add a 5- to 20-fold molar excess of NLS peptide to TRITC-BSA-sulfo-SMCC. Cover with foil and incubate on a rocking platform overnight at 4°C .

13. Microcentrifuge 10 min at $15,000 \times g$, 4°C , to remove insoluble material.

14. Concentrate to 0.75 ml in Centricon-30.

15. Load the concentrated TRITC-BSA-NLS onto the 15-ml Sephadex G-50 column equilibrated with 0.1 M sodium phosphate, pH 7.0. Elute with 0.1 M sodium phosphate, pH 7.0, collecting 1-ml fractions. Pool fractions containing the red eluate, which is the TRITC-BSA-NLS.

This column separates the final product, TRITC-BSA-NLS (faster and red) from the unconjugated NLS peptide (slower and colorless).

16. Dialyze (APPENDIX 3C) against a total of 3 liters of 20 mM HEPES (potassium salt), pH 7.3/110 mM potassium acetate at 4°C . Change the buffer three times, with the last change followed by overnight dialysis.

17. Centrifuge dialysate 10 min at $10,000 \times g$, 4°C . Save the supernatant. Remove 20 μl and save for SDS-PAGE analysis.

18. Determine protein concentration using the Bio-Rad assay (APPENDIX 3B).

19. Concentrate in Centricon-30 if necessary.

The concentration of the stock import substrate should be at least 40× the final concentration used in the import assay. The authors' TRITC-BSA-NLS conjugates typically exhibit maximal import at 5 to 10 µg/ml.

Analyze conjugates

20. Analyze aliquots collected at steps 2, 8, 11, and 17 by SDS-PAGE (UNIT 6.1).

Conjugation of sulfo-SMCC to BSA (without peptide) will result in an apparent increase in molecular weight on SDS-PAGE. Successful coupling of peptide to the sulfo-SMCC-BSA will cause a further decrease in mobility. The number of peptides conjugated can be roughly estimated by the change in molecular weight.

21. Divide the conjugate solutions into aliquots and snap freeze in liquid nitrogen. Store at −80°C (for up to a year).
22. When required, briefly thaw aliquot in 37°C water bath, dilute to 40× with TB, and microcentrifuge 20 min at 15,000 × g, 4°C. Transfer supernatant to a fresh tube. Store in a dark container at 4°C for up to 1 month.

SUPPORT PROTOCOL 3

PRODUCTION OF FLUORESCENT RECOMBINANT IMPORT SUBSTRATE: GFP-GST-NLS

As an alternative to coupling of BSA to the NLS and TRITC (see Support Protocol 2), the nuclear localization substrate can be a recombinant GFP-GST-NLS produced in *E. coli*.

Materials

LB medium (APPENDIX 2A) containing 100 µg/ml ampicillin
DH5α strain of *E. coli*
GST-GFP-NLS and GST-GFP-mutNLS constructs in *E. coli* (Dr. Manfred Lohka, Biological Sciences, University of Calgary, Calgary, Canada)
1 M IPTG in H₂O, sterile filtered
Phosphate-buffered saline (PBS; see recipe)
Protease inhibitors tablets (Boehringer Mannheim Complete, EDTA-free)
PBS/EDTA (see recipe)
10% (v/v) Triton X-100
Glutathione–Sepharose 4B (Pharmacia Biotech)
Glutathione/Tris (see recipe)
20 mM HEPES (potassium salt), pH 7.3/110 mM potassium acetate (prepare fresh)
Transport buffer (TB; see recipe)

Shaking incubator
Sorvall centrifuge with GSA and SS-34 rotors (or equivalents)
50-ml conical centrifuge tubes
French press or sonicator
Rocking platform
Disposable 2-ml column (Bio-Rad)
Centricon-30 (Amicon)

Additional reagents and equipment for dialysis (APPENDIX 3B), determining protein concentration (APPENDIX 3B), and SDS-PAGE (UNIT 6.1)

1. Inoculate a 250-ml culture of LB medium containing 100 µg/ml ampicillin with a single colony of *E. coli* DH5α containing GST-GFP-NLS. Grow overnight in a shaking incubator at 300 rpm, 37°C.

This 1-liter culture yields ~9 mg of GFP-GST-NLS.

2. Add the overnight culture to 750 ml of the LB medium. Mix and split into two flasks of 500 ml each. Add IPTG (from 1 M stock) to 0.4 mM, and continue shaking at 28°C for 24 hr.
3. Harvest cells by centrifuging 10 min in a GSA rotor at 4000 × g, 4°C. Discard the supernatant.

Pellets can be frozen at –80°C at this time if desired.

4. Resuspend pellets in a total of 15 ml of 1× PBS and transfer suspension to a 50-ml conical tube. Rinse centrifuge bottles with an additional 3 ml PBS and pool the wash with the suspension. Vortex thoroughly to completely resuspend bacterial pellet.
5. Dissolve one protease inhibitor tablet in 1 ml of 1× PBS/EDTA and add to suspension. Add 3 ml of 10% Triton X-100 (1% final), and bring volume to 30 ml with 1× PBS/EDTA. Vortex to mix.

Dissolve tablet in room temperature PBS/EDTA. Crush tablet with a spatula to aid in dissolution and vortex until dissolved.

6. Disrupt cells by passage through a chilled French press at 16,000 psi.

Chill the cylinder prior to use to minimize warming the sample. The authors find that lysis is most efficiently achieved with the French press. Alternately, cells can be lysed by sonication using three pulses of 1 min with a 1-min interval on ice after each pulse for cooling.

7. Clarify lysate by centrifuging 20 min at 16,000 × g, 4°C, in an SS-34 rotor or equivalent. Discard the pellet.
8. Gently resuspend glutathione-Sepharose 4B slurry in the stock bottle. Remove 2 ml (corresponding to ~1.5 ml of packed resin) and transfer to a 50-ml conical centrifuge tube. Centrifuge 2 min at 500 × g to sediment resin and remove supernatant. Resuspend the resin in 30 ml of 1× PBS/EDTA. Sediment resin by centrifuging 5 min at 500 × g. Discard supernatant.
9. Transfer clarified lysate from step 7 to the 50-ml conical centrifuge tube containing the glutathione–Sepharose 4B. Invert to mix and incubate with gentle rocking at 4°C for 2 hr.

Brief exposure of GFP to ambient light does not noticeably affect its brightness, but protect the GFP from light during any prolonged step.

10. In the cold room, pour resin/homogenate from step 9 into a 2-ml disposable column and collect flowthrough for future analysis. Wash the column with 50 ml of cold PBS. Minimize disturbance of the column bed during addition of PBS. After the last wash, allow the meniscus of the buffer to approach the top of the column bed, then stopper the column.

The presence of GFP should be particularly evident as the resin packs at the bottom of the column. The bedding will appear bright green.

11. Elute GFP-GST-NLS at room temperature with glutathione/Tris by adding 10 ml of glutathione/Tris to the top of the column bed. Unstopper column and collect 1-ml fractions of the eluate in 1.5-ml microcentrifuge tubes. Continue until the eluate is almost colorless and column bed appears nearly white. Place fractions on ice as they are collected and protect from light. Pool green fractions.
12. Dialyze (APPENDIX 3C) against a total of 3 liters of 20 mM HEPES (potassium salt), pH 7.3/110 mM potassium acetate at 4°C. Change buffer three times with the last change followed by overnight dialysis.

13. Centrifuge dialysate 20 min in an SS-34 at $10,000 \times g$. Save the supernatant.

Some protein may precipitate during dialysis.

14. Determine protein concentration using the Bio-Rad assay (APPENDIX 3B). Concentrate using Centricon-30 if necessary. Determine purity by SDS-PAGE (UNIT 6.1).

The authors typically find that the activity of this substrate in the nuclear import assay is maximal at 25 to 50 $\mu\text{g/ml}$. It is convenient to concentrate the substrate to at least 40 \times its estimated final concentration, corresponding to $\sim 2 \text{ mg/ml}$.

15. Aliquot, snap freeze in liquid nitrogen, and store at -80°C .

The GST-GFP-NLS can be stored at -80°C for long periods. The size of the aliquots depends on the size of the experiment.

16. Perform a preliminary import assay (see Basic Protocol) to determine the optimal concentration of import substrate.

Test several concentrations between 0 and 100 $\mu\text{g/ml}$. Too little substrate gives a faint signal, while too much substrate increases background and also decreases signal.

17. When required, thaw aliquot briefly in 37°C water bath, then dilute with TB to 40 \times the optimal concentration determined in step 16. Microcentrifuge 10 min at $15,000 \times g$, 4°C , to remove any precipitated protein, and transfer the supernatant to a fresh tube. Store at 4°C in a dark container (for up to a few weeks).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Digitonin solution

Stock solution (20 mg/ml):

40 mg digitonin (Calbiochem-Novabiochem)

Bring to 2 ml with DMSO

Store at room temperature for up to 1 month

Working solution: Dilute to 70 $\mu\text{g/ml}$ in transport buffer (TB; see recipe) on day solution is to be used.

DMEM/10% FBS

Dulbecco's Modified Eagle Medium (e.g., Life Technologies, 1 \times liquid, high glucose) containing:

10% fetal bovine serum (APPENDIX 2A)

10 mM HEPES (pH 7.55)

100 U/ml penicillin G sodium

100 $\mu\text{g/ml}$ streptomycin sulfate

Store up to 1 month at 4°C

Glutathione/Tris

2.5 ml 1 M Tris \cdot Cl, pH 8.0 (APPENDIX 2A; 50 mM final)

H_2O to 45 ml

76.8 mg reduced glutathione (Sigma; 5 mM final)

Adjust pH to 8.0 at room temperature with NaOH

Bring to 50 ml with H_2O

Divide into aliquots

Store up to several months frozen at -20°C

Glutathione noticeably alters the pH of the Tris buffer, which strongly affects the elution of some proteins.

Homogenization buffer

Prepare 10× stock by combining the following:

200 ml 1 M HEPES, pH 7.3 (use KOH to adjust the pH)

20 ml 1 M magnesium acetate, pH 7.3

Bring volume to 1 liter with H₂O

Filter sterilize using a 0.2- μ m filter

Store up to several months at 4°C

Prepare 1× working solution on day of use:

Dilute 10× stock to 1× with H₂O

Add 1 M dithiothreitol (DTT) stock (store frozen in aliquots at –20°C) to 2 mM final

Hydroxylamine-HCl, 1.5 M, pH 8.5

Dissolve 2.1 g hydroxylamine hydrochloride in 10 ml water. Adjust pH to 8.5 with 5 M NaOH and adjust volume to 20 ml with H₂O. Prepare fresh.

Import reaction mixture

~10 mg/ml *Xenopus* ovarian cytosol (see Support Protocol 1)

0.5 mM GTP: add from 20 mM GTP stock in transport buffer (TB; see recipe)

1 mM ATP: add from 20 mM ATP stock in transport buffer (TB; see recipe)

Import substrate (for approximate concentrations, see Support Protocols 2 and 3)

Transport buffer (TB; see recipe) to 40 μ l

Mix and store on ice until immediately before use.

The 20 mM ATP and GTP stock solutions are stored frozen in aliquots at –80°C.

Paraformaldehyde solution

Stock solution (6%): Under a fume hood, heat 150 ml of water to 60°C. Remove from heat and add 9 g paraformaldehyde. While stirring, add 2 drops 5 N NaOH from a Pasteur pipet. Continue stirring until dissolved. Store at 4°C for up to 1 month.

Working solution (3%): Dilute to 3% with 10× transport buffer (TB; see recipe) and water on the day of use.

PBS/EDTA, 4×

46 ml 0.2 M sodium phosphate, monobasic (NaH₂PO₄)

154 ml 0.2 M sodium phosphate, dibasic (Na₂HPO₄)

35.1 g NaCl

Bring to 900 ml with H₂O

Add 8 ml of 0.5 M EDTA

Adjust pH to 7.4 with NaOH

Bring to 1 liter with H₂O

Store at 4°C

Phenylendiamine mounting medium

Dissolve 100 mg *p*-phenylenediamine (an antifading agent) in 10 ml of 1× PBS (see recipe). Add 90 ml of glycerol and stir until homogenous. Divide into aliquots and store at –80°C. Warm to room temperature before use.

Prolonged exposure to light causes the mounting medium to turn brown.

Phosphate-buffered saline (PBS), 10×

2.3 mM NaH₂PO₄

7.7 mM Na₂HPO₄

150 mM NaCl

Store up to several months at 4°C

Transport buffer (TB)

Prepare 10× stock by combining the following:

200 ml 1 M HEPES, pH 7.3 (use KOH to adjust the pH)

275 ml 4 M potassium acetate, pH 7.3

20 ml 0.1 M magnesium acetate

3.8 g EGTA

Bring to 900 ml with H₂O and adjust pH to 7.3 with KOH or acetic acid

Bring volume to to 1 liter with H₂O

Filter sterilize using a 0.2- μ m filter

Store up to several months at 4°C (months)

Prepare 1× working solution on day of use:

Dilute 10× stock to 1× with H₂O (working concentration)

Add 1 M dithiothreitol (DTT) stock (store frozen in aliquots at –20°C) to 2 mM final

The 1× TB contains 20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, and 1 mM EDTA.

COMMENTARY

Background Information

Development of the permeabilized cell assay permitted a biochemical dissection of nuclear import mechanisms (Adam et al., 1990). This assay depends on the specificity of the detergent digitonin for cholesterol, which is present at higher concentrations in the plasma membrane than in the nuclear envelope (Colbeau et al., 1971). During the permeabilization process, the soluble contents of the cytoplasm leak out into the surrounding solution and are washed away. Included in these soluble contents are proteins and nucleotides (ATP and/or GTP) required for nuclear import. These must be re-added to permeabilized cells, in this case in the form of crude cytosol, for nuclear import to occur (Fig. 11.7.2A). Energy, in the form of nucleoside triphosphate (NTP), must also be added for most types of nuclear import to occur. While GTP alone appears to be required, the authors have included both ATP and GTP in this protocol. This is a common practice within the field when energy requirements are not a specific focus of the experiment. NTP can be removed from the import assay by addition of apyrase in place of ATP and GTP. The absence of NTP in the reaction mixture results in docking of the substrate at the NPC, yielding a characteristic rim pattern (Fig. 11.7.2B). Many kinds of nuclear import can also be inhibited by the addition of wheat germ agglutinin (WGA; Finlay et al., 1987; Fig. 11.7.2C), which binds to certain glycosylated NPC proteins (Hanover et al., 1987). This demonstrates that transport occurs through the NPC and is not the result of nuclear envelope damage.

There are a number of variations on the basic nuclear import assay. Several cell types have been used, but to date no significant differences have been found in import mechanics (see for instance Adam et al., 1990; Moore and Blobel, 1992; Adam and Adam, 1994). Cells in suspension can also be permeabilized, divided into aliquots, and frozen for future use (Leno et al., 1992; Görlich et al., 1994). The optimal digitonin concentration required for permeabilizing can, however, differ between cell types. At the ideal concentration, treatment of cells with digitonin results in permeabilization of the plasma membranes in the vast majority of cells (>95%), and nuclear envelope damage in very few cells (<5%). The integrity of the plasma membrane can be tested with DAPI, which is membrane impermeable and strongly stains DNA only in cells in which the plasma membrane has been permeabilized. The DAPI molecule is below the diffusion size limit of the NPC, and can therefore diffuse through the NPC into nuclei with intact nuclear envelopes. The integrity of the nuclear envelope can be tested with antibodies against nuclear components—i.e., lamina proteins or DNA itself (Adam et al., 1990). These antibodies cannot diffuse through the NPC, and will therefore only yield nuclear signal when the nuclear envelope has been damaged. Both the plasma membrane and the nuclear envelope are permeabilized with 0.1% Triton X-100.

There are also several variations on the duration of the nuclear import reaction and the temperature at which the reaction is incubated, extending up to 1 hr at 37°C. These longer

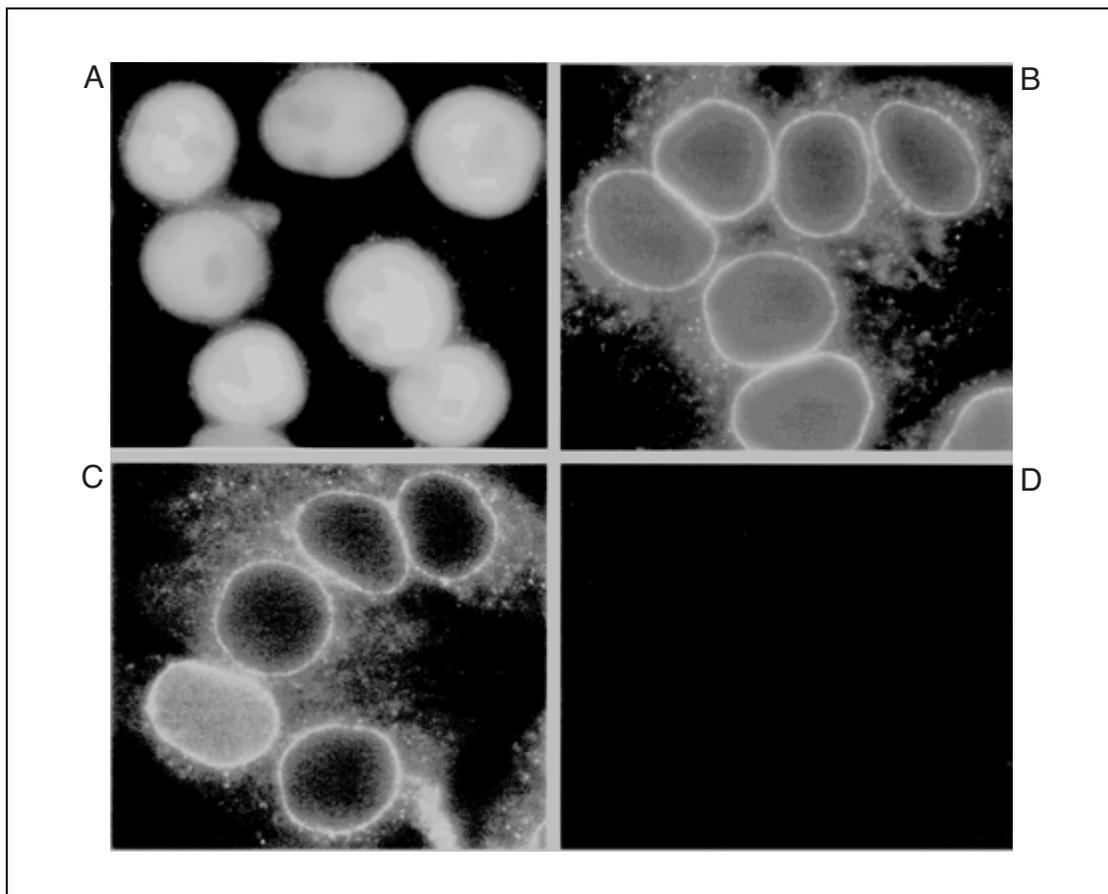


Figure 11.7.2 Fluorescence micrographs of the nuclear import assay performed under various conditions. All panels show representative nuclei from HeLa cells photographed and printed identically. **(A)** This positive control import reaction contained 10 mg/ml *Xenopus* cytosol, 0.5 mM GTP (0.5 mM), 1.6 mM ATP, and 50 μ g/ml GFP-GST-NLS import substrate in TB, and was incubated for 15 min at room temperature prior to washing and fixation. **(B)** Same as (A) but GTP and ATP were replaced with 50 U/ml apyrase (50 U/ml), which at this concentration hydrolyzes any NTP. Note strong “rim” staining, reflecting the presence of substrate docked at the nuclear envelope. The punctate cytoplasmic signal may represent annulate lamellae (Davis and Blobel, 1986). **(C)** Same as (A), but in the presence of WGA (500 μ g/ml), which binds to a subset of glycosylated NPC proteins and prevents import of NLS substrates. Note that the rim staining is still present but diminished. **(D)** Same as (A), but GFP-GST-NLS was replaced with GFP-GST-mutNLS. The mutant is neither imported into the nucleus nor bound to the nuclear envelope.

incubations should be performed in a humidified chamber to prevent the reaction mixture from evaporating. Long incubations may also require addition of an energy-regenerating system to maintain high NTP levels for the duration of the reaction. This regenerating system generally consists of 5 mM phosphocreatine and 20 U/ml creatine phosphokinase (final concentrations). The protocol given here is of relatively short duration by comparison, and addition of regenerating systems is not required.

Included is a support protocol describing the production of *Xenopus* ovarian cytosol (Moore and Blobel, 1992). While not as widely used as some other cytosols, it is simple and relatively inexpensive to produce. Alternatively, HeLa

cytosol or reticulocyte lysate can either be produced (Adam, 1998) or be purchased from the NIH. Many people also use *Xenopus* egg extract produced from ovulated eggs (Görlich et al., 1994). Note that in ovulated eggs the nuclear envelope is broken down and the NPC is disassembled, so the constituents of this extract differ from those in ovarian cytosol.

Four proteins have been purified from cytosol on the basis of their ability to restore import in permeabilized cells. Karyopherin α /importin α (Adam and Gerace, 1991) binds to the NLS on nuclear proteins, and karyopherin β /importin β (Adam and Adam, 1994) in turn binds to the karyopherin α -NLS protein heterodimer and docks the complex at the NPC,

probably through karyopherin β interactions with a subclass of NPC proteins (Radu et al., 1995). Ran, a small GTPase (Moore and Blobel, 1993), and p10/NTF2 (Moore and Blobel, 1994), another protein known to interact with NPC proteins, are required for translocation of the NLS protein through the NPC and into the nuclear interior. Karyopherins α and β are sensitive to oxidation (Adam and Gerace, 1991; Adam and Adam, 1994), and the continuous presence of DTT in cytosol purification and during the import assay helps assure that these components are active (glutathione performs this role *in vivo*). Ran is in the GDP-bound form in the cytosol, but GTP is required for import, and it appears that Ran must be converted to the GTP-bound state to release substrate into the nuclear interior. Formation of stable nucleotide-Ran complexes requires Mg^{2+} (Bischoff and Ponstingl, 1991) which is present throughout the procedure. Potassium acetate is the preferred salt, as the buffer is designed to mimic the cellular interior, which is high in potassium. The total salt concentration is ~ 150 mM, similar to the situation *in vivo*.

The second common objective in nuclear import studies is to identify the NLS(s) within a given protein responsible for its localization. There are three cautions with this analysis (reviewed by Jans and Hübner, 1996). First, a protein may contain more than one NLS. Second, NLS activity may require a modification of the protein (e.g., phosphorylation/dephosphorylation or ligand binding) and that modification may not occur in the *in vitro* system. Along the same line, some proteins have both NLSs and NESs (nuclear export signals), and the steady-state localization of that protein can be a dynamic balance between these activities. The dynamics of these NLS and NES activities can also be modulated by secondary modifications. Third, some proteins are “piggy-backed” into the nucleus by binding to proteins that contain an NLS, so a protein above the diffusion limit may be exclusively nuclear and yet have no intrinsic NLS at all.

An active NLS is most accurately identified by a two-pronged approach (e.g., Kalderon et al., 1984a,b; Lanford and Butel, 1984). First, the protein is scanned for regions containing NLS activity. Cleavage products of the cDNA are joined to a reporter (e.g., GFP-GST), and the resultant fusion proteins are tested for import activity in nuclear import assays. Regions of the protein that confer nuclear localization on the reporter can be examined in more detail to specify the exact peptide sequence required

for nuclear localization. There are no exact consensus sequences for NLSs, but there are some common themes that may be useful in their identification (Jans and Hübner, 1996). As mentioned above, this analysis may reveal more than one NLS. However, this analysis can also reveal NLSs that are not actually active in the native protein. There are portions of some proteins that act as NLSs in isolation, but which are not topologically exposed in the full-length protein and are, therefore, not active *in vivo*. For this reason, the putative NLS should also be mutated/deleted in the full-length protein to verify that inactivation of that putative NLS actually affects the localization of the protein.

This protocol is specifically designed to demonstrate nuclear import of test proteins and identify NLS(s) present in those proteins. Assays examining the intricacies of the nuclear import process itself require more specialized reagents, but are theoretically similar. In these studies, it is necessary to use more defined import reaction mixtures. This is most commonly achieved by depleting cytosol of certain import factors in order to determine their function, or, as is becoming more common, reconstituting nuclear import *de novo* using a complement of bacterially produced recombinant proteins.

Critical Parameters

The control import substrates are necessary to determine that the import assay is active. Typically, positive control substrates contain either an NLS like that of the SV40 T antigen (**PKKKRKV**; Kalderon et al., 1984a), or a bipartite NLS like that of nucleoplasmin (**KRPAATKKAGQAKKKKL**; Dingwall et al., 1988) in which two separate regions (shown here in bold) are required for activity. A longer peptide encoding the SV40 T antigen NLS is often used for coupling purposes (**CYTTPPKKKRKV**). In addition to the NLS, the cysteine is required for the reaction with sulfo-SMCC cross-linker. The tyrosine residue allows for monitoring the peptide by A_{280} during chromatography to ensure that excess peptide is separated from the import substrate after cross-linking. It also allows the peptide to be iodinated if so desired. There are two common mutant NLS sequences used in negative controls. One contains a critical lysine mutation that abolishes NLS activity (**PKTKRKV**; Lanford and Butel, 1984). The second is simply the reverse amino acid sequence of the wild-type NLS, which also has no NLS activity (Adam et al., 1989). This reverse NLS may be a better

negative control because the charge of the peptide is conserved; both the wild-type and the mutant are highly basic. This high degree of positive charge confers high electrostatic binding properties, in particular a tendency to non-specifically bind to DNA and proteins. Thus, the reverse sequence retains these nonspecific properties, unrelated to import activity, which may skew experimental results. These negative controls show neither rim staining (because they do not bind karyopherin α) nor nuclear import (Fig. 11.7.2D).

It is easiest to produce recombinant import substrates, such as GFP-GST-NLS, that encode both the fluorescent and targeting characteristics. The inclusion of GFP obviates the need to chemically attach fluorophores to the protein in order to detect nuclear import. The NLS directs the recombinant protein to the nucleus. The GST moiety serves a dual role. First, commercially available affinity resins greatly simplify the purification of this protein directly from bacterial lysates. Second, GST forms dimers under physiological conditions (reviewed in Mannervik and Danielson, 1988), raising the molecular weight of the import substrate to 106 kDa (2×53 kDa, the monomeric molecular weight of GST-GFP). This is well above the diffusion limit of the nuclear pore complex.

Alternatively, NLS peptides and fluorophores can be attached to BSA import substrate by chemical conjugation (Goldfarb et al., 1986). This method is a little more difficult. Both the sulfo-SMCC (which subsequently attaches to the peptide) and the TRITC react with lysines on the BSA, so it is important not to saturate the lysines with TRITC during the first reaction. Doing so will prevent subsequent binding of the sulfo-SMCC, and therefore prevent conjugation of the NLS (or mutant NLS) peptides. The terminal cysteine in the synthetic peptide is required for conjugation, because this residue reacts with the sulfo-SMCC on the BSA.

Many laboratories simplify this conjugation procedure by directly labeling nucleoplasmin (Newmeyer et al., 1986), which already encodes an NLS. In this manner a second reaction to attach NLS peptide is not required. The nucleoplasmin NLS is very protease sensitive, so negative control import substrate (lacking an NLS) can be produced by protease cleavage of the NLS from the nucleoplasmin, which is then referred to as nucleoplasmin core (Dingwall et al., 1982). In the authors' experience the nucleoplasmin-based import substrates have displayed higher background in the import assay,

but this can be overcome by the addition of high concentrations of nucleoplasmin core as a blocking agent (Görlich et al., 1994).

The experimental protein must also, of course, be detectable to measure import, either by using an antibody against the protein or by fluorescently labeling the protein directly. Both of these approaches have potential drawbacks. TRITC reacts with lysines, which are often critical amino acids within the NLS. Thus, it is important not to utilize a high molar excess of TRITC, lest an endogenous NLS be inactivated. Indirect immunofluorescence microscopy avoids this detection problem, but the additional steps required to perform immunofluorescence microscopy after the import assay can have detrimental effects on cell morphology. Care must be taken to treat the cells gently, particularly when adding solutions to the coverslips.

If the protein has been cloned, it can be produced as a recombinant attached to GFP-GST and detected directly. Alternatively, a recombinant protein can be produced with an antigen site attached (such as FLAG, 6 \times His, or HA) and detected by indirect immunofluorescence microscopy. The nuclear pore complex can allow diffusion of substrates $<50,000$ Da (Bonner, 1975; Paine et al., 1975), so the molecular weight of the experimental substrate must be greater than that limit. If not, the possible nuclear accumulation demonstrated in the import assay could be the result of diffusion of the substrate through the nuclear pores and subsequent binding to sites within the nuclear compartment. This would not be considered active nuclear import. Note that GST forms a dimer under physiological salt conditions, so the GST-GFP-NLS positive control, with a monomeric molecular weight of 53 kDa, is actually a dimer of 106 kDa, well above the diffusion limit of the NPC.

Troubleshooting

The import assay itself is quite forgiving. It is important to titer the import substrate and cytosol relative to one another for every new batch. Incorrect concentrations (either too high or too low) will decrease the resulting signal. Poor morphology is generally due to rough handling of the cells. Be careful with all manipulations involving the coverslips, especially when adding solutions. Always pipet down the side of the well and not directly onto the cells. If no cells are present, check that the fixative was made according to the directions. Addition

of excess NaOH to the paraformaldehyde causes cells to detach from the coverslip.

Likewise, it is unusual for a batch of *Xenopus* cytosol to lack activity. Low activity generally correlates to long preparation time or extended storage. The entire prep should take ~1.5 days, including dialysis, concentration, and freezing. Be sure that DTT is added fresh to solutions where indicated to maintain the karyopherins α and β in their active forms, and likewise that Mg^{2+} is present to maintain Ran activity.

TRITC-NLS-BSA is a little more difficult to make. The resulting import substrate stock, at a few milligrams per milliliter, should appear bright red. At the recommended ratio of fluorophore to BSA (~1.5:1) most of the TRITC should conjugate to the BSA. This should be obvious during the subsequent chromatography. If the first peak (BSA-TRITC) is darker than the second peak (TRITC), there is enough label on the BSA. Monitor each step by SDS-PAGE to verify that peptide is actually conjugated. There should be a noticeable molecular weight increase with the addition of the sulfo-SMCC, and a further increase with the addition of peptide. TRITC-BSA or TRITC-BSA-NLS can also be visualized in the SDS-PAGE gel, prior to fixation and staining of the gel, by exposing the gel to UV light. Too many NLS peptides conjugated to the BSA may result in relatively bright rim staining and relatively low levels of import showing strong nucleolar staining. Too few NLS peptides may decrease the rate of import.

GFP-GST-NLS production and purification is relatively straightforward. The NLS may be susceptible to proteolysis, so maintain the protein at 4°C throughout the procedure (except for the elution).

Anticipated Results

The anticipated yield for each of the support protocols is ~350 mg *Xenopus* ovarian cytosol (see Support Protocol 1), 8 mg TRITC-BSA-NLS (see Support Protocol 2), and 9 mg GST-GFP-NLS (see Support Protocol 3). After optimization of these components, assays using 36 coverslips can be performed, viewed and photographed in one day. It is recommended that all experimental samples be performed in duplicate, with negative controls (WGA, apyrase, and/or mutant NLS) and NLS positive control performed on each experiment. This leaves room for testing ~14 experimental samples (in duplicate).

Time Considerations

A typical import assay requires 2.5 hr to perform, with an additional 3 hr to record the results photographically. It should take ~1.5 days to prepare the *Xenopus* ovarian extract. Production of the TRITC-BSA-NLS requires 2.5 days and GFP-GST-NLS 3 days.

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In Vitro Translation Using HeLa Extract

UNIT 11.8

This unit describes the production and use of HeLa extract as a cell-free translation system. HeLa extract provides a useful alternative to rabbit reticulocyte lysate (RRL), the most widely used eukaryotic translation system (UNIT 11.2). HeLa extract has advantages over RRL in that it can translate mRNAs that cannot be translated in RRL, is more cap-dependent, has a higher capacity for faithful initiation at the correct start codon, and is likely more representative of translation in intact human cells. HeLa extract has been used for routine translation of mRNAs to characterize viral infections, for de novo synthesis of viruses, and for high-throughput drug screening. The HeLa cell-free translation system described here has applications in protein production, biotechnology, and for answering fundamental questions in biochemistry.

The following protocol describes the preparation of HeLa extract and its use in in vitro translation reactions (see Basic Protocol). The Basic Protocol is based on methods published over the past 25 years, but contains modifications that simplify the procedure, save time, reduce cost, and most importantly, provide highly reproducible translation signals for independent replicates. There is also a protocol for producing and purifying mRNA (see Support Protocol).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by the local radiation safety officer (also see APPENDIX 1D).

PRODUCTION AND USE OF mRNA-DEPENDENT CELL-FREE TRANSLATION SYSTEM FROM HeLa EXTRACT

**BASIC
PROTOCOL**

HeLa extract is produced by collecting HeLa cells grown in suspension, lysing the cells, removing cell debris, nuclei, and mitochondria, removing an inhibitory lipid layer, and exchanging the buffer by dialysis. Translation reactions are performed by adding an energy regeneration system, tRNAs, amino acids, and salts to the HeLa extract, Dounce homogenizing the mixture, removing the inhibitory lipid layer, adding purified mRNA, and incubating at 30°C for 3 hr. Translation signals are improved by removal of the lipid layer and treatment of the extract with calcium-dependent micrococcal nuclease to remove endogenous mRNAs. Dounce homogenizing the translation reaction prior to addition of mRNA provides a more homogenous mixture and greatly improves the reproducibility of the translation signal.

Materials

- HeLa S3 cells (ATCC #CCL 2.2)
- Joklik's modified Eagle medium for suspension cultures (SMEM)
- Heat-inactivated iron-supplemented calf serum (Sigma)
- 200 mM L-glutamine
- 10,000 U/ml penicillin
- 10,000 µg/ml streptomycin
- Hypotonic lysis buffer (see recipe)
- Dialysis buffer (see recipe)
- Diethylpyrocarbonate-treated H₂O (DEPCW; APPENDIX 2A)
- 1× phosphate-buffered saline (PBS; APPENDIX 2A)
- 0.4% (w/v) trypan blue stain
- Bradford Reagent (Bio-Rad)

**In Vitro
Reconstitution**

11.8.1

Contributed by Gary Witherell

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Supplement 6

1 mg/ml bovine serum albumin (BSA)
 10× translation mix (see recipe)
 0.1 M CaCl₂
 1 mg/ml micrococcal nuclease (Pharmacia or Fluka)
 0.2 M ethylenedis (oxyethylenetriolo) tetraacetic acid (EGTA), pH 7.5
 1 M and 2 M stocks of potassium acetate (KOAc), pH 7.5
 10 mM magnesium acetate [Mg(OAc)₂]
 1 mM amino acid mix minus methionine (Promega)
 1 mM methionine or ³⁵S-labeled methionine (15 mCi/ml; >1000 Ci/mmol; e.g.,
 NEN Life Sciences or Amersham)
 150 nM purified mRNA (capped or uncapped; see Support Protocol)

0.5, 1, and 6-liter glass spinner flasks (autoclaved)
 40-ml Wheaton Dounce homogenizer type A
 8-ml collodion dialysis bags, MWCO 12 kDa (Sartorius)
 50-ml plastic conical tubes
 30-ml glass centrifuge tubes, one of known weight
 500-ml or 1-liter centrifuge bottles
 Preparative centrifuge and rotor (e.g., Beckman J6-HC centrifuge and JS-4.2 rotor
 or equivalent)
 High-speed centrifuge and rotor (e.g., Beckman J2-21 centrifuge and JA-20 rotor
 or equivalent)
 2-ml screw-cap cryogenic vials with O-ring seal
 1-ml Wheaton Dounce homogenizer
 96-well microtiter plates

Additional reagents and equipment for assessing cell disruption by trypan blue dye
 exclusion (UNIT 1.1) and measurement of [³⁵S]methionine incorporation (UNIT 11.2)

NOTE: All solutions and equipment coming in contact with the cells or extract during the preparation must be sterile and at 4°C and aseptic technique should be used accordingly.

NOTE: All tissue culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

Grow HeLa cells

1. Grow 6 liters of HeLa S3 cells in suspension culture at 37°C with 5% CO₂ in SMEM supplemented with 10% (v/v) iron-supplemented calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Strictly maintain cell density between 2 × 10⁵ and 8 × 10⁵ cells/ml by passaging every other day. Transfer cells into larger spinner flasks as needed. Start initial 0.4-liter cultures with 2 × 10⁷ cells from a frozen stock.
2. The day before preparation of the extract, prepare hypotonic lysis buffer and dialysis buffer. Place buffers and any apparatus that will come into contact with cells or extract at 4°C. Autoclave 40-ml Dounce homogenizer and place at 4°C.
3. Place two collodion bags in separate 50-ml conical tubes and wash with DEPCW twice to remove residual ethanol. Fill tubes to the top with DEPCW and store at 4°C until ready for use.
4. Weigh one 30-ml glass centrifuge tube and place at 4°C.

The weight of the tube will be used to calculate the weight of the cell pellet.

5. Determine the cell density of the culture, and if necessary, dilute with fresh medium to give the desired density (4 to 5 × 10⁵ cells/ml) the next day.

Collect cells for HeLa extract

6. Obtain 6 liters of HeLa S3 cells in log-phase (4 to 5×10^5 cells/ml). Centrifuge cells 10 min at $2800 \times g$.
7. Wash cells three times with ice-cold $1 \times$ PBS: 50 ml for first wash and 25 ml for second and third washes. After each wash, pellet the cells by centrifuging 8 min at $640 \times g$. Use a pre-weighed 30-ml glass centrifuge tube for the third centrifugation. Remove buffer and weigh tube and pellet. Calculate cell volume assuming a density of 1 mg/ml.

The volume of the cell pellet should be ~10 ml.

8. Resuspend the cell pellet in 1.5 times its volume of hypotonic lysis buffer (~15 ml) and swell on ice for 10 min.
9. Homogenize cells with ~110 strokes of a 40-ml Wheaton Dounce homogenizer type A (tight). Check cell disruption visually or by dye exclusion using trypan blue (*UNIT 1.1*) after 40, 60, 70, 80, and 100 strokes.

The goal is to lyse >95% of the cells.

10. Transfer extract to 30-ml glass centrifuge tube. Remove cell debris and nuclei by centrifuging 5 min at $640 \times g$ for 5 min and then increasing the speed to $10,400 \times g$ for an additional 20 min to remove mitochondria.

The supernatant should be cloudy.

11. Carefully remove the upper white lipid layer if it is present.

This lipid material inhibits translation.

Prepare extract

12. Dialyze the extract for 2 to 3 hr in two collodion dialysis bags (8 ml, MWCO 12 kDa) against 1 liter of dialysis buffer to clean and replace buffer.
13. Transfer extract into a 30-ml glass centrifuge tube and centrifuge 10 min at $12,100 \times g$. Carefully remove the upper white layer.
14. Remove 2 μ l of extract and determine the protein concentration using the Bradford reagent (*APPENDIX 3B*). Use BSA to create a standard curve.

Typical extracts have protein concentrations of 20 to 30 mg/ml.

15. Transfer 380- μ l aliquots of extract to 2-ml screw-cap cryogenic tubes with O-rings and freeze and store in liquid nitrogen.

Prepare for translation reactions

16. Prepare translation mix, aliquot, and store at -80°C .
17. Autoclave 1-ml Dounce homogenizer and place at 4°C overnight.

Prepare extract for translation

18. Partially thaw vial containing 380 μ l of HeLa extract in hand and place on ice.
19. (*Optional*) Treat extract with calcium-dependent micrococcal nuclease. Add 10 μ l 0.1 M CaCl_2 and 2 μ l of 1 mg/ml micrococcal nuclease to 380 μ l of extract. Mix by pipeting gently up and down. Incubate 15 min at 25°C .

Optimal micrococcal nuclease concentration may vary with different suppliers and different lots (see Critical Parameters).

20. Place extract on ice and add 15 μ l of 0.2 M EGTA to chelate Ca^{2+} and inactivate the nuclease.

21. If micrococcal nuclease treatment is not performed, add 10 μl of 0.1 M CaCl_2 , 15 μl of 0.2 M EGTA, and 2 μl of DEPCW in place of nuclease.

Perform in vitro translation

22. Add 70 μl of translation mix. For internal ribosome entry site (IRES)-dependent translation add 30 μl of 1 M KOAc (pH 7.5) and 60 μl of 10 mM $\text{Mg}(\text{OAc})_2$. For non-IRES-dependent translation add 47 μl of 2 M KOAc (pH 7.5), 30 μl of 10 mM $\text{Mg}(\text{OAc})_2$, and 13 μl of DEPCW.

Optimal KOAc and $\text{Mg}(\text{OAc})_2$ concentrations may vary for different mRNAs.

23. For non-radioactive assays add 7 μl of 1 mM methionine and 7 μl of DEPCW. For radioactive assays add 14 μl (15 mCi/ μl) of [^{35}S]methionine.
24. Transfer extract to a 1-ml Wheaton Dounce homogenizer and homogenize with 15 strokes at 4°C.
25. Transfer the translation extract to a 1.5-ml microcentrifuge tube and microcentrifuge 10 min at 10,000 $\times g$ (e.g., 11,000 rpm in an Eppendorf model 5415C centrifuge), 4°C. Remove the upper white lipid layer.
26. Transfer extract to a clean 1.5-ml microcentrifuge tube and microcentrifuge again 10 min at 10,000 $\times g$ and remove the upper white lipid layer.
27. Transfer 22 μl of extract to a new 1.5-ml microcentrifuge tube or microtiter plate containing 3 μl of 150 nM mRNA and 3 μl of DEPCW. Incubate at 30°C for 3 hr.

Optimal mRNA concentrations may vary for different mRNAs. DEPCW may be replaced with test compound.

Microtiter plates should be covered to avoid evaporation.

28. Assay translation efficiency by reporter activity or [^{35}S]methionine incorporation (UNIT 11.2).

SUPPORT PROTOCOL

PRODUCTION AND PURIFICATION OF mRNA

mRNAs can be produced or purified from cells by a variety of methods. Purification of the mRNA results in higher translation signals and increased reproducibility for independent replicates. mRNA is purified by phenol/chloroform extraction, isopropanol precipitation, and desalting using a Microspin column. The integrity of the mRNA should be verified by agarose gel-electrophoresis prior to use.

Materials

Template: plasmid, synthetic, or PCR-amplified DNA
RNase-free DNase (optional)
0.5 M ammonium acetate
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (IAA)
24:1 (v/v) chloroform/IAA
Isopropanol
Diethylpyrocarbonate-treated H_2O (DEPCW; APPENDIX 2A)
10 mg/ml ethidium bromide or Stains-All (Kodak)
Microspin S-400 columns (Pharmacia)

Additional reagents and equipment for production of capped and uncapped mRNAs (UNIT 11.2) agarose gel-electrophoresis and spectrophotometric determination of RNA and DNA concentrations (APPENDIX 3)

1. Produce preparative amounts of both capped and uncapped mRNAs by transcription from plasmids, synthetic DNA templates, or PCR templates using bacteriophage RNA polymerases (UNIT 11.2).

Large-scale RNA production systems using bacteriophage RNA polymerases are available from Promega and Ambion. mRNAs may also be isolated from cells (UNIT 11.2) or chemically synthesized to a limited degree (Stiege and Erdmann, 1995).

2. (Optional) Remove DNA template by treatment with RNase-free DNase (UNIT 11.2).

The presence of template DNA does not affect the translation efficiency or mRNA quantitation.

3. Transfer mRNA to 1.5-ml microcentrifuge tube. Add ammonium acetate to the mRNA to a final concentration of 0.5 M. Remove protein by extraction with 1 vol 25:24:1 phenol/chloroform/IAA followed by extraction with 1 vol 24:1 chloroform/IAA.
4. Precipitate mRNA by adding an equal volume of isopropanol, incubating 15 min at -20°C , and microcentrifuging 15 min at $16,000 \times g$ (e.g., 14,000 rpm in an Eppendorf model 5415C centrifuge), 4°C .
5. Remove as much supernatant from the pellet as possible but do not dry the pellet. Resuspend the pellet in 100 μl of DEPCW.
6. Remove salts and nucleotides with a Microspin S-400 column. Resuspend resin by vortexing and pre-spinning column 1 min at $735 \times g$ (e.g., 3,000 rpm in an Eppendorf model 5415C centrifuge) to remove storage buffer. Place column in a clean 1.5-ml microcentrifuge tube and load 100 μl of mRNA sample to the top of the resin. Centrifuge 2 min at $735 \times g$ to recover mRNA.
7. Quantitate mRNA concentration by absorbance at 260 nm (APPENDIX 3) using an extinction coefficient of 25 ml/mg-cm.

The absorbance of template DNA is typically insignificant compared to the absorbance of mRNA.

8. Resuspend to 150 nM in DEPCW. Confirm integrity of mRNA by agarose gel-electrophoresis (APPENDIX 3) and staining with ethidium bromide or Stains-All. Store mRNA up to 1 month at -20°C .

REAGENTS AND SOLUTIONS

Use DEPC-treated deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Dialysis buffer

10 mM HEPES, pH 7.6, from a 1 M stock (see recipe)

90 mM potassium acetate (KOAc)

1.5 mM magnesium acetate [$\text{Mg}(\text{OAc})_2$]

Adjust to pH 7.2 with KOH

Filter sterilize

Store overnight at 4°C

Add 1 mM DTT just before use

1 M HEPES, pH 7.6

Add 11.2 g HEPES to 38 ml DEPCW. Adjust solution to pH 7.6 with KOH and final volume to 50 ml with DEPCW.

Hypotonic lysis buffer

10 mM HEPES buffer, pH 7.6, from a 1 M stock (see recipe)
10 mM potassium acetate (KOAc)
1.5 mM magnesium acetate [Mg(OAc)₂]
Adjust to pH 7.6 with KOH
Filter sterilize
Store overnight at 4°C
Add 2 mM DTT just before use

Translation mix, 10×

152 mM HEPES, pH 7.6, from a 1 M stock (see recipe)
80 mM creatine phosphate (Boehringer Mannheim), from a 1 M stock
200 µg/ml creatine phosphokinase (Boehringer Mannheim), from a 10 mg/ml stock
8 mM ATP dipotassium salt
480 µM GTP disodium salt
200 µg/ml calf liver tRNA, from a 10 mg/ml stock
100 µM amino acid mix minus methionine, from a 1 mM stock (Promega)
2 mM spermidine
16 mM DTT
diethylpyrocarbonate-treated H₂O (DEPCW; *APPENDIX 2A*)
Aliquot and store for up to 1 year at -80°C

Creatine phosphate and creatine phosphokinase stocks are prepared in DEPCW and stored at -20°C for several months.

Calf liver tRNA is purified by 25:24:1 phenol/chloroform/IAA extraction, 24:1 chloroform/IAA extraction, ethanol precipitation using 0.3 M sodium acetate (NaOAc). Resuspend the pellet to 10 mg/ml in DEPCW and store for up to 1 year at -20°C.

COMMENTARY

Background Information

A variety of mammalian, yeast, plant, and bacterial cell-free extracts have been used to assay translation in vitro (*UNIT 11.1*; Villa-Komaroff et al., 1974; Iizuka and Sarnow, 1997; Jermutus et al., 1998). Cell-free extracts contain ribosomes, translation factors, aminoacyl tRNA synthetases, and other macromolecular components required for translation. Extracts are generally supplemented with amino acids, tRNAs, energy sources, salts, and energy regeneration systems. RRL and HeLa extract are the most commonly used mammalian translation systems. RRL has advantages in that it is simple to use, will translate both capped and uncapped mRNAs, and is commercially available in large amounts. The HeLa cell-free translation system has advantages in that it is more cap-dependent, will translate mRNAs that do not translate well in RRL, and is more likely to represent translation in intact human cells.

HeLa extract has been prepared and used for in vitro translation reactions for over 25 years,

using a large variety of protocols (Villa-Komaroff et al., 1974; Celma and Ehrenfeld, 1975; Rose et al., 1978; Lee and Sonenberg, 1982; Molla et al., 1991; Carroll and Lucas-Lenard, 1993). The protocols used to prepare HeLa extract are similar in that they involve growing, collecting, and lysing cells, removing the cell debris, nuclei, and mitochondria, and storing the extract. The protocols differ in the type of serum used to grow the cells (horse, fetal bovine, calf), the cell lysis method (Dounce homogenization or detergent), lysis buffer composition and amount used, and the method used to exchange the buffer (dialysis, column chromatography, or none). In some protocols additional steps (freeze/thaw), treatments (micrococcal nuclease), or reagents (translation mix, buffer, or glycerol) are added before storage. In some cases these protocols fail to consistently produce useable extract, and the translation signals from in vitro translation reactions may vary 2- to 10-fold for independent replicates (Carroll and Lucas-Lenard, 1993; G. Witherell,

unpub. observ.). The method described here is a simplified protocol that saves time, lowers cost, and produces large amounts of HeLa extract. The quality of the HeLa extract produced is consistent from one preparation to another and the translation signals are highly reproducible.

The availability of a highly reproducible HeLa cell-free translation system has many applications in basic research, protein production, and biotechnology. Many of the applications performed with RRL and wheat germ extract (*UNIT 11.1*) have been or could be performed with HeLa extract. These applications include analysis and characterization of molecular interactions, functions, and structure of synthesized proteins. When studying biological processes, HeLa extract provides a translation system that is more cap-dependent, has a higher capacity for faithful initiation at the correct start codon, and is likely to be more predictive of results in intact human cells. HeLa extract has been used to translate mRNAs on a routine basis and is particularly useful for translation of mRNAs that do not translate well in RRL.

Human rhinovirus and enterovirus mRNAs are examples of mRNAs that translate poorly in RRL, producing low yields and a high degree of aberrant translation initiation (Belsham and Sonenberg, 1996; Hunt et al., 1999). Translation initiation of human rhinovirus and enterovirus mRNAs does not occur by a conventional 5' cap-dependent translation mechanism. Cap-dependent translation, used by the vast majority of cellular mRNAs in eukaryotes, involves the interaction between components of the cell's translation machinery and the m⁷G capped 5' end of the mRNA (Dever, 1999). The m⁷G cap, found on all nonorganellar mammalian mRNAs, is added post-transcriptionally. Translation initiation begins with the association of the 43S initiation complex (consisting of the 40S ribosomal subunit, charged initiator tRNA, and several initiation factors) with the m⁷G cap structure. The "scanning model" describes the two-dimensional movement of the 43S initiation complex along the RNA, from the 5' cap to the start codon of the protein coding region (Dever, 1999). To facilitate ribosome scanning, cellular 5' untranslated regions (5' UTRs) are typically <100 nucleotides in length, lack extensive secondary structure, and lack non-initiating AUG start codons.

In contrast, human rhinovirus and enterovirus 5' UTRs lack a 5' terminal m⁷G cap structure, are several hundred nucleotides long, contain a high degree of secondary structure,

and contain multiple noninitiating AUG codons preceding the authentic start codon of the polyprotein (Borman and Jackson, 1992). These features preclude ribosomes from binding the 5' end of the viral RNA and scanning to the polyprotein start codon. Rather than by a scanning mechanism, translation initiation of human rhinovirus and enterovirus mRNAs occurs by a mechanism in which the 43S initiation complex locates the AUG start codon of the protein coding region by binding directly to a region of the viral 5' UTR termed the internal ribosomal entry site, or IRES (Borman and Jackson, 1992). Human rhinovirus and enterovirus mRNAs can be translated under conditions in which cellular translation is inhibited. In fact, human rhinoviruses and enteroviruses encode a proteinase that is involved in the irreversible inactivation of 5' end-dependent translation, resulting in shut-off of host cell translation. IRES-dependent translation in these viruses has been shown to require HeLa specific factors that stimulate translation and correct aberrant initiation (Svitkin et al., 1996; Hunt et al., 1999). HeLa extract supports all steps of enterovirus replication including translation, protein processing, RNA replication, and viral assembly and can therefore be used for de novo synthesis of infectious virus (Molla et al., 1991).

The high reproducibility of the HeLa cell-free translation system makes it suitable for high-throughput drug screening. RiboGene has used HeLa extract to identify defined chemical entities, natural product extracts, antisense oligonucleotides, antisense oligoribonucleotides, and RNA decoys that inhibit human rhinovirus and coxsackievirus IRES-dependent translation (G. Witherell, unpub. observ.). RiboGene has also used mRNA translation in HeLa extract as a selectivity assay to identify compounds that specifically inhibit viral, fungal, and bacterial translation systems (G. Witherell, unpub. observ.). Application of the HeLa translation system to screen for inhibitors or stimulators of other viral or cellular idiosyncratic translation mechanisms may lead to the discovery of new drugs for human diseases.

Critical Parameters

There are several critical parameters for the successful preparation of HeLa extract. The growth of the HeLa cells must be strictly maintained between 2×10^5 and 8×10^5 cells/ml. Allowing the cells to become more concentrated or more dilute typically results in less predictable growth cycles and a less efficient extract. Healthy cells should double every 24 to 36 hr.

Cells are grown in iron-supplemented calf serum, rather than fetal bovine serum, to reduce cost. Substitution of fetal bovine serum with iron-supplemented calf serum does not affect cell health or growth of the cells. The night before the preparation is to take place the cells are counted and a calculated amount of fresh medium is added to feed the cells and ensure that they will be at the correct concentration and volume the following morning. In addition, fresh medium is added to ensure that the cells are growing at optimal rates when harvested. Monitor the cell growth carefully and harvest between 4×10^5 and 5×10^5 cells/ml. Failure to adequately lyse the cells by Dounce homogenization, or resuspending the cell pellet in more than 1.5 times the packed-cell volume with hypotonic lysis buffer, results in a low protein concentration and a less active or inactive extract. Dialyzing the extract in collodion bags is preferred over dialysis tubing because they are firm bags that can be suspended in the dialysis beaker, saving significant time and greatly increasing the recovery of extract. Removing the inhibitory white lipid layer before and after dialysis is essential. The extract appears to maintain activity longer with storage in liquid nitrogen than at -80°C . Once the extract is thawed it is best not to reuse it. Each freeze/thaw cycle reduces the activity to approximately one-half the original activity. Extract may be stored as a cocktail with translation mix and salts at -80°C , for a few days or weeks, with little loss of activity.

Obtaining reproducible signals in the *in vitro* translation reaction requires Dounce homogenizing the translation cocktail and removing the upper white lipid layer in two subsequent centrifuge steps before addition of mRNA. Micrococcal nuclease treatment is not necessary for IRES-dependent translation but may stimulate scanning-dependent translation, depending on the mRNA. The pH of potassium acetate must be adjusted to 7.6 to maintain the pH of the reaction. Incubation temperature is optimal at 30°C , decreasing dramatically if the temperature is increased or decreased only a few degrees. Translation signals tend to be very low during the first hour of incubation, increase linearly over the next 2 hr, plateau at 3 hr, and then decrease after 4 hr. Addition of nuclease inhibitors has little effect on the translation signal, suggesting that the extract is largely nuclease free.

The purity and quality of reagents in the HeLa translation reaction is important. Micrococcal nuclease quality varies greatly between

suppliers and even between lots, with some lots unusable due to contamination with other ribonucleases. Therefore, the optimal concentration of micrococcal nuclease must be determined for each supplier and each lot of micrococcal nuclease. Dipotassium ATP is used rather than disodium ATP to avoid high concentrations of sodium that could potentially reduce the translation efficiency of the HeLa extract. However, disodium ATP may be substituted for dipotassium ATP under most translation conditions. Purification of the mRNA and removal of salts with a Microspin column significantly improves the translation signal. Calf liver tRNA must be purified before use.

Suggested translation conditions for viral IRES-dependent translation are 50% (v/v) HeLa extract, 16 nM RNA, 40 mM potassium acetate, and 0.8 mM magnesium acetate for 3 hr at 30°C . Translation conditions for scanning-dependent translation are similar except that 126 mM potassium acetate and 0.4 mM magnesium acetate are suggested. Salts are added in addition to endogenous salts present in the extract. For radioactive assays, ^{35}S -labeled methionine may be substituted with a variety of commercially available labeled amino acids (e.g., Amersham).

During extract preparation, cells, extract, and all solutions and apparatus coming in contact with cells or extract, should be kept at 4°C unless otherwise specified. Latex or polyvinyl chloride gloves should be worn when handling extract, and only DEPCW used in buffers and reactions, to avoid contamination with ribonucleases.

Troubleshooting

A poor translation signal can typically be overcome by optimizing the translation conditions. Different mRNAs may require dramatically different translation conditions. The most critical factors for optimizing translation are Mg^{2+} , K^+ , and mRNA concentrations.

The absence of a translation signal is typically due to degradation of the mRNA, an impaired energy regeneration system, or low protein concentration in the extract. Sources for ribonucleases include water, micrococcal nuclease stocks, and hands. An impaired energy regeneration system can typically be overcome with a new stock of creatine phosphokinase.

Anticipated Results

The yield of final extract should be ~10 to 15 ml per 6 liters of cells. Successful extracts contain between 20 and 30 mg/ml of protein. There is a positive correlation between protein concentration and translation efficiency.

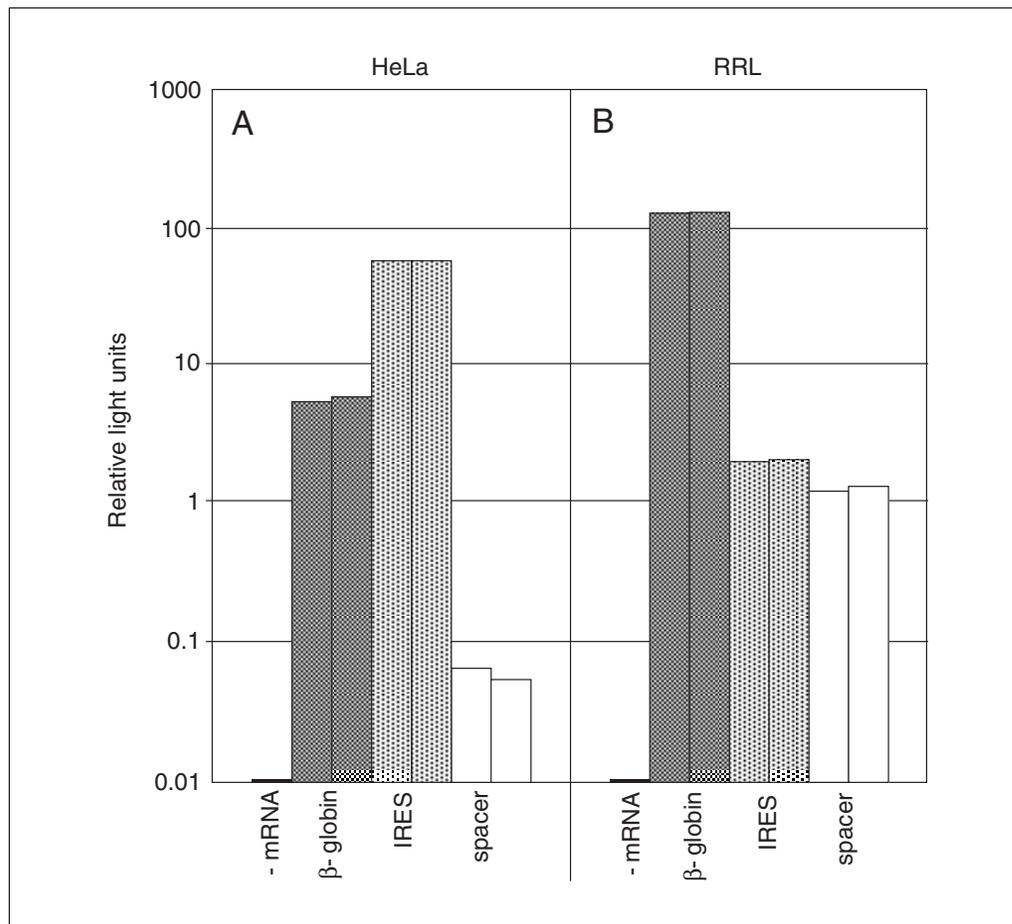


Figure 11.8.1 HeLa extract supports both viral IRES-dependent and human scanning-dependent translation. **(A)** HeLa *in vitro* translation reactions were performed as described in the Basic Protocol. **(B)** RRL translation reactions were performed as described previously (Witherell et al., 1995). Luciferase activity in relative light units (RLUs) was quantitated as a measure of translation. Translation reactions of luciferase driven by the β -globin 5' UTR (β -globin), rhinovirus IRES (IRES), and a nonfunctional spacer sequence (spacer) are shown. A control reaction was performed in the absence of mRNA (-mRNA). RRL, rabbit reticulocyte lysate.

HeLa extract translates both scanning-dependent and IRES-dependent mRNAs (see Fig. 11.8.1). Translation of a monocistronic mRNA containing the human β -globin 5' untranslated region (5' UTR) driving translation of the luciferase reporter gene was used to represent human scanning-dependent translation. Translation of a dicistronic mRNA containing the human rhinovirus IRES (Borman and Jackson, 1992) driving translation of the luciferase reporter gene was used to represent viral IRES-dependent translation. A control dicistronic mRNA containing a non-functional spacer sequence in place of the rhinovirus IRES was used as a control mRNA. Uncapped mRNAs were prepared by *in vitro* transcription with T7 RNA polymerase using the MEGAscript kit (Promega) and purified as described in the Support Protocol. HeLa extract was prepared and *in vitro* translation reactions performed as de-

scribed in the Basic Protocol. After incubation, luciferin reagent (Analytical Bioluminescence) was added and the relative light units (RLUs) quantitated using a Dynatech ML3000 luminometer. Luciferase activity was used as a direct measure of translation, and results were confirmed with a [35 S]-methionine incorporation assay (data not shown).

The HeLa cell-free translation system provides efficient and highly reproducible signals for both IRES- and scanning-dependent translation. Luciferase signals obtained from *in vitro* translation reactions were highly reproducible, with <5% variation for independent replicates (Fig. 11.8.1A). This degree of reproducibility is comparable to that of RRL (Fig. 11.8.1B). *In vitro* translation reactions using HeLa extract produced high luciferase activity from mRNAs containing the rhinovirus IRES and β -globin 5' UTR (Fig. 11.8.1A). Translation driven by the

rhinovirus IRES was greater than 10,000-fold above background (Fig. 11.8.1A; compare IRES to -mRNA) and 1000-fold above translation driven by the nonfunctional spacer sequence (Fig. 11.8.1A; compare IRES to spacer). The low translation signal produced with the spacer sequence is likely due to a low level of aberrant initiation in the HeLa extract. These results demonstrate that the rhinovirus IRES is functional in HeLa extract. As expected, the rhinovirus IRES was not functional in RRL, producing a translation signal similar to that of the spacer sequence (Fig. 11.8.1B; compare IRES to spacer). RRL is known to lack certain factors required for rhinovirus IRES-dependent translation (Hunt et al., 1999). Translation driven by the spacer sequence was 20-fold higher in RRL than in HeLa extract (compare spacer in Fig. 11.8.1A to Fig. 11.8.1B), demonstrating that RRL initiates translation at incorrect start codons far more often in RRL than HeLa extract. Translation driven by the β -globin 5' UTR in HeLa extract was greater than 1000-fold above background (Fig. 11.8.1A; compare β -globin to -mRNA), but 10-fold lower than rhinovirus IRES-dependent translation (Fig. 11.8.1A; compare β -globin to IRES). This was not surprising since the translation conditions were optimized for IRES-dependent translation rather than scanning-dependent translation. In addition, HeLa extract contains factors that limit translation of uncapped (non IRES-dependent) mRNAs (Svitkin et al., 1996). The translation signal produced by the β -globin 5' UTR is far higher when capped mRNA and optimized conditions are used (data not shown). Translation of luciferase driven by the β -globin 5' UTR was stimulated 26-fold in RRL compared to HeLa extract (compare β -globin in Fig. 11.8.1A to Fig. 11.8.1B). This result is consistent with the increased ability of uncapped mRNAs to translate in RRL (Svitkin et al., 1996).

Time Considerations

It takes ~4 days to grow the HeLa cells to 6 liters from a 400-ml starter culture. Preparation of the extract takes ~6 hr. Translation reactions take ~1 hr to set up followed by a 3-hr incubation.

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Analysis of Eukaryotic Translation in Purified and Semipurified Systems

This unit describes analysis of eukaryotic translation in purified and semipurified systems. Sucrose gradients are used to separate ribosomal complexes to analyze eukaryotic translation by reconstituting the steps in initiation (see Basic Protocol 1 and Alternate Protocols 1 and 2). While gel-filtration chromatography is excellent for resolution of low-molecular-weight components ($M_r < 50,000$ Da), sucrose gradients are most useful with high-molecular-weight complexes, often in excess of 1,000,000 Da. The large size of ribosomes has made sucrose gradients the method of choice for purification of ribosomes and ribosomal subunits. More importantly, this method has also proven highly useful for analyzing the formation of intermediates in translation initiation and identifying the protein translation factors associated with initiating ribosomes. Much of the current understanding of the sequential steps involved in translation initiation has been obtained by utilizing sucrose gradients in conjunction with purified translation factors to analyze translation *in vitro*.

Sucrose gradients are useful for other purposes as well. Because they provide an entirely liquid system, losses due to the sticking of material to a solid matrix are nonexistent. Thus, the gradients may be used in a preparative manner (see Support Protocol 1). Determining relative sedimentation coefficients can be accomplished by using analytical sucrose gradients (see Support Protocol 2).

Sucrose gradients have also been widely used to study translational regulation in intact cells. Interpretation rests on the assumption that the distribution of an mRNA in different complexes (e.g., polysomes versus free mRNP) is a reflection of the rate-limiting step for translation. For most situations, initiation is the affected step and the association of mRNA with polyribosomes is affected (Hershey, 1991; see Basic Protocol 2 and Alternate Protocol 3). Ribosomal complexes from cultured cells can also be purified for biochemical studies (see Basic Protocol 2 and Alternate Protocol 4). Yeast cells can also be used as source material (see Support Protocol 3).

A term that is often associated with sucrose gradients is density (as in sucrose density gradients), which is misleading. For the experiments described in this unit, density is not a consideration. The materials to be separated are either proteins, RNA, or both, and as such will all pellet if the centrifugation is carried out long enough. Thus, an early consideration is the type of gradient (i.e., 5% to 20% versus 10% to 40% sucrose) and the length of time that centrifugation will be necessary (3 to 24 hr). The advantage of gradients that have a linear increase in sucrose concentration is that, due to the retarding effects of both the density and the viscosity of the solution, the distance a particle sediments is linear with time. Thus, if an 80S particle has traveled a third of the way down the gradient in time t , then in time $2t$ the particle will have traveled twice as far. This facilitates fine tuning of the time required for optimal resolution of components. However, the optimal time must be determined empirically for each rotor and bucket, even when identical g forces are considered, due to variability in length of centrifuge tubes.

The protocols in this unit are primarily focused on the sedimentation of large particles (>40S), although they could be adapted for smaller ones (4S to 25S; see Support Protocol 1). The two Basic Protocols focus on *in vitro* experiments with particles of 40S to 80S (see Basic Protocol 1) and on the analysis of particles from cultured cells, which range in size from 40S to 300S (see Basic Protocol 2). Basic Protocol 1 has been the primary method for analysis of intermediate steps in the formation of eukaryotic 80S initiation complexes, which contain 40S and 60S subunits, mRNA (or AUG codon), and Met-tRNA_i. Basic Protocol 2 has been used to study cell culture systems and ascertain under specific

conditions whether there has been a change in the initiation or elongation rate of protein synthesis. At the same time, this protocol also allows one to determine if there has been a selective change in the distribution of various mRNAs in polysomes.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

NOTE: Diethylpyrocarbonate (DEPC)-treated water (*APPENDIX 2A*) should be used for all protocols in this unit.

**BASIC
PROTOCOL 1**

**IDENTIFYING INTERMEDIATES IN THE FORMATION OF PROTEIN
SYNTHESIS INITIATION COMPLEXES**

Sucrose gradients have been a key methodology to analyze the binding of Met-tRNA_i, mRNA, and factors to 40S and 80S ribosomes because of the resolving power achieved. Free components are resolved from either of several 40S complexes (40S, 43S, and 48S; see Alternate Protocol 1), 60S, or 80S complexes (see Alternate Protocol 2). No other technique accomplishes this resolution. The procedure below describes an example of the methodology for isolation of a 43S complex (40S subunits bound to eIF2, eIF3, AUG, and Met-tRNA_i, but not mRNA); with some simple modifications, 48S or 80S complexes can be identified in a similar manner. For the preparation of [³H]Met-tRNA_i, eukaryotic initiation factor 2 (eIF2), and eIF3, see Merrick (1979a,b) and Grifo et al. (1983) and references therein.

Materials

- 10% and 40% (w/v) sucrose gradient solutions (see recipe)
- 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
- 1 M KCl
- 100 mM MgCl₂ (see recipe)
- 100 mM dithiothreitol
- 100 A₂₆₀ units/ml AUG
- 100 mM GTP (see recipe)
- 150 mM phosphoenolpyruvate
- 3000 IU/ml pyruvate kinase
- 10 μM [³H]Met-tRNA_i (15 Ci/mmol)
- 16 μM 40S subunits (see Support Protocol 1)
- 8 μM eucaryotic initiation factor 2 (eIF2)
- 15 μM eIF3
- Aqueous scintillation solution (e.g., Ecoscint, National Diagnostics)
- 10% (w/v) trichloroacetic acid (TCA) solution, ice cold
- Acetone
- SDS sample buffer (see recipe)
- Gradient maker
- Refrigerated high-speed centrifuge (e.g., Beckman L7-55)
- Swinging bucket rotor and appropriate tubes (e.g., Beckman SW56 rotor with 5-ml tubes)
- UV absorbance detector and chart recorder
- Syringe or peristaltic pump
- Fraction collector (e.g., ISCO model 640 gradient fractionator)
- 90°C oven or heating block
- Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*)

1. Use a gradient maker to prepare linear 10% to 40% sucrose gradients in 5-ml SW56 centrifuge tubes. Use 2.5 ml each of 10% and 40% sucrose gradient solutions to prepare the gradient. Fill the tubes to within $\frac{1}{4}$ inch (6.4 mm) from the top (see Fig. 5.3.1). Keep gradients at 4°C.

It is important to have the tube filled nearly to the top, as tubes that are only 80% filled tend to collapse. To determine the correct volumes for other tubes, fill a tube with water to within $\frac{1}{4}$ inch and measure the volume contained.

The gradients may be stored overnight at 4°C prior to use. It is important that the gradients be cold at the beginning of the experiment.

2. Combine the following components in a reaction mixture (100 μ l total):

2 μ l 1 M Tris·Cl, pH 7.5 (final 20 mM)
10 μ l 1 M KCl (final 100 mM)
3 μ l 100 mM MgCl₂ (final 3 mM)
1 μ l 100 mM dithiothreitol (final 1 mM)
2 μ l 100 A₂₆₀ units/ml AUG (final 0.2 units)
0.8 μ l 100 mM GTP (final 0.8 mM)
2.7 μ l 150 mM phosphoenolpyruvate (final 4 mM)
0.15 μ l 3000 IU/ml pyruvate kinase (final 0.5 IU)
3 μ l 10 μ M [³H]Met-tRNA_i (30 pmol)
2 μ l 16 μ M 40S subunits (30 pmol)
6 μ l 8 μ M eIF2 (50 pmol)
7 μ l 15 μ M eIF3 (100 pmol).

Incubate 15 min at 30°C and then chill on ice.

While the inclusion of most of the above ingredients is standard, the inclusion of phosphoenolpyruvate and the enzyme pyruvate kinase are not. These two components make up an energy regenerating system that converts any GDP to GTP (i.e., phosphoenolpyruvate + GDP \rightarrow pyruvate + GTP). This is important for translation initiation experiments, as eIF2 has a 100-fold higher affinity for GDP than GTP. Therefore, any GDP present in the original GTP stock solution or generated during the incubation is quite inhibitory.

It was experimentally determined that the inclusion of 0.1 mM GDPNP in the gradients improved the yield of 43S complexes. The rationale is that GTP is lost from the ternary complex at a slow rate. This loss labilizes the Met-tRNA_i, and it dissociates from the 40S subunit. By having guanylyl-5'-imidodiphosphate (GDPNP; Calbiochem) or GTP in the gradient, the complex of eIF2·Met-tRNA_i is able to bind GDPNP (or GTP) and reform the ternary complex, which is more stably bound to the 40S subunit.

3. Carefully layer the chilled reaction mixture onto the top of the 10% to 40% sucrose gradient and carefully place the tube in the rotor. Make sure the tubes are balanced in the rotor. Centrifuge gradients 150 min at 270,000 \times g (55,000 rpm in SW56 rotor), 4°C.

To avoid disturbing the gradients, great care should be taken when buckets are placed in and removed from the rotor, and when the rotor is placed in and removed from the centrifuge.

If the number of samples is less than the number of positions in the rotor, all of the other positions must be occupied by similar gradients in buckets, but lacking a sample for analysis.

Slow acceleration should be used if the centrifuge has that capability. Too fast of an acceleration causes mixing of the sample with the gradient or loss out of the tube. At the end of the run, normal braking is slow enough to not disturb the gradients (i.e., it is not necessary to coast to a stop).

If an SW56 swinging bucket rotor is not available, it is possible to calculate conditions for other rotors. For rotors with the same size bucket (4.4 to 5 ml; e.g., Beckman SW65L, SW50L, SW39L, SW60Ti, SW50.1), the relative *k* factor can be used to correct for time and speed (see Beckman Instruments, <http://beckmancoulter.com>; McEwen, 1967). The general formula is: (time × rpm of SW56)/SW56 *k* factor = (time × rpm of other rotor)/*k* factor of other rotor, where the *k* factor of the SW56 rotor is 55. Thus, to find the equivalent centrifugation using an SW39L rotor (*k* = 112), the calculation would be: (150 min × 55,000 rpm)/55 = (x min × 39,000 rpm)/112, giving x = 431 min. For centrifugation using alternate sucrose gradients, see McEwen (1967).

4. Carefully take the rotor out of the centrifuge, remove tubes, and place the tubes on ice.

Warming causes thermal mixing and adversely affects the resolution of the gradients.

5. Unload samples from the tube by one of two alternative methods shown in Figure 11.9.1. In either case, use an in-line UV absorbance detector and chart recorder to monitor absorbance, and collect ~20 to 30 fractions (e.g., twenty-five 200- μ l fractions) with a fraction collector.

The tRNA and ribosomal RNAs will show up well by monitoring absorbance at 254 or 260 nm.

At this point, the radioactivity in each fraction can be determined (step 6a). Alternatively, the location of eIF2 and eIF3 proteins can be determined by SDS-PAGE (steps 6b to 9b).

To measure radioactivity

- 6a. Mix 100 μ l of each fraction with 5 ml of an aqueous scintillation solution and measure radioactivity by scintillation spectrometry (see Fig. 11.9.2).

For the above example, two peaks of radioactivity should be observed, one of low molecular weight representing free [³H]Met-tRNA_i and a second, more rapidly sedimenting peak representing the labeled 43S complex (40S subunits, eIF2, GTP, Met-tRNA_p, eIF3, AUG).

To detect proteins

- 6b. Mix a 50- to 100- μ l aliquot from each fraction with 1 ml ice-cold 10% TCA solution in a microcentrifuge tube and incubate 30 min on ice to precipitate the protein.
- 7b. Pellet protein by microcentrifuging 20 min at 10,000 × *g*, 4°C.
- 8b. Discard supernatant, wash pellet twice with 1 ml acetone to remove residual TCA, and allow to dry.
- 9b. Add 20 μ l SDS sample buffer to the pellet, mix, heat 10 min at 90°C, and then analyze by SDS-PAGE (UNIT 6.1; Laemmli, 1970).

If the sample is yellow in color, not all of the trichloroacetic acid was removed. Add 1 to 3 μ l of 1 M Tris-Cl, pH 8, until the sample is blue.

The peptide bands diagnostic for each factor can be visualized after the gel has been stained with Coomassie blue (UNIT 6.5). In general, only those peptide bands that are >40 kDa can be visualized in the 43S region of the gradient, as the 40S subunit has numerous subunits with molecular weights generally <40 kDa.

An alternate approach to identify the proteins of interest is possible if antibodies are available. The proteins are transferred to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane (immunoblotting; UNIT 6.2). The fractions containing the protein of interest are identified using standard methods for antibody binding and detection.

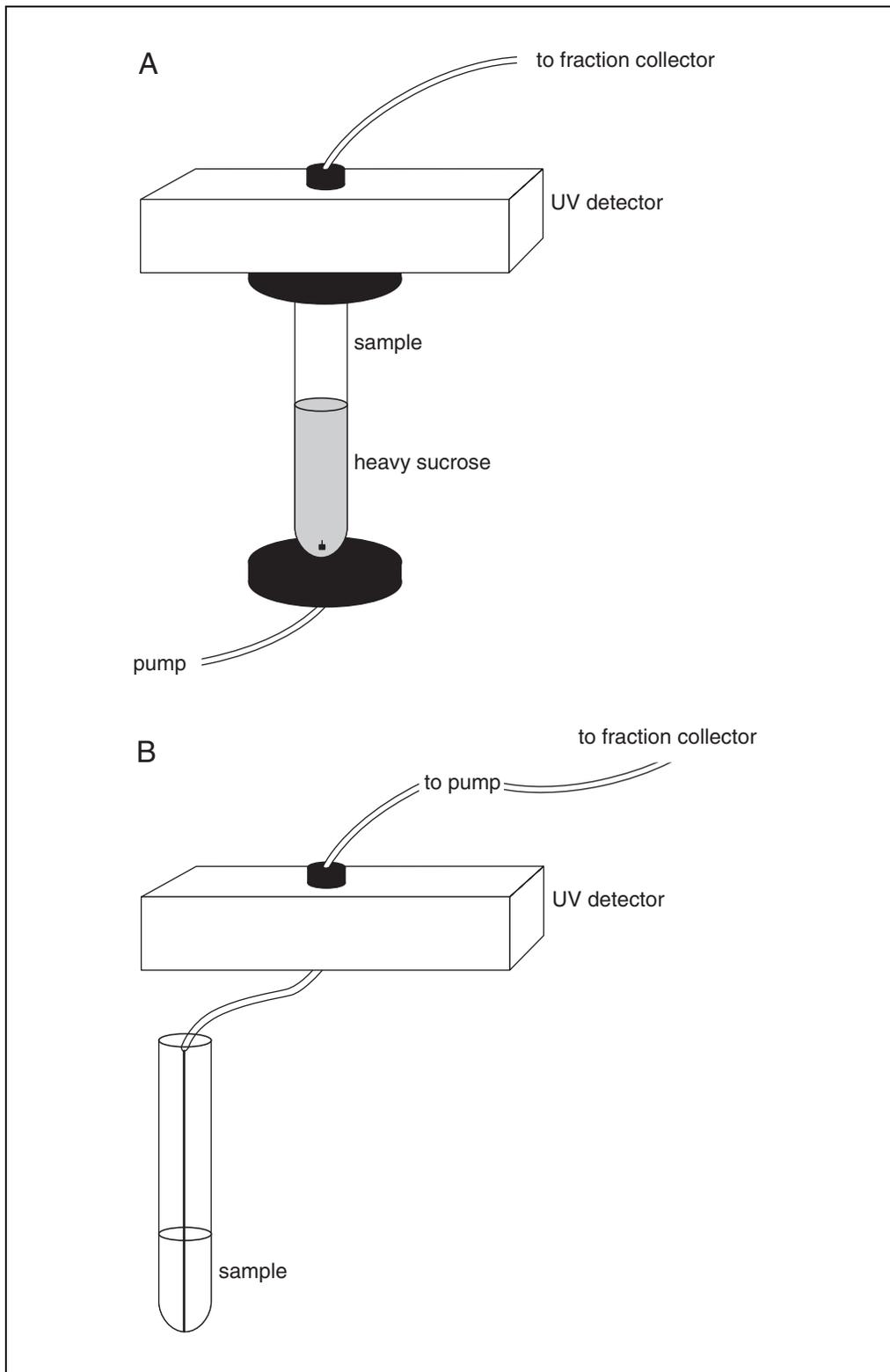


Figure 11.9.1 Methods for collecting sucrose gradients. In the example shown in **(A)**, the top of the centrifuge tube is fitted into an adaptor that fits into the UV detector. A dense sucrose solution (i.e., >50% w/v) is introduced into the bottom of the centrifuge tube with the aid of a tube-piercing apparatus that seals the bottom of the tube. As the heavy sucrose is introduced, the gradient is displaced upwards through the UV detector and then to a fraction collector. In the example shown in **(B)**, a thin metal tube is carefully inserted to the bottom of the centrifuge tube. The sample is pumped out of the bottom of the tube, through the UV detector, and then to the fraction collector.

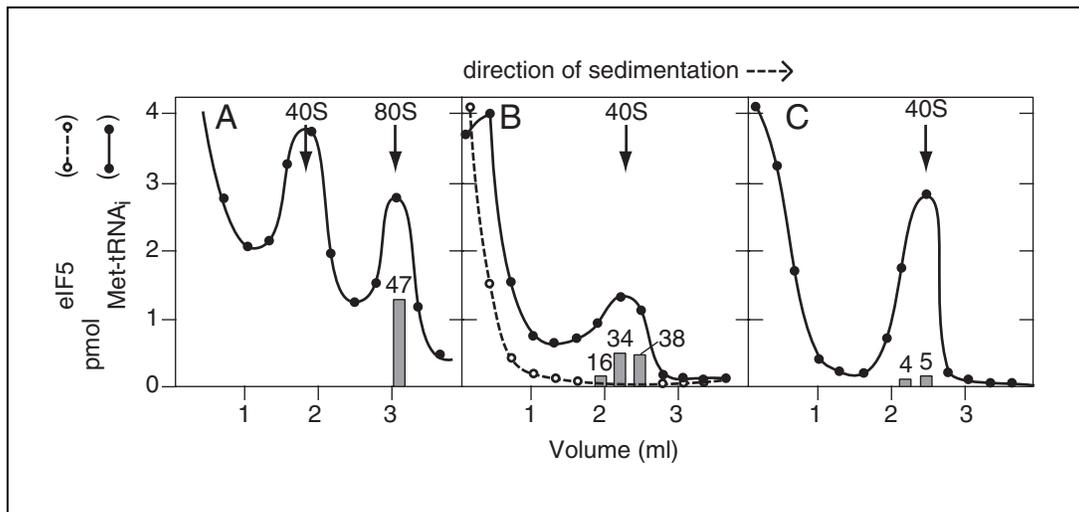


Figure 11.9.2 Reactivity of Met-tRNA_i bound to 40S and 80S initiation complexes. In this experiment, eIF2, eIF3, AUG, GTP, [³H]Met-tRNA_i, and 40S subunits were incubated 15 min at 30°C in a 100-μl volume. After incubation, the reaction was layered onto a 10% to 35% sucrose gradient (buffered in 20 mM Tris·Cl, pH 7.5, 100 mM KCl, and 5 mM MgCl₂). After centrifugation at 55,000 rpm at 4°C for 140 minutes, the gradients were fractionated (250-μl fractions) by displacement with 40% sucrose (Fig. 11.9.1A). Aliquots were sampled for radioactivity (closed circles) and for reactivity of Met-tRNA_i with puromycin (expressed as a percentage of cpm bound Met-tRNA_i, shaded bars). **(A)** Also included in the incubation were eIF5 and 60S subunits. **(B)** [¹⁴C]eIF5 (open circles) was present in the incubation; 60S subunits were added to test for reactivity with puromycin. **(C)** GDPNP replaced GTP in the incubation; eIF5 and 60S subunits were added to test for reactivity with puromycin. (Adapted from Peterson et al., 1979b.) This figure represents an experiment where different complexes are resolved by sucrose gradients. Met-tRNA_i, present in all experiments, was monitored by liquid scintillation spectrometry. The ability of bound Met-tRNA_i to form methionyl-puromycin was determined separately. Previous studies had shown that formation of methionyl-puromycin required 40S and 60S subunits, AUG, eIF2, eIF3, eIF5, and Met-tRNA_i. eIF1A and eIF5A stimulate this process as well.

ALTERNATE PROTOCOL 1

FORMATION OF 48S PREINITIATION COMPLEXES

Using the same sucrose gradients, a variety of other components can be added to determine their influence on the binding of tRNA and mRNA to 40S subunits to form 48S preinitiation complexes (Benne and Hershey, 1978; Merrick, 1992). These include the substitution of globin mRNA (or an mRNA transcript of choice made with T7 polymerase) for AUG, the addition of ATP (required to obtain binding of mRNA to 40S subunits), or the addition of other translation initiation factors (most importantly eIF4A, eIF4B, eIF4F, and eIF4H to obtain maximal binding of mRNA to 40S subunits). As long as 60S subunits are not added, all of the products should be either free components or components bound to 40S subunits. Depending on additions, the 40S complexes could have the following characteristics: (1) 40S subunits: no factors, Met-tRNA_i, or mRNA are bound; (2) 43S complexes: eIF2, eIF3, AUG, and Met-tRNA_i are bound, but not mRNA; (3) 48S complexes: eIF2, eIF3, mRNA, and Met-tRNA_i are bound. The procedure is identical to that in Basic Protocol 1, except that the reaction in step 2 is replaced with the one in step 2a below.

Additional Materials (also see Basic Protocol 1)

- 1 M KCl
- 1 M Tris·Cl, pH 7.5 (APPENDIX 2A)
- 100 mM dithiothreitol

2a. Prepare a reaction for formation of 48S complexes (total 100 μ l):

2 μ l 16 μ M 40S subunits (40 pmol)
3 μ l 10 μ M [3 H]Met-tRNA_i (30 pmol)
7 μ l 15 μ M eIF3 (100 pmol)
6 μ l 8 μ M eIF2 (50 pmol)
3 μ l 100 A₂₆₀ units/ml of AUG (0.3 units)
10 μ l 1 M KCl
2 μ l 1 M Tris·Cl, pH 7.5
1 μ l 100 mM dithiothreitol
4 μ l 100 mM MgCl₂
1 μ l 100 mM GTP
61 μ l H₂O.

Incubate 15 min at 30°C and then chill on ice.

It has been the authors' experience that, when using purified translation factors and 40S subunits, factors other than eIF2 and eIF3 are not found associated with the above complexes. However, since their initial purification is based on their association with polysomes, it is likely that either the ribosomal subunits are damaged or that an unidentified factor that stabilizes the association of these other factors with the 40S subunit is lost during purification.

If the presence of mRNA in the 48S complexes is to be studied, 10 μ l of a 100 μ g/ml solution of mRNA and 1 μ l of 100 mM ATP should be substituted for the AUG codon. In addition, translation factors eIF4A, eIF4B, and eIF4F should be added (Merrick, 1979a; Grifo et al., 1983).

FORMATION OF 80S INITIATION COMPLEXES

When 60S subunits are also added, it is possible to obtain complexes that contain 40S subunits, 60S subunits, or 80S subunits. The gradient conditions described above should allow the separation of all of these complexes. Polysomes, if formed, will be found pelleted at the bottom of the tube. If the original reaction mixture contains GDPNP, then no 80S complexes will be seen, and usually more 40S complexes are seen than with GTP. In the presence of GTP, 43S (with AUG) or 48S (with mRNA) complexes may be converted to 80S complexes (40S and 60S subunits, mRNA, Met-tRNA_i) if eIF1A, eIF5, and eIF5A (Merrick, 1979a; Grifo et al., 1983) are present in the original reaction mixture. The isolated 80S complexes should be reactive with puromycin to form methionyl puromycin (see Fig. 11.9.2).

Additional Materials (also see Basic Protocol 1)

16 μ M 60S subunits (see Support Protocol 1)
70 μ M eucaryotic initiation factor 1A (eIF1A)
70 μ M eIF5A
20 μ M eIF5
1 M Tris·Cl, pH 7.5 (APPENDIX 2A)
1 M KCl
100 mM dithiothreitol
100 mM KPO₄, pH 8.0
Ethyl acetate
Scintillation solution (e.g., Econofluor)
13 \times 100-mm test tubes
Scintillation vials

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Separate and analyze complexes

1. Prepare 10% to 40% sucrose gradients in 5-ml SW56 tubes (see Basic Protocol 1, step 1).
2. Prepare a reaction for the formation of 80S complexes (total 100 μ l):

2 μ l 16 μ M 40S subunits (final 40 pmol)
2 μ l 16 μ M 60S subunits (final 40 pmol)
3 μ l 10 μ M [3 H]Met-tRNA_i (final 30 pmol)
7 μ l 15 μ M eIF3 (final 100 pmol)
6 μ l 8 μ M eIF2 (final 50 pmol)
0.7 μ l 70 μ M eIF1A (final 50 pmol)
0.7 μ l 70 μ M eIF5A (final 50 pmol)
0.5 μ l 20 μ M eIF5 (final 10 pmol)
3 μ l 100 A₂₆₀ units/ml AUG (final 0.3 units)
2 μ l 1 M Tris-Cl, pH 7.5
10 μ l 1 M KCl
1 μ l 100 mM dithiothreitol
4 μ l 100 mM MgCl₂
1 μ l 100 mM GTP
57 μ l H₂O.

Incubate 15 min at 30°C and then chill on ice.

3. Separate subunits and analyze radioactivity as described (see Basic Protocol 1, steps 3 to 6a).

If desired, protein content may be determined for fractions at this point.

Determine puromycin reactivity of peaks

4. Mix 100 μ l of each fraction in a 13 \times 100-mm test tube with 100 μ l reaction buffer II (200 μ l total). Incubate 15 min at 37°C.
5. Terminate reaction by sequential addition of 0.9 ml of 100 mM KPO₄, pH 8.0, and 3 ml of ethyl acetate. Vortex vigorously for 30 sec.
6. Separate aqueous and organic phases by centrifuging 10 min at 1000 \times g (~2000 rpm), room temperature, in a tabletop centrifuge.
7. Carefully remove 2 ml of the organic phase (upper phase, containing the product methionylpuromycin) to a scintillation vial. Add 10 ml scintillation solution and measure radioactivity by scintillation spectrometry.

The reactivity of the [3 H]Met-tRNA_i in the 80S peak can be determined from the ratio of the amount of radioactivity obtained as methionylpuromycin relative to the radioactivity added to the reaction mixture in step 4 (equal to the radioactivity of the 100- μ l aliquot measured in step 3).

SUPPORT PROTOCOL 1

ISOLATION OF RIBOSOMAL SUBUNITS USING PREPARATIVE SUCROSE GRADIENTS

The standard preparative use of sucrose gradients is for the isolation of 40S and 60S ribosomal subunits (Merrick, 1979b), although any macromolecular component with an S value of 10S or larger may be a candidate for purification by this method.

Materials

Rabbit reticulocyte lysate (Green Hectares)
Standard sucrose solution (see recipe)
KCl
Subunit buffer (see recipe)
5% and 20% (w/v) sucrose gradient solutions (see recipe)
0.25 M sucrose solution (see recipe)
Refrigerated high-speed centrifuge (e.g., Beckman L7-55)
Type 35 rotor and 70-ml polycarbonate centrifuge tubes (Beckman)
Ti60 or Ti70 rotor and 26-ml polycarbonate centrifuge tubes (Beckman)
Gradient maker
SW27 rotor and 32-ml polyallomer or cellulose nitrate centrifuge tubes (Beckman)

Prepare salt-washed ribosomes

1. In 70-ml polycarbonate centrifuge tubes for a Beckman type 35 rotor, centrifuge 420 ml rabbit reticulocyte lysate 4 hr at $\sim 100,000 \times g$ (35,000 rpm), 4°C.
2. Decant and discard supernatant. Rinse the surface of the polysomal pellets once with 3 ml standard sucrose solution.
3. Add 5 ml standard sucrose solution to each tube and tease the pellet using a glass stirring rod to give a solution of dissolved and partially dissolved polysomes.
4. Transfer contents of all tubes to a beaker in ice with a stir bar. Stir the polysome solution until all of the particles have dissolved (no more than 30 min).
5. Measure the volume of the polysome solution and add solid KCl to a final concentration of 0.5 M. Stir 30 min until KCl has dissolved completely.
6. Pour into 26-ml polycarbonate centrifuge tubes for a Ti60 or Ti70 rotor. Centrifuge 2 hr at $180,000 \times g$ (50,000 rpm in Ti60 or Ti70 rotor), 4°C.
7. Decant supernatant and save as an enriched source of protein synthesis initiation factors (Merrick, 1979a). Store frozen at -120°C until needed (stable up to several years).
8. Dissolve pellet in a minimal amount of standard sucrose solution.

This dissolved pellet is referred to as "salt-washed ribosomes." They may be stored frozen at -120°C .

Prepare ribosomal subunits

9. Dissolve salt-washed ribosomes in sufficient subunit buffer to achieve a concentration of 50 A_{260} units/ml.
10. Incubate 10 min on ice and then 10 min at 37°C.

Puromycin causes release of the nascent peptide chains that stabilize ribosomal subunit interactions in translating polysomes during the initial high-salt wash. Release of the nascent chains followed by exposure to high salt causes the 80S ribosomes to dissociate into the 40S and 60S subunits.

11. Prepare 5% to 20% sucrose gradients in 32-ml SW27 polyallomer centrifuge tubes as described above (see Basic Protocol 1, step 1).
12. Layer 2- to 3-ml samples onto sucrose gradients and centrifuge 8 hr at $95,000 \times g$ (27,000 rpm in SW27 rotor), 4°C.

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13. Collect the gradients in sixty 0.5-ml fractions, monitoring A_{260} with a UV detector.
The region used to obtain 40S subunits represents the front two-thirds of the 40S peak, while the region used for 60S subunits represents the middle half of the 60S peak. In this manner, 40S subunits are not contaminated with 60S subunits and 60S subunits are not contaminated with either 40S subunits or 80S subunits.
14. Concentrate individual subunits by centrifuging 18 hr at $170,000 \times g$ (50,000 rpm in a Ti60 rotor), 4°C .
15. Dissolve pelleted subunits in a minimal amount of 0.25 M sucrose solution. Once the pellets are dissolved, determine the concentration of the subunits and adjust to $16 \mu\text{M}$ ($135 A_{260}$ units/ml for 40S subunits and $270 A_{260}$ units/ml for 60S subunits). Store in 100- μl aliquots at the vapor temperature of liquid nitrogen for up to 2 years.

MONITORING THE POSITION OF THE RIBOSOME ON GLOBIN mRNA

This protocol describes a method called “toeprinting” that is used for locating the position of the mRNA on the 40S subunit. For this assay (Anthony and Merrick, 1992), the mRNA of interest is hybridized to a radiolabeled oligonucleotide (≥ 20 nucleotides) that is complimentary to a region of the mRNA located ~ 50 nucleotides $3'$ of the initiating AUG. In this case, the reaction is described for globin mRNA as an example.

Additional Materials (also see *Basic Protocol 1*)

- mRNA for globin or other gene of interest
- ^{32}P -end-labeled oligonucleotide primer: e.g.,
5'-TCACCACCAACTTCTTCCAC-3' for globin (5000 Ci/mmol), or primer appropriate to gene of interest
- Micrococcal nuclease-treated rabbit reticulocyte lysate (Promega)
- 1 M HEPES-KOH, pH 7.5
- 100 and 500 mM $\text{Mg}(\text{CH}_3\text{COOH})_2$
- 100 mM dithiothreitol
- 10 mM anisomycin
- 10% and 35% (w/v) sucrose gradient solutions (see recipe)
- 100 mM each dATP, dGTP, dCTP, dTTP
- AMV reverse transcriptase
- 1:1 (v/v) phenol/chloroform (*APPENDIX 3*)
- Ethanol
- 3 M potassium acetate, pH 5.0
- SW56 rotor and 5-ml polyallomer centrifuge tubes (Beckman)
- Additional reagents and equipment for DNA sequencing gels (*APPENDIX 3*)

Label message

1. For ten reactions, place 15 μCi of ^{32}P -labeled oligonucleotide primer and 10 μg globin mRNA in a microcentrifuge tube, place the tube in a small beaker of boiling water, and incubate 1 min.
The probe should be a 20- to 30-oligonucleotide sequence complimentary to a portion of the mRNA of interest and located ~ 50 nucleotides $3'$ of the initiating AUG.
2. Place the beaker on the bench top and allow to cool to room temperature (~ 30 min).
3. Prepare reaction mixtures (150 μl each):

50 μ l micrococcal nuclease-treated rabbit reticulocyte lysate
 2.25 μ l 1 M HEPES·KOH, pH 7.5 (final 15 mM)
 15 μ l 1 M KCl (final 100 mM)
 6.0 μ l 100 mM Mg(CH₃COOH)₂ (final 24 mM)
 1.5 μ l 100 mM dithiothreitol (final 1 mM)
 1.5 μ l 10 mM anisomycin (final 0.1 mM)
 1/10th volume of mRNA-oligonucleotide duplex (1 μ g mRNA, 1.5 μ Ci oligonucleotide)
 1 μ l 100 mM dithiothreitol
 2 μ l 100 mM ATP
 1 μ l 100 mM GTP
 55 μ l H₂O.

Incubate 15 min at 30°C and then chill 2 min on ice.

4. Prepare 10% to 35% sucrose gradients in 5-ml SW56 centrifuge tubes as described above (see Basic Protocol 1, step 1).
5. Layer the reaction on the sucrose gradients and centrifuge 150 min at 270,000 \times g, 4°C.
6. Collect 0.2-ml fractions.

Reverse transcribe message

7. Combine the following:

200- μ l aliquots of gradient fractions
 0.2 mM each dATP, dGTP, dCTP, dTTP
 4 U AMV reverse transcriptase.

Incubate 30 min at 37°C.

The concentrations of components in the sucrose gradient solution are designed to be compatible with the ionic and divalent ion requirements of the reverse transcriptase.

8. Extract reactions with an equal volume of 1:1 (v/v) phenol/chloroform. Separate phases by centrifuging 10 min at 1000 \times g, 4°C. Remove the aqueous (upper) phase and add 0.1 vol 3 M potassium acetate, pH 5.0 and 2.5 vol ethanol. Let stand overnight at -20°C. Collect the precipitated nucleic acid by centrifuging 20 min at 10,000 \times g, 4°C. Decant the aqueous/ethanol and allow tube to dry.
9. Resolve products on an 8% (w/v) acrylamide DNA sequencing gel (APPENDIX 3). Compare with a sequence ladder obtained by performing an extension reaction in the presence of dideoxynucleotides.

For the bottom sequencing gel, mix 10 ml of 8% acrylamide/urea bottom solution (see recipe), 40 μ l of 10% ammonium persulfate, and 15 μ l TEMED. For the top sequencing gel, mix 35 ml of 8% acrylamide/urea top solution (see recipe), 120 μ l of 10% ammonium persulfate, and 50 μ l TEMED.

10. Quantify the amount of mRNA in each complex (48S or 80S) based upon the radioactivity from the [³²P]primer.

DETERMINATION OF RELATIVE SEDIMENTATION COEFFICIENTS USING ANALYTICAL SUCROSE GRADIENTS

Sucrose gradients are a standard means for determining the relative sedimentation coefficient of proteins (for example see Merrick and Anderson, 1975) and to get a rough estimate of molecular mass. The advantage of this method over use of the analytical ultracentrifuge (which is much more accurate; see UNIT 5.3) is that either crude or pure

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samples can be analyzed. The location of the desired protein can be determined by biological assay, by band pattern on SDS gels (*UNIT 6.1*), by antibody detection (immunoblotting; *UNIT 6.2*), or, in some instances, by ligand binding (far western blot; *UNIT 17.2*). For these determinations, 5% to 20% sucrose gradients are used. These lower concentrations of sucrose reflect the much smaller *S* values of proteins relative to ribosomal subunits. Buffer composition and ionic strength should be optimized for the protein in question, either for preservation of activity or for suitability in the subsequent assay. A variety of proteins can be used as markers, although a convenient set of proteins includes ovalbumin (3.55*S*), aldolase (7.8*S*), catalase (11.2*S*), and β -galactosidase (16.1*S*). As the shape of the protein will influence the *S* value (greater asymmetry will decrease the *S* value relative to a symmetric protein of the same molecular weight), this value is often used in conjunction with the Stokes radius, which can be determined by gel filtration (*UNIT 5.5*). The formula $M_r = 6\pi\eta Nsa/(1 - \bar{v}\rho)$ allows for the determination of the molecular weight, where η is viscosity, *N* is Avogadro's number, *s* is the sedimentation coefficient, *a* is the Stokes radius, \bar{v} is the partial specific volume of the protein, and ρ is the solvent density. In this formula, the apparent increase in molecular weight that asymmetry causes in the determination of the Stokes radius is balanced by the decrease in *S* value that asymmetry induces.

Additional Materials (also see *Basic Protocol 1*)

5% and 20% (w/v) sucrose gradient solutions in suitable buffer (e.g., 20 mM Tris-Cl, pH 7.5/100 mM KCl/1 mM dithiothreitol)

Protein standards in same buffer:

10 mg/ml ovalbumin (3.55*S*)

10 mg/ml alddase (7.8*S*)

10 mg/ml catalase (11.2*S*)

10 mg/ml β -galactosidase (16.1*S*)

Unknown protein

1. Prepare 5% to 20% sucrose gradients in 5-ml SW56 centrifuge tubes as described above (see *Basic Protocol 1*, step 1).
2. Mix 50 to 100 μ l of each protein standard together to yield 200 to 400 μ l of standard mixture containing 10 mg/ml of each marker.

Prepare 200 μ l for SW rotors with small volumes (~5 ml/tube) or 400 μ l for SW rotors with large volumes (~12 ml/tube).

3. Layer standard mixture on top of a 5% to 20% sucrose gradient.
4. Layer 100 to 300 μ l unknown protein on top of a separate 5% to 20% sucrose gradient or mix with one or more of the standard proteins if an internal standard is desired.

*If an internal standard is used, it is best to choose a standard that is anticipated to have a significantly higher or lower *S* value, if possible.*

5. Centrifuge 4.5 hr at $300,000 \times g$ (65,000 rpm in SW56 rotor), 4°C.
6. Place the tubes on ice.
7. Collect twenty-five 0.2-ml fractions.
8. Analyze fractions by precipitating and electrophoresing the proteins (see *Basic Protocol 1*, steps 6b to 9b) or by assaying fractions directly for biological activity to determine the distance the protein has sedimented.
9. Determine the distance sedimented for each of the standard proteins by measuring UV absorbance at 280 nm as the gradient is removed from the tube or by analyzing aliquots of each fraction by SDS-PAGE (see *Basic Protocol 1*, steps 6b to 9b).

10. Construct a plot of $S_{20,w}$ of the standards versus the distance migrated (e.g., as milliliters or fraction tube number). Determine the S value for the unknown protein based upon its distance sedimented.

The plot should be a straight line.

The $S_{20,w}$ is the rate of sedimentation for a particle analyzed in distilled water at 20°C. Normally, one would have to correct for the usual centrifugation conditions, which would include both salt and temperature differences. However, as the protein standards are measured under the same conditions as the unknown, this correction is not necessary. For proteins that contain extensive sugar or lipid modifications, there will be a slight error as their partial specific volumes will not be 0.73 g/ml.

ANALYSIS OF TRANSLATION IN CULTURED CELLS

Initiation of translation is rate-limiting for translation of most mRNAs and is subject to regulation at multiple steps (Hershey, 1991). Thus, most changes in the translational efficiency of a given mRNA can be assessed by determining the percentage of that mRNA associated with actively translating polysomes. An approach to assess the relative amount of an individual mRNA in polysomes is outlined below.

The procedures described in this protocol have been broadly applied to analyze translation in adherent cells (BALB/c 3T3, Chinese hamster ovary, normal rat kidney, and C6 glioma cells), suspension cells (Friend erythroleukemia, Da3, and 32D hematopoietic cell lines), and in yeast (*Saccharomyces cerevisiae*). However, while broadly applicable, the necessity of lysing cells under conditions that do not denature ribosomes may preclude analysis of cells that have high concentrations of endogenous nucleases and that require strong denaturants (such as guanidinium or TriZol reagent) for preparation of intact RNA.

Materials

- 10% and 50% (w/v) sucrose gradient solutions (see recipe)
- Cultured cells or yeast lysate (see Support Protocol 3)
- PBS (APPENDIX 2A) or other neutral, buffered isotonic solution
- TMK₁₀₀ lysis buffer (see recipe), ice cold
- Tris-buffered, water-saturated phenol: for buffering, use 1 M Tris-Cl, pH 7.5 (APPENDIX 2A)
- Chloroform
- Ethanol
- RNA-loading buffer (see recipe)
- Gradient maker
- Refrigerated high-speed centrifuge (e.g., Beckman L7-55)
- Swinging bucket rotor and appropriate tubes (e.g., Beckman SW28.1 rotor and 17-ml tubes)
- 15-ml polyethylene tubes with caps
- UV absorbance detector and chart recorder
- Syringe or peristaltic pump
- Fraction collector (e.g., ISCO model 640 gradient fractionator)
- Water bath or heating block at 65°C
- Additional reagents and equipment for analyzing RNA on agarose or acrylamide gels and for northern blotting (APPENDIX 3)

NOTE: Experiments involving RNA require careful precautions to prevent contamination and RNA degradation (see APPENDIX 2A).

NOTE: All procedures are carried out on ice with solutions that have been precooled to 4°C.

Prepare gradients

1. Prepare a linear sucrose gradient in a 17-ml SW28.1 centrifuge tube using a gradient maker and 8.15 ml each of 10% (light) and 50% (heavy) sucrose gradient solutions. Store gradient on ice, or at 4°C, until lysate is ready for loading.

The addition of nuclease inhibitors to the sucrose gradients is not necessary. Heparin has occasionally been added to gradients as a nonspecific inhibitor of nucleases. However, this practice is discouraged because heparin competes with RNA for nucleic acid-binding proteins, and 80S ribosomal/mRNA complexes are not stable in heparin (W.C. Merrick and J.O. Hensold, unpub. obser.).

For analysis of interactions of mRNAs (or proteins) with individual ribosomal subunits, insufficient separation is obtained using 10% to 50% sucrose gradients. For analysis of these smaller complexes, 10% to 25% sucrose gradients should be used.

Prepare lysate

- 2a. *For suspension cultures:* Collect cells in a 15-ml sterile centrifuge tube. Rinse three times in 10 ml ice-cold PBS, centrifuging 10 min at 1500 × g, 4°C, each time. Proceed to step 3.

For optimal results, it is best to empirically determine the number of cells necessary for analysis. While increasing the load can increase detection of proteins or RNAs of low abundance, there is a resultant loss of resolution. For practical purposes, 2 to 4 × 10⁷ cells provides optimal resolution on 10% to 50% gradients, while still allowing for detection of most proteins or RNAs of interest by immunoblotting or northern blotting, respectively. For analysis of more slowly sedimenting complexes on 10% to 25% gradients, the number of cells is increased by 1.5 to 2 fold.

- 2b. *For adherent cultures:* Rinse adherent cells three times with 10 ml ice-cold PBS, detach cells with a rubber policeman, and transfer to a microcentrifuge tube. Pellet cells by microcentrifuging 10 min at 1500 × g, 4°C. Proceed to step 3.

Trypsin should be avoided because it may affect cell signaling and its use is not practical at the low temperatures employed.

- 2c. *For yeast:* Grow and lyse yeast cells as described below (see Support Protocol 3). Proceed to step 5.

3. Discard supernatant and lyse cell pellet by repeated pipetting in 400 μl ice-cold TMK₁₀₀ lysis buffer.

RNase inhibitors may be added to the lysis buffer (see Critical Parameters).

4. Transfer lysed pellet to a microcentrifuge tube and pellet nuclei by microcentrifuging 5 min at 10,000 × g, 4°C.

Fractionate lysate

5. Remove supernatant and layer onto the 10% to 50% sucrose gradient. For yeast, load 10 A₂₅₄ units lysate per gradient.
6. Centrifuge 4 hr at 100,000 × g (27,000 rpm in SW28.1 rotor), 4°C.

The methods described have been optimized for use with the SW28 rotor and SW28.1 buckets. This rotor accommodates a centrifuge tube that is 15% longer than that accommodated by the SW41 rotor, and thus provides better resolution of ribosomal complexes. However, for most purposes the separations achieved with the SW41 rotor are adequate. Centrifugation conditions for the SW41 and other rotors can be found in a variety of references (Steel and Jacobson, 1987; Kaspar and Gehrke, 1994; Proweller and Butler, 1994).

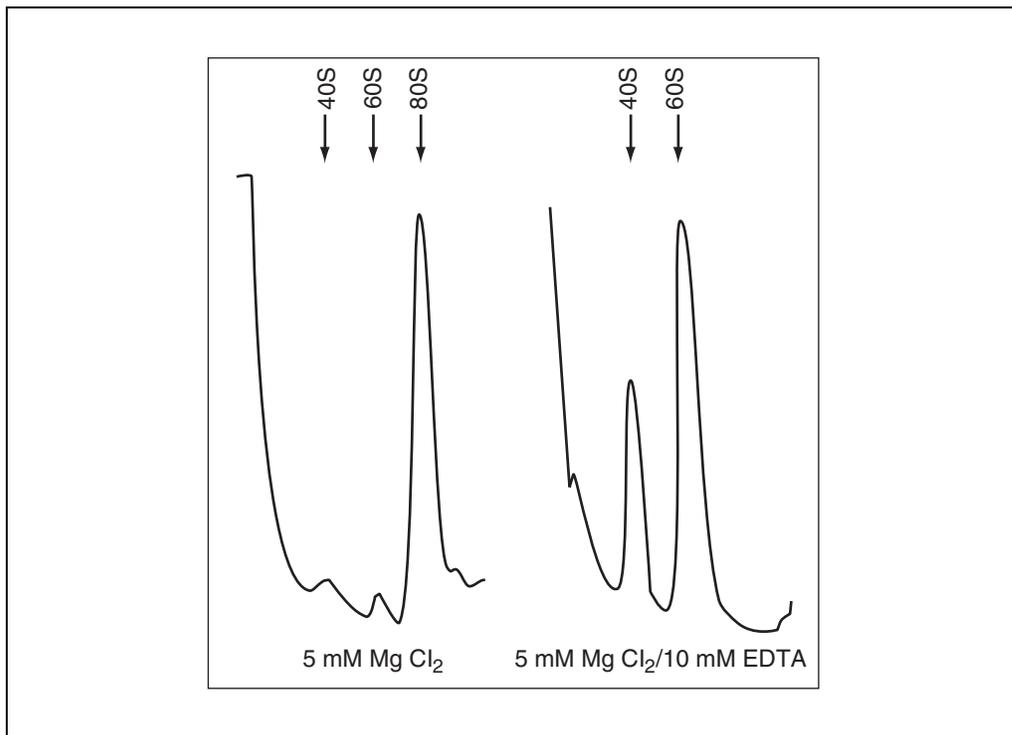


Figure 11.9.3 Sedimentation of ribosomes from erythroleukemic cells in 10% to 50% and 10% to 25% sucrose gradients. Erythroleukemic cells in log phase of growth were lysed as described in the text, and post-nuclear supernatants were loaded onto 10% to 25% sucrose gradients. The gradients were collected with an ISCO model 640 gradient fractionator by displacement from the bottom with 60% sucrose into a flow cell (Fig. 11.9.1A). UV absorbance was continuously monitored during the collection. The gradients contained 20 mM HEPES-KOH, pH 7.4, 5 mM MgCl₂, 100 mM KCl, and 2 mM dithiothreitol. In the experiment shown in the far right panel, 10 mM EDTA was added to the post-nuclear supernatants and to the gradients. The positions of sedimentation of the 40S, 60S, and 80S ribosomes are indicated by arrows above the figures. These assignments were confirmed by analysis of the rRNA content of the inclusive fractions. The direction of sedimentation is from right to left.

For analysis of 40S to 80S complexes in 10% to 25% sucrose gradients, centrifugation is 16 hr at 55,000 × g (20,000 rpm in SW28.1 rotor), 4°C. This completely pellets the polysomes while separating the 40S, 60S, and 80S complexes into distinct fractions (see Fig. 11.9.3). By increasing the sedimentation speed of these gradients to 23,000 rpm, 80S complexes are also pelleted, and separation of 43S from 48S (preinitiation) complexes is possible.

7. Collect gradients in 5-ml polyethylene tubes containing 1 ml Tris-buffered, water-saturated phenol using a fraction collector with continuous monitoring of absorbance at 254 nm (see Fig. 11.9.1).

For most purposes, fractionation of the gradients into fourteen 1.2-ml fractions provides sufficient detail to determine the position of sedimentation of mRNAs in the gradient.

To ensure that the amount of RNA in each fraction is representative of its relative distribution in the gradient, it is imperative that equal volumes of phenol and chloroform are added to the fractions. The amount of aqueous phase recovered following phase separation must also be constant.

Extract RNA

8. Immediately following collection, tightly cap the tubes, vortex to mix the phases, and place on ice until all fractions are collected.

9. Add 1 ml chloroform to each tube, mix phases by vortexing, and separate phases by centrifuging 15 min at $3000 \times g$, 4°C , in a tabletop centrifuge.

The stepwise addition of phenol and then chloroform, rather than addition of a phenol/chloroform solution, facilitates mixing of the phenol with the viscous sucrose solution.

Alternatively, the distribution of proteins in gradient fractions can be analyzed. In this case, the fractions are collected directly into microcentrifuge tubes containing $\frac{1}{9}$ volume of 100% trichloroacetic acid (e.g., 133 μl for a 1.2-ml fraction). The pelleted proteins are recovered and analyzed by SDS-PAGE (UNIT 6.1).

10. To ensure equal recovery of extracted RNA from the fractions, transfer a constant volume of the aqueous phase (for example, separate the aqueous phase into two 500- μl fractions) to 1.5-ml microcentrifuge tubes and precipitate with 2 vol ethanol.

The salt concentration in the buffer is sufficient for precipitation of RNA.

The precipitated RNA can be stored for extended periods of time (3 to 6 months) in ethanol at -20°C prior to analysis.

11. Pellet RNA by microcentrifuging 20 min at $10,000 \times g$, 4°C .

Analyze RNA content

12. Carefully aspirate the ethanol-buffered sucrose solution, taking care not to disturb the RNA pellet. Resuspend pellet in 20 μl RNA-loading buffer.

Excess sucrose remaining with the pellet can inhibit migration of the RNA out of the wells, particularly in the fractions that contain the highest concentration of sucrose. Complete removal of the sucrose can be facilitated by a second, brief (i.e., 30-sec) centrifugation in a microcentrifuge and aspiration of the residual supernatant.

13. Denature RNA by heating 15 min at 65°C .
14. Load the entire sample onto either an agarose or acrylamide gel prepared for electrophoresis (APPENDIX 3).

Care must be taken to ensure that the entire RNA pellet is loaded onto the gel if the results are to be reflective of the actual distribution of RNA across the gradient fractions.
15. Blot the gel for northern blot hybridization and hybridize (APPENDIX 3).

ALTERNATE PROTOCOL 4

PURIFICATION OF RIBOSOMAL COMPLEXES FROM CULTURED CELLS FOR BIOCHEMICAL ASSAYS

When it is necessary to analyze ribosomal complexes in their native state, collection into either phenol or TCA is not possible. Collection of pooled polysomes is simplified by sedimentation in 10% to 25% sucrose gradients, since these conditions will pellet complexes that are larger than 80S.

Additional Materials (also see Basic Protocol 2)

Cultured cell lysate (Basic Protocol 2) or yeast lysate (Support Protocol 3)
25% sucrose gradient solution (buffered as for 10% and 50% solutions in Basic Protocol 2)
TMK₁₀₀ lysis buffer (see recipe) without detergent
22% (w/v) sucrose solution (see recipe)
Swinging bucket rotor and tubes (e.g., Beckman SW50.1 rotor and 5-ml tubes)

1. Prepare and fractionate lysates as described (see Basic Protocol 2, steps 1 to 6), but use 10% and 25% sucrose gradient solutions and centrifuge 16 hr at $55,000 \times g$ (20,000 rpm in SW28.1 rotor), 4°C .

2. Solubilize polysomal pellet in 0.4 ml TMK₁₀₀ buffer without detergent.

To obtain other complexes (such as individual subunits or monosomes), the relevant fractions from the 10% to 25% gradient are removed from the fractionator and placed on ice as soon as they are collected. The pooled fractions are then diluted in TMK₁₀₀ lysis buffer.

3. Wash ribosomes and concentrate by centrifuging through 22% (w/v) sucrose solution in 5-ml tubes for 16 hr at 110,000 × g (34,000 rpm in an SW50.1 rotor), 4°C.

This pellet contains all complexes larger than 40S.

4. Dissolve pelleted ribosomes and subunits in 0.25 M sucrose solution and store up to 2 years in liquid nitrogen.

PREPARATION OF YEAST LYSATES

Preparation of lysates from yeast follows general, previously described methods (Baim et al., 1985; Moritz et al., 1991).

Materials

Yeast culture

Breaking buffer (see recipe)

Dry acid-washed glass beads (425- to 600- μ m; Sigma)

1 mg/ml cyclohexamide

Spectrophotometer

Additional reagents and equipment for growing yeast (UNIT 1.6)

1. Grow 100-ml yeast cultures to an OD₆₀₀ of ~1 (log phase; UNIT 1.6).
2. Pellet cells by centrifuging 5 min at 1500 × g, 4°C.
3. Resuspend pellet in 5 ml sterile 4°C water containing 100 μ g/ml cyclohexamide, incubate 1 min on ice, and pellet again.
4. Resuspend pellet in 500 μ l breaking buffer and 400 μ l acid-washed glass beads. Vortex eight times for 20 to 30 sec each, with 30-sec incubations on ice between vortexing steps.
5. Pellet beads and cellular debris in a refrigerated microcentrifuge 8 min at 10,000 × g, 4°C.
6. Transfer supernatant to a fresh tube and repeat centrifugation.
7. Measure the absorbance of an aliquot of the sample at 254 nm (nucleic acid).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Acrylamide solution, 40% (w/v)

380 g acrylamide

20 g *N,N*-methylenebisacrylamide

H₂O to 600 ml

Store up to 2 months at 4°C

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Acrylamide/urea bottom solution, 8% (w/v)

40 ml 40% (w/v) acrylamide solution (see recipe)
90 ml 5× TBE electrophoresis buffer (APPENDIX 2A)
92 g urea
20 g sucrose
10 mg bromphenol blue
H₂O to 200 ml
Store up to 2 days at room temperature

Acrylamide/urea top solution, 8% (w/v)

100 ml 40% (w/v) acrylamide solution (see recipe)
50 ml 5× TBE electrophoresis buffer (APPENDIX 2A)
230 g urea
H₂O to 500 ml
Store up to 2 days at room temperature

ATP, 100 mM

Dissolve ATP to 100 mM in 20 mM Tris·Cl, pH 7.5 (APPENDIX 2A). Check the concentration by measuring A₂₅₉. Store 100-μl frozen aliquots, preferably at -80°C, for up to 6 months.

A 1:100 dilution of this solution (1 mM) should have an absorbance of 15.4 at 259 nm.

Breaking buffer

20 mM HEPES·KOH, pH 7.4
2 mM MgCl₂
100 mM KCl
14.4 mM 2-mercaptoethanol
100 μg/ml cyclohexamide
Store up to 2 weeks at -70°C

GTP, 100 mM

Dissolve GTP to 100 mM in 20 mM Tris·Cl, pH 7.5 (APPENDIX 2A). Check the concentration by measuring A₂₅₂. Store 100-μl aliquots frozen, preferably at -80°C, for up to 6 months.

A 1:100 dilution (1 mM) should have an absorbance of 13.7 at 252 nm.

MgCl₂, 100 mM

Since most bottles of MgCl₂ tend to contain visible H₂O (i.e., the crystals appear wet), assume that the original manufacturer's weight is correct and dissolve the entire contents of the bottle in H₂O to the desired concentration.

Recently, the authors have substituted magnesium acetate [Mg(CH₃COO)₂] for MgCl₂, as Mg(CH₃COO)₂ comes as a dry powder. However, since acetate is a great carbon source for bacteria or fungi, the solution should be autoclaved and stored cold.

RNA-loading buffer

20 mM MOPS, pH 7.0
8 mM sodium acetate
1 mM EDTA
50% (v/v) formamide
6.5% (v/v) formaldehyde
5% (v/v) glycerol
0.025% (w/v) bromphenol blue
0.025 (w/v) xylene cyanol
Store up to 2 months at 4°C

SDS sample buffer

1 mM dithiothreitol
80 mM Tris·Cl, pH 6.8 (APPENDIX 2A)
0.002% (w/v) bromphenol blue
2% (w/v) sodium dodecyl sulfate (SDS)
10% (v/v) glycerol
Store up to 6 months at -20°C

Standard sucrose solution

0.25 M sucrose
20 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
0.1 mM EDTA
1 mM dithiothreitol
Store up to 6 months at -20°C

Subunit buffer

50 mM HEPES·KOH, pH 7.5
2 mM $\text{Mg}(\text{CH}_3\text{COO})_2$
1 mM puromycin
500 mM KCl
Prepare fresh
If necessary, may be stored 1 day at 4°C

Sucrose gradient solutions, 5% and 20% (w/v)

5% or 20% (w/v) sucrose
50 mM HEPES·KOH, pH 7.5
5 mM $\text{Mg}(\text{CH}_3\text{COO})_2$
2 mM dithiothreitol
500 mM KCl
0.1 mM EDTA
Prepare fresh immediately before use

These solutions are used to prepare gradients for isolating 40S and 60S ribosomal subunits (see Support Protocol 1).

Sucrose gradient solutions, 10% and 35% (w/v)

10% or 35% (w/v) sucrose
60 mM NaCl
6 mM $\text{Mg}(\text{CH}_3\text{COOH})_2$
50 mM Tris·Cl, pH 8.4 (APPENDIX 2A)
10 mM dithiothreitol
Prepare fresh immediately before use

The solutions are used to prepare gradients for toeprinting (see Alternate Protocol 3).

Sucrose gradient solutions, 10% and 40% (w/v)

10% or 40% (w/v) sucrose
20 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
100 mM KCl
5 mM MgCl_2
100 μM GDPNP
Prepare fresh immediately before use

These solutions are used to prepare gradients for isolating ribosomal complexes (see Basic Protocol 1 and Alternate Protocols 1 and 2). They may be stored overnight at 4°C , but GDPNP should be added just before use.

Sucrose gradient solutions, 10% and 50% (w/v)

10% or 50% (w/v) sucrose
100 mM KCl
5 mM MgCl₂
2 mM dithiothreitol
20 mM HEPES·KOH, pH 7.4
Prepare in diethylpyrocarbonate (DEPC)-treated water (APPENDIX 2A)
Store up to 3 months at 4°C

These solutions are used for analyzing translation in cultured cells (see Basic Protocol 2). General procedures to limit contamination with exogenous nucleases (use of gloves, buffer preparation with ultrapure reagents and DEPC-treated water; see APPENDIX 2A) should be employed at all times.

Sucrose solution, 22% (w/v)

22% (w/v) sucrose
100 mM KCl
5 mM MgCl₂
2 mM dithiothreitol
20 mM HEPES/KOH, pH 7.4
Prepare in diethylpyrocarbonate (DEPC)-treated water (APPENDIX 2A)
Prepare immediately before use

This solution is used for preparing ribosomal complexes for biochemical assays (see Alternate Protocol 4).

Sucrose solution, 0.25 M

0.25 M sucrose
1 mM dithiothreitol
0.2 mM EDTA
10 mM KCl
1 mM Mg(CH₃COO)₂
Store up to 6 months at -20°C

TMK₁₀₀ (Tris/Mg²⁺/K⁺) lysis buffer

10 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
5 mM MgCl₂
100 mM KCl
1% (v/v) Triton X-100
0.5% (w/v) deoxycholate
Prepare in diethylpyrocarbonate (DEPC)-treated water (APPENDIX 2A)
1 U/ml placental ribonuclease inhibitor, added fresh just before use
2 mM dithiothreitol, added fresh just before use
Store up to 3 to 6 months at 4°C

General procedures to limit contamination with exogenous nucleases (use of gloves, buffer preparation with ultrapure reagents and DEPC-treated water; see APPENDIX 2A) should be employed at all times.

COMMENTARY

Background Information

Translation of an mRNA can be conveniently separated into several discrete steps. Translation initiation is the sequential association of an mRNA with a 40S ribosomal subunit to form a 48S preinitiation complex, which is followed by joining of the 60S subunit to form

an 80S ribosome. This complex can then initiate the repetitive cycle of tRNA binding, peptide bond formation, and translocation that defines elongation. Repeated episodes of initiation and elongation on an individual mRNA result in the formation of polysomes, which can include over fifteen translating ribosomes per

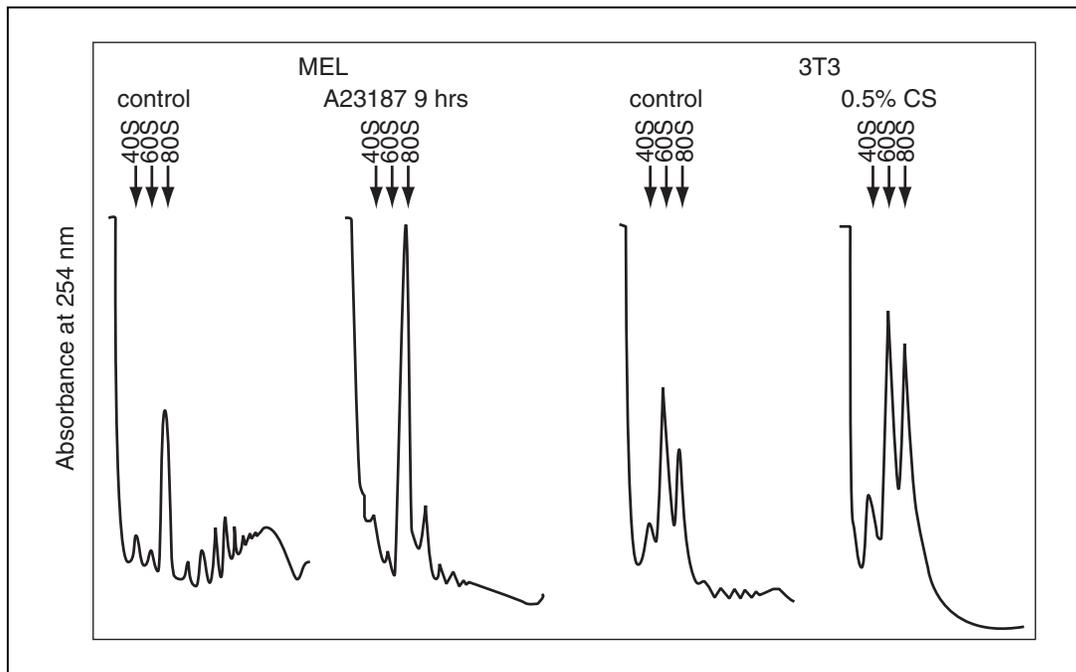


Figure 11.9.4 Serum-starved 3T3 cells and erythroleukemic cells exposed to inducers of differentiation demonstrate evidence of a decrease in the rate of translation initiation. Murine erythroleukemic (MEL) cells were grown for 9 hr in the presence or absence of the inducer of differentiation A23187 (0.75 $\mu\text{g/ml}$). BALB/c 3T3 cells were grown to confluence, and then either (1) replated in fresh medium containing 10% calf serum (control), or (2) washed with PBS, refed with medium containing 0.5% calf serum (0.5% CS), and incubated for an additional 18 hr. Cell lysates were prepared for analysis on 10% to 50% sucrose gradients as described in the text. Gradients were collected with an ISCO 640 gradient fractionator with continuous monitoring of the UV absorbance at 254 nm (Fig. 11.9.1A). Positions of 40S, 60S, and 80S ribosomal peaks are indicated by arrows above each tracing. The direction of sedimentation is from right to left.

mRNA. Due to the quantal increase in complex size as 40S and 60S subunits are added to an individual mRNA, it is possible to identify a number of different translational complexes by sucrose gradients. Using the techniques described here, it is possible to resolve a number of different complexes including uninitiated mRNAs (such as 7S globin mRNAs), 40S subunits (43S complexes), 48S preinitiation complexes, 60S subunits, 80S ribosomes, and polysomal complexes containing up to eight to ten ribosomes/mRNA.

Interpreting results of sucrose gradient separation of cell lysates

The distribution of ribosomes or mRNAs across a gradient ultimately reflects the combined rates of initiation, elongation, and termination of translation. Therefore, it is possible for different combinations of effects on these processes to produce similar changes in distribution. For example, a decrease in initiation rate would be expected to decrease the size of the average polysome as well as to decrease the

total amount of polysomes (and polysomal mRNAs; e.g., Fig. 11.9.4). However, a similar result would be expected if initiation rate remained constant while elongation and termination rates were increased. Therefore, the results are best interpreted in the context of an independent determinant of translation, for example, as determined by the incorporation of radioactive amino acids into protein.

Suggested approaches for demonstrating physical association of mRNAs or proteins with ribosomes

To demonstrate that the proteins or RNAs found in individual fractions are in fact components of ribosomal complexes, several different approaches are commonly used. Digestion of extracts with RNase prior to their sedimentation can be used to confirm that the identified proteins are components of a ribonucleoprotein (RNP) complex. However, this approach does not distinguish between different types of RNPs and is not useful if the component of interest is itself an RNA. A more generally

useful approach takes advantage of the magnesium requirement for formation of higher-order ribosomal structures (Spirin, 1986). Addition of EDTA (5 mM in excess of $MgCl_2$) to the gradients results in complete dissociation of polyribosomes, 80S ribosomal complexes, and 40S ribosomal/mRNA preinitiation complexes (and also dissociates the 5S and 5.8S RNAs from the 60S ribosomal subunit). However, since most RNA/protein interactions are not dependent on magnesium, the presence of EDTA does not disrupt sedimentation of less complex RNPs. EDTA (10 mM) may be added to the postnuclear supernatant prior to loading on the gradient. (Its presence before separation of the nuclei results in nuclear dissolution.) An example of the effect of EDTA on ribosome sedimentation in 10% to 25% gradients is shown in Fig. 11.9.3.

A functional approach to demonstrate translation of an mRNA that cosediments with polysomes is to determine the sensitivity of the complex to puromycin. This inhibitor occupies the "A" site of the elongating ribosome, terminates translation, and causes release of the prematurely terminated peptide. Puromycin treatment therefore results in the "collapse" of polysomes, with an associated increase in monosomes and disomes, as determined by UV absorbance of the fractionated RNA. A similar effect should be evident for individual mRNAs in the fractions. For the purposes of these experiments, a 10-min incubation at 37°C in normal growth medium containing 100 μ M puromycin is sufficient to observe the effects of this agent on translation.

As an alternative approach to determining translational activity, the stability of the ribosomal complexes in high concentrations of monovalent cations has occasionally been employed. This technique derived from the early observations that: (1) 80S fractions frequently contain a high content of ribosomal subunits, (2) radioactive amino acid incorporation does not occur in 80S fractions (Warner et al., 1963), and (3) 80S ribosomes can be dissociated into 40S and 60S complexes by sedimentation in 500 mM KCl (Martin and Hartwell, 1970; Zylber and Penman, 1970; Martin, 1973). On the basis of these observations, salt-labile subunits were considered to be "vacant couples" that did not contain bound mRNA. The addition of KCl at a concentration of 500 mM to the standard gradient conditions described in these protocols is sufficient to dissociate 80S ribosomal complexes in both mammalian cells and yeast, while polyribosomes remain intact (Martin and

Hartwell, 1970; Zylber and Penman, 1970; Martin, 1973). However, while published data from this laboratory is consistent with the observations that salt-labile 80S complexes are translationally inactive, the authors have demonstrated that these complexes contain mRNA stably bound to the 40S ribosomal subunit (Hensold et al., 1996). Thus, while stability in 500 mM KCl can be used as a surrogate marker for translational activity, it cannot be assumed that these complexes do not contain mRNA, although the significance of these 80S complexes remains to be established.

When assessing the presence of a protein in a given ribosomal complex, it is important to keep in mind that the absence of a protein from a fraction does not exclude its existence in that complex. For example, elongation factors are not routinely found in polysomal fractions. Thus, loosely adherent proteins may be lost during sedimentation through sucrose or under the conditions used for lysis.

Critical Parameters

Cell translation is rapidly responsive to a variety of signals that are generated from both external and internal signaling pathways (Hershey, 1991; Merrick, 1992). Thus, care must be taken to ensure that extract preparation does not superimpose independent effects upon the cells to be assayed. For this reason, as a first step in the preparation of extracts, cells should be rapidly equilibrated in ice-cold PBS to cause a general arrest of cellular processes. This also serves to inhibit endogenous nucleases that may be released during extract preparation. Thus, it is critical that all steps in extract preparation are performed on ice or in refrigerated centrifuges. While some protocols for polysome analysis include cyclohexamide (50 to 100 μ g/ml) to prevent "run-off translation" from polysomes, this is unnecessary if the extracts are maintained at 4°C during their preparation. Further, transient elevations in temperature that occur while the cells are exposed to cyclohexamide results in polysome profiles with characteristics of inhibited elongation. Thus, if cyclohexamide is included in buffers, precautions must be taken to avoid exposure to temperatures above 4°C.

The presence of ribonuclease inhibitors in the lysis buffer is also not absolutely necessary, since it is possible to fractionate intact RNA from MEL and 3T3 cells without added nuclease inhibitors as long as the extracts are prepared and fractionated at 4°C. For cell lines that have higher levels of endogenous RNases, lysis

buffer should be supplemented with placental ribonuclease inhibitor (1 U/ μ l) or RNase Block (Stratagene; Kaspar and Gehrke, 1994). The inclusion of deoxycholate in the lysis buffer increases the yield of polysomes, presumably due to the dissolution of the cytoskeleton that occurs in the ionic detergent. However, if ribosomal complexes are to be isolated for use in biochemical assays, it may be advisable to avoid this detergent, since it is more denaturing than nonionic detergents.

When assessing the distribution of mRNAs (or proteins) in gradient fractions, it is important that the amount of material recovered from each fraction be representative of the actual amount of material present in that fraction. As described here, this is ensured when care is taken to maintain equal volumes throughout all steps of the extractions. Further, it is equally important that the pellets be resuspended in a constant volume, and that either all of the material or a constant fraction of the volume be loaded onto gels, if the distribution of material is to be representative of its actual distribution across the gradient. Alternatively, a radiolabeled standard may be added to each fraction (such as an *in vitro*-transcribed RNA) prior to recovery of the RNA. Any variability in recovery of extracted RNA can then be corrected by loading equal amounts of the recovered radioactive RNA, as determined by scintillation counting of an aliquot of each fraction.

Troubleshooting

Translation in lysates

Translation in a system reconstituted from fractionated components is very inefficient and the molar yield of product is often on the same order of magnitude as the input level of ribosomes and factors. Reaction rates are also considerably reduced, especially for elongation. To circumvent this problem, unfractionated reticulocyte lysate can be used (see *UNIT 11.2*). However, in this case any added component must compete with the endogenous equivalent. Suggested approaches for the use of these lysates are discussed below.

The individual steps in initiation can be monitored by determining the binding of specific translation factors to either 40S, 60S, or 80S ribosomes. Purified factors can be radiolabeled by reductive methylation (Peterson et al., 1979a), or the factor may be translated *in vitro* in the presence of radioactive amino acids (Joshi et al., 1994). Alternatively, association of factors with specific translational complexes

could be monitored by immunoblotting (*UNIT 6.2*), as previously described. Met-tRNA_i may be monitored by addition of exogenously labeled [³⁵S]Met-tRNA_i. However, the aminoacyl linkage in Met-tRNA_i is one of the least stable and generally has a half-life of ~8 to 10 min. Thus, under normal protein synthesis conditions, the percentage of radioactive Met-tRNA_i falls quite rapidly. The stability of the initiator tRNA can be increased considerably by the addition of a formyl group (using *E. coli* transformylase) to yield fMet-tRNA_i, but this initiator tRNA is utilized about a third as well as the normal Met-tRNA_i.

For the analysis of mRNAs, radioactive mRNAs can be conveniently transcribed *in vitro* using bacterial polymerases (SP6, T7, or T4) in the presence of an mRNA cap analog, m⁷GpppG. By setting the ratio of cap analog to GTP at 5:1 to 10:1, most of the transcripts emerge with an m⁷G cap. The use of lysates that have been treated with micrococcal nuclease (Pelham and Jackson, 1976) reduces the level of endogenous mRNAs to the point where there is little competition for translation as long as the level of added transcript is <1 μ g. The use of *in vitro*-transcribed RNAs allows one to systematically alter the RNA sequence/structure to determine the influence of either specific structural elements (stem-loops of specific sequences) or context (i.e., the nucleotides surrounding the AUG start codon) on the formation of 40S or 80S initiation complexes. If desired, the binding site of the ribosome/subunit to the mRNA can be mapped by toeprinting, as described previously (Anthony and Merrick, 1992). Both the 40S and 80S regions can be toeprinted. While it might appear ideal to perform toeprinting in the original translation mixture without gradient fractionation, it has been observed that it is not possible to reverse transcribe in the crude lysate. This may be due to the presence of phosphatases or other undefined inhibitors in the lysate.

Given that lysates contain all the ingredients necessary for complete polypeptide chain synthesis, normally most of the components end up in polysomes, and thus it is difficult to identify useful intermediate complexes. In this case, the use of inhibitors may facilitate their identification (Peterson et al., 1979a,b; Safer et al., 1979). To block the joining of the 40S and 60S subunits, the lysates can be supplemented with the drug edeine (which is increasingly difficult to obtain) or by the inclusion of GDPNP (as subunit joining requires the hydrolysis of the GTP in the ternary complex

eIF2-GTP-Met-tRNA_i). The presence of GTPNP also ensures that no elongation will take place. In the presence of these inhibitors, these experiments yield complexes that are either 40S, 43S, or 48S. Similarly, to monitor the flow of components (mRNA or Met-tRNA_i) into 80S complexes, anisomycin (an inhibitor of elongation) is used. It should be mentioned that in these studies with a specific inhibitor there will be a relative buildup of complexes at or before the point of the block. Inhibitors are therefore useful in magnifying the signal at individual steps.

Translation in intact cells

The most common problem in using gradients to analyze translation in cells is degradation of the RNA. If this occurs during extract preparation, the result will be a decrease in the amount of polysomal material, due to cleavage of the mRNA between translating ribosomes. If this is severe, it may be apparent on the UV absorbance tracing during collection of the gradient. Lesser degrees of degradation may only become apparent during subsequent gel electrophoresis of the RNA and be detected as widespread “nicking” of the rRNA by ethidium bromide staining of the gel. More subtle nicking may only be detected following northern blot hybridization and visualization of individual mRNAs. Suggestions to decrease nuclease activity during extract preparation are discussed. It is worth reemphasizing that keeping the cells and extracts on ice and precooling the gradients and centrifuge to 4°C before centrifugation provides an important safeguard against nuclease activity, even in the absence of exogenously added RNase inhibitors.

If nicking is detectable in only one or two fractions, this is usually due to a nuclease that was inadequately removed following phenol and chloroform extraction of the gradient fractions. Ribonuclease can be recovered from polysome preparations (Caruccio and Ross, 1994), so the most likely cause of this problem is recovery of an incompletely (or reversibly) denatured nuclease from the interface during the phenol and chloroform extractions. In this case, greater care should be taken to avoid disturbing the interface, including removing a smaller volume of the aqueous fraction. In either case, if evidence of even partial RNA degradation is detected, the gradients should be repeated, since any conclusions obtained from even partially degraded complexes would be invalid.

When recovering proteins from TCA precipitates of gradient fractions, difficulty is

sometimes encountered in solubilizing the proteins in the first one or two fractions. This is generally due to the high concentration of protein in these fractions. Complete solubilization may not be possible in the small volume of sample buffer necessary for loading the entire sample in a single well of a gel. In this case, it is best to increase the volume to ensure complete solubilization of the proteins and load a predetermined percentage of this volume in the well. This should then be taken into account when quantifying the results determined by immunoblotting.

During preparation of cell extracts, nuclear dissolution may occur due to the presence of the ionic detergent deoxycholate. This will be manifest as an increase in viscosity of the extract and the lack of a detectable nuclear pellet following centrifugation of the initial lysate. The concentration of deoxycholate used in Basic Protocol 2 was empirically determined for MEL cells, and cells differ in their sensitivity to this detergent. Thus, while 1% Triton X-100/0.5% deoxycholate is a useful starting point, the optimal concentration for other cells may need to be empirically determined. In addition, since mixed micelles of Triton X-100 and deoxycholate are formed in the extraction buffer, it is important that these detergents be well mixed in the lysis buffer before addition to the cells.

Anticipated Results

In analyzing the distribution of ribosomes or mRNA in cells, it is important to avoid preconceived notions of how the profile should appear. Most knowledge of translational mechanisms derives from insights gained from studies in reticulocyte lysates, and although these observations have been generally applicable to cells, it is likely that additional mechanisms of regulation have yet to be identified. For example, while phosphorylation of ribosomal protein S6 is one of the most highly conserved sequelae of mitogenic stimulation, the biochemical effect of this modification has yet to be conclusively established. Recent evidence suggests that this may influence translation of mRNAs that have a 5′-terminal oligopyrimidine tract (5′-TOP; Jefferies et al., 1994).

Changes in translation rate may also be due to changes in total ribosomal content (Rudland and de Asua, 1979). Recent studies in yeast suggest that ribosomal abundance may differentially affect mRNA translation (Proweller and Butler, 1997). However, unless care is taken

to standardize the amount of lysate loaded for total cell number, changes in cellular ribosomal abundance would not be detected by assessing the distribution of ribosomes across a gradient. In this case, it may be best to use alternative means to quantitate changes in ribosome content, such as blot hybridization with radiolabeled probes to rRNAs. In this case, RNA extractions and gel loading should be standardized for cell number.

Cell type differences in translational regulation are also likely to occur. The expression of the two isoforms of eIF4A demonstrate tissue-specific differences in their expression (Nielsen and Trachsel, 1988), and the mRNA levels of eIF4B and eIF4H are quite variable in different tissues (Richter et al., 1999). In addition, translational regulation may be abnormal in transformed cells (Rinker-Schaefer et al., 1992; Sonenberg, 1993) and thus in immortal cell lines. To emphasize these cell type differences in the distribution of ribosomes in sucrose gradients, Figure 11.9.4 illustrates differences between two murine cell lines. These differences are independent of cell growth conditions and cannot be attributed to differences in commonly identified means of translational regulation: phosphorylation of the initiation factors eIF2, eIF4E, or the 4E-binding protein PHAS I. Analysis of other cell types in this laboratory has identified at least two other distinct patterns of ribosomal distribution in mammalian cell lines. It is likely that the continued analysis of cellular growth and differentiation will provide insights into novel mechanisms of translational regulation.

Time Considerations

The time required for completion of these experiments will be largely dependent on the centrifugation time, since the 3 to 16 hr required for this step will be longer than any of the preceding or ensuing steps. For analyzing translation in cells, the preparation of the extracts should take 15 to 20 min, and a similar period of time should be allotted for pouring the gradients. Gradients can be poured the preceding day and stored at 4°C overnight without any ill effects. The time required for extraction of RNA or protein from the gradient fractions will depend on the number of gradients and the number of fractions collected from each gradient. In general, this should take 1 to 3 hr for most experiments.

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Preparation and Use of Interphase *Xenopus* Egg Extracts

UNIT 11.10

Xenopus interphase egg extracts (see Basic Protocol 1 and Alternate Protocol 1) are useful as sources of cytoplasmic and organelle material for in vitro reconstitution of cellular events such as nuclear assembly (see Basic Protocol 2) from demembrated sperm chromatin (see Support Protocol 2), nuclear protein import (see Basic Protocol 3), and nuclear DNA replication (see Basic Protocol 4 and Alternate Protocol 2). To characterize the molecular bases of these and other processes, the extracts can be modified by immunodepletion (see Support Protocol 3) or protein addition (see Support Protocol 4). Eggs are obtained from primed female frogs (see Support Protocol 1). These extracts can also be modified to support cycling (see UNIT 11.11) and apoptosis (see UNIT 11.12). To study meiotic maturation, extracts can be prepared from oocytes (see Basic Protocol 5).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

PREPARATION OF INTERPHASE EGG EXTRACTS

Prior to preparation of an interphase extract, the frog is injected and eggs are laid overnight in 100 mM NaCl (see Support Protocol 1). The mature *Xenopus* eggs are arrested in metaphase of meiosis II and therefore have high mitotic kinase or cyclin B/cdc2 activity. Egg lysis by centrifugation causes a transient release of Ca²⁺ from intracellular stores, resulting in proteolysis of cyclin B and subsequent loss of cdc2 kinase activity. After inactivation of cyclin B/cdc2, the extract moves from M phase to S phase. Addition of cycloheximide to the extract prevents synthesis of any new proteins, most notably cyclin B. Therefore, the extract remains in interphase of the cell cycle. Upon addition of sperm chromatin and an ATP regenerating system, membrane vesicles present in the extract fuse to the chromatin to form interphase nuclei (see Basic Protocol 2) which are capable of replicating their DNA (see Basic Protocol 4) and have intact nuclear pore complexes.

**BASIC
PROTOCOL 1**

Materials

- Eggs obtained from female frogs, in 100 mM NaCl (see Support Protocol 1)
- 2% (w/v) L-cysteine free base (Sigma) in H₂O
- 0.25× modified Ringer's solution (MMR), pH 7.7 (see recipe for 10×)
- Egg lysis buffer, pH 7.7 (see recipe)
- 5 mg/ml cytochalasin B (Calbiochem) in DMSO
- 5 mg/ml aprotinin (Roche Diagnostics) in H₂O
- 5 mg/ml leupeptin (Roche Diagnostics) in H₂O
- 10 mg/ml cycloheximide in H₂O
- 200-ml beaker
- 100-mm glass petri dish
- Pasteur pipets
- Dissecting microscope
- 15-ml conical polypropylene centrifuge tubes
- IEC Clinical centrifuge
- Sorvall HB-4 swinging bucket rotor
- 18-G needle attached to 5-ml syringe

**In Vitro
Reconstitution**

11.10.1

Wash and dejelly eggs

1. Collect eggs in a 200-ml glass beaker and pour off any excess solution.
2. Rinse eggs once in 2% L-cysteine, pH 8.0, then continue to incubate in 100 ml of 2% L-cysteine solution per frog for ~5 min at room temperature, gently swirling the eggs periodically.

Before incubation with cysteine solution, a transparent jelly coat, which prevents the eggs from directly contacting each other, will surround each individual egg. Once eggs are dejellied, they will pack closely together

3. Pour the cysteine solution off of the dejellied eggs and wash eggs in 0.25× MMR by decantation to remove debris. Repeat 2 times. Pour eggs into a 100-mm glass petri dish.
4. Preferably working under a dissecting microscope, remove bad eggs from the petri dish with a Pasteur pipet.

Mature Xenopus eggs have a darkly pigmented half on top and a light half on the bottom. The dark half of the egg should have a small white spot present in the middle created by breakdown of the oocyte nucleus (germinal vesicle breakdown) during the process of oocyte maturation. This spot can be seen by eye but is more easily seen using a dissecting microscope. Anything without this white spot is an oocyte and should be removed. Any eggs that are white, enlarged, or not uniformly pigmented should also be removed.

5. Wash eggs 2 to 3 times, each time by pouring egg lysis buffer on them and carefully pouring the buffer off, minimizing the number of eggs that are spilled out.

Lyse eggs

6. Transfer eggs with an inverted Pasteur pipet (with the bulb on the thin, pointed end) into a 15-ml polypropylene tube.

The Pasteur pipets must be inverted because a wide-mouthed pipet is required to avoid lysing the eggs.

7. Centrifuge eggs 15 sec in a clinical centrifuge at $400 \times g$. Remove extra buffer from the top of the packed eggs by decantation.

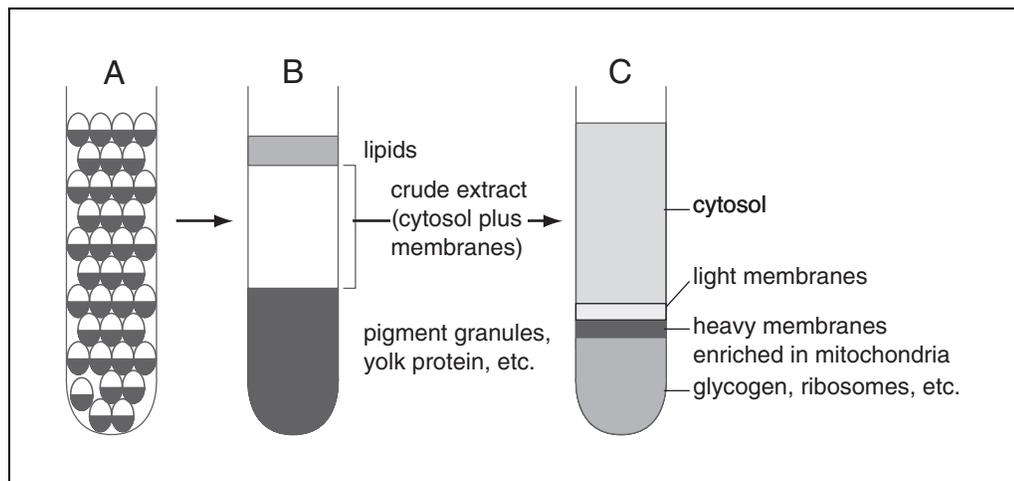


Figure 11.10.1 Preparation of the interphase egg extract. Eggs are packed in tubes by low-speed centrifugation (A) and then are centrifuged further at $12,000 \times g$ to produce layers containing lipids, crude extract, and pigment granules (B). The crude extract can be further fractionated into cytosol, light membranes, and a heavy membrane fraction enriched in mitochondria (C).

8. On the basis of the volume of the packed eggs, add the following directly to the top of the packed eggs:

5 µg/ml aprotinin (1:1000 dilution of 5 mg/ml stock)
5 µg/ml leupeptin (1:1000 dilution of 5 mg/ml stock)
5 µg/ml cytochalasin B (1:1000 dilution of 5 mg/ml stock)
5 µl/ml of 10 mg/ml cycloheximide (50 µg/µl final)

Aprotinin and leupeptin inhibit proteases, cytochalasin B inhibits actin polymerization, and cycloheximide inhibits protein synthesis.

9. Lyse eggs by centrifuging 15 min at $12,000 \times g$, 4°C, in Sorvall HB-4 swinging bucket rotor.

The lysed eggs will be separated into three layers, a yellow lipid layer on top, the crude interphase extract in the middle, and a dark pellet (see Fig. 11.10.1B).

Collect extract

10. Remove the crude interphase extract by piercing the side of the polypropylene tube with an 18-G needle attached to a 5-ml syringe.

The crude extract contains cytoplasm, light membrane from the ER and nuclear envelope, ribosomes, and heavy membrane organelles including mitochondria.

When incubated at room temperature in the presence of sperm chromatin and an ATP-regenerating cocktail, the crude extract supports nuclear reconstitution (see Basic Protocol 2). However, this extract will not support nuclear assembly after freeze/thawing.

The extract can be stored ~1 hr on ice.

PREPARING FRACTIONATED INTERPHASE EXTRACTS

Using the following protocol, the crude interphase egg extract may be separated by high-speed centrifugation into discrete cytoplasmic, light-membrane, and heavy-membrane (mitochondrially-enriched) fractions. The cytosolic and light-membrane fractions can be snap-frozen in liquid nitrogen, stored at -80°C, and later reconstituted with sperm chromatin for nuclear assembly.

Additional Materials (also see Basic Protocol 1)

Crude interphase extract (see Basic Protocol 1)
2.5-ml Ultraclear centrifuge tubes
TLS centrifuge and TLS-55 rotor (Beckman) or equivalent

1. If this has not been done already (see Basic Protocol 1, step 8), supplement the isolated crude interphase extract with aprotinin and leupeptin (1:1000 dilution of 5 mg/ml stock) and cytochalasin B (1:1000 dilution of 5 mg/ml stock).
2. Transfer extract to a 2.5-ml Ultraclear centrifuge tube. Centrifuge the crude extract 70 min in a Beckman TLS-100 centrifuge at $250,000 \times g$ (55,000 rpm in TLS-55 rotor), 4°C.

The extract will separate into a clear cytoplasmic portion with a pale yellow membrane fraction below. A dark membrane fraction consisting of mitochondria and other organelles will lay below the light membranes (Fig. 11.10.1C).

3. Keeping extract on ice, remove clear cytoplasmic fraction with 200-µl pipet tip, taking care not to disrupt the light membrane layer. Retain both the cytoplasmic fraction and membrane layer and keep on ice.

ALTERNATE PROTOCOL 1

In Vitro Reconstitution

11.10.3

4. Centrifuge cytoplasmic fraction again, in a new 2.5-ml Ultraclear centrifuge tube, 25 min at $250,000 \times g$, 4°C . Collect the top cytoplasmic fraction as in step 3, divide into aliquots (50 or 100 μl /microcentrifuge tube), freeze in liquid nitrogen, and store at -80°C until ready for use.

Steps 5 and 6 should be performed while the above centrifugation is in progress.

5. Using a micropipettor with a cut-off or wide-bore pipet tip, carefully remove the light membrane fraction from the membrane layer (step 3), leaving dark membranes undisturbed. Dilute all of the light membrane in 1.5 ml egg lysis buffer and store on ice until ready to perform step 7.
6. Add 0.42 g sucrose to 5 ml egg lysis buffer to create $2\times$ sucrose/egg lysis buffer. Chill to 4°C .
7. When ready to centrifuge the membranes, place the diluted membranes (step 5) into 2.5-ml Ultraclear centrifuge tube. Underlay the membranes with $2\times$ sucrose/egg lysis buffer by releasing it from a Pasteur pipet at the bottom of the membrane-containing tube. Add enough buffer to fill the tube.

A clear layer will easily be seen below the membranes.

The $2\times$ sucrose/egg lysis buffer acts as a cushion to remove any proteins peripherally or nonspecifically associated with the membranes.

8. Centrifuge 20 min at $26,000 \times g$ in the TLS-55 rotor, 4°C .

After centrifugation, the membrane will be in a pellet at the bottom of the tube.

9. Pipet off the clear buffer until only the membrane fraction remains in the tube. With a wide-bore pipet tip, divide membranes into aliquots (5 to 10 μl per 0.5-ml microcentrifuge tube), flash freeze, and store at -80°C until ready for use.

If there are any contaminating mitochondria/heavy membranes in the light membrane fraction, they will appear as a dark spot in the center of the light membrane pellet. By gently flicking the tube, the light membrane will dislodge, leaving an adherent mitochondrial pellet. It is important to avoid contaminating the light membrane with this fraction; therefore, should any dark spot be apparent, carefully use a wide-bore pipet tip to remove the light membranes to a separate tube before aliquoting.

SUPPORT PROTOCOL 1

INJECTION OF FROGS TO OBTAIN EGGS

Xenopus laevis mature females can be obtained from a number of commercial sources, including Nasco, Xenopus I, and Xenopus Express (see *SUPPLIERS APPENDIX*). Frogs should be kept in clean, dechlorinated tap water, at a maximal density of one frog per liter (though lower densities are desirable). Although dechlorination can be achieved by storing water in an open tank for 24 hr with aeration, it is faster to pass the water through a carbon filter. Optimal egg production is maintained by housing frogs at 18°C . Frogs can be fed a variety of foods, but the easiest is frog brittle from Nasco or Purina trout chow (1 g/frog, 3 to 4 days/week). Water should be changed on the days following feeding.

To produce eggs, frogs are primed with pregnant mare serum gonadotropin and are then induced to lay eggs at least 2 days later by injection of human chorionic gonadotropin.

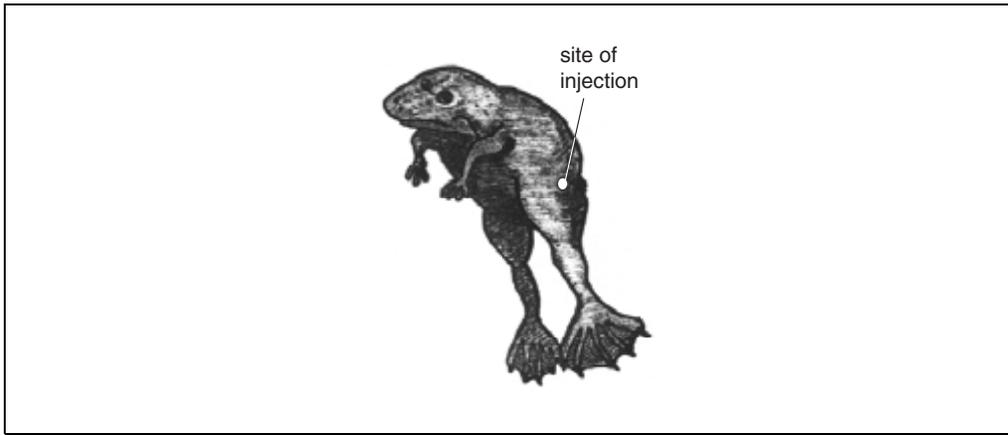


Figure 11.10.2 *Xenopus* injection site. The dorsal lymph sac, where the frog is injected, lies at the juncture of the torso and the upper portion of the leg. There is a loose fold of skin at this location under which the needle is slipped. Do not push the needle down into the muscle.

Materials

- Female frogs, mature (e.g., Nasco, *Xenopus* I, or *Xenopus* Express)
- 100 mM NaCl (25 g NaCl/5 liters H₂O)
- 200 U/ml pregnant mare serum gonadotropin (PMSG; Calbiochem): reconstitute powder in sterile water to allow final injection volume of 0.5 ml (i.e., 200 U/ml final concentration); store in aliquots indefinitely at -20°C
- 1000 U/ml human chorionic gonadotropin (HCG; Sigma or Amersham/USB): reconstitute powder in sterile water to allow final injection volume of 0.5 ml (i.e., 1000 U/ml final concentration); store up to 1 month at 4°C
- 1× modified Ringer's solution (MMR; optional; see recipe)

Water tanks
 1-ml syringes
 25-G needles

1. For priming frogs, prepare a tank containing 100 mM NaCl to house frogs after injection.

Primed frogs should be kept at a density of ~1 frog/liter.

2. Using a 25-G needle attached to a 1-ml syringe, inject frogs subcutaneously into the dorsal lymph sac (see Fig. 11.10.2) with 0.5 ml of 200 U/ml PMSG (total of 100 U). Return primed frogs to the tank prepared in step 1.
3. After a 2- to 3-day interval, induce egg laying by injecting frogs with 0.5 ml of 1000 U/ml HCG (total of 500 U), by the same route as PMSG injection (step 2). After injection, place each frog in 5 liters of 100 mM NaCl (or place the frog in 1× MMR if the eggs are to be used for preparation of cycling or CSF extracts; see UNIT 11.11).

If more than one frog is injected, it is best to put each frog in its own container as the quality of eggs varies from frog to frog.

4. Approximately 16 to 22 hr after HCG injection (if frogs are kept at 18°C), remove the frog to a clean water tank and pour the eggs into a beaker.

It is advisable to observe the frogs for 1 to 2 days for signs of infection at the site of injection.

5. Sort through the eggs and remove any bad eggs and oocytes.

A "good egg" is one with even, unmottled pigmentation. A white pinpoint-sized spot, indicative of oocyte maturation, should be visible in the center of the darkly pigmented hemisphere of the mature egg. A particularly "bad" egg will appear as a white puffy ball, with the boundary between the hemispheres entirely obscured.

NUCLEAR ASSEMBLY IN THE INTERPHASE EGG EXTRACT

When sperm chromatin or other DNA templates (e.g., λ DNA) are added to interphase *Xenopus* egg extracts, membrane vesicles present in the extract bind to the surface of the chromatin, fuse, and incorporate nuclear pore components to form fully functional synthetic nuclei. These nuclei are competent to replicate their DNA (see Basic Protocol 4), transport macromolecules (see Basic Protocol 3), and undergo apoptotic nuclear fragmentation (see UNIT 11.12). This protocol describes the steps in setting up a nuclear assembly reaction using sperm chromatin as a template. This can be done using either crude interphase extract (Basic Protocol 1) or previously stored fractionated cytosol and light membranes (Alternate Protocol 1).

Materials

Interphase extract: crude (see Basic Protocol 1) or fractionated (see Alternate Protocol 1)

0.2 M phosphocreatine (store in small aliquots at -20°C)

5 mg/ml creatine kinase (store in small aliquots at -20°C)

0.2 M ATP (store in small aliquots at -20°C)

Sperm chromatin (see Support Protocol 2)

Fixative for visualizing nuclear assembly (see recipe)

Microscope slides

18-mm² glass coverslips

Fluorescence microscope

Prepare mix for nuclear assembly

1. To either crude extract or fractionated cytosol add phosphocreatine (from 0.2 M stock) to a final concentration of 20 mM, creatine kinase (from 5 mg/ml stock) to 50 $\mu\text{g}/\text{ml}$, and ATP (from 0.2 M stock) to 20 μM .

Alternatively, It is also acceptable to use the 20 \times energy regenerating mix described in UNIT 11.11.

2. If fractionated extract is being used, add light membranes to the cytosol at a 1:10 dilution.

This approximates the amount of membrane present in the original crude extract.

3. After vigorous mixing of the sperm chromatin preparation by repeated pipetting, add sperm chromatin to the crude or fractionated extract at the desired concentration and mix extract/sperm by up-and-down pipetting.

Generally, these reactions are set up in 500- μl microcentrifuge tubes with a total volume <100 μl . Egg extracts will support the formation of up to 8000 to 9000 nuclei/ μl . As more and more chromatin is used, the nuclei will become smaller in size due to depletion of nuclear-forming membranes. Below 50 nuclei/ μl , it becomes increasingly difficult to locate nuclei by microscopy.

4. Incubate 60 min to 2 hr at room temperature.

Nuclear formation in crude extracts is considerably faster than in the fractionated extract. In a robust extract, large nuclei can be seen in the crude extract within 60 min. Equivalently sized nuclei will form in the fractionated extract in \sim 2 hr.

Assess formation of nuclei

5. Mix the assembly reaction by gently pipetting up and down several times using a wide-bore or cut-off 200 μl pipet tip.

This evenly suspends the nuclei which, especially as they grow larger, tend to settle out.

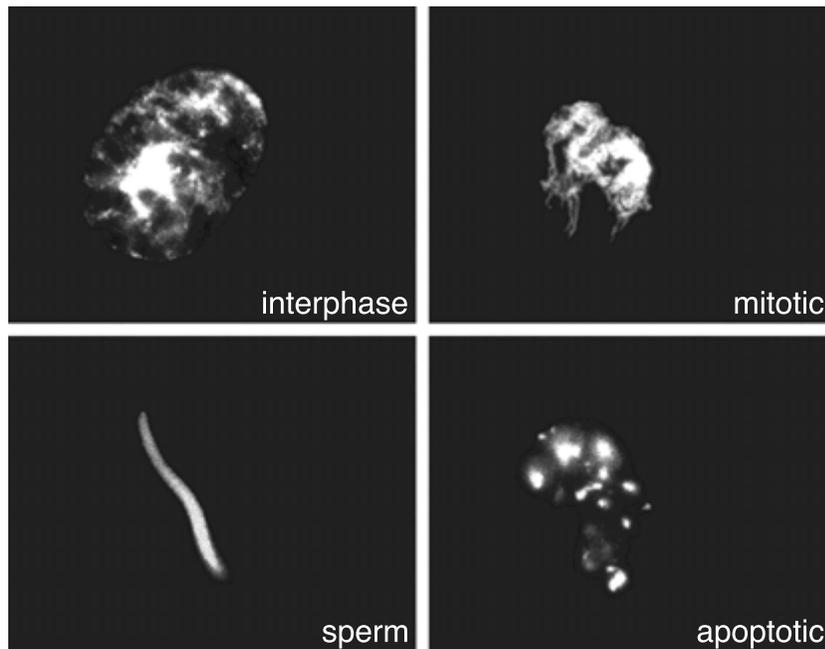


Figure 11.10.3 Nuclei in *Xenopus* egg extracts. Typical nuclei formed in *Xenopus* interphase extracts are shown as well as mitotic extracts (note condensed chromosomes) and apoptotic extracts (note fragmented nucleus). Also shown is a *Xenopus* sperm head prior to nuclear formation.

- Remove a 4- μ l aliquot of the assembly reaction mixture using a cut-off tip and spot it onto a glass microscope slide. Add 1 μ l of fixative and gently swirl it into the sample by circling the pipet tip. Slowly and very gently lay a glass coverslip (18-mm²) over the sample and examine the nuclei under the fluorescence microscope.

The nuclei undergo a series of morphological changes as nuclear formation progresses. In a crude extract, after ~30 min, the newly formed nuclei will be small and oval in shape. The DNA is still quite condensed, and the nucleus should appear to be a solid light blue (from Hoechst binding) when observed in the UV channel. As the DNA decondenses further, the blue staining will be less homogeneous, with black “gaps” eventually appearing (e.g., Fig. 11.10.3). The formation of an intact nuclear envelope is evaluated by the ability of intact nuclei to exclude the high-molecular-weight FITC-dextran (which can be added to the fixative). Since the dextran does not contain a nuclear localization signal, once a fully closed nuclear membrane has formed, this large molecule will not enter the nucleus.

As an alternative, the membrane can be visualized with 3,3'-dihexyloxycarboxyanine (DHCC; Kodak; 1 μ g/ml included in the fix in place of FITC dextran). This is a fluorescent, lipophilic dye that is visualized in the fluorescein channel. In early stages of assembly, membrane vesicles are bound on the chromatin surface and begin to fuse in patches. This results in an uneven and discontinuous appearance of the forming membrane. Once the membrane is complete, it “smooths out” over the nuclear surface and will appear as an even, unbroken rim around the DNA (note that this can also be observed as a dark phase-dense line around the nucleus using phase-contrast microscopy). There will be a fair amount of background fluorescence in the surrounding cytosol due to membrane vesicles which have not been (or cannot be) incorporated into nuclei (see Fig. 11.10.3).

**PREPARATION OF DEMEMBRANATED SPERM CHROMATIN TO USE AS
TEMPLATES FOR NUCLEAR ASSEMBLY**

Male *Xenopus* testes provide an abundant source of easily prepared chromatin to use as templates for nuclear assembly in the extract. Male frogs, which can be purchased from the same suppliers as the females (see Support Protocol 1), are most visibly distinguished from females by their relatively small size and by the presence of darkly pigmented patches on their forearms. Because frogs are external fertilizers, they produce large amounts of sperm. Consequently it is reasonable to expect to obtain 1 to 2×10^7 sperm heads per frog. These are generally used for nuclear formation in egg extracts at anywhere from 100 to 4000 per μl , depending upon the application. This protocol has been adapted from one developed by Drs. C. Macaulay and D. Forbes (University of California, San Diego).

Materials

- 4 to 5 male *Xenopus* (e.g., Nasco, Xenopus I, or Xenopus Express)
- 0.1% (w/v) Tricaine (see recipe)
- Extraction buffer (see recipe), 4°C
- Extraction buffer (see recipe) containing 200 mM, 2 M, 2.3 M, and 2.5 M sucrose
- 5 mg/ml aprotinin (Roche Diagnostics) in H₂O
- 5 mg/ml leupeptin (Roche Diagnostics) in H₂O
- Dithiothreitol (DTT)
- Triton X-100
- Sucrose, ultrapure
- Bovine serum albumin (BSA; fraction V)

- Dissecting tools including scissors and 2 pairs of sharp forceps
- 60-mm glass petri dish
- 15-ml conical polypropylene tubes
- Tabletop centrifuge
- Refrigerated centrifuge with Sorvall HB-4 swinging-bucket rotor or equivalent
- 2.5 ml Beckman Ultraclear centrifuge tubes
- TL-100 tabletop centrifuge and TLS-55 rotor (Beckman) or equivalent

- Additional reagents and equipment for counting with a hemacytometer (UNIT 1.1)

Dissect testes

1. Anesthetize male frogs by immersion in 0.1% Tricaine (or immobilize in ice water) and sacrifice either by cervical dislocation or by pithing, followed by cutting through the spinal cord. Using dissection scissors, cut through the skin into the peritoneum along the midline of the frog.

As indicated in the diagram (Fig. 11.10.4), the testes are located on either side of the midline in the center of the abdominal cavity. They are beige-colored 0.5-cm organs.

2. After pushing the liver aside, grasp the fatty material at mid-abdomen and pull gently until the testes emerge on either side of the midline. Gently cut them free of adherent tissue with either a forceps or scissors, and place in a 60-mm glass petri dish containing 4°C extraction buffer.

To prepare a sizeable stock of sperm, testes should be isolated from 4 to 5 frogs and then placed in the petri dish containing extraction buffer.

3. Using 2 pairs of sharp forceps, mince the testes into small pieces and transfer them to a 15-ml conical polypropylene tube.

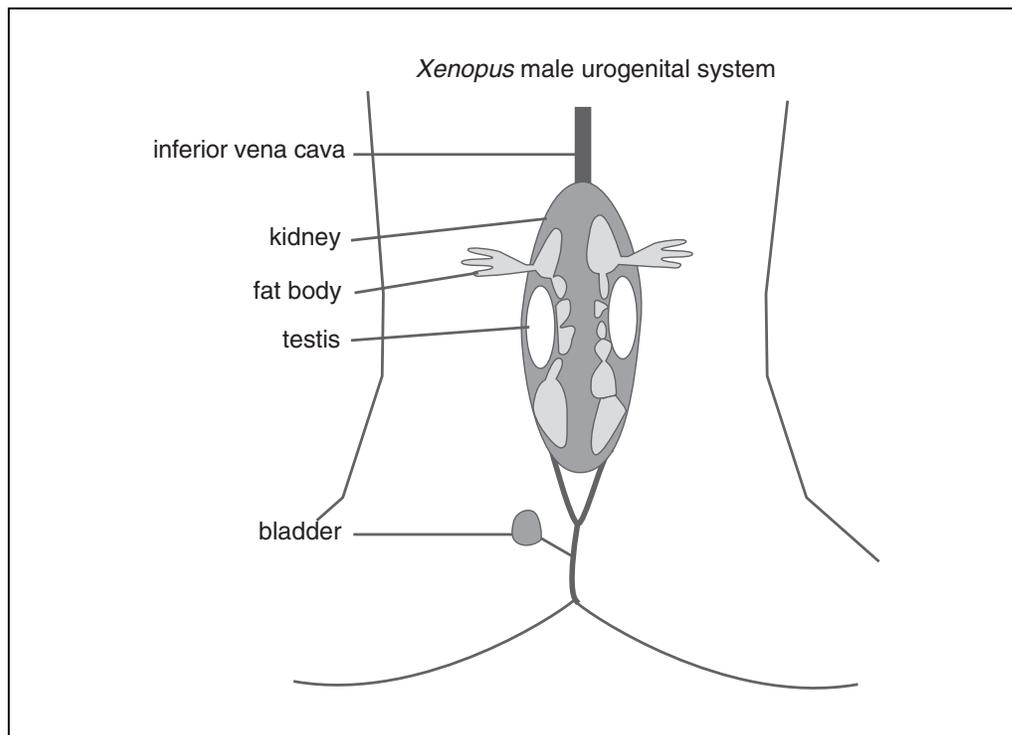


Figure 11.10.4 Schematic of the male *Xenopus* urogenital system. Testes are two white organs flanking the midline.

- Vortex the minced testes vigorously and pellet the larger pieces by gentle centrifugation (10 sec at $\sim 200 \times g$, room temperature in a clinical tabletop centrifuge).

Isolate sperm heads

- Remove the supernatant to a new tube and add 3 ml extraction buffer supplemented with 200 mM sucrose to the pellet. Vortex 1 min, and briefly recentrifuge for 10 sec at $200 \times g$, room temperature. Combine the supernatants and repeat the extraction of the pellets 2 to 3 times until the supernatant is not very cloudy.
- Centrifuge the combined supernatants 50 sec at $450 \times g$, room temperature, to pellet any remaining large pieces of tissue. Transfer the supernatants to a 15-ml tube and centrifuge 10 min at $2600 \times g$ (4000 rpm in a Sorvall HB4 rotor), room temperature.
- Prepare gradients by adding 0.2 ml extraction buffer containing 2.5 M sucrose to each of four 2.5-ml Beckman Ultraclear tubes and overlaying with 1.7 ml of extraction buffer containing 2.3 M sucrose.
- Resuspend sperm in 0.8 ml extraction buffer containing 2 M sucrose and overlay gently onto the top of the sucrose gradients (0.2 ml per tube). After stirring the interface between the sperm and the 2.3 M sucrose with a pipet tip, centrifuge the sucrose gradients in a TL-100 tabletop ultracentrifuge, 25 min at $93,000 \times g$ (33,000 rpm a TLS-55 rotor), 4°C .
- Aspirate the top half of the gradient, which contains contaminating red blood cells. Transfer the lower half of the gradient to a new tube.

Although the majority of the sperm bands on top of the 2.3 M sucrose cushion, the authors generally keep the entire lower half of the gradient.
- Dilute sperm to 12 ml with extraction buffer containing 200 mM sucrose and pellet the sperm by centrifuging 10 min in a swinging-bucket rotor at $4100 \times g$ (5000 rpm in a Sorvall HB4 or HB6 rotor or equivalent), 4°C .

Demembranate sperm

11. Supplement extraction buffer with additives at the following concentrations:

200 mM sucrose
5 µg/ml aprotinin (add from 5 mg/ml stock)
5 µg/ml leupeptin (add from 5 mg/ml stock)
1 mM DTT
0.4% (v/v) Triton X-100.

Resuspend the pellet in 1 ml of this supplemented extraction buffer/200 mM sucrose.
Incubate 30 min on ice.

This serves to demembranate the sperm.

12. Prepare sucrose cushions in two 1.5-ml microcentrifuge tubes by adding:

0.5 ml extraction buffer
0.5 M sucrose
5 µg/ml leupeptin
5 µg/ml aprotinin
1 mM DTT
3% (w/v) bovine serum albumin (BSA; fraction V).

Overlay each sucrose cushion with one-half of the sperm preparation from step 11 and centrifuge 10 min in a clinical tabletop centrifuge at $870 \times g$, room temperature, to pellet the sperm.

13. Supplement extraction buffer with additives at the following concentrations:

200 mM sucrose
3% (w/v) bovine serum albumin (BSA; fraction V)
5 µg/ml aprotinin (add from 5 mg/ml stock)
5 µg/ml leupeptin (add from 5 mg/ml stock)
1 mM DTT.

Remove the supernatant from the pellets obtained in step 12 and resuspend the sperm pellet in 0.1 ml of the supplemented extraction buffer prepared in this step. Transfer the resuspended sperm to a clean microcentrifuge tube.

At this point, it is important to avoid contaminating the preparation with residual Triton X-100 from the sides of the original tube.

Prepare final sperm suspension

14. Resuspend the sperm chromatin pellet in a total of ~0.25 ml of the supplemented extraction buffer/200 mM sucrose prepared in step 13.
15. Count the sperm using a hemocytometer and dilute the suspension to 50,000 to 100,000 sperm/µl. Snap freeze the sperm in small aliquots in liquid nitrogen and store at -70°C .

The typical yield is $1-2 \times 10^7$ sperm per frog.

At this point, the sperm are discrete masses of tightly condensed chromatin, unbounded by membranes.

NUCLEAR PROTEIN IMPORT IN VITRO

Once nuclei have been formed from interphase extracts, they can be assayed for the ability to import nuclear proteins by the addition to the extract of a fluorescently labeled import substrate (see *UNIT 11.7*). This provides a relatively rapid, visual assay that can be used to confirm that nuclei in the extract are intact and functional (see Basic Protocol 2). Nuclear transport can be monitored over time by quantitation of the amount of substrate that accumulates in the nucleus at various time points.

This following protocol can be used to quantitate nuclear import for comparison between different conditions or extracts, or simply to assess functional competence of nuclei in which another parameter is being altered.

Materials

Interphase egg extract: crude (see Basic Protocol 1) or fractionated (see Alternate Protocol 1)

ATP-regenerating system (see Basic Protocol 2)

Xenopus sperm chromatin (see Support Protocol 2)

Nuclear import substrate (*UNIT 11.7*)

Fix 1 (see recipe for fixatives for visualizing nuclear protein import)

16% (w/v) paraformaldehyde

Fix 2 (see recipe for fixatives for visualizing nuclear protein import)

Microscope slides

Coverslips

Fluorescence microscope equipped with:

64× objective (e.g., Zeiss 64× planapochromat, large aperture)

CCD camera

Frame grabber

Computer running image analysis software (e.g., NIH Image)

Additional reagents and equipment for assembling nuclei (see Basic Protocol 2)

1. Assemble nuclei using sperm chromatin and the interphase extract, along with the ATP-regenerating system (phosphocreatine, creatine kinase, and ATP; see Basic Protocol 2, step 1 and materials list), or add ~1000 nuclei per μl of extract from another source (e.g., rat liver nuclei) into the extract. Incubate 30 min, at room temperature.
2. After ~30 min incubation, check that nuclei have been formed (see Basic Protocol 2, step 5).

Where time course is not to be performed

- 3a. Once nuclei are formed, add the nuclear import substrate (*UNIT 11.7*) to the assembly reaction and mix by gently pipetting. Incubate 30 min, or longer, at room temperature.

The exact amount of substrate to add must be determined empirically, depending upon the particular batch of substrate and the purpose of the assay. For a simple determination of function, RITC import substrate should be added at a final concentration of ~5 $\mu\text{g}/\text{ml}$ (1/200 dilution). For quantitation of a time course of import, the concentration should be somewhat lower in order to keep the signals within a linear response range over the time of the assay.

- 4a. After the nuclei have been incubated with import substrate for 30 min or longer, remove and fix aliquots, using Fix 1, to detect uptake of the substrate. Store fixed samples in the dark at 4°C, if necessary.

Fixation is essentially immediate upon exposure to Fix 1.

For quantitation of the time course of import

- 3b. Set up an assembly reaction (see above) of sufficient volume to allow for removal of at least 5- μ l aliquots at each timepoint.

Quantitation of a import assay requires at least 15 to 20 min. For this reason, when performing a time course, it is best to fix each sample with 16% paraformaldehyde and then quantitate import after all samples have been collected.

- 4b. Immediately after the addition of import substrate, remove an aliquot from the reaction (5 μ l) and transfer to a microcentrifuge tube on ice. Fix this sample (T_0) by adding 1.5 μ l of 16% paraformaldehyde to bring the final concentration to 3.7% paraformaldehyde. Mix gently with a cut-off pipet tip. Store fixed samples in the dark at 4°C. At desired time points, continue to remove aliquots and fix as above.
5. Prepare samples for microscopy immediately before use. Place 4 μ l of the fixed aliquot on a slide, and add 1 μ l of Fix 2. Gently place a coverslip over the sample.

As the nuclei grow larger, they become increasingly susceptible to breakage when prepared for microscopy so the coverslip must be placed on the sample very gently and gradually. Breakage of a nucleus during processing can allow leakage of imported substrate. For this reason, the exclusion of FITC-dextran is used to identify intact nuclei.

6. Quantitate transport immediately after adding the coverslip and score only nuclei that exclude the FITC-dextran.

Because of the possibility of nuclear breakage, finding sufficient scorable nuclei can be a significant problem and thus, if possible, it is best to have large enough aliquots that more than one sample can be prepared if needed.

7. For quantitation, observe nuclei using a 64 \times objective on a microscope equipped with CCD camera, frame grabber, and computer running image analysis software.

The authors use a Zeiss 64 \times planapochromat large aperture objective which is very light sensitive.

NIH Image is available at no cost and is sufficient for this application, although more sophisticated and expensive software packages are available.

8. Once an appropriate nucleus is identified, capture the image and encircle the outline of the nucleus on the computer using the mouse. Determine the average fluorescence intensity within the nucleus, as well as the nuclear area using the software. Multiply average fluorescence by the area to yield the total nuclear fluorescence.

BASIC PROTOCOL 4

DNA REPLICATION WITH CONTINUOUS LABELING

DNA replication assays can be done using unfractionated interphase extracts (Basic Protocol 1), fractionated, reconstituted interphase extracts (Alternate Protocol 1), or cycling extracts (UNIT 11.11). While it takes ~1 hr to replicate 1000 synthetic nuclei/ μ l of crude extract, it can take 2 to 4 hr to fully replicate nuclei in a reconstituted extract. Although the protocol detailed below is useful for measuring replication of sperm chromatin nuclei in the extract, it can also be used to measure plasmid replication rates in the extract, and to examine replication of intact nuclei from other sources (e.g., nuclei prepared from tissue culture cells) added to extracts. It is worth bearing in mind the fact that these templates may replicate more slowly than sperm chromatin nuclei and that plasmid replication, in particular, will be slowed by inefficient nuclear formation (a prerequisite for replication in this system).

Two distinct replication assay protocols, continuous and pulse labeling (see Alternate Protocol 2) which depend upon the incorporation of radiolabeled nucleotides (e.g.,

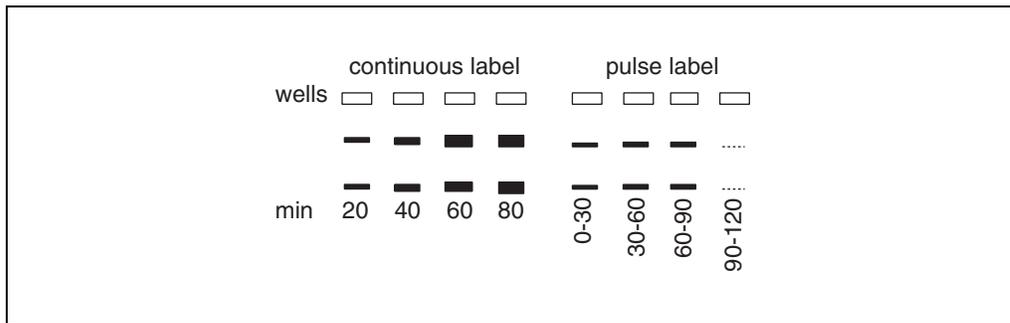


Figure 11.10.5 The banding pattern of labeled DNA following replication of DNA in egg extracts. When the products of a DNA replication reaction in *Xenopus* egg extract are resolved by agarose gel electrophoresis, two distinct bands are apparent (shown here schematically). In the case of continuous labeling, the intensity of these bands increases with time as increasingly more label is incorporated into DNA. In the case of pulse labeling, incorporation generally peaks and then diminishes as replication is completed (hence, as shown schematically here, no incorporation of label is seen at the later time point).

[α - 32 P]dCTP) into replicating DNA, are routinely used. Direct inclusion of these nucleotides in extracts, withdrawal of aliquots, and resolution by agarose gel electrophoresis will give an estimate of total DNA replication, which will increase over time. Conversely, replication can be measured by inclusion of radiolabeled nucleotide in aliquots of extract over specific time intervals (e.g., 0 to 20 min, 20 to 40 min, 40 to 60 min, etc., after the start of nuclear formation) to compare, for instance, the replication that has occurred in one time interval with that which occurs in another. This “pulse replication” methodology is a more sensitive method than continuous labeling for comparing replication efficiency after different experimental treatments of the extract (e.g., depletion of particular components, addition of inhibitors; see Support Protocol 3 and Support Protocol 4, respectively).

When using sperm chromatin as the replication template, there should be two radiolabeled bands of replicated DNA. The pattern of incorporation differs when the two different replication protocols are used, as would be expected for continuous or pulsed incorporation of label (see schematic, Figure 11.10.5).

Materials

- Interphase extract (see Basic Protocol 1)
- 0.2 M phosphocreatine (store in small aliquots at -20°C)
- 5 mg/ml creatine kinase (store in small aliquots at -20°C)
- 0.2 M ATP (store in small aliquots at -20°C)
- Demembrated sperm chromatin (see Support Protocol 2)
- 10 $\mu\text{Ci}/\mu\text{l}$ [α - 32 P]dCTP (3000 Ci/mmol)
- Replication stop buffer (see recipe)
- 10 mg/ml proteinase K (Worthington)
- Additional reagents and equipment for agarose gel electrophoresis (APPENDIX 3A) and autoradiography or phosphorimaging (UNIT 6.3)

1. To interphase extract add phosphocreatine (from 0.2 M stock) to a final concentration of 20 mM, creatine kinase (from 5 mg/ml stock) to 50 $\mu\text{g}/\text{ml}$, and ATP (from 0.2 M stock) to 2 mM.
2. For replication assays using sperm chromatin, pipet the demembrated sperm chromatin vigorously to eliminate clumping and add directly to extract at a final concentration of 100 to 4000 sperm heads/ μl of extract.

3. Add [α - 32 P]dCTP (or one of the other three deoxynucleotides in radiolabeled form) directly to the extract at 0.1 μ Ci/ μ l of extract and incubate at room temperature.
4. Withdraw 10- μ l aliquots at 20-min intervals into an equal volume of replication stop buffer.
5. Add 3 μ l of 10 mg/ml proteinase K stock to each aliquot and incubate 2 hr at 37°C.
6. Resolve samples on a 1% agarose gel.

Any of the standard agarose gel running buffers will work fine.

Loading the gel may be difficult if proteinase K treatment is not complete as the DNA will tend to adhere to the pipet tip.
7. Dry the gel and expose to film for autoradiography or develop by phosphorimager (UNIT 6.3).

ALTERNATE PROTOCOL 2

PULSE-LABELING DNA TO ASSESS REPLICATION

For this protocol, the replication reaction is initiated in the absence of labeled precursor. At defined time points (e.g., at 15- or 30-min intervals), samples of the reaction mixture are taken. Radiolabeled precursor is added to the sample for a defined period to measure replication during that time period.

Additional Materials (also see Basic Protocol 4)

XB buffer (see recipe)

1. Dilute 1 part [α - 32 P]dCTP in 4 parts XB buffer and add 0.5 μ l (1 μ Ci) of the resulting mix to an empty microcentrifuge tube for each time point to be taken. Do this for each condition to be tested (e.g., control extract, extract with added protein of interest, extract with depleted protein of interest, etc.). Let stand at room temperature.

These are the assay tubes. To use fractionated extracts which have been reconstituted (the ultra S extract with added membrane), it should be suitable to set up assay tubes for the following intervals: 0 to 30 min, 30 to 60 min, 60 to 90 min, 90 to 120 min, and 120 to 150 min. For crude interphase extracts, it is better to have shorter time points; for example: 0 to 15 min, 15 to 30 min, 30 to 45 min, 45 to 60 min, 60 to 75 min, 75 to 80 min. Leave at room temperature.

2. Set up the reactions (Basic Protocol 4, steps 1 and 2). Place on ice.

These are the master sample tubes.

3. To start the assay, move the master sample tubes to room temperature and remove 5 μ l from each master sample tube to the first of each assay tube containing the [α - 32 P]dCTP (e.g., the 0 to 30 min assay tube for fractionated extract or 0 to 15 min assay tube for unfractionated extract tube). Continue incubating the master sample and assay tubes at room temperature.
4. At the end of the specified time interval (e.g. 30 min or 15 min), add 5 μ l of replication stop buffer and remove sample to ice. Withdraw 5 μ l more from each of the master sample tubes and transfer to the next assay tube in the series (e.g., 30 to 60 min, 15 to 30 min). Again, stop the reaction by addition of replication stop buffer at the end of the specified time interval. Continue this until all time intervals have been done.

This will give you "snapshots" of replication that occurred during each time interval.

It is worthwhile to examine the morphology of nuclei in the samples at several time points during the experiment (see Basic Protocol 2) to make sure experimental treatments have not compromised nuclear architecture, which might in itself impair replication.

5. Digest samples with proteinase K and resolve by agarose gel electrophoresis as in Basic Protocol 4.

PREPARATION OF OOCYTE EXTRACT

Mature *Xenopus* Stage VI oocytes are physiologically arrested in prophase of the first meiotic division and therefore contain inactive cdc2/cyclin B complexes. Progesterone treatment of prophase I–arrested oocytes triggers meiotic maturation, concomitant with activation of the cdc2/cyclin B kinase. An additional Ser/Thr kinase, mos, is also required for meiotic maturation of oocytes. The oocyte extracts described below mimic several features of in vivo prophase I–arrested oocytes, including the ability of mos to evoke activation of MAP kinases.

Materials

Female frogs, mature (e.g., Nasco, *Xenopus* I, or *Xenopus* Express)
0.1% (w/v) Tricaine (see recipe)
Modified Barth's (MB) with and without calcium (see recipe)
1 mg/ml collagenase A (Roche Diagnostics) in MB (see recipe) without calcium
EB buffer (see recipe)
1000× CLAP protease inhibitor cocktail (see recipe)
1000× (5 mg/ml) cytochalasin B in DMSO

Dissecting microscope
Dissecting scalpel
Sharp forceps
50-ml plastic tubes
Tube rotator
Clinical centrifuge
Tabletop ultracentrifuge and swinging-bucket rotor
1-ml syringe
18-G needle

Dissect out ovary

1. Place female mature frog in 1 liter of 0.1% (w/v) tricaine until she is immobile, usually 30 to 60 min.

Oocytes must first be dissected out of the anesthetized female frog

2. Place the frog on her back atop a bed of ice. Swab the abdomen first with deionized water and then with ethanol. Clean and sterilize all instruments with ethanol.
3. Make a small incision (~1 cm) in the abdomen, vertically and slightly off center. Cut through the skin and muscle layers.

The oocytes, present in great abundance, should be readily apparent at this stage.

4. Gently tease oocytes out of the abdominal cavity and excise with a scalpel or scissors. Place oocytes in a petri dish containing MB without calcium.

Precoating the dish with 6% (w/v) poly-HEMA in ethanol (dilute 12% poly-HEMA from Polysciences to 6% in ethanol) will prevent oocytes from sticking to the plastic dish. Simply pour the poly-HEMA solution onto the surface of the dish to coat, then pour off and allow to air dry in a hood. The dishes can be stored at room temperature (with lids).

5. Tear ovary (the mass of oocytes) into chunks of ~50 oocytes. Store oocytes that are not going to be used immediately in MB plus calcium at 18°C.

Free oocytes

6. Place ~10 ml oocytes in 50-ml tubes containing 30 ml collagenase A solution. Place on a tube rotator. Periodically check oocytes under the dissecting microscope until the majority have fallen away from the follicular cells.

Since prolonged treatment with collagenase can damage the oocytes, it is worthwhile to observe them carefully and remove them as soon as defolliculation is complete.

7. Remove and save the collagenase at -20°C .

Collagenase A solution can be reused 3 to 4 times.

Wash and sort oocytes

8. Wash the oocytes extensively with MB minus calcium. Fill the 50-ml tube containing the oocytes with buffer, wait a few seconds for the larger, full-grown oocytes to settle and then, using a wide-bore pipet, aspirate the upper layer of buffer which contains the smaller, immature oocytes. Repeat this process five times to remove collagenase and to select for mature oocytes.
9. After washing, put oocytes in a petri dish for scanning with a dissecting microscope. Remove any oocytes that appear to be unhealthy (lysed or mottled), immature, or which have undergone germinal vesicle breakdown (GVBD).

If oocytes have undergone GVBD, a white spot will appear at the top, center of the dark hemisphere of the oocyte.

If oocytes are not to be used immediately to make an extract, store in MB plus calcium in a petri dish at 18°C for up to 2 days.

10. Carefully transfer oocytes to 50-ml conical tubes with the wide-end of a glass Pasteur pipet (place the bulb on the sharp end of the pipet, after snapping off most of the end). Wash twice with EB buffer using the method described in step 8.
11. Prepare 10 ml EB containing protease inhibitor cocktail CLAP (1:1000 dilution of stock solution). Wash oocytes with EB/CLAP using the method described in step 8.
12. Transfer oocytes to a thin-walled 2.5-ml polyallomer centrifuge tube for use in a tabletop ultracentrifuge rotor (e.g., for the Beckman TLS-55 rotor for the TL-100). Drop the filled small tube into a 15-ml centrifuge tube and pack the oocytes by gently spinning in a clinical centrifuge at $400 \times g$ for 30 sec. Remove as much buffer as possible from the surface of the oocytes to prevent eventual dilution of the extracts.

Prepare oocyte extract

13. Lyse oocytes by centrifugation in a TL-100 ultracentrifuge (or equivalent), 15 min at $15,000 \times g$ in a Beckman TLS-55 SW Ti rotor, 4°C .

After centrifugation, three layers will be apparent: a black/green bottom layer (yolk platelets, mitochondria, etc.), a milky middle layer (oocyte extract), and a yellow top layer (lipids). The oocyte extract is a mixture of cytosolic and membranous components.

14. Using a 1-ml syringe with an 18-G needle, puncture the side of the tube at the base of the oocyte extract. Remove this layer and transfer to a second thin-wall centrifuge tube.
15. Add $1000\times$ cytochalasin B to a final concentration in extract of $50 \mu\text{g/ml}$ (i.e., 1:1000 dilution of stock). Centrifuge as in step 13.
16. Transfer the large middle extract layer as in step 14 to a microcentrifuge tube and discard the pellet.
17. Divide the extract into $100\text{-}\mu\text{l}$ aliquots in microcentrifuge tubes and snap freeze in liquid nitrogen. Store aliquots at -80°C .

IMMUNODEPLETION OF EXTRACTS

The ease with which components can be added to or depleted from *in vitro* reconstitution reactions is one of the chief advantages of using these systems to study biological processes. This protocol provides general guidelines for immunodepleting proteins from *Xenopus* egg extracts.

Complete immunodepletion of proteins from the egg extract often requires multiple rounds of depletion using a good, high-affinity antibody. One of the primary concerns is that depletion itself may cause artifactual perturbations in the process being studied. Therefore, it is critical to have a preimmune or IgG-depleted extract as a control. Moreover, it is important to avoid excessive dilution of the extract during the depletion procedure, since this may impair the proper functioning of the extract.

Materials

Antibody/antiserum
Preimmune serum or control IgG
Protein A–Sepharose
Phosphate-buffered saline (PBS; see recipe)
Bovine serum albumin (BSA), fraction V
XB buffer (see recipe)

1. Prepare antibody beads by incubating the affinity-purified antibody with protein A Sepharose in PBS containing 2 mg/ml BSA fraction V (also see *UNIT 7.2*). Prepare preimmune serum (control) beads in the same manner.

If crude serum, rather than affinity-purified serum is being used, the protein A–Sepharose can be incubated directly with the serum without addition of BSA.

Control protein A–Sepharose beads should be incubated with preimmune sera. If affinity-purified antibody is being used, the control can be pure IgG linked to protein A–Sepharose. While approximate volumes depend upon the antisera being used, ~15 μ l of packed protein A–Sepharose can be incubated with ~10 μ g of IgG in 200 μ l of PBS containing 2 mg/ml BSA.

2. Pellet the beads in a microcentrifuge and remove all liquid.
3. *For crude or cycling extracts only:* Wash beads very rapidly with 100 μ l or less of fresh extract that will be used for depletion experiments. Again, remove all liquid.

Add extract to the beads, shake, centrifuge, and remove the extract; do this all in a cold room at 4°C. This allows the interstitial buffer of the beads to be replaced with extract and helps to avoid dilution of the extract to be depleted.

4. Add 100 μ l of extract to be depleted to the 15 μ l of packed antibody beads and rotate at 4°C for 40 min.
5. Pellet the beads by microcentrifuging and remove the supernatant to a new tube of antibody beads, washed as above.
6. Rotate at 4°C for 40 min and microcentrifuge. Remove the extract to a fresh tube.

At this stage the supernatant contains a twice-depleted extract. A third (or even fourth) round of depletion may be necessary to completely deplete the protein of interest. The extent of depletion should be assessed by immunoblotting an aliquot of the depleted extract with the antibody used for the depletion or with an alternative antibody directed against the same protein. Occasionally, it is impossible to deplete a protein of interest, but addition of antibody directly to the extract has a “blocking” effect on the process being examined. If this effect can be reversed by adding an excess of the antigen, this can be an indication that the protein participates in the process under study.

ADDITION OF PROTEIN TO EXTRACTS

Addition of wild-type or mutant recombinant proteins to egg extracts often provides a relatively rapid assay of protein function. As with antibody addition, one of the most important considerations is adding a sufficient amount of protein to achieve an effect without excessively diluting the extract.

Materials

Recombinant protein of interest
XB buffer (see recipe)

Additional reagents and equipment for dialysis and microconcentration (*APPENDIX 3C*)

1. Dialyze or microconcentrate recombinant protein of interest into XB buffer.
2. Add proteins at more than a 1:5 dilution into the extract; if possible, a 1:10 or greater dilution is preferable.

When using in vitro-translated proteins, the reticulocyte lysate will not grossly disrupt extract function if added directly to the extract at 1:10 or greater dilutions.

This extract can be used for studying protein-protein interactions or for looking at biochemical reactions occurring in oocytes specifically. For example, MAPK can be activated in oocytes by addition of exogenous MOS protein.

REAGENTS AND SOLUTIONS

*Use deionized distilled water in all recipes and protocol steps. For common stock solutions see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.*

CLAP protease inhibitor cocktail, 1000×

Prepare in DMSO:

10 mg/ml chymostatin

10 mg/ml leupeptin

10 mg/ml antipain

10 mg/ml pepstatin

Store in aliquots indefinitely at -70°C

EB buffer (for preparation of oocyte extract; Basic Protocol 5)

42.68 g sucrose (0.25 M final)

10 ml of 5 M NaCl (0.1 M final)

1.25 ml of 1 M MgCl_2 (2.5 mM final)

2.4 g HEPES (20 mM final)

Adjust pH to 7.2 with NaOH and bring the volume to 500 ml with water.

Store until cloudy at 4°C

Egg lysis buffer, pH 7.7

250 mM sucrose

2.5 mM MgCl_2

1 mM DTT

50 mM KCl

10 mM HEPES

Prepare fresh

Extraction buffer (for preparation of demembrated sperm chromatin; Support Protocol 2)

10 mM HEPES, pH 7.4
80 mM KCl
15 mM NaCl
5 mM MgCl₂
1 mM EDTA
Store until cloudy at 4°C

Fixative for visualizing nuclear assembly (Basic Protocol 2)

10 mM HEPES, pH 7.5
200 mM sucrose
1 µg/ml Hoechst 33258
1 mg/ml FITC-dextran
12% (w/v) paraformaldehyde
Store at -20°C wrapped in foil

Fixatives for visualizing nuclear protein import (Basic Protocol 3)

Fix 1:

1 µl of 1 M HEPES, pH 7.5 (10 mM final)
10 µl of 2 M sucrose (200 mM final)
1 µl of 1 mg/ml Hoechst 33258 (bisbenzimidazole; Calbiochem; 1 µg/ml final)
10 µl of 10 mg/ml fluorescein isothiocyanate-dextran (FITC-dextran, 150 kDa, Sigma; 1 mg/ml final)
77 µl of 16% paraformaldehyde (methanol-free, Polysciences; 12% final)

After opening 16% paraformaldehyde vial, divide solution into aliquots and store at -20°C.

Fix 2: (for use on previously fixed samples)

10 mM HEPES, pH 7.5
200 mM sucrose
1 µg/ml Hoechst 33258
1 mg/ml FITC-dextran
3.7% (w/v) paraformaldehyde
Store at -20°C wrapped in foil

Modified Barth's (MB) solutions

Prepare the following stock solutions:

Stock solution A:

64 g NaCl
1 g KCl
2.5 g NaHCO₃
44.5 g HEPES

Adjust pH to 7.6 with NaOH and bring the volume to 500 ml with water. Store in 20-ml aliquots at -20°C.

Stock solution B:

0.95 g Ca(NO₃)₂·4H₂O
0.75 g CaCl₂·2H₂O
1.23 g MgSO₄ anhydrous
Bring volume to 500 ml with water.
Store in 20-ml aliquots at -20°C

continued

Stock solution B without Ca:

Weigh out 1.23 g anhydrous MgSO₄ and bring volume to 500 ml with water. Store 20-ml aliquots at -20°C.

For Modified Barth's with calcium:

460 ml H₂O
20 ml stock solution A (see above)
20 ml stock solution B (see above)
2.5 ml 10 mg/ml gentamycin solution (Sigma)
Store at 4°C

For Modified Barth's without Ca:

460 ml H₂O
20 ml stock solution A (see above)
20 ml stock solution B without Ca (see above)
2.5 ml 10 mg/ml gentamycin solution (Sigma)
Store up to several months at 4°C

Modified Ringer's solution (MMR), pH 7.7, 10×

1 M NaCl
20 mM KCl
10 mM MgSO₄
25 mM CaCl₂
5 mM HEPES, pH 7.8
0.8 mM EDTA
Store indefinitely at room temperature
Dilute 10× MMR to 0.25× MMR in water (1:40) before extract is made
~200 ml of 0.25× MMR are required to make the interphase extract.

Phosphate buffered saline (PBS)

8 g NaCl
0.2 g KCl
0.1 g CaCl₂
0.1 g MgCl₂·6H₂O
1.15 g NaH₂PO₄·2H₂O
0.2 g KH₂PO₄
Add water to 1 liter
Store indefinitely at room temperature

Replication stop buffer

80 mM Tris·Cl, pH 8.0 (APPENDIX 2A)
8 mM EDTA
0.13% phosphoric acid
10% Ficoll
5% (w/v) SDS
0.2% (w/v) bromphenol blue
Store at -20°C

Tricaine, 0.1% (w/v)

Dissolve 1 g 3-aminobenzoic acid ethyl ester (Tricaine; Sigma) in 1 liter water.
Prepare fresh.

XB buffer

50 mM sucrose
100 mM KCl
0.1 mM CaCl₂
1 mM MgCl₂
10 mM potassium HEPES, pH 7.7
Prepare fresh

COMMENTARY

Background Information

The *Xenopus* egg is a very large cell (>0.5 mm in diameter) which, upon fertilization, gives rise to a 4000-cell embryo without any input from gene transcription and without any cell growth. In practical terms, this means that the egg contains a stockpile of cellular components poised for the assembly of cellular structures and for the execution of diverse cellular functions. While the normal somatic cell may contain the same range of cellular components as the egg, the vast excess of “uncommitted” components makes the egg an ideal source of material for in vitro reconstitutions.

There are multiple advantages to using the *Xenopus* egg cell-free system to study complex cellular processes such as cell cycle progression, DNA replication, apoptosis, nuclear envelope assembly and disassembly, and nuclear trafficking. In particular:

1. Because *Xenopus* are external fertilizers, each animal lays a large number of eggs, providing large amounts of homogeneous, cell cycle-synchronous material.
2. Pharmacological agents, recombinant proteins, and antibodies can be directly added to the in vitro extract, allowing direct manipulation and analysis of the biological process under examination.
3. Egg extracts are easily accessible to biochemical fractionation, facilitating immediate identification of factors involved in the process of interest.
4. Complex processes can be broken down into “partial reactions” that can be dissected individually (e.g., how does the first step in chromatin condensation occur?). Taken together, these features offer the opportunity to dissect even the most complex of cellular functions.

Extracts in interphase of the cell cycle

The key regulator of entry into mitosis in all eukaryotes studied to date is the Ser/Thr kinase cdc2/cyclin B (reviewed in Nurse 1990; Lew and Kornbluth 1996). This kinase complex is

believed to catalyze mitotic cellular rearrangements either directly or indirectly through the phosphorylation of critical cellular substrates. Although cdc2 itself is inactive in its monomeric form, binding of the cyclin subunit activates cdc2 to promote mitosis. *Xenopus* eggs are physiologically arrested in metaphase of meiosis II with high cdc2/cyclin B kinase activity. Crushing these eggs by centrifugation releases Ca⁺² from internal stores, leading to proteolysis of the cyclin subunit and loss of cdc2-associated kinase activity. Consequently, at the time of preparation, egg extracts transit from M phase to interphase of the cell cycle (S phase). Since cyclin B protein must be newly synthesized for each cell cycle, cycloheximide addition to extracts prevents reaccumulation of cyclin protein, and hence the extracts remain in interphase (Basic Protocol 1).

S phase extracts prepared from *Xenopus* eggs can spontaneously form nuclei upon addition of chromatin (Basic Protocol 2). While the most commonly used source of chromatin for these reconstitutions is demembrated sperm chromatin prepared from the testes of male *Xenopus* (see Support Protocol 2), even lambda phage DNA will suffice to provide a template for nuclear assembly, since it, too, will be packaged into chromatin by the extract (Forbes et al., 1983; Hartl et al., 1994). Although the precise molecular details of nuclear assembly have not yet been described in full, nuclear formation in the extract generally involves (1) swelling of the chromatin mediated, at least in part, by the nucleoplasmin protein, (2) binding of membrane vesicles to the surface of the chromatin, (3) fusion of bound membranes to form enclosed nuclear structures, and (4) further decondensation of the enclosed chromatin (Sheehan et al., 1988; Wilson and Newport, 1988; Philpott et al., 1991). At some point during this progression, functional nuclear pores are incorporated into the synthetic nuclei, allowing bidirectional nuclear transport of macromolecules. Indeed, it has been shown that nuclear pores can be incorporated de novo

into fully enclosed nuclear structures (Macaulay and Forbes, 1996).

Double-stranded DNA templates added to extracts will undergo a single round of semi-conservative replication (see UNIT 11.11), provided they are assembled into nuclear structures (Blow and Laskey, 1986; 1988). In contrast, single-stranded templates will replicate even in the absence of nuclear structure, concurrently assembling into chromatin (Mechali and Harland, 1982).

Although primases in the extract will prime DNA synthesis from single-stranded templates, primers preannealed to the single-stranded DNA will be incorporated during replication. As is the case for nuclear assembly, the DNA sequence of templates to be replicated in the extract is not important, as highlighted by the ability of lambda DNA to replicate efficiently. This suggests that initiation at specific sequences may not be a feature of early embryonic DNA replication in *Xenopus*, though proteins critical for replication in all eukaryotes studied are of demonstrated importance in *Xenopus* (e.g., Orcs; Carpenter et al., 1996).

Nuclear transport and Xenopus egg extracts

Nuclei formed in egg extracts or added exogenously from another source are capable of supporting nuclear import of proteins. Nuclei can be assayed for the ability to import nuclear proteins by the addition to the extract of a fluorescently labeled nuclear import substrate (see Basic Protocol 3; Newmeyer et al., 1986; Finlay et al., 1989). This assay is often used as a relatively rapid and visual confirmation that nuclei are intact and functional (see Basic Protocol 2), and that they can be used to assess the rate of nuclear transport by quantitating the increase in nuclear fluorescence over time. Like nuclear transport in tissue culture cells, import into reconstituted nuclei is dependent upon temperature, energy in the form of ATP or GTP (which are readily interconverted in the extract), and a number of cytoplasmic protein factors. The specific functions of individual cytoplasmic factors have been most extensively studied using tissue culture cells which have been digitonin-treated to permeabilize their plasma membranes. This leaves the nuclear envelope intact, removes the cytoplasmic contents, and allows direct addition of proteins or cytosol to the environment outside the nucleus (see UNIT 11.7). Indeed, *Xenopus* extracts from eggs or oocytes have proven to be a convenient source of cytoplasmic material in such assays (e.g., Moore and Blobel, 1993; Gorlich et al.,

1994). However, the unique advantage of the *Xenopus* extract in analysis of nuclear transport is the ability to manipulate the composition of the nuclear pore complex. Because the proteins that will make up the nuclear pore complex are found in the extract as soluble, individual components or small complexes, they are accessible for immunodepletion with specific antibodies (see Support Protocol 3; Finlay and Forbes, 1990; Powers et al., 1997). These depleted extracts are then used to form nuclei, and the effect of such “biochemical mutants” on nuclear transport can be monitored. In this manner, it is possible to address the function of individual proteins of the nuclear pore. Such depletion experiments can be performed on proteins involved in any process that is recapitulated in the extract and have proven invaluable in the study of cell cycle regulation and DNA replication.

Critical Parameters

The goal of extract preparation is to make a concentrated extract as close to the original composition of the cell as possible. Therefore, it is critical to remove as much of the buffer as possible after packing the eggs, even if it means loss of some of the eggs. During dejellying, it is important to wait just until the eggs settle against one another without a separation imposed by the jelly coat; however, dejellying the eggs for too long will result in damage to the egg, which can compromise extract function. It is also critical to handle the eggs gently until the time of centrifugation. Therefore, eggs should be transferred between containers either by direct pouring or by pipetting with a wide-bore cut-off and fire-polished (or upside-down) Pasteur pipet. If the eggs break prior to centrifugation, the extract will be sub-optimal.

Avoiding excessive dilution of the extract is also critical to the successful functioning of immunodepletion or protein addition experiments. For immunodepletions, this is accomplished by adding extract directly to packed beads (after washing and removal of all buffer) which have already been linked to antibody. It may require multiple immunodepletions to remove a protein of interest; therefore, it is important to examine the depleted extract by immunoblotting to confirm that the protein in question has actually been removed. It is also imperative that antibodies containing sodium azide not be used for the immunodepletions. This will disrupt extract function.

During preparation of light membranes, it is important to avoid contamination with the dark,

mitochondrial fraction. Since the light membranes are usually used after some time in frozen storage, contaminating mitochondria will lyse upon thawing, releasing cytochrome *c*. Any contaminating cytochrome *c* will induce activation of apoptotic proteases, or caspases (see UNIT 11.12), which will destroy nuclear function in the extract.

Many laboratories that prepare extracts also refrain from feeding any of the frogs once they have been primed. Any regurgitated food or stomach contents from the injected frogs may damage the eggs, compromising the extract.

To avoid damage to the sperm chromatin that will be used in the *in vitro* reconstitutions, it is important that they not be demembrated for longer than the allotted 30 min. In addition, it is best to avoid contamination with the red blood cells present in the gradient. These are much smaller than the sperm nuclei, and can produce confusion when examining samples under the microscope (e.g., it can be difficult to distinguish a nucleus with tightly condensed chromatin from a small red cell nucleus).

In examining nuclei, particularly in nuclear import experiments, it is important not to disrupt nuclear integrity. Therefore, when preparing slides, it is best to use a cut-off pipet tip and very gently lower the coverslip to the slide with a forceps.

Anticipated Results

These protocols can be used to produce extracts arrested in interphase. Upon addition of sperm chromatin, nuclei competent to transport macromolecules should be formed; these nuclei should replicate their DNA and undergo mitotic DNA condensation in response to addition of exogenous cyclin B.

Time Considerations

Interphase extracts require ~30 min to prepare and must be used within 2 hr of preparation if they are to preserve the ability to perform complex functions such as DNA replication and nuclear import. The fractionated egg extract requires an additional 2 hr of preparation time, but can be frozen indefinitely at -80°C .

Nuclear assembly in these extracts takes a variable amount of time. However, expect to see nice round nuclei in crude interphase extracts ~1 hr after the beginning of incubation at room temperature; similar nuclei can take up to 2 hr to form in a fractionated interphase extract. The kinetics of nuclear import in these two extracts are also different, with nuclear accumulation of a fluorescent import substrate to

detectable levels requiring approximately twice as much time in a fractionated as in a crude extract.

Although preparation of the oocyte extract appears superficially as simple as the crude egg extract, sorting through the oocytes to remove mottled oocytes or oocytes that have undergone GVBD can be quite time-consuming. It is best to budget a full day to prepare an oocyte extract and perform a basic experiment using the extract.

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Analysis of the Cell Cycle Using *Xenopus* Egg Extracts

UNIT 11.11

This unit describes the preparation of cycling extracts that recapitulate, in vitro, the cell cycle of the intact fertilized egg. These extracts oscillate between interphase and mitosis, reproducing many of the in vivo events of the cell cycle including DNA replication, cyclin B synthesis and degradation, cdc2/cyclin B activation and inactivation, nuclear envelope breakdown and reformation, and chromosome condensation.

Preparation of a cycling extract requires activation of mature *Xenopus* eggs (see Basic Protocol 1). Extracts prepared in the presence of a calcium chelator are arrested in mitosis (see Basic Protocol 2), but these extracts can resume cycling upon addition of calcium (see Support Protocol 3). Extract stably arrested in mitosis requires not only a calcium chelator, but also a general phosphatase inhibitor (see Basic Protocol 3). It is also possible to drive interphase extracts into mitosis with the addition of recombinant cyclin. Changes in nuclear morphology (see Support Protocol 1) and in the extent of histone H1 phosphorylation (see Support Protocol 2) are used to follow cell cycle progression in these various extracts.

PREPARATION OF THE CYCLING EXTRACT

Mature *Xenopus* eggs are activated to provide an extract that cycles between interphase (S phase) and mitosis. Freshly collected eggs are activated in a specially constructed activation chamber and lysed for a crude cycling extract.

**BASIC
PROTOCOL 1**

Materials

- 1× and 0.2× MMR (UNIT 11.10)
- Unfertilized eggs laid in 1× MMR (UNIT 11.10)
- Versilube F-50/silicone oil (see note in Basic Protocol 2)
- XB buffer (UNIT 11.10)
- CL protease inhibitor cocktail (see recipe)
- 2% (w/v) cysteine (see recipe)
- 5-ml polyallomer centrifuge tubes for Beckman SW 55 Ti rotor
- Activation chamber (see Fig. 11.11.1)
- 12 V (AC) power supply with a toggle switch to rapidly control the delivery of current
- Wide-bore Pasteur pipet
- 15-ml centrifuge tubes
- Clinical centrifuge
- Ice-water bath
- Beckman floor model ultracentrifuge (with a SW 55 Ti rotor or equivalent)
- 18-G needle attached to a 5-ml syringe
- 1.5-ml microcentrifuge tubes and microcentrifuge at 4°C
- Additional reagents and equipment for frog injection (UNIT 11.10)

NOTE: Versilube F-50 silicon oil is the oil traditionally used in preparing cycling extracts. However, the company has recently replaced this with an M-20 oil, which purportedly has the same properties; this has not yet been tested extensively.

**In Vitro
Reconstitution**

11.11.1

Collect and wash eggs

1. Inject frogs (*UNIT 11.10*, Support Protocol 1), but place the frogs in $1\times$ MMR after injection, so that the eggs will be laid in this solution.
2. The next morning, pour off the eggs into a beaker, rinse them once in room temperature distilled water, and then let them stand in water for 10 min.

This facilitates the subsequent activation step.

3. Place 1 ml of Versilube F-50 oil into each of two 5-ml polyallomer centrifuge tubes for the Beckman SW 55 Ti rotor.

It may be necessary to prepare more than two tubes to accommodate the eggs of one frog.

4. In a separate tube, mix 10 ml XB buffer with CL protease inhibitor cocktail to a final concentration of $10\ \mu\text{g/ml}$. Add 3 ml of this to each of the oil-containing tubes.

This solution should sit on top of the oil layer.

5. Incubate the eggs in 2% cysteine for ~ 5 min until the jelly coat is removed and rinse three times with $0.2\times$ MMR.

Activate eggs

6. Fill the activation chamber with $0.2\times$ MMR so that the upper electrode will contact the solution. Pour in the eggs. For activation, deliver a 3-sec pulse (12 V AC), followed by a 5-sec pause and a subsequent 3-sec pulse. At the time of activation, start a timer (in the “counting up” mode) to keep the total time elapsed from activation until step 10 to <15 min.

The activation chamber, made of Plexiglas, should be $\sim 11\ \text{cm}^2$ and 5 cm high (Fig. 11.11.1). The bottom of the chamber and the underside of the chamber lid are covered with stainless steel plate electrodes.

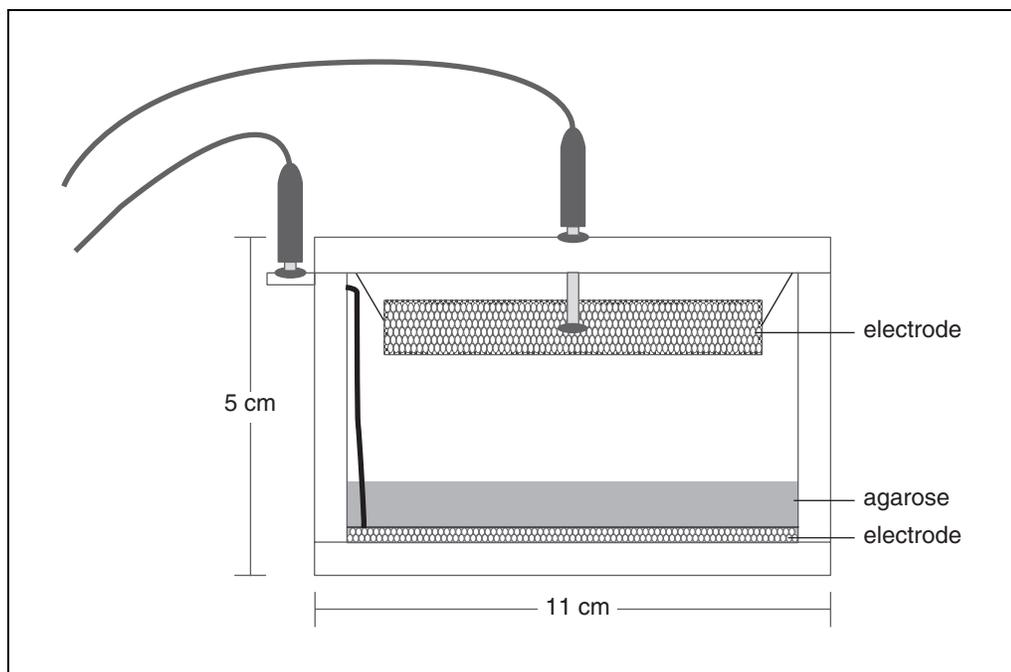


Figure 11.11.1 The activation chamber. The chamber is 11 cm on a side and 5 cm high and made of Plexiglas. The bottom of the chamber and the underside of the lid are covered with stainless steel plate electrodes. There is a pad of 0.2% (w/v) agarose in $0.2\times$ MMR buffer covering the bottom of the chamber. Eggs and $0.2\times$ MMR buffer are poured onto the agarose pad at the base of the chamber. The electrodes are attached via leads to a 12-V AC power supply. It is critical that the buffer make contact with the upper plate electrode in order for current to pass through the chamber, thereby activating the eggs.

steel plate electrodes. A pad of 0.2% agarose prepared in 0.2× MMR is poured onto the bottom of the chamber.

Successful activation should induce the contraction of the pigment in the animal hemisphere. If the eggs are examined from above with the dark side up, a white rim (from the vegetal hemisphere) can be seen around the circumference of the egg after activation.

7. After activation, pour the eggs into a petri dish and rinse three times with XB buffer.
8. Using a wide-bore Pasteur pipet, drop the eggs gently into the centrifuge tubes containing oil and buffer, taking care to avoid dilution of the buffer in the tube with the buffer surrounding the eggs.
9. Place the tubes containing the eggs into 15-ml centrifuge tubes and centrifuge in a clinical centrifuge for 30 sec at $400 \times g$, followed by 20 sec at $1350 \times g$, room temperature.

The eggs will have spun through the F-50 oil, resulting in elimination of excess buffer.

10. Remove all excess buffer and oil from above the eggs with a Pasteur pipet.

Lyse eggs

11. Continue incubation at room temperature until 15 min post-activation, then incubate a further 15 min in an ice-water bath.
12. Lyse the eggs by centrifuging them 10 min at $12,000 \times g$, 4°C (in a SW 55 Ti swinging-bucket rotor or equivalent).

The extracts will now be fractionated into three layers: lipid, extract (cytoplasm plus membrane), and yolk, from top to bottom.

13. Collect the extract layer (cytosol plus membrane) by piercing through the side of the tube using a 18-G needle attached to a 5-ml syringe.
14. Place the extract into a 1.5-ml microcentrifuge tube and microcentrifuge 10 min at $12,000 \times g$, 4°C , to remove pigment granules and other particulate material.

This extract should be used immediately for experiments requiring cycling extract; it should not be stored.

PREPARATION OF CSF-ARRESTED EXTRACTS

Xenopus eggs contain a cytoplasmic factor, termed cytostatic factor (CSF), that helps to stabilize the mitotic state, at least in part, through stabilization of the mitotic regulator cyclin B. Extracts prepared in the presence of a calcium chelator will maintain high levels of both CSF activity and cyclin B proteins. Extracts prepared in this manner are referred to as “CSF-arrested” extracts and are often used to study biochemical/morphological events of mitosis (but keep in mind that these are actually meiotically arrested extracts). While these extracts are in M phase at the time of preparation, they can be released into interphase by addition of calcium, leading to loss of CSF activity and destruction of cyclin B. Although these extracts will then behave like oscillating, or cycling extracts (see Basic Protocol 1), progressing to a subsequent mitosis as cyclins are synthesized, they tend to arrest at the next mitosis, rather than continuing to cycle, presumably because of residual, incompletely destroyed, CSF activity.

Materials

- Xenopus* eggs in 1× MMR
- 2% (w/v) cysteine (see recipe)
- 0.2× MMR (UNIT 11.10)

BASIC PROTOCOL 2

In Vitro Reconstitution

11.11.3

XB buffer (UNIT 11.10)
Versilube F-50/silicon oil (Andpak-EMA)
0.5 M EGTA stock solution, pH 8.0
1 M MgCl₂ stock solution
CL protease inhibitor cocktail (see recipe)
2% (w/v) cytochalasin B (Calbiochem)

5-ml polyallomer tubes for Beckman SW 55 Ti rotor (or equivalent)
Sawed-off and fire-polished Pasteur pipet
15-ml centrifuge tube
Clinical centrifuge
Floor model high-speed centrifuge (e.g., Sorvall, Beckman)
Beckman SW 55 Ti swinging-bucket rotor, or equivalent
5-ml syringe with an 18-G needle attached
Polycarbonate ultraclear microcentrifuge tubes (Beckman)
Microcentrifuge, 4°C

NOTE: Versilube F-50 silicon oil is the oil traditionally used in cycling extracts. However, the company has recently replaced this with an M-20 oil, which purportedly has the same properties; this has not yet been tested extensively.

Collect and wash eggs

1. Dejelly unfertilized eggs previously laid into 1× MMR in 2% cysteine and rinse with 0.2× MMR (see Basic Protocol 1).

The amount of material to use depends on the amount of extract needed; 1 ml eggs yields ~0.5 ml packed eggs which yields ~0.25 ml extract.

2. Wash eggs three times in XB buffer.
3. Wash eggs three times in XB buffer containing 5 mM EGTA and an additional 1 mM MgCl₂.
4. Add 1 ml Versilube F-50 oil to each of two 5-ml polyallomer centrifuge tubes for an SW 55 Ti centrifuge (or equivalent).
5. In a clean tube, supplement a 10-ml aliquot of XB with EGTA to 5 mM, MgCl₂ to 1 mM, and add the CL protease inhibitor cocktail and cytochalasin B to a final concentration of 10 µg/ml each. Pipet 3 ml of this solution onto the top of the F-50 oil.

Isolate eggs

6. Using a sawed-off and fire-polished Pasteur pipet, drop the eggs into the centrifuge tubes containing oil and buffer until the tube is full.

In lieu of a sawed-off pipet, use a short Pasteur pipet “backwards,” with the sharp, long end inside the pipet bulb and the wide end to pick up the eggs. It is important that the eggs not be lysed at this step, so it is not advisable to use a narrow-bore pipet.

7. Place these egg-containing tubes inside 15-ml centrifuge tubes and centrifuge 40 sec in a clinical centrifuge at 400 × g, room temperature.

This packs the eggs on the bottom of the tubes and the F-50 oil separates them from excess buffer.

8. Remove excess buffer and all of the oil with a Pasteur pipet.

Lyse eggs

9. Lyse the eggs by centrifuging 10 min at $12,000 \times g$, 4°C , in an SW 55 Ti (or equivalent) rotor.

The extracts will now be fractionated into three layers, from top to bottom: a bright yellow lipid-containing layer, a brown extract layer (cytoplasm and membrane), and a dark greenish layer containing yolk and pigment granule.

10. Collect the extract layer (middle, brown layer) using an 18-G needle and a 5-ml syringe.
11. Place the extract into a microcentrifuge tube and microcentrifuge 10 min at $12,000 \times g$, 4°C . Using a needle, again withdraw the extract layer and place on ice.

This extract can be used for mitotic reconstitutions or released into interphase by addition of CaCl_2 to a range of final concentrations between 0.1 and 0.4 mM (see Support Protocol 3).

PREPARING A MITOTIC EXTRACT

To make an extract more stably arrested in M phase than the CSF-arrested extract, it is necessary to include not only calcium chelators, but also a general phosphatase inhibitor. Together, these buffer components not only prevent the proteolytic destruction of cyclin B, but also maintain mitotic protein phosphorylation in the extract.

Materials

Xenopus eggs laid in 100 mM NaCl
2% (w/v) cysteine (see recipe)
Mitotic buffer (see recipe)
AL protease inhibitor cocktail (see recipe)
5 mg/ml cytochalasin B stock (see recipe)
Mitotic buffer (see recipe) supplemented with 250 mM sucrose (optional)
15-ml polycarbonate Ultraclear centrifuge tubes
Clinical centrifuge
High-speed floor model centrifuge (e.g. Sorvall or Beckman)
Large-capacity swinging-bucket rotor (e.g. Sorvall HB-4 or HB-6)
18-G needle attached to a 5-ml syringe

1. Collect eggs laid in 100 mM NaCl. Dejelley them in 2% cysteine, wash, and sort them (see UNIT 11.10, Basic Protocol 1).
2. After dejellying, rinse the eggs three times with mitotic buffer. Pour the eggs into a 15-ml polycarbonate Ultraclear centrifuge tube.
3. Pack the eggs by centrifuging 20 sec at $400 \times g$, room temperature, in a clinical centrifuge and remove all excess buffer with a Pasteur pipet.

As in the interphase extract, it is better to lose a few eggs than to leave buffer behind.

4. Add the AL protease inhibitor cocktail to $5 \mu\text{g/ml}$ and the 5 mg/ml cytochalasin B stock to $5 \mu\text{g/ml}$.

Both reagents are diluted 1:1000 in the total egg volume.

5. Centrifuge the packed eggs 10 min at $12,000 \times g$ (10,000 rpm in Sorvall HB-4 rotor), 4°C .

As for the interphase extract, the mitotic extract will appear as a brownish layer sandwiched between a bright yellow lipid layer and a dark green layer.

BASIC PROTOCOL 3

In Vitro Reconstitution

11.11.5

6. Collect the brown extract layer, containing both cytoplasm and membrane, by inserting an 18-G needle attached to a 5-ml syringe through the wall of the centrifuge tube at the base of the extract layer.
7. *Optional:* Further separate the crude mitotic extract described above into cytoplasmic and membrane fractions using the method described for preparing fractionated interphase extracts (see *UNIT 11.10*, Alternate Protocol 1). In this case, use mitotic buffer supplemented with 250 mM sucrose to make a sucrose cushion for membrane fractionation.

Note that this extract is an excellent source of mitotically vesiculated membranes.

DRIVING INTERPHASE EXTRACTS INTO MITOSIS

An interphase egg extract will not spontaneously enter mitosis because the added cycloheximide prevents synthesis of the cyclin proteins required for mitotic entry. To drive these extracts into mitosis, one can add recombinant forms of mitotic cyclins (e.g., cyclin B) that will associate with and activate the mitotic kinase, cdc2.

Since mitotic cyclins are degraded after cdc2 activation, mutant variants of cyclin lacking the signal for ubiquitination (the “destruction box”) must be used if the extract is to be permanently arrested in mitosis (e.g., Murray et al., 1989). An alternative method to induce mitosis after nuclear assembly is to dilute the extract 1:1 with mitotic extract (see Basic Protocol 3). This provides active cdc2/cyclin complexes that will catalyze disassembly of the preformed nuclei.

Materials

Interphase extract (crude or fractionated) containing newly formed nuclei (see *UNIT 11.10*)

Recombinant cyclin protein (Desai et al., 1995)

Additional reagents and equipment for nuclear disassembly (*UNIT 11.10*)

1. After nuclear formation in either a crude or fractionated interphase extract, add recombinant cyclin protein (produced in either bacteria or baculovirus, e.g., Desai et al., 1995) to the extract and incubate at room temperature.

It should take 30 to 60 min to observe nuclear envelope breakdown.

It is best to titrate the added proteins over a range of concentrations since preparations vary in their potency.

Typically, 15 to 20 nM recombinant cyclin B will drive extracts into mitosis.

2. To monitor nuclear disassembly, observe nuclei using the same methodologies described for nuclear assembly (see *UNIT 11.10*).

In this case, the nuclear envelope will break down, as observed by loss of a dark ring by phase-contrast microscopy, loss of nuclear exclusion of the FITC-dextran, and loss of binding of the DHCC. More strikingly, the chromatin condenses into discrete packets (Fig. 11.10.3). Although prolonged incubation allows visualization of discrete chromosomes, in general, the mitotic DNA stained with Hoechst 33258 appears as a tangled, spaghetti-like mass.

Although nuclear morphology is a good indicator of conversion into mitosis, the activity of added cyclin can be confirmed by assaying the kinase activity of the bound cdc2, using histone H1 as an exogenous substrate (see Support Protocol 2).

GENERATING A REPLICATION CHECKPOINT IN VITRO

**ALTERNATE
PROTOCOL**

It is imperative that cells not enter mitosis prior to the completion of replication. To prevent this from occurring, cell-cycle checkpoints monitor the status of DNA replication and prevent onset of mitosis until DNA replication is finished. If DNA replication is artificially blocked using polymerase inhibitors, cells will arrest in G₂, prior to mitotic entry. This form of cell cycle regulation can be recapitulated in vitro using cycling extracts and replicating sperm chromatin. While a permanent arrest prior to entry into mitosis can be achieved if extracts are supplemented with DNA replication inhibitors, the cell cycle can also be delayed by simple addition of large numbers of nuclei to the extract. Indeed, as increasing amounts of sperm chromatin are added, S phase and the time prior to entry into M phase lengthen concomitantly. Extracts will remain arrested prior to entry into mitosis until the DNA has been completely replicated.

Materials

- Sperm chromatin (*UNIT 11.10*)
- 20× energy-regenerating mix (see recipe)
- Cycling extract (see Basic Protocol 1)
- 5 mg/ml aphidicolin stock solution (Sigma) in high-quality DMSO (e.g., Pierce)
(see recipe)

1. Supplement cycling extract with 20× energy-regenerating mix to a final concentration of 1×. Add sperm chromatin to a final concentration of 500 nuclei/μl extract. Supplement the reaction with 50 μg/ml aphidicolin and mix thoroughly. Incubate at room temperature and monitor the cell cycle state (see Support Protocol 1).

Approximately 50 μl cycling extract is used per sample.

Aphidicolin is a DNA polymerase inhibitor that will prevent replication of the sperm chromatin, thereby generating a cell cycle arrest prior to entry into mitosis. Note that the extract may not function well if the final DMSO concentration (the solvent base for the aphidicolin) in the extract exceeds 1% (v/v). To confirm that the failure to enter mitosis is due to the DNA replication checkpoint, extracts can be supplemented with 5 mM caffeine, which will override the checkpoint and drive the extracts into mitosis. Add the caffeine from a 0.5 M stock solution in water. It is necessary to boil the mixture to prepare the stock solution, since caffeine will only enter solution at this concentration if boiled.

MONITORING THE CELL CYCLE STATE OF EXTRACTS

**SUPPORT
PROTOCOL 1**

As the cycling extract progresses through the cell cycle, nuclei form, DNA replication proceeds, and, after replication is complete, the extract enters mitosis. This series of events can be monitored by observation of nuclear morphology, by biochemical assessment of cdc2/cyclin kinase activity or phosphorylation state, and by direct measurements of DNA replication (see *UNIT 11.10*).

Materials

- Cycling extract
- Sperm chromatin (*UNIT 11.10*)
- 20× energy regenerating mix (see recipe)

1. To observe nuclear morphology through the cell cycle, supplement extracts with sperm chromatin (~500 nuclei/μl), and add a 1:20 dilution of the 20× energy regenerating mix. Mix the extract thoroughly by pipetting and incubate at room temperature. At 10-min intervals, withdraw samples for observation by fluorescence microscopy (see *UNIT 11.10*, Basic Protocol 2).

**In Vitro
Reconstitution**

11.11.7

Since the extract is fairly viscous, it is best to gently invert the sample periodically to ensure uniform distribution of the nuclei. Initially, samples should appear identical to those described in UNIT 11.10 for interphase nuclei. These should then progress abruptly into mitosis, as described for nuclei in UNIT 11.10, Basic Protocol 4, and Figure 11.10.3. The transition from interphase into mitosis should occur between 60 and 120 min after the start of the room temperature incubation.

2. Use histone H1 as an exogenous substrate for cdc2/cyclin B (see Support Protocol 2). In general, withdraw aliquots of extract at 10-min intervals and snap freeze in liquid nitrogen for later histone H1 kinase assays.

The peak in histone H1-directed kinase activity typically appears ~10 min before observable chromatin condensation or nuclear envelope breakdown. Oscillations in histone H1 kinase activity occur even in extracts without sperm chromatin (or any other source of DNA).

As an alternative means of assessing the cell cycle status of the extract, one can measure the tyrosine phosphorylation state of cdc2. Phosphorylation of cdc2 at its two negative regulatory sites, Thr 14 and Tyr 15, is high in interphase and then drops precipitously as cdc25 dephosphorylates cdc2 at the time of mitotic entry. Therefore, aliquots of extract withdrawn at 10-min intervals can be resolved by SDS-PAGE (UNIT 6.1) and immunoblotted (UNIT 6.2) with anti-phosphotyrosine antisera.

SUPPORT PROTOCOL 2

ASSAYING HISTONE H1 KINASE ACTIVITY

Since entry into mitosis is regulated by the cyclin-dependent kinase, cdc2, progression into mitosis in the egg extract is often assayed by measuring cdc2 activity using histone H1 as an exogenous substrate. Aliquots of extracts are withdrawn at closely spaced time intervals (10 to 15 min) from either cycling extracts (see Basic Protocol 1) or interphase extracts converted to mitosis using recombinant cyclin protein (see Basic Protocol 4).

Materials

Extract: cycling (see Basic Protocol 1) or interphase driven into mitosis (see Basic Protocol 4)
EB buffer (UNIT 11.10)
Liquid nitrogen
10× kinase buffer (see recipe)
1 mg/ml histone H1 (Roche Diagnostics)
500 μM protein kinase inhibitor peptide (PKI; Sigma)
0.2 M ATP
5 mCi/ml [γ -³²P]-ATP (3000 Ci/mmol)
2× SDS sample loading buffer (APPENDIX 2A)

1. Dilute aliquots of extract to be assayed 1:1 with EB buffer (e.g., 2 μl of extract in 2 μl EB buffer will suffice) and place immediately into liquid nitrogen. Then transfer the frozen aliquots to a -80°C freezer without thawing.
2. Prepare the following kinase cocktail so there is sufficient amount for 10 μl per kinase reaction (i.e., for each aliquot of extract to be assayed). Prepare a master mix, multiplying the following amounts by the number of reactions plus 1 for pipetting error.

1 μl 10× kinase buffer
1 μl 1 mg/ml histone H1
0.4 μl 500 μM PKI
0.1 μl 0.2 M ATP
4-8 μCi [γ -³²P]ATP
Water to 10 μl.

3. For each sample to be assayed, add 10 μ l of kinase cocktail to a fresh microcentrifuge tube.
4. Transfer samples to be assayed from the -80°C freezer directly into a container containing finely crushed dry ice.

The samples should be arrayed in the order in which they will be assayed.

5. To the first sample to be assayed (containing 2 μ l extract and 2 μ l EB buffer) add 240 μ l EB buffer and pipet up and down rapidly. As soon as the sample is thawed, transfer 10 μ l of the mixture to the corresponding tube containing 10 μ l of kinase cocktail and start a timer counting upwards.
6. Stop the reaction after 10 min by adding 20 μ l of standard 2 \times SDS sample loading buffer.

Since reactions proceed for 10 min, for multiple aliquots start and stop reactions at staggered 1-min intervals.

7. Store samples by freezing or electrophorese directly on an 11% SDS-polyacrylamide gel (UNIT 6.1).

Since most of the radioactive ATP runs below the dye front, it is best not to run the gel until the dye front reaches the bottom to avoid contamination of the SDS-running buffer. Indeed, it is best to cut the gel off at the dye front before drying and exposing the gel. After autoradiography, histone will be identifiable as a very closely spaced radiolabeled doublet.

RELEASE OF CSF-ARRESTED EXTRACTS AND THEIR PROGRESSION INTO INTERPHASE

SUPPORT PROTOCOL 3

To determine if removal or addition of particular components from an extract has a specific effect on exit from mitosis, it is easiest to use CSF extracts that can transit from mitosis to interphase in a controlled manner. Supplementation of CSF extracts with calcium drives them into interphase. If these extracts are supplemented with sperm chromatin, they will form nuclei and initiate DNA replication in the same manner as in cycling extracts described in Basic Protocol 1. However, the released CSF extracts have a tendency to arrest on re-entry into mitosis, unlike the cycling extracts that will continue through multiple cycles.

Materials

CSF-arrested extract (see Basic Protocol 2)
20 \times energy-regenerating mix (see recipe)
Sperm chromatin (UNIT 11.10)
1 M calcium chloride
Fixative for visualizing nuclear assembly (UNIT 11.10)
Cycloheximide (optional)

1. Supplement CSF extract with 20 \times energy-regenerating mix to a final concentration of 1 \times .
2. Add sperm chromatin to a final concentration of 100 to 500 nuclei/ μ l.
3. Determine the minimal concentration of calcium required to drive the extract into mitosis. Supplement small aliquots of extract containing energy-regenerating mix and sperm chromatin with 1 M CaCl_2 to 0.1 mM, 0.2 mM, or 0.4 mM and incubate at room temperature.

While adding calcium to this extract will release it into interphase, the precise concentration of calcium required varies somewhat from extract to extract. Hence, to perform a large experiment it is probably worthwhile to first determine the minimal concentration of calcium required to drive a particular extract into interphase.

In Vitro Reconstitution

11.11.9

- At 10-min intervals, remove a 4- μ l sample and add 1 μ l Hoechst 33258 to stain the DNA. Observe using fluorescent microscopy. Identify the lowest concentration of CaCl_2 that allows good release of CSF-arrested extract into interphase.

Stain nuclei as described in UNIT 11.10, except FITC-dextran may be omitted here.

In the absence of calcium addition, condensed mitotic chromosomes should be easily visible by fluorescence microscopy after 20 to 30 min of room-temperature incubation. For the calcium-released samples, find the lowest concentration allowing good release from CSF arrest into interphase. At the appropriate concentration, the sperm chromatin should decondense to form membrane-enclosed nuclei (see UNIT 11.10).

- Optional:** To arrest these extracts in interphase after calcium addition, add cycloheximide to a final concentration of 50 μ g/ml at the time of calcium addition.

The crude CSF-arrested extract can be further separated into cytoplasmic and membrane fractions (see UNIT 11.10, Alternate Protocol 1) and stored at -80°C . The freeze/thawed cytoplasmic fraction of CSF extracts can fully support chromosome condensation and spindle formation.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

AL protease inhibitor cocktail

Dissolve aprotinin to 10 mg/ml in water and leupeptin to 10 mg/ml in water. Mix these two solutions 1:1 to give a final 5 mg/ml solution. Store at -70°C .

Aphidicolin stock solution, 5 mg/ml

Dissolve aphidicolin to 5 mg/ml in high-quality DMSO (e.g., Pierce). Store several months at -70°C .

CL protease inhibitor cocktail

Dissolve leupeptin to 10 mg/ml in distilled water and chymostatin to 10 mg/ml in DMSO and mix them at a ratio of 1:1 to give a final concentration of 5 mg/ml. Store at -70°C .

Cysteine, 2%

Prepare 2% (w/v) cysteine in water. Adjust pH to 8.0 with NaOH. Prepare fresh for each use.

Cytochalasin B stock solution, 5 mg/ml

Prepare a 5 mg/ml stock of cytochalasin B (Calbiochem) in DMSO. Store up to 1 year at -20°C .

Energy-regenerating mix, 20 \times

150 mM creatine phosphate

20 mM ATP

2 mM EGTA, pH 7.7

20 mM MgCl_2

Store up to several months at -20°C , but avoid repeated freeze/thaw

Kinase buffer, 10 \times

200 mM HEPES, pH 7.3

5 mM EGTA

10 mM MgCl_2

Store indefinitely at 4°C , unless it becomes cloudy

Mitotic buffer

24 ml 1 M β -glycerophosphate, pH 7.3
12 ml 0.5 M EGTA, pH 8.0
4.5 ml 1 M MgCl_2
100 μl 1 M DTT
Add water to 100 ml
Adjust pH to 7.3 with NaOH
Store indefinitely at 4°C, unless a precipitate forms

COMMENTARY

Background Information

Eggs arrested in metaphase have not only high levels of *cdc2/cyclin B* activity but also a cytoplasmic component, CSF (cytostatic factor), which stabilizes this activity. During normal fertilization, sperm entry promotes the destruction of CSF, initiating the oscillation between S and M phases seen in early embryonic development. Destruction of CSF and cyclin B can be achieved synchronously in a large population of eggs by electrical stimulation of the eggs (see Basic Protocol 1). If cycloheximide is not added to these extracts (so that protein synthesis can occur), extracts made from these electrically activated eggs faithfully recapitulate the embryonic cell cycle oscillations with respect to nuclear envelope formation and breakdown, DNA replication, cyclin accumulation and destruction, and *cdc2* kinase oscillations (Murray and Kirschner, 1989; Murray et al., 1989). Indeed, in these extracts, DNA replication occurs only once per cell cycle and multiple rounds of replication occur only because they are separated by intervening mitosis.

Although cycling extracts are useful for studying the basic cell cycle machinery, they can also be used to reconstitute more complex phenomena, such as the operation of cell cycle checkpoints. When cellular events regulated by the cell cycle machinery malfunction or do not occur on time, subsequent cell cycle events are delayed. For example, if DNA replication is inhibited or DNA is damaged, mitosis will not occur, preventing chromosome segregation and almost certain breakage of partially replicated or damaged chromosomes. In cycling extracts, if nuclei are formed in the presence of the DNA polymerase α inhibitor, aphidicolin, mitosis will not occur since replication cannot be completed. This cell cycle arrest can be overridden by caffeine, an agent known to override this cell cycle checkpoint in intact cells, reinforcing the idea that the extract uses the same biochemical pathways to implement the cell cycle arrest as are used in vivo (Dasso and Newport, 1990).

Hence, this system can be used to unravel the biochemical pathways that underlie the operation of the DNA replication checkpoint.

Extracts in mitosis

Although synthesis of cyclin will drive a cycling extract into mitosis, the extract will not remain in mitosis since cyclin is destroyed by regulated ubiquitination and proteasome-mediated degradation. To study mitotic functions in isolation (e.g., nuclear disassembly, chromosome condensation), inclusion of EGTA in the buffer used during egg lysis results in chelation of the Ca^{+2} normally released upon egg lysis, thereby preventing the loss of CSF and *cdc2/cyclin B* activity, and locking these extracts in M phase (Glotzer et al., 1991; Murray et al., 1989). If Ca^{+2} is then added to these “CSF-arrested extracts,” they can be released into interphase. Because these extracts are competent to synthesize protein, cyclin will accumulate, driving this extract into a subsequent mitosis (see Support Protocol 3). These extracts are excellent for the study of mitotic enzyme activities and for microscopic and biochemical analysis of mitotic chromosome condensation and spindle assembly. In addition, they have recently been used to great effect in the purification of proteins interacting with mitotic chromosomes (Hirano and Mitchison, 1994). Ca^{+2} addition allows controlled release of CSF extracts into interphase, making this extract the preferred choice for examining the M to S phase transition. This feature has been exploited to study the biochemistry of the “spindle checkpoint” that prevents anaphase progression if chromosomes are not properly attached to the mitotic spindle (Minshull et al., 1994). The CSF extract is also particularly useful if removal and addition of cellular components is desired prior to commencement of DNA replication.

If β -glycerophosphate (as well as EGTA) is included in the egg lysis buffer, the mitotic state is stabilized indefinitely, not only by making CSF and cyclin impervious to degradation, but

also by stabilizing mitotic phosphorylations. These “mitotic” extracts (see Basic Protocol 3) can be fractionated into cytosolic and membranous components, which can be stored indefinitely and retain the mitotic character of the original extract.

An alternative method to prepare extracts arrested in mitosis is to convert S phase extracts to the mitotic state by addition of a recombinant cyclin B whose “destruction box” (the region of the protein that renders it susceptible to proteolytic degradation) has been removed (see Basic Protocol 4; Solomon et al., 1990). This leads to constitutively high levels of *cdc2/cyclin B* activity with a consequent mitotic arrest.

Critical Parameters

Excessive dilution of the cycling extract will interfere with its ability to cycle. This is probably because protein synthesis in these extracts is sensitive to dilution, and cyclin synthesis is required to complete the various cell cycle transitions. When preparing the cycling extract, it is imperative that all of the eggs in the preparation have been properly activated. If a significant proportion of the eggs have not undergone activation after the electrical pulse, the extract probably will not cycle unless the unsuccessfully activated eggs are removed. Rarely, a batch of eggs will be contaminated with small numbers of immature oocytes; these lack the white spot characteristic of eggs (resulting from germinal vesicle breakdown and displacement of pigment granules) and should be removed before extract preparation.

When triggering a checkpoint in the extract, the aphidicolin should be prepared so as not to exceed 1% final DMSO concentration in the extracts. Higher amounts of DMSO will impair extract function. If caffeine is being used to override the replication checkpoint, make sure it is fully in solution and do not place on ice (to avoid precipitation of the caffeine).

To monitor progression of the cell cycle in the *in vitro* extract, it is convenient to assay kinase activity directed against histone H1 (catalyzed by *cdc2/cyclin B* complexes). Each time point should be assayed immediately after thawing. If the whole time course is thawed at once and then assayed, the peaks of H1-directed kinase activity will be lost.

After preparing the CSF extract, it is worthwhile to test that it can be successfully released from metaphase into interphase with the desired concentration of calcium. Occasionally, release does not occur, indicating a requirement for higher calcium levels or indicating that the

extract is nonfunctional. The easiest way to determine if release can occur is to supplement the extract with sperm chromatin and examine samples by Hoechst staining and fluorescence microscopy.

When driving an extract into mitosis using exogenous cyclin, bear in mind that cyclin preparations vary in their potency to induce mitosis. All batches of cyclin should be titered with regard to mitosis-inducing activity. If a nondestructible cyclin B is used, the extract will remain arrested in mitosis. With native cyclin B, the extract will complete mitosis and return to interphase concomitant with cyclin destruction.

Anticipated Results

Cycling extracts should complete interphase, go into mitosis, and come out again two or three times. Histone H1 kinase activity should peak ~10 min before visual breakdown of added nuclei. In the CSF extract, added sperm chromatin should look like tangled threads. Upon addition of calcium, these should now form round nuclei similar to those formed either in interphase extracts or at the start of a cycling extract incubation.

Time Considerations

Once beginning a cycling extract, it is not possible to stop at any point. It is particularly important that the time from egg activation to incubation on ice not exceed 15 min. The cycling extract can remain on ice for 1 to 2 hr before use, but it cannot be frozen for later use.

For assaying progression of the cycling extracts using histone H1 kinase assays, it is critical that all of the time points be incubated for precisely the same amount of time; hence, the authors have recommended starting the sample incubations at 1-min intervals so that they can be subsequently stopped at 1-min intervals.

Entry into mitosis in interphase extracts supplemented with recombinant cyclin can take a variable amount of time, depending on the particular extract and preparation of cyclin. However, it is advisable to aim for an amount of cyclin that takes 30 to 45 min to promote mitotic entry. If, after 60 min, entry into mitosis is not observed, it is likely that the extract is not responsive, that suboptimal levels of cyclin have been used, or that the preparation is inactive.

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Analysis of Apoptosis Using *Xenopus* Egg Extracts

UNIT 11.12

Recapitulation of the apoptotic phenotype in the *Xenopus* egg extract system was first described by Newmeyer et al. (1994) where it was noted that prolonged incubation of the crude interphase extract resulted in extracts that were capable of undergoing apoptosis characterized by chromosome condensation and nuclear fragmentation. The presence of a subcellular fraction enriched in mitochondria is essential for the induction of apoptosis in the extracts. These extracts share many of the biochemical characteristics described in apoptotic cells from other systems, such as caspase activation and DNA fragmentation. In addition, apoptosis in these extracts can be inhibited by addition of caspase inhibitors as well as the anti-apoptotic protein Bcl-2.

PREPARATION OF APOPTOTIC EXTRACTS AND ASSESSING APOPTOSIS

**BASIC
PROTOCOL**

This unit describes preparation of an apoptotic extract from a crude interphase extract (see Basic Protocol) and an extract fractionated into latent and execution phases (see Alternate Protocol). An apoptotic extract can also be reconstituted from a fractionated interphase extract (UNIT 11.10) and purified mitochondria (see Support Protocol 2). Protocols are also included for monitoring apoptotic progression in the extract either by following activation of apoptotic proteases (caspases; see Support Protocol 1) or by assessing translocation of cytochrome *c* from the mitochondria to the cytosol (see Support Protocols 3 and 4).

In essence, the apoptotic extract does not differ from the crude interphase extract described in UNIT 11.10. Prolonged incubation of the extract at room temperature will result in release of mitochondrial cytochrome *c*, activation of caspases, and if nuclei are present, nuclear fragmentation. Therefore most other applications using crude extract require that incubation times not exceed 3 to 4 hr or the extract may enter apoptosis confounding analysis of the process under study (e.g., nuclear transport, nuclear envelope assembly). However, extracts lacking mitochondria (e.g., the fractionated interphase extract; UNIT 11.10) will not enter apoptosis even after prolonged incubation.

Materials

Crude interphase extract (UNIT 11.10)
0.2 M phosphocreatine
0.5 mg/ml creatine kinase
0.2 M ATP
Hoechst 33258 (Bisbenzimidazole H 33258; Calbiochem)
Fluorescence microscope

Additional reagents and equipment for preparation of *Xenopus* crude interphase extract (UNIT 11.10)

1. To a crude interphase extract, add 0.2 M phosphocreatine to a concentration of 20 mM (1:10), 0.5 mg/ml creatine kinase to a concentration of 5 μ g/ml (1:100 dilution), and 0.2 M ATP to a concentration of 2 mM (1:100) to create an ATP-regenerating system. Mix and incubate this extract 4 to 8 hr at room temperature.

Since extracts undergo apoptotic nuclear fragmentation at slightly different times, it is a good idea to start observing the nuclei at 0.5-hr intervals starting at ~3 hr after beginning the room temperature incubation.

**In Vitro
Reconstitution**

11.12.1

2. Determine the onset of apoptosis by observing synthetic nuclei stained with Hoechst 33258 and visualized using fluorescence microscopy.

Nuclei assembled around sperm chromatin in the extract can be used as morphological indicators of apoptosis. For assembly of nuclei in vitro and slide preparation, see UNIT 11.10.

At the time of apoptosis, condensation of the chromatin and nuclear fragmentation are clearly visible (see Figure 11.10.3).

For a more quantitative assessment of apoptosis, it is easiest to measure activation of the apoptotic proteases, or caspases in the extract. It is generally possible to detect caspase activity from 2 to 3 μ l of apoptotic extract (see Support Protocol 1). It is not necessary to include nuclei in an extract when measuring apoptosis by caspase activation or cytochrome c release.

It is also possible to measure mitochondrial cytochrome c release in these extracts; this is tightly correlated with the onset of apoptosis (see Support Protocol 3).

ALTERNATE PROTOCOL

SEPARATING APOPTOSIS INTO LATENT AND EXECUTION PHASES

This protocol separates the apoptotic reaction into two steps. The first step is the latent phase where a reaction or series of reactions that prepare the extract for apoptosis takes place. The second step of the apoptotic reaction is the execution phase where the apoptotic phenotype characterized by caspase activation, DNA degradation, and nuclear fragmentation is manifested.

The latent phase requires only the cytosolic and light membrane fractions of an interphase extract. Because this extract lacks the mitochondrial fraction, it cannot support caspase activation or full-blown apoptosis. However, when the cytoplasmic and membrane components are combined and allowed to incubate at room temperature for 2.5 hr, a "latent" apoptotic activity develops that is able to accelerate apoptosis in an extract that contains mitochondria. It is likely that activities which trigger mitochondrial cytochrome c release develop during the latent incubation. After transfer to the execution extract containing mitochondria, the latent extract triggers release of mitochondrial cytochrome c to the cytosol, which then serves as a cofactor in caspase activation.

The execution phase of the apoptotic reaction requires the presence of mitochondria, which are provided by the crude extract. Once a latent extract has been incubated for the appropriate time, 1/10 vol is transferred into a crude extract (execution extract) containing nuclei and the apoptotic morphology of the nuclei is observed after 60 to 120 min. The advantage of setting up the apoptotic reaction in this manner is that signaling molecules operating during the latent phase of apoptosis (prior to detectable caspase activation) can be studied separately from those involved in the apoptotic dismantling of cellular components. The fractionated extract is also easier to immunodeplete (see UNIT 11.10), since the soluble extract can be depleted in the absence of membrane components and then later reconstituted with membrane.

Materials

- Freshly obtained *Xenopus* eggs, washed with egg lysis buffer (UNIT 11.10)
- 0.2 M phosphocreatine
- 0.5 mg/ml creatine kinase
- 0.2 M ATP
- 20 \times energy-regenerating mix (UNIT 11.11)
- Demembrated sperm chromatin (UNIT 11.10)
- Hoechst 33258 (Bisbenzimidazole H 33258; Calbiochem)

15-ml polypropylene tubes
5-ml syringe and 18-G needle

Additional reagents and equipment for preparing crude and fractionated interphase extracts (*UNIT 11.10*)

Prepare a fractionated interphase extract

To create a latent extract, a fractionated interphase extract is made exactly as described in *UNIT 11.10*, Basic Protocol 1, and Alternate Protocol 1, with the following variations.

1. Once the eggs are washed with egg lysis buffer and transferred to 15-ml polypropylene tubes (see step 6 of Basic Protocol 1 in *UNIT 11.10*), keep them on ice until crushed (lysed) by centrifugation (step 9 in that protocol), and keep them on ice again at the completion of the lysis centrifugation, until high-speed centrifugation (performed in step 2 of Alternate Protocol 1, *UNIT 11.10*).
2. After removing the crude extract from the 15-ml polypropylene tube with a 5-ml syringe and 18-G needle, do not supplement the crude extract with additional protease inhibitors or cytochalasin B (i.e., skip step 1 of Alternate Protocol 1 in *UNIT 11.10*).
3. Proceed with the steps required for making a fractionated interphase extract (see *UNIT 11.10*, Alternate Protocol 1).

Prepare the latent extract

4. Thaw an aliquot of fractionated cytosol and membrane (*UNIT 11.10*). Recombine 10 μ l of membrane with 100 μ l of cytosol.
5. Add 0.2 M phosphocreatine to 20 mM (1:10), 0.5 mg/ml creatine kinase to 5 μ g/ml (1:100), and 0.2 M ATP to 2 mM (1:100). Mix and incubate 2.5 hr at room temperature.

Prepare execution extract

6. Make fresh crude interphase extract (see *UNIT 11.10*, Basic Protocol 1).
7. Dilute 20 \times energy regenerating mix to 1 \times in fresh crude extract.

This energy mix is used in the execution extract because it does not dilute the extract as much as separate addition of phosphocreatine, creatine kinase, and ATP.

8. Dilute demembrated sperm chromatin to 1000 nuclei/ μ l in the execution extract.

Mix extracts and assess for apoptosis

9. Transfer 1/10 vol latent extract into execution extract (e.g., 5 μ l latent extract into 50 μ l execution extract). Mix by flicking tube.
10. Incubate at room temperature. Withdraw samples at 10-min intervals and stain with Hoechst 33258 (as for visualizing nuclear protein import; see *UNIT 11.10*, Basic Protocol 3). Examine for apoptotic nuclei.

Nuclei should form by 30 min. Nuclei usually undergo apoptosis between 60 and 120 min after transfer.

MEASURING CASPASE 3–LIKE ACTIVITY

When apoptosis is activated in the egg extracts, it is possible to detect a caspase activity with a substrate specificity very similar to that seen for mammalian caspase 3, one of the “effector” caspases. Measurement of this activity provides an easy way to quantitate apoptosis in the extract, particularly to compare apoptosis in extracts subjected to different experimental treatments.

In vitro caspase assays are based on the cleavage of synthetic peptide substrates of the caspases linked to either chromophores or fluorophores. Upon cleavage of the substrate by relevant caspases from the apoptotic extract, release of the chromophore or fluorophore can be detected by fluorimeter or spectrophotometer. In the case of caspase 3, the synthetic substrate, Ac-DEVD-pNA is used.

Materials

Assay buffer (see recipe), room temperature
Apoptotic egg extract (see Basic Protocol or Alternate Protocol)
Cleavage substrate: 2 mM Ac-DEVD-pNA (see recipe)
Cleavage inhibitor: 20× Ac-DEVD-CHO (see recipe)
96-well microtiter plate

1. For each sample/time point to be assayed, place 85 μ l of room temperature assay buffer in a well of a 96-well microtiter plate. Keep at room temperature.

Assays are done in a 96-well plate to facilitate reading of multiple samples.

2. Add 1 to 5 μ l of the apoptotic egg extract to be assayed to the well.
3. Add 10 μ l of caspase substrate, 2 mM Ac-DEVD-pNA. Mix by tapping the sides of the plate. Incubate 1 hr in a 37°C incubator.
4. Stop the assay by addition of 5 μ l of caspase inhibitor, 20× Ac-DEVD-CHO. Read all samples at 405 nm at the very end of the experiment.

Alternatively, read the samples immediately using a microtiter plate reader at 405 nm.

Addition of the caspase inhibitor stops further development of the caspase assay, allowing samples to be read later. If the peptide inhibitor is not added, the caspase assay will continue to develop, making it impossible to compare samples from different time points.

PREPARATION OF MITOCHONDRIA FROM XENOPUS EGG EXTRACTS

As noted in the Basic Protocol, the mitochondrial fraction of *Xenopus* egg extracts is absolutely required for in vitro apoptosis. When the cytosol and light membrane fractions of the extract (UNIT 11.10) are combined, the reconstituted extract will not enter apoptosis. However, if a purified heavy membrane fraction enriched in mitochondria is added, the reconstituted extract becomes competent to support apoptosis. Indeed, it has been reported that adding twice the concentration of mitochondria found endogenously in the crude extract results in the production of a robust and reproducible apoptotic extract (Faure et al., 1997). This is almost certainly due to the fact that mitochondrial cytochrome *c* release, which triggers activation of apoptotic proteases, is the downstream target of many apoptotic signaling pathways. Therefore, increasing the mitochondrial concentration results in increased cytochrome *c* release and more rapid apoptosis. The following protocol describes a procedure, adapted from the laboratory of Dr. Don Newmeyer of La Jolla Institute of Allergy and Immunology, to prepare a heavy membrane fraction enriched in mitochondria.

Materials

Fractionated interphase extract (Alternate Protocol 1 in *UNIT 11.10*)

Percoll solutions (see recipe)

MIB buffer (see recipe)

2.5-ml ultracentrifuge tubes (e.g., Beckman)

Beckman TL-100 table-top ultracentrifuge and TLS-55 swinging-bucket rotor

Additional reagents and equipment for preparing fractionated *Xenopus* interphase extract (*UNIT 11.10*)

1. Harvest the cytosol and light membrane fractions of a fractionated interphase extract. After removing the light membranes, gently remove the heavy membrane fraction using a cut-off pipet tip and a P-200 micropipettor. Place the material on ice.

The mitochondrial/heavy membrane fraction is the brown opaque layer that lies directly below the light membrane layer. The translucent layer directly below the heavy membranes consists largely of pigment granules and glycogen.

2. For each sample, prepare a Percoll step gradient by slowly adding successive 0.45 ml layers of each of the following Percoll solutions to a 2.5-ml tube for the TLS-55 swinging-bucket rotor: 42%, 37%, 30%, and 25%.

These solutions must be layered on top of each other slowly to prevent mixing.

3. Add the entire mitochondrial layer from the extracts of eggs of one to two frogs on top of the gradient. Centrifuge 25 min at $53,500 \times g$ (25,000 rpm in TLS-55 rotor), 4°C , without the brake.

After centrifugation, any contaminating light membranes will be at the top of the Percoll gradient. Heavy membranes/mitochondria will form a discrete dark band between Percoll concentrations.

4. Withdraw the heavy membranes/mitochondria using a Pasteur pipet. Resuspend them in 50 vol MIB buffer. Mix by gently inverting the tube.

5. Pellet the mitochondria by centrifuging 10 min at $1700 \times g$, 4°C .

6. Discard the supernatant and resuspend the mitochondrial pellet in an equal volume of MIB buffer to give a $10\times$ stock for addition to reconstituted extract.

*Although some laboratories use frozen stocks of mitochondria, freeze/thaw can result in puncturing of mitochondria. Should this occur, direct cytochrome *c* leakage from mitochondria may produce rapid, unregulated apoptosis upon addition to extracts. This is obviously not useful for studying regulation of the apoptotic process. Consequently, the authors prefer to use the mitochondrial fraction on the day of preparation. The entire procedure takes ~ 2.5 hr.*

CYTOCHROME *c* RELEASE ASSAYS

In many systems, execution of the apoptotic program depends upon release of cytochrome *c* from the mitochondria. Once released to the cytoplasm, cytochrome *c* serves as a cofactor in activation of the apoptotic proteases, or caspases. Indeed, addition of purified cytochrome *c* to *Xenopus* egg extracts results in rapid caspase activation and fragmentation of subcellular structures (e.g., nuclei, endoplasmic reticulum). Therefore, one way to assess apoptotic progression in the *Xenopus* egg extract is through measurement of mitochondrial cytochrome *c* release. This can be done in crude egg extracts or, alternatively, purified mitochondria (see Support Protocol 2) can be mixed with cytosol from the fractionated interphase extract (*UNIT 11.10*), which will also support mitochondrial cytochrome *c* release.

SUPPORT PROTOCOL 3

In Vitro Reconstitution

11.12.5

Materials

Crude extract for apoptotic reaction (see Basic Protocol)

0.1- μ m ultrafree-MC filters (Millipore)

Anti-cytochrome *c* antibody (Pharmingen)

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and immunoblotting (UNIT 6.2)

1. At various time intervals (e.g., 0.5-hr to 1-hr intervals are generally sufficient), withdraw 25- μ l aliquots of crude extract for apoptotic reaction and pass through a 0.1- μ m ultrafree-MC filter.

*This removes both mitochondria and other particulate matter. The supernatant above the filter should contain any cytochrome *c* that has been released into the cytosol.*

2. Separate 7 μ l of the supernatant retained above the filter by SDS-PAGE (UNIT 6.1) and immunoblot (UNIT 6.2) with anti-cytochrome *c* antibody.

CYTOCHROME *c* RELEASE ASSAY USING PURIFIED MITOCHONDRIA AND CYTOSOL

Under some circumstances it is desirable to prepare cytosol and mitochondria separately for reconstitution of cytochrome *c* release. For example, separate preparations are used when immunodepleting the cytosol of a particular protein to determine whether the protein in question affects cytochrome *c* release. Moreover, using purified mitochondria it is possible to determine if a given recombinant protein can induce mitochondrial cytochrome *c* release in the absence of accessory cytosolic components. Under these circumstances, egg lysis buffer containing the protein of interest would be substituted for cytosol in this protocol.

Materials

Heavy membrane fraction containing mitochondria (see Support Protocol 2)

Cytosolic fraction containing energy regenerating mix (UNIT 11.10)

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and immunoblotting (UNIT 6.2)

1. Combine 14 μ l of the heavy membrane fraction containing mitochondria with 170 μ l cytosol containing an energy-regenerating mix. Incubate at room temperature.
2. At 0.5-hr intervals, withdraw 25- μ l aliquots. Filter, electrophorese (UNIT 6.1), and immunoblot (UNIT 6.2) the supernatant for cytochrome *c*.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Assay buffer

50 mM HEPES, pH 7.4

100 mM NaCl

0.1% (w/v) CHAPS

1 mM EDTA

10% (v/v) glycerol

10 mM dithiothreitol, added fresh before each use

Store indefinitely at 4°C

Table 11.12.1 Preparation of Percoll Solutions for Gradients

Ingredient	% Percoll			
	42%	37%	30%	25%
MIB buffer concentrate ^a	0.612 ml	0.612 ml	0.612 ml	0.612 ml
Percoll	1.05 ml	0.92 ml	0.75 ml	0.25 ml
Water	0.838 ml	0.968 ml	1.14 ml	1.263 ml

^aSee recipe for MIB buffer.

Cleavage inhibitor: Ac-DEVD-CHO, 20×

Prepare 0.1 mM or 0.05 mg/ml stock of Ac-DEVD-CHO (Biomol) in DMSO. Dilute stock 1:50 in assay buffer (see recipe). Store up to several months at -20°C .

Cleavage substrate: Ac-DEVD-pNA, 2 mM

Dissolve 1.3 mg/ml Ac-DEVD-pNA (Biomol) in assay buffer (see recipe) for a 2 mM final concentration. Store in light-proof tube at -20°C .

MIB buffer

Prepare MIB buffer concentrate:

50 mM sucrose
 10 mM KCl
 10 mM sodium succinate
 10 mM HEPES adjusted to pH 7.5 with KOH
 5 mM EGTA
 210 mM mannitol
 0.5 mM dithiothreitol, added fresh before each use
 Store indefinitely at 4°C

To prepare MIB buffer: Dilute 2.45 ml MIB buffer concentrate to 10 ml final volume in water.

Discard MIB buffer concentrate if microbial growth is detected.

Percoll solutions

Prepare Percoll solutions according to Table 11.12.1.

COMMENTARY

Background Information

Recently, a cell-free extract of *Xenopus* eggs that can support apoptosis was described (Newmeyer et al., 1994). The preparation of these extracts and assays of apoptosis are described in this unit (see Basic Protocol and Alternate Protocol). The ability to use eggs as a source of material to reconstitute apoptotic processes in vitro most likely reflects the in vivo process of oocyte atresia, wherein oocytes that have matured but have not been laid due to a lack of appropriate hormonal stimuli, are systematically resorbed by apoptosis.

As is the case in vivo, the apoptotic nuclear fragmentation seen in the in vitro extract of *Xenopus* eggs is characterized by a series of stereotyped phenotypic changes in subcellular

structures. The extract offers the opportunity to examine nuclear events of apoptosis and also to study biochemically the regulation and activation of proteases critical for apoptotic execution. In extracts, as in vivo, the onset of apoptosis is marked by the condensation of DNA, after which the condensed DNA is distributed into many small membrane-enclosed structures (see Figure 11.10.3). Gel electrophoresis reveals the characteristic “laddering” of DNA, indicative of internucleosomal cleavage. Nuclear fragmentation in these extracts is accompanied by the activation of apoptotic proteases, the caspases (Newmeyer et al., 1994; Cosulich et al., 1996), and this is completely inhibited by addition of the broad range apoptotic inhibitor, bcl-2 protein (Bellamy et al., 1995; Villa et

al., 1997; Chao and Korsmeyer, 1998). By these criteria, the extract faithfully recapitulates a number of the biochemical events of in vivo apoptosis. Additionally, other known positive regulators of apoptosis (e.g., *Drosophila* reaper protein, activated caspases, and cytochrome *c*) and negative regulators of apoptosis (e.g., tetrapeptide caspase inhibitors) behave in the *Xenopus* extract as in other systems, again supporting the notion that the extract accurately reconstitutes the apoptotic process (Evans et al., 1997; Kuwana et al., 1998). Coupled with the fact that elements of the apoptotic machinery have proven to be well conserved from worms to man, these observations make it highly likely that the *Xenopus* system will provide an excellent model for understanding key events in the regulation of apoptosis, as has been the case for the study of cell cycle progression, DNA replication, nuclear transport, and nuclear architecture.

Critical Parameters

Although most extracts will eventually enter apoptosis, the time required varies a great deal from extract to extract. A CSF-arrested extract or an extract otherwise in mitosis is extremely refractory to apoptosis.

When handling mitochondria to be used for apoptotic or cytochrome *c* release assays, they must be treated gently to prevent breakage. Freeze-thawing the mitochondria can cause them to leak cytochrome *c*; therefore, fresh mitochondria are optimal for apoptosis experiments.

In extracts containing in excess of 1000 sperm nuclei/ μ l, the nuclei may fragment very slowly, despite relatively high levels of caspase activity. Therefore, to use nuclear fragmentation as an indicator of apoptosis, nuclei should be kept at concentrations <1000 sperm nuclei/ μ l.

When resolving samples by SDS-PAGE for anti-cytochrome *c* immunoblots, it is worth noting that many of the commercially available molecular weight markers include cytochrome *c* as one of the molecular weight standards. This band will also "light up" with the cytochrome *c* antibodies, providing a convenient size marker for the cytochrome *c* on immunoblots.

Anticipated Results

Extracts entering apoptosis should fragment added nuclei and activate DNases that can cleave the added DNA. Caspase activity should be detectable prior to visible chromatin fragmentation, and this activity should be preceded by observation of cytochrome *c* release from the mitochondria to the cytoplasm. Expect to

obtain a volume of mitochondria \sim 1/20 the volume of the starting crude extract.

Time Considerations

The time for an extract to enter apoptosis varies enormously depending upon the extract. Although the typical extract takes 4 to 6 hr to enter apoptosis, extracts can enter apoptosis in as little as 1 hr or after as long as 12 hr. When doing an experiment dependent upon an extract having entered apoptosis, it is advisable to monitor apoptotic progression, either through examination of nuclei in the extract or through measurement of caspase activity. The authors have also observed that extract containing large numbers of nuclei may be slower to enter apoptosis than extracts lacking nuclei. Cytochrome *c* release from mitochondria will precede caspase activation slightly.

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Mitotic Spindle Assembly In Vitro

UNIT 11.13

This unit presents in vitro assays for studying the process of mitotic spindle assembly using *Xenopus laevis* egg extracts. These protocols all make use of cytosolic factor (CSF)-arrested extracts. CSF extracts arrested in metaphase are open to biochemical study and manipulation and allow for extensive cytological analysis of experiments. A detailed protocol for the preparation of CSF extracts can be found in UNIT 11.11, and the authors' modifications are given in Support Protocol 1.

The basic protocols in this unit can be used to assay the roles of different cellular factors in the many processes and events that must take place to properly assemble a mitotic spindle. All of these assays are performed in a test tube environment. The researcher combines CSF extract with factors that generate mitotic microtubule structures, such as centrosomes or dimethyl sulfoxide (DMSO), which give rise to mitotic asters (see Basic Protocol 1), sperm nuclei (see Basic Protocols 2 and 3), or DNA beads (see Basic Protocol 4), which lead to the formation of spindles. Rhodamine-labeled tubulin (see Support Protocol 2) is added to allow quick and easy visualization by fluorescence microscopy of microtubule growth and organization in samples taken at various time points. Experimental conditions are created by pretreating the extract to specifically inactivate or remove proteins of interest (see UNIT 11.11 and Support Protocol 3). Finally, the results of individual experiments are preserved by transferring the extract reactions to coverslips for fixation and storage (see Support Protocol 4). This same fixation method also allows for immunofluorescence analysis of individual experiments (UNIT 4.3).

The first basic protocol (see Basic Protocol 1) describes a method for assembling microtubule asters in extracts using either DMSO or purified centrosomes. Although spindles are not formed in these reactions, they are useful, because of their simplicity of method and mechanism, for studying microtubule nucleation, dynamics, and organization. Subsequent protocols build on the same basic reaction, but substitute sperm nuclei (see Basic Protocols 2 and 3) or DNA beads (see Basic Protocol 4) to promote spindle formation. Purified *Xenopus* sperm nuclei added to the CSF extract support assembly of monopolar spindles ("half-spindles"), which eventually fuse to form bipolar spindles. This protocol is useful as a fast and simple assay of spindle assembly in the extract. A more physiological method for assembling spindles involves cycling the extract containing sperm nuclei through interphase and back into mitosis, allowing the chromosomes and centrosome to duplicate (see Basic Protocol 3). After reentry into a mitotic state, the extract assembles true bipolar spindles. This protocol allows the researcher to study the importance of interphase events in spindle assembly. The last basic protocol (see Basic Protocol 4) describes a method for assembling spindles in extract around DNA-coated beads (for preparation see Support Protocol 5). These beads are sufficient to support spindle assembly in the extract and yet lack centromeric DNA sequences and centrosomes. This protocol, therefore, allows study of the spindle assembly process in a more simplified system that depends on the microtubule-stabilizing and -organizing capacity of mitotic chromatin.

NOTE: Schematic diagrams illustrating the anticipated time course of extract reactions are shown in Figure 11.13.1, while actual micrographs of asters and spindles are shown in Figure 11.13.2.

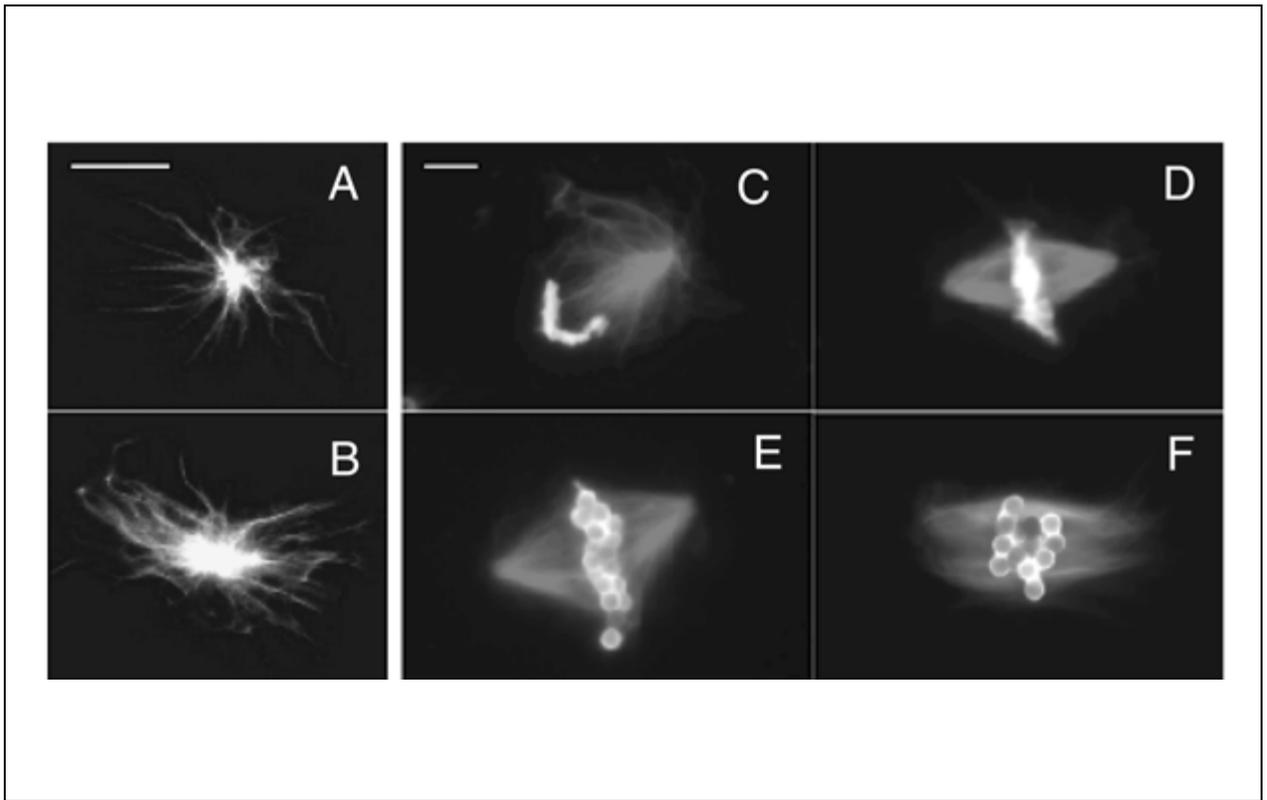


Figure 11.13.2 Examples of asters and spindles visualized by fluorescence microscopy. **(A)** Centrosome-nucleated aster. **(B)** DMSO aster. **(C)** Sperm half-spindle. **(D)** Sperm bipolar spindle. **(E)** DNA bead spindle. **(F)** DNA bead spindle assembled in the presence of antibody 70.1 that recognizes dynein intermediate chain. Microtubules are visualized by incorporation of rhodamine-labeled tubulin and appear gray. DNA is seen in the DAPI channel and appears white. Bar is equal to 5 μm .

1.5-ml microcentrifuge tubes

Wide-orifice 1- to 200- μl pipet tips

Water bath

Microscope slides and 18 \times 18-mm square coverslips

Fluorescence microscope with 40 \times or 63 \times lens and rhodamine/Hoechst filter sets

1. On ice, prepare a 1:10 dilution of rhodamine tubulin stock by adding 1 μl stock to 10 μl CSF extract. Prepare the reaction tube on ice by adding 1.25 μl of the 1:10 dilution of rhodamine tubulin to 25 μl CSF extract (1:20) in a 1.5-ml microcentrifuge tube to give a final reaction mixture with a tubulin dilution of 1:200.

IMPORTANT NOTE: Always mix and transfer extract and extract reactions with wide-orifice (or cut-off) pipet tips, and avoid generating bubbles. It is important to use 1.5-ml tubes and not let reaction volumes exceed 100 μl , to permit better gas exchange.

For multiple reactions, dilute labeled tubulin stock, to a concentration of 0.10 to 0.15 mg/ml (1:200), into the total volume of extract needed, then aliquot into 25- μl reactions.

2. Add 1.25 μl of anhydrous DMSO or purified centrosomes.

Centrosome stock should be $\sim 5 \times 10^8$ centrosomes/ml.

3. Prepare a 20 $^{\circ}\text{C}$ bath by adding sufficient ice to bring a room temperature bath to the appropriate temperature. Add additional ice, as needed, to maintain temperature.

IMPORTANT NOTE: Reactions are initiated by placing samples at 20 $^{\circ}\text{C}$. Do not place samples on ice before fixation, as this will cause the microtubules to depolymerize.

**In Vitro
Reconstitution**

11.13.3

- 4a. *For centrosome reactions:* Incubate for 5 to 15 min then take a “squash sample” by transferring 1 μ l of the reaction to a microscope slide. Carefully place 5 μ l of spindle fix on top of the drop of extract and squash by gently overlaying with an 18 \times 18-mm coverslip.

Always mix reactions by pipetting gently before removing “squash samples” for mounting on slides.

For best results, lower the coverslip at an angle and let it touch the extract/fixative drop before releasing. Because preservation of microtubule structures is somewhat variable, take duplicate samples.

Two or three coverslips can be fit side-by-side on each slide.

Analysis using the rhodamine channel should reveal red asters of microtubules emanating from single focal points. Aster size will increase with increasing incubation time.

- 4b. *For DMSO reactions:* Incubate for 5 to 30 min then take a 1- μ l sample of the reaction to fix and squash as described above.

At early time points extensive microtubule polymerization is apparent. By 20 min, asters have organized, and these contain many more microtubules than centrosome asters.

Analysis using the rhodamine channel should reveal red asters of microtubules emanating from single focal points. Aster size will increase with increasing incubation time.

5. Seal the edges of the coverslips with nail polish and store squash samples in the dark at 4°C.

Alternatively, reaction mixtures can be spun onto coverslips, fixed, and mounted (see Support Protocol 4).

Spin-downs and squash samples, if sealed, can be stored at 4°C for months to years after the experiment is performed.

6. Analyze on a fluorescence microscope with 40 \times or 63 \times lens and rhodamine/Hoechst filter sets.

Analysis using the rhodamine channel should reveal red asters of microtubules emanating from single focal points. Aster size will increase with increasing incubation time.

BASIC PROTOCOL 2

ANALYZING SPERM DNA “HALF-SPINDLE” REACTIONS

“Half-spindle” reactions provide a fast and easy assay for spindle assembly in an extract. This method of spindle assembly proceeds in a nonphysiological manner. The centrosome of a sperm nucleus nucleates a single microtubule aster that interacts with sperm chromosomes, forming a monopolar (half-) spindle. Half-spindles then fuse pairwise to form bipolar spindles; however, these reactions are still quite sensitive to perturbations in the extract, making them quite useful for studying the roles of specific proteins and factors in spindle assembly. CSF extract is combined with demembrated *Xenopus* sperm nuclei (for preparation, see *UNIT 11.10*) and rhodamine-labeled tubulin. Incubation at 20°C allows microtubule polymerization and spindle assembly. Fluorescence microscopy analysis of squash samples will reveal a few fully-formed bipolar spindles and many half-spindles as early as 15 to 30 min after the start of incubation; however, a reaction is not considered “complete” until at least 45 or 60 min. At this point, an unperturbed extract of good quality will have produced bipolar spindles around 75% to 95% of the sperm nuclei that it contains. Reactions can then be spun down onto coverslips for fixation, immunofluorescence analysis, and long-term storage (see Support Protocol 4).

Materials

- 20 to 30 mg/ml rhodamine tubulin stock (Murray, 1991)
- CSF extract (*UNIT 11.11*; also see Support Protocol 1)
- 20 \times demembrated sperm nuclei (~100 sperm/ μ l extract; *UNIT 11.10*)

Spindle fix (see recipe)

Nail polish

1.5-ml microcentrifuge tubes

Water bath

Microscope slides and 18 × 18-mm square coverslips

Fluorescence microscope with 40× or 63× lens and rhodamine/Hoechst filter sets

1. On ice, prepare a 1:10 dilution of rhodamine tubulin stock by adding 1 μl stock to 10 μl CSF extract. Prepare the reaction tube on ice by adding 1.25 μl of the 1:10 dilution of rhodamine tubulin to 25 μl CSF extract (1:20) in a 1.5-ml microcentrifuge tube to give a final reaction mixture with a tubulin dilution of 1:200.

IMPORTANT NOTE: Always mix and transfer extract and extract reactions with wide-orifice (or cut-off) pipet tips, and avoid generating bubbles. It is important to use 1.5-ml tubes and not let reaction volumes exceed 100 μl, to permit better gas exchange.

For multiple reactions, dilute labeled tubulin stock, to a concentration of 0.10 to 0.15 mg/ml (1:200), into the total volume of extract needed, then aliquot into 25-μl reactions.

2. Add 2.5 μl of 20× demembrated sperm nuclei (~100 sperm/μl extract).

More concentrated sperm stocks can be diluted to 20× in 100 mM KCl/150 mM sucrose/1 mM MgCl₂, flash frozen, and stored in aliquots at -80°C.

3. Incubate the reaction for 15 min in a 20°C water bath.

The bath is prepared by adding sufficient ice to bring a room temperature bath to 20°C (see Basic Protocol 1, step 3) and adding additional ice as necessary to maintain temperature.

4. Prepare a squash sample by transferring 1 μl of the reaction to a microscope slide. Carefully place 5 μl of spindle fix on top of the drop of extract and squash by gently overlaying with an 18 × 18-mm coverslip. Analyze samples on a fluorescence microscope with 40× or 63× objective and rhodamine/Hoechst filter sets. Seal with nail polish.

Always mix reactions by pipetting gently before removing “squash samples” for mounting on slides.

For best results, lower the coverslip at an angle and let it touch the extract/fixative drop before releasing. Because preservation of microtubule structures is somewhat variable, take duplicate samples.

Two or three coverslips can be fit side-by-side on each slide.

Analysis of early time points on a fluorescence microscope should reveal condensed chromatin structures in the channel associated with microtubules in early stages of organization visible in the rhodamine channel.

5. Take additional squash samples at 30, 45, and 60 min to assess spindle assembly as described above. Analyze samples, seal with nail polish and store in the dark at 4°C.

By 30 min, samples should contain condensed chromatin structures associated with astral arrays of microtubules focused into a single pole.

At 45 to 60 min samples should also contain half-spindles that have fused to produce bipolar spindles that resemble those produced by the more physiological “cycling” reactions described in the next protocol (see Basic Protocol 3). At this point the reaction is complete. Continued incubation will lead to the formation of large aggregates of spindles.

Alternatively, reaction mixtures can be spun onto coverslips, fixed, and mounted as described (see Support Protocol 4).

Spin-downs and squash samples, if sealed, can be stored at 4°C for months to years after the experiment is performed.

ANALYZING SPERM DNA “CYCLING” REACTIONS

This protocol provides an assay for the importance of interphase events (i.e., DNA replication, centrosome duplication, and chromatin packaging) in the process of spindle assembly. Unlike half-spindle reactions, this protocol cycles the extract through interphase and back into mitosis. Any sperm nuclei present during the reaction undergo chromatin decondensation, DNA replication, and centrosome duplication. Returning the extract to mitosis promotes spindle assembly through the more physiological pathway of microtubule nucleation and growth from duplicated centrosomes around a replicated set of chromosomes containing duplicated kinetochores.

In this protocol, as in half-spindle reactions (see Basic Protocol 2), CSF extract is combined with rhodamine-labeled tubulin and demembrated *Xenopus* sperm nuclei. After a 10-min incubation at 20°C, calcium is added to the reaction to promote entry into interphase. After 80 min of further incubation at 20°C, nuclei will appear large and round, and microtubules should be long and abundant. Addition of fresh CSF extract to the reaction drives the reaction back into mitosis and spindle assembly begins. Spindles are formed 45 to 60 min after addition of fresh CSF extract, and, as with previous reactions, can be spun down onto coverslips (see Support Protocol 4).

Materials

- 20 to 30 mg/ml rhodamine tubulin stock (see Support Protocol 2)
- CSF extract (*UNIT 11.11*; also see Support Protocol 1)
- 20× demembrated sperm nuclei (~100 sperm/μl extract; *UNIT 11.10*)
- 10× calcium solution (see recipe)
- Spindle fix (see recipe)
- 20°C water bath
- Microscope slides and 18 × 18–mm square coverslips
- Fluorescence microscope with 40× or 63× lens and rhodamine/Hoechst filter sets

Prepare reaction

1. On ice, prepare a 1:10 dilution of rhodamine tubulin stock by adding 1 μl stock to 10 μl CSF extract. Prepare the reaction tube on ice by adding 1.25 μl of the 1:10 dilution of rhodamine tubulin to 25 μl CSF extract (1:20) in a 1.5-ml microcentrifuge tube to give a final reaction mixture with a tubulin dilution of 1:200.

IMPORTANT NOTE: Always mix and transfer extract and extract reactions with wide-orifice (or cut-off) pipet tips, and avoid generating bubbles. It is important to use 1.5-ml tubes and not let reaction volumes exceed 100 μl, to permit better gas exchange.

For multiple reactions, dilute labeled tubulin stock, to a concentration of 0.10 to 0.15 mg/ml (1:200), into the total volume of extract needed, then aliquot into 25-μl reactions.

2. Add 2.5 μl of 20× sperm nuclei (~100 sperm/μl extract).
3. Incubate the reaction 10 min in a 20°C water bath.

The bath is prepared by adding sufficient ice to bring a room temperature bath to 20°C (see Basic Protocol 1, step 3) and adding additional ice as necessary to maintain temperature.

Release extract into interphase

4. Add 5 μl 10× calcium solution and mix well to release extract into interphase.

Calcium leads to the degradation of the cell cycle regulatory proteins cyclin B and CSF, causing inactivation of the mitotic cdc2 kinase and exit from mitosis.

5. Incubate the reaction 80 min in a 20°C water bath.

6. Transfer 1 μl of the reaction to a microscope slide. Carefully place 5 μl of spindle fix on top of the drop of extract and squash by gently overlaying with an 18 \times 18-mm coverslip. At 30 and 80 min after calcium addition, check that the extract is entering interphase. Analyze on a fluorescence microscope with 40 \times or 63 \times objective and rhodamine/Hoechst filter sets.

If sperm nuclei do not appear to be decondensing at 30 min after calcium addition, add an additional 2.5 μl (1/20 dilution) of 10 \times calcium.

By 80 min, nuclei are visible in the DAPI channel and should appear large, round, and uniform, while microtubules should be long and abundant.

Drive the reaction into mitosis

7. At 90 min post calcium addition, add 0.5 vol (25 μl) of fresh CSF extract to the reaction. Continue incubation at 20°C.

Addition of fresh extract will send the reaction back into mitosis.

8. Take squash samples at 15, 30, 45, 60, and 90 min after addition of fresh CSF extract to assess the spindle assembly reaction. Examine the samples using a fluorescence microscope with 40 \times or 63 \times objective and rhodamine/Hoechst filter sets.

Bipolar spindles should be visible by 45 min after addition of fresh CSF extract.

9. Seal the edges of the coverslips with nail polish and store squash samples in the dark at 4°C.

Alternatively, entire reaction mixtures can be spun onto coverslips, fixed, and mounted (see Support Protocol 4).

ANALYZING DNA-BEAD REACTIONS

This protocol assays the ability of an extract to support spindle assembly around chromatin in the absence of centrosomes or special chromosomal sequences. This reduces the system even more towards a minimal set of necessary components and allows for detection of otherwise subtle phenotypes (such as those resulting from the disruption of motor proteins) in the absence of the strong structural cues normally provided by the centrosomes.

DNA-coupled Dynabeads are retrieved on a magnet, washed with CSF extract to remove the storage buffer, and resuspended in fresh CSF extract. After 10 min of incubation at 20°C, the reaction is sent into interphase by the addition of calcium. Incubation for 2 hr at 20°C allows for proper assembly of chromatin on the beads. Addition of fresh CSF extract and incubation at 20°C for 30 min brings the reaction back into mitosis. The beads are retrieved using a magnet, or flash frozen for later use. After removal of the extract, the beads are resuspended in fresh CSF extract. The reaction is again incubated at 20°C. Bipolar spindles should form after 30 to 90 min of incubation, and, as with previous protocols, reactions can then be spun down onto coverslips for fixation, immunofluorescence analysis, and long-term storage.

Materials

- DNA beads (see Support Protocol 5)
- CSF extract (*UNIT 11.11*; also see Support Protocol 1) with and without 1:200 rhodamine-labeled tubulin (Support Protocol 2)
- 10 \times calcium solution (see recipe)
- Spindle fix (see recipe)
- 0.5-ml microcentrifuge tubes

BASIC PROTOCOL 4

In Vitro Reconstitution

11.13.7

Magnetic particle concentrator (MPC; Dynal)
20°C water bath
Microscope slides and 18 × 18–mm square coverslips
Fluorescence microscope with 40× or 63× lens and rhodamine/Hoechst filter sets

1. Transfer 3 µl of DNA beads (about 0.5 µg DNA) to a 0.5-ml microcentrifuge tube and place on a magnetic particle concentrator (MPC) on ice. Remove supernatant using a pipet. Be careful not to disturb the pellet. Wash beads by resuspending them in 20 µl of CSF extract.
2. Retrieve beads using the magnet, pipet off supernatant, and resuspend in 100 µl CSF extract. Transfer to a 1.5-ml microcentrifuge tube and incubate 10 min in a 20°C water bath.

The bath is prepared by adding sufficient ice to bring a room temperature bath to 20°C (see Basic Protocol 1, step 3) and adding additional ice as necessary to maintain temperature.

3. Send CSF extract into interphase by adding 10 µl of 10× calcium solution. Incubate for 2 hr in a 20°C water bath, mixing periodically.

Mixing the reactions periodically (every 20 to 30 min) is important to prevent aggregation of beads into large clumps.

4. Return the extract containing the beads to mitosis by adding 50 µl of fresh CSF extract. Incubate for 30 min at 20°C.

At this point the entire reaction can be flash frozen in liquid nitrogen and stored at –80°C. This provides a stock of preformed chromatin beads thereby avoiding the lengthy chromatin-assembly steps.

5. Incubate the bead mixture on ice for several minutes. Retrieve the beads by incubating in the MPC (magnet) on ice for 10 to 15 min.

Due to the high viscosity of the extract, bead retrieval is slow. To accelerate the process, mix by pipetting the mixture every few minutes, keeping the tube on the MPC.

6. Remove the supernatant with a pipet. Resuspend the beads in 100 µl of fresh CSF extract containing rhodamine-labeled tubulin at a 1:200 dilution. Incubate at 20°C.

7. Take squash samples to monitor the spindle assembly process by transferring 1 µl of the reaction to a microscope slide. Carefully place 5 µl of spindle fix on top of the drop of extract and squash by gently overlaying with an 18 × 18–mm coverslip. Analyze on a fluorescence microscope with 40× or 63× objective and rhodamine/Hoechst filter sets.

Spindle assembly requires between 30 and 90 min, depending on the extract.

8. Seal the edges of the coverslips with nail polish and store squash samples in the dark at 4°C.

Alternatively, entire reaction mixtures can be spun onto coverslips, fixed, and mounted (see Support Protocol 4).

PREPARATION OF CSF EXTRACT

The authors' method for extract preparation, presented here, is very similar to that presented in *UNIT 11.11*, but is somewhat more simple; i.e., oil is not used, eggs are crushed by centrifugation in a Sorvall HB-4 or HB-6 rotor, a second clarifying centrifugation is not performed, cytochalasin D is used instead of cytochalasin B, and a different mix of protease inhibitors is used. If the extract is to be cycled, EGTA should be omitted from the 20× energy mix.

Materials

Xenopus eggs, dejellied (*UNIT 11.10*)

Extract buffer (XB; see recipe)

CSF-XB (see recipe)

10 mg/ml LPC (see recipe)

10 mg/ml cytochalasin D in DMSO: store in aliquots at -20°C

20× energy mix (*UNIT 11.11*)

400-ml beaker

Polished, cut-off glass pipet

SW-50 tubes (Beckman)

13-ml adapter tubes (Sarstedt)

Clinical centrifuge

Sorvall centrifuge and HB-4 or HB-6 rotor with rubber adapters for 15-ml tubes

18-G needle and 1-ml syringe

Additional reagents and equipment for injecting frogs, and collecting and dejelling eggs (*UNIT 11.10*)

1. Inject frogs, collect eggs in 400-ml beaker, and dejelly (*UNIT 11.10*).
2. Wash dejellied eggs several times with 50 to 100 ml extract buffer (XB) and then twice with 50 to 100 ml CSF-XB (XB containing 5 mM EGTA and 1 mM MgCl_2). Swirl beaker gently and decant as much buffer as possible between washes.

Preparation of a good extract is critical for the success of in vitro aster and spindle assembly reactions. Pay close attention to the protocols describing how to collect, handle, and dejelly eggs (UNIT 11.10).

3. Perform one final wash with 50 ml CSF-XB to which LPC has been added to a final concentration of 10 $\mu\text{g/ml}$.
4. Using a polished, cut-off glass pipet, rinsed with CSF-XB, load eggs into SW-50 tubes containing 1 ml CSF-XB with 10 $\mu\text{g/ml}$ LPC, to which 10 μl of 10 mg/ml cytochalasin D has been added (100 $\mu\text{g/ml}$ final).

Immerse glass pipet into solution before expelling eggs, so that they do not contact air.

5. Transfer SW-50 tubes containing eggs to 13-ml Sarstedt adapter tubes containing 0.5 ml water to prevent tubes from collapsing.
6. Centrifuge in a room temperature clinical centrifuge at $150 \times g$ for 1 min, then at $700 \times g$ for 30 sec. Remove all buffer from the top of the packed eggs with a cut-off glass pipet.

Removal of all the buffer is essential to obtain concentrated cytoplasm.

7. Place the tubes in an HB-4 or HB-6 rotor containing rubber adapters. Centrifuge 15 min at $16,460 \times g$ (10,000 rpm), 16°C , to crush eggs.

- Place tubes on ice. Pierce tube at the bottom of the yellow cytoplasmic layer with an 18-G needle attached to a 1-ml syringe, with the opening directed upwards. Collect cytoplasmic layer.

Some of the grayish layer just below the cytoplasm should also be collected, but avoid the gray layer above the cytoplasm.

- Dilute 10 mg/ml LPC to 1:1000, 10 mg/ml cytochalasin D to 1:500, and 20× energy mix to 1× in the cytoplasmic layer.

If extract is to be cycled through interphase, omit EGTA from the 20× energy mix. The extract can be kept on ice for up to 6 hr before use.

SUPPORT PROTOCOL 2

PREPARATION OF RHODAMINE-LABELED TUBULIN

In the assays described in this unit, microtubules are visualized by incorporation of fluorescently-labeled tubulin subunits. This allows for easy analysis of the reaction without the need for immunostaining techniques. Combining a succinimidyl ester fluorochrome with pure bovine brain microtubules produces fluorescent tubulin derivatives (Hyman et al., 1991). The activated esters are thought to label random surface lysines and give good labeling stoichiometry and yield of functional tubulin. A variety of different fluorochromes can be used, including tetramethylrhodamine, X-rhodamine, Texas Red, and fluorescein. The following protocol requires 100 mg of purified tubulin for the eventual recovery of around 20 mg of labeled product.

Phosphocellulose column-purified tubulin is generally recovered and stored frozen in column buffer. After thawing the tubulin, 20× conversion buffer is added to convert this buffer to BRB80, a buffer that better promotes microtubule polymerization. Addition of GTP and glycerol, and incubation at 37°C, induces polymerization of the tubulin into microtubules. The microtubules are then pelleted, gently resuspended in labeling buffer, and incubated with the fluorochrome while still polymerized. After the addition of quenching buffer to end the reaction, the microtubules are again pelleted to remove unincorporated fluorochrome. The pellets are depolymerized and the resulting soluble tubulin subunits are separated from denatured protein by centrifugation. The recovered supernatants are incubated to again polymerize microtubules, which are then pelleted and depolymerized. After a final centrifugation, the tubulin-containing supernatants are frozen in small aliquots in liquid nitrogen and stored at –80°C until needed for use in extract reactions.

Materials

- 100 to 200 mg phosphocellulose column-purified tubulin, frozen (Ashford et al., 1998)
- 20× conversion buffer, ice-cold (see recipe)
- 200 mM GTP
- Glycerol, 37°C
- Labeling cushion (see recipe)
- Labeling buffer, 37°C (see recipe)
- Tetramethylrhodamine succinimidyl ester (Molecular Probes)
- Dimethyl sulfoxide (DMSO), anhydrous (Sigma)
- BRB80 cushion, 37°C (see recipe)
- Quenching buffer, 37°C (see recipe)
- IB, ice-cold (see recipe)
- 2× and 1× BRB80, ice-cold (see recipe for 5×)

37°C water bath
Ultracentrifuge with Ti50 rotor and polycarbonate screw-cap centrifuge tubes, prewarmed to 35°C (Beckman)
3-ml wide-orifice plastic transfer pipets
Wash bottle with H₂O
TLA100 table-top ultracentrifuge with TLA100.3 rotor, TL100 rotor, and polycarbonate centrifuge tubes, prewarmed to 35°C (Beckman)
Sonicator
Additional equipment and reagents for determining protein concentration by Bradford assay (*APPENDIX 3B*)

Prepare reaction mixture

1. Thaw phosphocellulose column-purified tubulin aliquots quickly in a 37°C water bath until mostly liquid. Transfer tubes to ice and measure the volume in a graduated cylinder.

The starting tubulin amount should be at least 100 mg.

2. Add 1/20 vol ice-cold 20× conversion buffer to convert the column buffer to BRB80.
3. Add 200 mM GTP to 1 mM, and 0.5 vol glycerol, 37°C.
4. Mix well and cover with Parafilm. Immediately transfer to a 37°C water bath and incubate for 45 min to polymerize tubulin.

Mix the tubulin periodically during this incubation. Bubbles should form and remain suspended in the viscous microtubule solution.

5. During the incubation, load polycarbonate screw-cap centrifuge tubes appropriate for a Ti50 rotor with 5 ml of labeling cushion. Prewarm along with Ti50 rotor to 37°C.

Perform first warm spin

6. Load polymerized tubulin on top of cushions with a 3-ml wide-orifice plastic transfer pipet. Ultracentrifuge 1 hr at 242,000 × g (50,000 rpm), 35°C.

Keep polymerized tubulin warm at all times, for best results perform this step in a 37°C warm room. Fill each tube with 5 ml sample.

Prewarm labeling buffer to 37°C at this step.

7. After centrifuging, keep tubes at 37°C. Aspirate down to the cushion. Wash the interface with water by filling each tube gently with water from wash bottle, then aspirate the wash and the cushion.

This step removes inactive tubulin subunits.

8. Resuspend pellets in a minimal volume (50 µl each) of labeling buffer, 37°C, by drawing up and down into a cut-off pipet tip, and keep at 37°C. Wash tubes with 100 µl of labeling buffer, 37°C, to recover the remaining tubulin. Combine all resuspensions and the wash. Note the total volume, which should be around 1.5 ml.

During this step, be sure to keep the microtubules at 37°C to maintain polymerization. The high pH of the labeling buffer is best for high-stoichiometry labeling, but it is not favorable for tubulin polymerization.

9. Prepare 100 mM tetramethylrhodamine succinimidyl ester in anhydrous DMSO. Add 1/10 volume 100 mM tetramethylrhodamine succinimidyl ester to the resuspended microtubules and vortex gently.

As an alternative to 100 mM tetramethylrhodamine succinimidyl ester, a different succinimidyl ester fluorochrome can be used instead.

Tetramethylrhodamine succinimidyl ester reacts with water and is not stable for storage.

10. Incubate 45 min at 37°C. Gently vortex the mixture every few minutes.

Vortexing is especially important during the first few minutes of incubation. Do not vortex vigorously or the microtubules will depolymerize.

11. Add 1.5 ml BRB80 cushion to polycarbonate tubes appropriate for a TLA100.3 rotor.

BRB80 cushion, rotor, and tubes should all be prewarmed to 37°C. During this step the quenching buffer can be prewarmed to 37°C as well.

12. Stop the labeling reaction (step 10) by adding 2 vol of quenching buffer, 37°C. Mix the reaction well and keep at 37°C.

Perform second warm spin

13. Load the mixture onto the prewarmed BRB80 cushions with a 3-ml wide-orifice plastic transfer pipet. Ultracentrifuge in a TLA 100.3 rotor for 30 min at $265,070 \times g$ (70,000 rpm), 35°C. After the centrifugation, begin cooling the centrifuge to 4°C.

14. Aspirate the supernatants, washing the interface as before (step 8). Place both the rotor and the tubes on ice.

15. Resuspend each pellet in 100 μ l ice-cold IB to depolymerize the microtubules. Pool the resuspensions. Draw up and down in a pipet to break up tubulin clumps. Incubate the mixture on ice for 15 min.

Depolymerization can be accelerated by gentle sonication on ice for 1 to 2 sec pulses.

16. Add 1 vol of ice-cold 2 \times BRB80, and sonicate briefly. Check the tubulin concentration by Bradford analysis (*APPENDIX 3B*). If it exceeds 30 mg/ml, dilute tubulin with 1 \times BRB80 to 25 mg/ml before proceeding to the next step.

Perform first cold spin

17. Load depolymerized tubulin into a tube appropriate for the TLA100.3 rotor on ice. Centrifuge 15 min at $265,070 \times g$ (70,000 rpm), 4°C. After the centrifugation, begin warming the centrifuge to 35°C.

18. Recover the supernatant and transfer to a 15-ml conical tube. Add 0.5 vol 37°C glycerol and GTP to 1 mM. After recovering the supernatants begin warming the rotor to 37°C.

19. Polymerize the microtubules by incubating 45 min at 37°C.

Mix the reaction periodically during this step.

20. During the incubation add 1.5 ml of BRB80 cushion to tubes appropriate for a TLA100.3 rotor, and begin warming to 37°C.

Perform third warm spin

21. Load the polymerized microtubules onto the prewarmed cushions with a wide orifice (i.e., a cut 200- to 1000- μ l pipet tip). Centrifuge 20 min at $265,070 \times g$ (70,000 rpm), 35°C. After the centrifugation, begin cooling the centrifuge to 4°C. Also begin cooling a TLA100 rotor and polycarbonate tubes to 4°C.

22. Aspirate the supernatants and resuspend each pellet in 100 μ l of ice-cold IB.

23. Add 1 vol cold 2 \times BRB80 and sonicate briefly. Check the concentration by Bradford assay (*APPENDIX 3B*). If necessary, dilute labeled tubulin to 30 mg/ml with cold 1 \times BRB80.

Perform final cold spin

24. Load mixture into TLA100 tubes on ice. Centrifuge 15 min at $265,070 \times g$ (70,000 rpm), 4°C.
25. Recover the supernatant on ice and determine tubulin concentration by Bradford assay (*APPENDIX 3B*), and record.
26. Freeze in 1- μ l and 50- μ l aliquots in liquid nitrogen and store at -80°C .

The 1- μ l aliquots are for use with extract reactions. The 50- μ l aliquots may be thawed when needed, split into 1- μ l aliquots, flash frozen, and stored at -80°C .

MOTOR DISRUPTION

The following protocol illustrates a technique that can be used to assess the function of an individual protein in spindle assembly using the *Xenopus* egg extract system. The protocol describes how to disrupt the microtubule-based motor cytoplasmic dynein, which is required for organization of spindle poles. Disruption is achieved by adding inhibitory antibodies, which have been shown to inhibit association of dynein to microtubules in spindles (Heald et al., 1997). Antibodies dialyzed against extract buffer (XB) can be added to the CSF extract either before initiating the reaction, or after microtubule structures have formed. The effects of dynein disruption are monitored by squash or spin-down analysis.

Materials

Dynein intermediate chain antibody 70.1 (Sigma), as ascites
Unreactive antibody (e.g., mouse IgG or ascites; Sigma)
Extract buffer (XB; see recipe)

12,000 to 14,000 mol.-wt. cut-off dialysis tubing
4°C microcentrifuge

Low-volume concentrators with mol. wt. 50,000 cut-off (Microcon)

1. Dialyze 0.5 ml of 70.1 ascites against two changes of 1 liter XB, 4 hr each, using 12 to 14 kDa cut-off dialysis tubing at 4°C. Concentrate to 15 to 20 mg/ml using microconcentrator in a microcentrifuge at 4°C.

Dynein inhibition requires a relatively high concentration of this antibody.

Dialysis into XB removes any sodium azide, buffer, or salts that could disrupt the extract.

2. Add 1.25 μ l diluted rhodamine tubulin and 2.5 μ l dynein intermediate chain antibody 70.1 to 25 μ l CSF extract (a dilution of 1:10). Mix well and incubate on ice for 10 to 20 min. Prepare a control sample containing a similar amount of control antibody such as mouse IgG in parallel.
3. Proceed with the aster or spindle assembly protocol (see Basic Protocols 1 to 4).

Antibody can also be added to extract after reactions are complete. For dynein inhibition the same effects are observed.

4. Analyze squash or spin-down samples taken at different time points, and compare the phenotypes observed in the antibody-treated samples with the control samples.

SUPPORT PROTOCOL 3

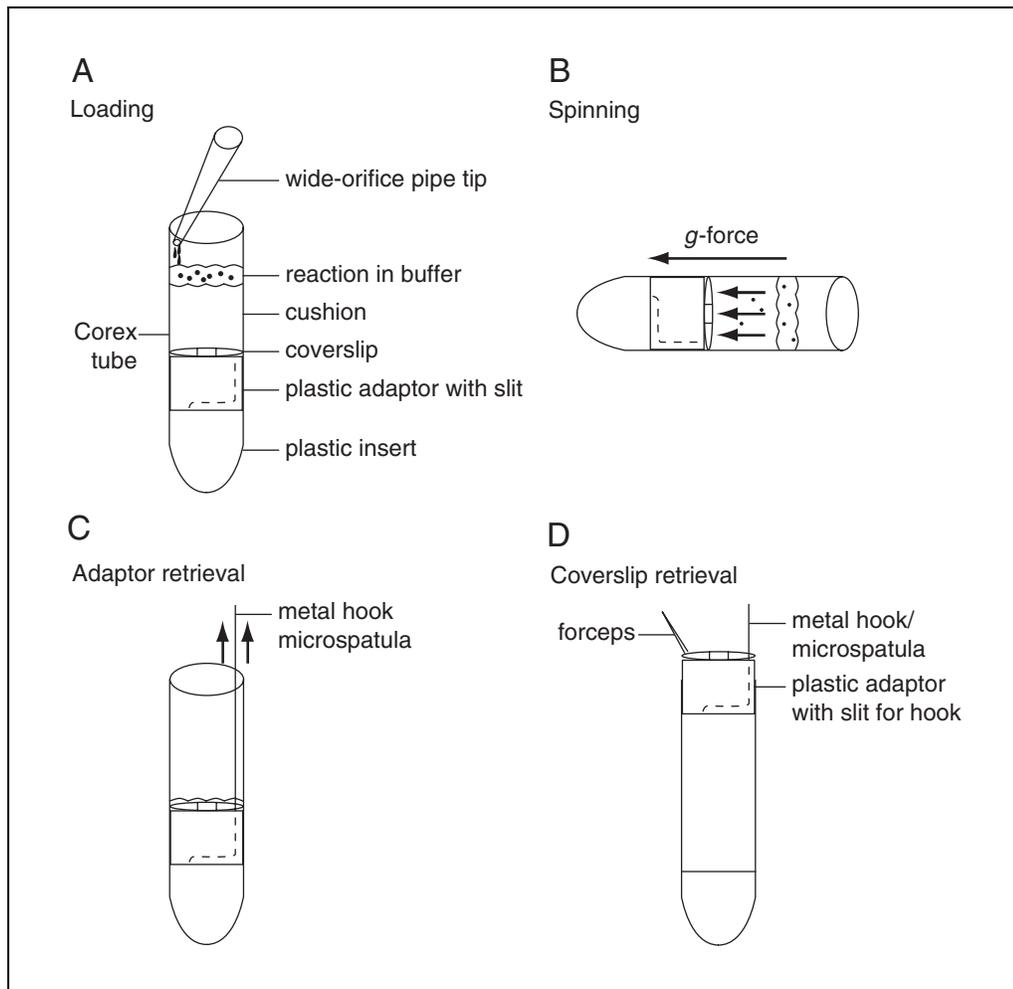


Figure 11.13.3 Schematic showing how spin-down tubes work to transfer spindles or asters from extract to coverslips for immunofluorescence and long-term storage. **(A)** Using a wide-orifice pipet tip, the extract reaction is layered on top of a glycerol cushion in a modified Corex tube containing a plastic insert and adapter with a coverslip on top. **(B)** Centrifugation in a swinging bucket rotor pellets asters and spindles through the cushion onto the coverslip. **(C)** After the cushion is aspirated, the adaptor is retrieved using a metal hook or spatula that fits into groove on one side of the adaptor. **(D)** The coverslip is retrieved from the top of the adaptor with forceps and transferred to -20°C methanol to fix.

**SUPPORT
PROTOCOL 4**

REACTION SPIN-DOWNS

Spin-downs allow an entire reaction mixture to be transferred onto glass coverslips, fixed, and, if desired, subjected to immunostaining techniques. Because reactions are diluted into detergent and spun through a cushion, much of the soluble material is removed, including unincorporated fluorochrome-labeled tubulin subunits. This procedure dramatically reduces background fluorescence compared to squash samples.

Reactions to be processed are first diluted with the appropriate spin-down dilution buffer. The mixture is then gently layered over the appropriate spin-down cushion in a modified Corex centrifuge tube containing a 12-mm coverslip (Mitchison and Kirschner, 1984; Fig. 11.13.3). Centrifugation in a swinging-bucket rotor pellets spindles and other insoluble structures like asters, free nuclei, and DNA beads onto the coverslips. The coverslips are then fixed in methanol, washed in PBS/0.1% NP-40, immunostained if necessary, stained with Hoechst DNA dye, mounted onto slides, and sealed with nail polish. Slides are then observed on a fluorescence microscope and, over the long-term, stored in the dark at 4°C .

Additional Materials (also see Basic Protocol 1)

Extract reaction
30% or 15% spin-down dilution buffer (see recipe)
40% or 25% spin-down cushion (see recipe)
Methanol, -20°C
Wash buffer: PBS, sterile/0.1% (v/v) NP-40
PBS, sterile/3% (w/v) BSA
Primary and secondary antibodies in PBS, sterile/3% BSA (e.g., GTU88 anti γ -tubulin and FITC-conjugated goat anti-mouse; Sigma)
10 mg/ml Hoechst dye, in water
Mounting medium (see recipe)

1.5-ml microcentrifuge tube
Modified 15-ml Corex tube containing 12-mm round coverslip (Aladin Enterprises; see Fig. 11.13.3)
Wide-orifice 200- to 1000- μl pipet tips
Sorvall centrifuge, HB-4, HB-6, or HS-4 rotor and appropriate rubber adapters
Metal hook or microspatula
Watchmaker's forceps
Ceramic staining rack
Glass staining dish
Incubation chamber: parafilm cut to fit in bottom of 150-cm round plastic tissue culture dish with lid

NOTE: When using this protocol for either type of aster reaction (see Basic Protocol 1), substitute 15% spin-down dilution buffer and 25% spin-down cushion in place of those called for below.

1. Transfer up to 50 μl extract reaction to a 1.5-ml microcentrifuge tube and add 1 ml of 30% spin-down dilution buffer.

Add the dilution buffer quickly and evenly to avoid clumping.

2. Layer the mixture over 5 ml of 40% spin-down cushion in a 15-ml modified Corex tube containing a 12-mm round coverslip using a wide-orifice 200- to 1000- μl pipet tip.

Use care when layering. The mixture will form a distinct layer over the cushion.

3. Centrifuge tubes 15 min at $17,000 \times g$ (10,000 rpm) in an HB-4 or HB-6 rotor, 16°C .

If large numbers of samples are generated, an HS-4 rotor with 16-tube capacity can be used. Run the HS-4 centrifuge at $5930 \times g$ (6,000 rpm) for 25 min, 16°C .

4. Aspirate supernatant and gently pull out plastic support with a metal hook or microspatula.

5. Pick up each coverslip off of support using watchmaker's forceps and place in ceramic staining rack in methanol, -20°C , in glass staining dish for 5 min.

Take care to be aware which side of the coverslip is facing up. Once the proper orientation has been lost it can be very difficult to reestablish.

6. Transfer each coverslip right side up to Parafilm in an incubation chamber, placing it in a drop of wash buffer. Gently push each coverslip through the drop so that it adheres to the Parafilm.

7. Block coverslips to reduce background by aspirating wash buffer and adding 200 μl sterile PBS/3% BSA to each coverslip. Incubate 10 min at room temperature.

8. *Optional:* If desired, stain coverslips with primary and secondary antibodies in PBS/3% BSA for 15 min each. After each antibody incubation wash coverslips four times with copious amounts of wash buffer.
9. Stain DNA with 10 mg/ml Hoechst dye diluted 1:2000 in wash buffer (5 µg/ml final) for 1 min. After four washes in wash buffer, place coverslips upside down on a 3-µl drop of mounting medium on microscope slide. Aspirate excess mounting medium and seal coverslip edges with nail polish. Store slides in the dark at 4°C for up to one year.

DNA-COATED BEADS

The use of bead-bound plasmid DNA allows for assays of spindle assembly in a more minimal system. DNA beads are produced using the high-affinity interaction between biotin and streptavidin molecules to couple plasmid DNA to paramagnetic Dynabeads. Large batches of DNA beads can be produced and stored for long periods at 4°C until needed for use in extracts.

Purified plasmid DNA is cut with restriction enzymes to produce a linear DNA fragment containing two sticky ends. Klenow is used to fill in one end with biotin-conjugated nucleotides, while the other is filled in with thionucleotides to help prevent exonuclease digestion. After removing enzyme and unincorporated nucleotides, the biotin-labeled DNA is combined with streptavidin-coated Dynabeads in a specialized binding solution. Overnight incubation at 16°C on a rotator provides sufficient time for binding. After incubation, the beads are washed, resuspended, and stored in bead buffer. The amount of DNA bound is quantified by spectrophotometric analysis of the binding reaction supernatant before and after incubation with the beads.

Materials

- 50 µg plasmid DNA (<5 kb) purified by column (Qiagen) chromatography
- Appropriate restriction enzymes (e.g., *NotI*, *BamHI*)
- TE buffer, pH 8, sterile (*APPENDIX 2A*)
- Klenow DNA polymerase (exo⁻) and buffer (New England Biolabs)
- Nucleotides: biotin-dATP (Life Technologies), biotin-dUTP (Clontech), thio-dCTP, and thio-dGTP (Pharmacia)
- G-50 Nick columns (Pharmacia)
- Washing and binding solutions from Kilobase BINDER kit (Dynal)
- Streptavidin Dynabeads from Kilobase BINDER kit (Dynal)
- Bead buffer (see recipe)

- Magnetic particle concentrator (MPC; Dynal)
- Rotator at 16°C

- Additional reagents and equipment for ethanol precipitation and restriction endonuclease digestion of plasmid DNA (see *APPENDIX 3A*), and quantification by absorption spectroscopy (*APPENDIX 3D*)

Prepare plasmid DNA

1. Prepare plasmid DNA by Qiagen-style column purification.

While the sequence of the DNA is not important, the plasmid should be more than 5 kb to effectively induce chromatin assembly in extracts.
2. Cut 50 µg of the DNA with two restriction enzymes (see *APPENDIX 3A*) that have unique sites in the polylinker to produce one short and one long DNA fragment.

The choice of the enzyme should be such that one end of the long fragment terminates in an overhang containing guanines and cytosines, while the other contains only adenines and thymidines (e.g., NotI, BamHI).

3. Ethanol precipitate the linearized DNA (see APPENDIX 3A), and resuspend in 25 μ l TE buffer, pH 8. Quantify recovery by A_{260} measurement (APPENDIX 3D).
4. Prepare fill-in reaction containing 1 \times Klenow buffer, 30 μ g linearized DNA, 50 μ M nucleotides (biotin-dATP, biotin-dUTP, thio-dCTP, and thio-dGTP), 20 U Klenow polymerase, and sufficient H₂O to make the final volume 70 μ l. Incubate for 2 hr at 37°C.
5. Remove unincorporated nucleotides and small DNA fragments, using a Pharmacia G-50 Nick column, following manufacturer's instructions. Quantify recovery by A_{260} measurement (APPENDIX 3D).

The DNA is eluted in a large volume (400 μ l), but the recovery is better than with spin columns.

Couple DNA to beads

6. Prepare coupling mix by combining 400 μ l biotinylated DNA (step 4) and 400 μ l binding solution. Set aside 25 μ l of the coupling mix for later evaluation of coupling efficiency.
7. Prepare 4 μ l of streptavidin Dynabeads for each microgram of DNA to be coupled according to manufacturer's instructions.
8. Retrieve beads using the magnetic particle concentrator (MPC). Wash once with 5 vol binding solution (600 μ l for 120 μ l beads). Retrieve the beads and resuspend them in coupling mix containing DNA.
9. Incubate bead/coupling mixture for several hours (or overnight) on a rotator at 16°C.

Retrieve and wash beads

10. Retrieve the beads using the MPC. Save the supernatant. Compare the A_{260} of the supernatant to that of the sample taken before coupling (step 3) to determine the amount of DNA immobilized.

Typically two-thirds of the DNA is coupled.

11. Wash beads twice with 500 μ l washing solution, and then twice with 500 μ l bead buffer. After the last wash, resuspend the beads in sufficient bead buffer such that the final concentration of immobilized DNA is 1 μ g/5 μ l of beads. Store at 4°C for up to one year.

If beads are "clumpy," pass through a 12-G needle.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Bead buffer

- 29.2 g NaCl (2 M final)
- 0.30 g Tris base (10 mM final)
- 73 mg EDTA, free acid (1 mM final))
- 200 ml H₂O
- Adjust pH to 7.6 with 2 M HCl
- H₂O to 250 ml
- Store up to 1 year at room temperature

BRB80, 5×

30.24 g PIPES free acid (0.4 M final)
0.26 ml 4.9 M MgCl₂ (5 mM final; Sigma)
0.48 g EGTA (5 mM final)
200 ml H₂O

While stirring, add KOH pellets until the PIPES dissolves. Adjust to pH 6.8 with 10 M KOH. Add H₂O to 250 ml. Sterilize by filtration. Store up to 1 year at 4°C.

BRB80 cushion

60 ml glycerol (60% v/v final)
20 ml 5× BRB80 (1× final; see recipe)
20 ml H₂O
Sterilize by filtration
Store up to 1 year at 4°C

Calcium solution, 10×

1 ml 400 mM CaCl₂ (4 mM final)
20.4 μl 4.9 M MgCl₂ (1 mM final; Sigma)
0.75 g KCl (100 mM final)
H₂O to 100 ml
Store in aliquots indefinitely at -20°C

Conversion buffer, 20×

47.6 g PIPES free acid (630 mM final)
857 μl 4.9 M MgCl₂ (16.8 mM final; Sigma)
0.5 ml 0.5 M EGTA (1 mM final)
200 ml H₂O

While stirring, add KOH pellets until the PIPES dissolves. Adjust the pH to 6.8 with 10 M KOH, and bring the volume to 250 ml with water. Sterilize by filtration. Store up to 1 year at 4°C.

CSF-XB

198 ml extract buffer (XB; see recipe)
2 ml 0.5 M K-EGTA (5 mM final); store indefinitely in 2-ml aliquots at -20°C
40.8 μl 4.9 M MgCl₂ (1 mM final; Sigma)
Prepare fresh on day of use

The 0.5 M K-EGTA is made by dissolving 9.51 g EGTA in 40 ml water, pH is adjusted to 7.7 with 10 M KOH, and the volume is adjusted to 50 ml.

Extract buffer (XB)

25 ml 20× XB salts (see recipe)
12.5 ml 2 M sucrose (50 mM final); store in aliquots at -20°C
5 ml 1 M HEPES (0.1 M final), adjusted to pH 7.7 with 10 M KOH
H₂O to 500 ml
Make up fresh on day of use

IB

1.852 g monopotassium glutamate (50 mM final)
15 mg free glutamic acid (0.5 mM final)
20.4 μl 4.9 M MgCl₂ (Sigma; 0.5 mM final)
H₂O to 200 ml
Sterilize by filtration
Store in 50-ml aliquots indefinitely at -20°C

Labeling buffer

40 ml glycerol (40% v/v final)
2.38 g HEPES, free acid (0.1 M final)
20 μ l 4.9 M MgCl₂ (1 mM final)
200 μ l 0.5 M EGTA (1 mM final)
20 ml H₂O

Adjust the pH to 8.6 with 10 M NaOH. Add water to 100 ml. Sterilize by filtration.
Store up to 6 months at 4°C.

Labeling cushion

60 ml glycerol (60% v/v final)
20 ml 0.5 M HEPES (0.1 M final)
20 μ l 4.9 M MgCl₂ (1 mM final)
200 μ l 0.5 M EGTA (1 mM final)
Adjust pH to 8.6 with 10 M NaOH

H₂O to 100 ml
Sterilize by filtration
Store up to 6 months at 4°C

LPC, 10 mg/ml

Combine leupeptin, pepstatin, and chymostatin to a concentration of 10 mg/ml each in DMSO. Store in aliquots at -20°C.

Mounting medium

9 ml glycerol (90% v/v final)
1 ml 0.2 M Tris·Cl, pH 8 (APPENDIX 2A; 10% v/v or 0.02 M final)
Store up to 1 year at room temperature

Quenching buffer

20 ml 5× BRB80 (2× final; see recipe)
20 ml glycerol (40% v/v final)
5 ml 1 M potassium glutamate (100 mM final)
H₂O to 50 ml
Make up fresh on day of use

Spin-down dilution buffer, 30%, 15%

30 ml glycerol (30% v/v final)
1 ml Triton X-100 (1% v/v final)
20 ml 5× BRB80 (1× final)
H₂O to 100 ml
Store up to 1 year at room temperature

For 15% spin-down dilution buffer, only add 15 ml glycerol.

Spin-down cushion, 40%, 25%

200 ml glycerol (40% v/v final)
100 ml 5× BRB80 (1× final)
H₂O to 500 ml
Sterilize by filtration

Store up to 1 year at 4°C

For 25% spin-down cushion, only add 125 ml glycerol.

Spindle fix

600 μ l 80% glycerol (48% v/v final)
300 μ l 37% formaldehyde (11% final)
100 μ l 10 \times MMR (1 \times final; *UNIT 11.10*)
0.5 μ l 10 mg/ml Hoechst dye (5 μ g/ml final; Sigma)
Store up to 1 week at room temperature

Best prepared fresh on day of use.

XB salts, 20 \times

74.6 g KCl (2 M final)
2.04 ml 4.9 M MgCl₂ (Sigma; 20 mM final)
147 mg CaCl₂ (2 mM final)
H₂O to 500 ml
Sterilize by filtration
Store up to 1 year at 4°C

COMMENTARY

Background Information

The function of the mitotic spindle is essential for proper segregation of chromosomes during cell division. At the onset of mitosis, the radial interphase array of microtubules breaks down, and then forms a bipolar spindle around condensed chromosomes. Microtubules from each pole attach to sister chromatids and align them in the center of the spindle before segregating them to opposite poles during anaphase. Spindle assembly is a complex process, requiring intricate regulation of microtubule dynamics (Andersen, 1999; Walczak, 2000) and of microtubule and chromosome movements mediated by multiple different microtubule-based motor proteins (Heald and Walczak, 1999; Mountain and Compton, 2000).

Spindle assembly and function has been studied in a variety of model organisms, including genetic systems such as yeast, *C. elegans*, and *Drosophila*, and in cultured cells; however, *Xenopus* egg extracts provide the only system in which spindle assembly has been reconstituted in vitro. This is possible because eggs are synchronized in the cell cycle, and concentrated cytoplasm can be obtained without harsh disruption techniques. Pioneered by Lohka, Masui, and Maller (Lohka and Masui, 1983; Lohka and Maller, 1985), *Xenopus* egg extracts have been optimized to study cell cycle transitions (Murray and Kirschner, 1989), spindle assembly (Sawin and Mitchison, 1991), and anaphase (Shamu and Murray, 1992; Murray et al., 1996), as well as many other cellular processes (see *UNITS 11.10 to 11.12*).

Xenopus eggs are arrested in metaphase of meiosis II, a “mitotic-like” division, by cytostatic factor (CSF), which is thought to be the

product of the *c-mos* protooncogene (Sagata et al., 1989). This calcium-sensitive activity is preserved if extracts are prepared in the presence of EGTA, a calcium chelator. CSF-arrested extracts allow experimentation in mitotic cytoplasm, without transiting through the cell cycle. CSF extracts can be induced to cycle by the addition of calcium, which causes the degradation of CSF and cyclin B, thereby inactivating the mitotic cdc2 kinase and promoting entry into interphase.

The power of egg extracts derives from the extensive structural and functional analyses, facilitated by the “open” nature of the system, which nevertheless maintains many properties of intact cytoplasm. Individual components can be manipulated in order to compromise their function by techniques such as immunodepletion (*UNIT 11.10*) or antibody addition (see Support Protocol 3). The excellent cytology of the system permits detailed analysis of the effects of the disruption on spindle assembly. In addition, the system has been ideal for dissecting subsets of processes required for spindle assembly, including microtubule nucleation, dynamics, chromosome condensation, and kinetochore assembly (Belmont et al., 1990; Verde et al., 1990; Hirano and Mitchison, 1991; Zheng et al., 1995; Desai et al., 1997).

The protocols in this unit highlight several of the reactions possible in *Xenopus* egg extracts, and serve to illustrate some of the mechanisms underlying spindle assembly. Microtubule nucleation and organization can be assayed using aster reactions (see Basic Protocol 1); however, there is an important distinction between the two agents used to induce aster formation. Centrosomes are found in most so-

matic cells and serve as focal microtubule nucleation sites that organize spindle poles. In contrast, DMSO, like the microtubule-stabilizing drug taxol, leads to random polymerization of microtubules, which are subsequently organized into focused asters in mitotic cytoplasm (Verde et al., 1991). This process is dependent on microtubule-based motors, including cytoplasmic dynein (Heald et al., 1997) and is thought to mimic the formation and organization of spindle poles (Gaglio et al., 1996, 1997).

Reactions containing *Xenopus* sperm nuclei (Basic Protocols 2 and 3) introduce another level of complexity to the reaction. The centrosome associated with each sperm nucleus nucleates microtubules that interact with sperm chromosomes. In a CSF extract, this leads directly to the formation of mitotic structures. If the reaction is cycled through interphase, the chromosomes and centrosomes duplicate and subsequent spindle assembly is more physiological. DNA bead spindle assembly reactions (see Basic Protocol 4) are similar to female meiosis in that both pathways occur in the absence of centrosomes and illustrate the microtubule-stabilizing force of mitotic chromatin (Heald et al., 1996). Such a mechanism of spindle assembly has been termed “self-organization,” because obvious bipolar cues are absent. This system has been particularly useful in dissecting the roles of different microtubule-based motors in setting up the bipolar spindle structure (Walczak et al., 1998).

Therefore, CSF extracts provide a system to examine both centrosome-directed assembly and the self-organization mechanisms of spindle assembly. This system is also amenable to immunofluorescence techniques. Spindle assembly reactions can be easily transferred from reaction tubes onto coverslips, fixed, and stained for desired components.

Critical Parameters and Troubleshooting

The single most important factor in successful *in vitro* spindle assembly reactions is the quality of the extract; therefore, special attention should be paid to protocols outlining frog handling and extract preparation (UNIT 11.10). Once prepared, extract should always be transferred with wide-orifice pipet tips to minimize disruption. Vortexing or any violent agitation of extract or reactions should always be avoided. Flash-frozen extracts can be used in these assays, but generally give less reproducible results. Freeze/thawing can disrupt proteins and rupture vesicles, both of which lower

the quality of the extract, and can result in poorly organized microtubule structures or loss of CSF arrest.

The authors find that the most common problem with these assays relates to the inconsistency of the extracts. Although the quality of eggs is by far the most important parameter for a good extract, it is nevertheless problematic to predict when an extract will be of sufficiently low quality to preclude use in these assays. When setting up an experiment involving manipulation of the extract, it is advisable to first run a standard half-spindle reaction to assess the quality of the extract. Running such a reaction will reveal the level of “mitotic” arrest, and the speed and quality of spindle assembly, before extensive time or scarce resources are invested. If assays that require cycling the extract through interphase are to be run, it is advisable to run a test “cycling” reaction with sperm nuclei. After the normal addition of calcium, squash samples can be taken to monitor the extract’s ability to properly enter interphase, which is not always reproducible. In this case, a second addition of calcium may be made 10 to 30 min after the first addition. Non-extract-related problems frequently involve observations such as high background in spin-downs or squashes, absence of structures in squash samples, and the “stickiness” of DNA beads. High background in squash samples can be caused by a concentration of rhodamine-labeled tubulin in the reaction that is too high, the reagent should then be used at a higher dilution. High background in spin-down samples normally results from improper immunofluorescence techniques and can usually be remedied in subsequent reactions by titration of the antibodies used and more extensive washes or blocking incubations. A lack of observable structures in squash samples usually results from inadequate mixing of reactions before taking samples, as spindles tend to settle to the bottom of the tube. Problems with “stickiness” and aggregation of DNA beads can be avoided by occasional mixing of bead reactions during both the chromatin assembly and spindle assembly steps. Additionally, if a particular batch of DNA beads has proven to be quite “sticky,” they should be passed through a 27-G needle immediately before being used for “bead spindle” reactions.

Anticipated Results

The consistency of results from these assays depends upon the quality of the CSF extracts, which is variable even under normal circum-

stances, as *Xenopus* have seasonal variations in egg yields. Extracts may exhibit differences in the amount of time necessary for spindle assembly, and microtubule and chromosome morphology can also vary from experiment to experiment; therefore, reactions should always be repeated several times to ensure interpretable results. With experience, a user of the system can run assays that show clear and reproducible differences between control and experimental reactions. With good extracts and effective disruption of target proteins, these assays can provide up to hundreds of scorable structures (spindle or aster) per coverslip.

Time Considerations

The assays described in this unit work best with freshly prepared extracts, which require ~2 hr of preparation. Once extracts are prepared, the assays generally take from 15 min for aster reactions, to 4 hr for bead-spindle reactions, to run to completion. Chromatin beads can also be prepared ahead of time and stored frozen in extract, reducing the time for this assay to that of a sperm spindle reaction. If reactions are spun down, an additional 30 to 60 min must be added depending upon whether immunofluorescence is also performed. Spindowns and squash samples, if sealed, can be stored in the dark at 4°C for months to years after the experiment is performed.

Preparation of DNA beads is generally a 2-day project only because of the need for long incubations for restriction digests and coupling. Once prepared, the beads can be kept for months to years at 4°C without significant loss of activity in extracts.

The rhodamine-labeled tubulin preparation requires considerable attention and a full day to complete, and, for best results, it should not be performed simultaneously with other experiments.

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Key References

Murray, 1991. See above.

This article documents the Xenopus system and is the basis for many of the protocols presented here.

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Analysis of RNA Export Using *Xenopus* Oocytes

UNIT 11.14

Microinjection of *Xenopus* oocytes has long been a tremendously useful tool in the study of nucleocytoplasmic transport, as well as many other aspects of intracellular function. A mature *Xenopus* oocyte (stage V, VI) is an extremely large cell (~1 mm). Indeed, following maturation, the oocyte (now egg) will respond to fertilization by dividing into thousands of embryonic cells with no significant increase in total cellular volume. The large size of the *Xenopus* oocyte makes it possible to quantitatively address questions in cell biology on a single-cell basis. For instance, in the case of nucleocytoplasmic transport, it is uniquely possible to definitively monitor the nucleocytoplasmic distribution of a wide range of transport substrates simultaneously in individual cells. When studying RNA export, this panel typically includes transcripts that represent different classes of RNA, such as tRNA, snRNA, and mRNA. The experimental strategy is straightforward: transport cargos of choice are injected into either the nucleus or cytoplasm; then, at different points over time, each oocyte nucleus is dissected away from its respective cytoplasm. These physically divided pools of nuclear and cytoplasmic components are rapidly processed and analyzed for specific proteins or RNAs.

This unit will focus on the analysis of RNA export using oocyte microinjection. The procedure for the injection itself is described along with a method for recovering RNA from oocyte nuclear and cytoplasmic fractions (Basic Protocol). Obtaining the oocytes is a simple surgical procedure and is also presented (Support Protocol 1). Additional information is included to describe how RNA cargo can be synthesized and labeled in vitro prior to injection (Support Protocol 2).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer; also see *APPENDIX 1D*.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: Experiments involving RNA require careful technique to prevent contamination (see *APPENDIX 2A*). All equipment should be RNase free and all solutions should be RNase free or made with DEPC-treated water.

ANALYSIS OF RNA EXPORT IN MICROINJECTED *XENOPUS* OOCYTES

Although the nucleus is not visible in an intact oocyte, material can be delivered specifically to this intracellular compartment because the position of the nucleus is relatively fixed within the cell (Fig. 11.14.1). Thus, by orienting the oocyte with respect to the needle and then pushing the needle a fixed distance into the oocyte, there is a high probability of hitting the nucleus. One procedure that can be used to ensure that the nucleus is in an obvious place is to centrifuge the oocytes. This causes the nucleus to rise to the top of the oocyte (just under the animal pole). It also can cause a slight clearing in the pigment granules at this central point, creating a bull's eye for the injection needle. This makes injections initially easier to master; however, with practice, this step is not necessary (it simply takes a little more effort to learn how far the needle must penetrate the oocyte when the nucleus has not been spun to the surface). In either case, it is important

**BASIC
PROTOCOL**

**In Vitro
Reconstitution**

11.14.1

to include markers that will confirm a nuclear hit. One marker is a visual tag, namely a high-molecular-weight dye. This allows the accuracy of the hit to be assessed at the time of dissection. Depending on the experimental design, more than one dye can be used in successive injections to create a color mix indicative of two nuclear hits. In the case of RNA export, the other useful marker is an RNA that is not an export (or import) cargo. Selective recovery of this marker with the nuclear fraction confirms that RNA was delivered to the nucleus and that the dissection was performed accurately.

Not only does the large size of the *Xenopus* oocyte facilitate the process of nuclear microinjection, but this feature is also key to the analysis of RNA export following microinjection. To analyze the localization of RNA, each oocyte is physically dissected into nuclear and cytoplasmic fractions, and RNA is prepared from these fractions. To begin this process, individual oocytes are retrieved, blotted dry, and then placed under oil prior to dissection. The nucleus is removed by puncturing a small hole in the middle of the animal hemisphere and then applying slight pressure to the oocyte with forceps. Due to the relatively light density of the nucleus compared to other contents of the cell, it will emerge first from this hole. A needle can then be used to guide the nucleus away from the cytoplasm. Performing this dissection under oil ensures that there will be no loss of material from the nucleus during the process (Lund and Paine, 1990). The nucleus and cytoplasm can then be pipetted out of the oil and into a microcentrifuge tube containing the appropriate buffer. Proteins are removed by a combination of proteinase K digestion and phenol extraction. The RNA is then precipitated and electrophoresed on a denaturing acrylamide gel.

Materials

- RNA sample (see Support Protocol 2)
- Xenopus* oocytes (see Support Protocol 1)
- Light mineral oil (e.g., Fisher Scientific)
- RNA harvest buffer (see recipe)
- 4 mg/ml proteinase K solution: 1:4 (v/v) 20 mg/ml proteinase K (e.g., Ambion, Worthington) in RNA harvest buffer, prepared fresh
- 5:1 (v/v) acid phenol/chloroform, pH 4.5 (Ambion)
- 20 mg/ml glycogen or seedNA (Amersham Pharmacia Biotech)
- RNA sample buffer (see recipe)
- Glass capillary tubes, 1-mm o.d., 0.75-mm i.d., 10-cm length (e.g., Sutter Instruments)
- Needle-pulling apparatus (e.g., Sutter Instruments micropipet puller, model P-87)
- Pico-injector, basic model (e.g., Model PL1-100, Medical Systems Corp.)
- Nitrogen

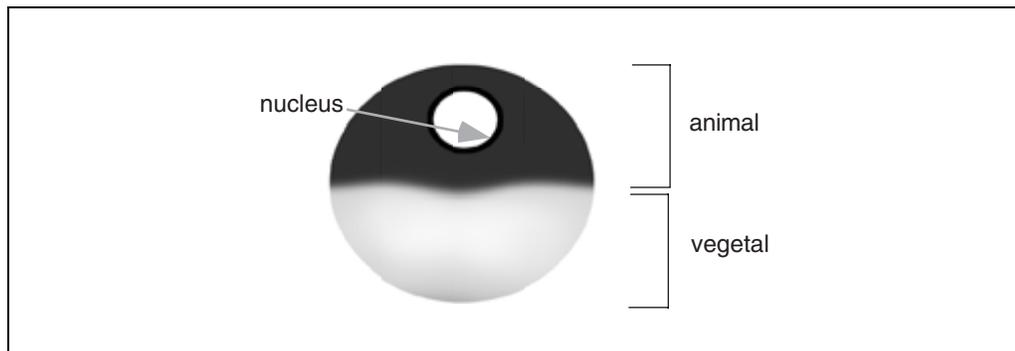


Figure 11.14.1 Stage VI oocyte with approximate location of nucleus and animal and vegetal hemispheres indicated.

Dumont no. 3 and Dumont SS forceps (Fine Science Tools), including one pair of no. 3 forceps set aside for use with oil
Dissecting microscope (e.g., MZ7.5, Leica) with optional cold stage (e.g., Labworks # Equipment Service, Inc.; also requires circulating cold bath), 4°C 18°C incubator
Disposable transfer pipets (e.g., Fisher Scientific)
Whatman 3MM filter paper, cut into ~0.5-in × 3-in strips
Mid-sized polystyrene weighing dishes
1-ml syringes
27.5-G needles
100- μ l glass capillary tubes with 25- μ l microcaps (e.g., Drummond Scientific)
Additional reagents and equipment for phenol/chloroform extraction, ethanol precipitation (APPENDIX 3A), and polyacrylamide gel electrophoresis of RNA (UNIT 6.1)

Make injection needle

1. Wearing gloves to protect against RNase contamination, clamp a glass capillary tube into a needle-pulling apparatus. Adjust the apparatus and make needles with a 20- μ m-diameter tip.

This type of instrument automatically heats the capillary tube and pulls it in two. Each half is then considered a needle and has an extremely fine tapered point.

There may be some variability in settings, depending on the instrument and the heating filament. With the Sutter Flaming/Brown micropipet puller, model P87, the authors have used the following parameters with success: P100, heat 340, velocity 120, time 150, and pressure 225.

It is best to do this step ahead of time. Several needles can be pulled and then stored indefinitely in a large plastic petri dish. The needles should be anchored by embedding the center of the needle in a small piece of clay affixed to the dish. Depending on design (i.e., whether successive injections, different cargo, etc), each experiment may take 4 to 10 needles. It is possible to reuse needles after extensive rinsing with DEPC-treated water. The needles do break easily, but with experience this becomes less of a problem.

2. Mount a needle into the injection arm of a pico-injector according to manufacturer's instructions. Place the threaded metal cuff over the back of the needle, followed by a freshly cut silicon gasket. Push the needle into the arm until gasket seals. Then slide the metal collar onto the injection-arm threads and screw firmly in place.

These directions will vary with the specific type of injection apparatus.

3. Gently break the sealed tip of the needle by grasping the tip with a pair of Dumont no. 3 forceps and gently turning them perpendicular to the needle.

The opening at this point should be <20 μ m. The seal must be broken before the sample can be loaded.

4. Test the seal by pulling up some sterile water into the needle and then ejecting the water completely.
5. Spot ~3 μ l RNA sample onto a piece of Parafilm and draw sample up into the needle using the load feature of the pico-injector. Avoid air bubbles.

Alternatively, the needle can be backfilled using a gel-loading tip before it is mounted on the injection arm. If the needle is immobilized in an upright position for several minutes, the sample will slide into place at the tip through capillary action.

6. Adjust the size of the needle tip such that the ejected volume is 5 to 20 nl by successively breaking the tip with forceps and checking the ejected drop size under a microscope. Use the eye-piece reticule to compare the drop to a previously calculated conversion between drop diameter and volume.

The desired ejected volume depends on the particular injection. Although the cytoplasm can be injected with up to 50 nl, it is best to divide this total volume into more than one drop or injection.

Drop size is dependent on three main factors: the pressure of nitrogen (or air) in the output, the duration of the output, and the needle tip size. Generally, for an ~7-nl drop, the authors keep the pressure and time at ~15 psi and 100 msec, respectively, although these parameters can be used to make fine adjustments in volume.

Some pico-injectors also have a back-pressure feature that allows a constant low-level pressure to be continuously applied. This prevents dilution of the sample that can result from small amounts of buffer influx through the needle tip due to capillary action. Depending on the viscosity of the sample, back pressure is generally ~1 psi but should be adjusted empirically such that a minimal amount of sample flows out of the needle to counteract capillary flow into the needle.

There are alternative methods of perfecting the needle. One important technique is beveling the needle. This procedure can be found in Terns and Goldfarb (1998).

Inject oocytes

7. Chill *Xenopus* oocytes ~10 min on ice.

Chilling the oocytes serves two purposes: (1) it stops active nucleocytoplasmic transport, allowing the transport experiment to be synchronized by the shift back to 18°C, and (2) it causes microtubules to depolymerize, allowing the nucleus to move towards the surface of the oocyte, which creates a more reproducible target.

If not using a cold-stage, it is usually sufficient to work with ice-cold buffer and inject oocytes in small enough groups that they are returned to 4°C or 18°C without significant warming. Alternatively, an outer petri dish with ice-water can be used to keep the oocytes in chilled buffer if this is critical (for many experiments, the kinetics are slow enough that this is not a critical point).

Oocytes can also be centrifuged (15 min, 1000 × g) to ensure that the nucleus is directly under the outer surface of the oocyte (Terns and Goldfarb, 1998). However, this will also cause the nucleus to flatten out, restricting the range of depth for an accurate nuclear injection.

8. Bring a petri dish with oocytes into the field of view of the microscope and push the oocytes into a group at one side of the dish using a pair of Dumont SS forceps.

Alternatively, a mesh grid can be used to organize and orient the oocytes.

9. Hold one oocyte with forceps and orient the animal hemisphere (dark pigmented half) toward the needle such that the central axis of the oocyte is aligned with the path of the needle. For cytoplasmic injection, aim to hit the vegetal hemisphere, to assure avoiding the nucleus.

The yield of RNA recovery per oocyte is quite efficient, so knowing the detectability of the input material should allow one to almost directly extrapolate to the number of oocytes needed in the harvest. One to two oocytes worth of material is typically sufficient for one lane of a gel. Since it is good to have duplicate samples and since not every injection will lead to a clean hit and a clean dissection, it is recommended to inject at least 10 to 12 oocytes per sample, more if multiple time points are being analyzed.

10. Lower the needle to the oocyte and advance the needle into the oocyte.

This works best when a bit of pressure is applied to the oocyte with the forceps, creating a taut surface.

11. Deliver material by depressing the foot pedal of the pico-injector.

12. Retract needle and push oocyte to opposite side of the petri dish.

13. Repeat process on remaining oocytes. Return oocytes to an 18°C incubator until the desired time point (typically between 30 min and 18 hr).

Some experiments require harvesting oocytes injected with the same material at different time points, in order to analyze kinetics.

Intermittently between injections, a drop of sample should be ejected directly into the buffer to confirm that the needle has not become blocked. With experience, observing this drop also gives a rough idea of the ejection volume. If there is any concern that the volume has decreased, the drop size should be measured at high magnification.

Dissect oocytes

14. Using a disposable transfer pipet, place a few oocytes onto a strip of Whatman 3MM filter paper and allow buffer to absorb.
15. Gently nudge an oocyte with a second strip of filter paper until the oocyte sticks to the second strip. Using Dumont no. 3 forceps set aside for working with oil, immediately place oocyte into a mid-sized polystyrene weighing dish containing a layer of light mineral oil. Repeat until six or seven oocytes are in the oil.

It is important to remove the right amount of liquid at this point, as any delay in transfer of the oocyte will result in dehydration, but premature transfer of the oocyte will result in a residual aqueous layer that is problematic during dissection. It may help to have a drop of oil on the forceps used to transfer oocytes from the filter paper to the oil.

16. To dissect the nucleus away from the cytoplasm, hold the oocyte with the same forceps and puncture the center of the animal hemisphere using a 27.5-G needle attached to a 1-ml syringe.
17. Gently squeeze the oocyte with the forceps until the nucleus emerges. Use the back side of the needle to brush the nucleus away from the cytoplasm.

Some practice is required to know how much pressure to apply during dissection. The size of the hole punctured in the oocyte is important. It should be large enough to allow the nucleus to pass through intact and yet small enough to exclude cytoplasm while the nucleus is emerging.

18. Using a 100- μ l glass capillary tube and 25- μ l microcap, transfer nucleus and remaining cytoplasm (enucleated oocyte) to separate microcentrifuge tubes containing 165 μ l RNA harvest buffer. Keep samples on ice (if processing within the hour or so) or frozen until ready for further processing.

The samples are relatively stable once frozen, but for longer-term storage, proceed through RNA harvest step. Even then, analysis within a few days is optimal due to the radioactive nature of the sample.

The cytoplasm can also be transferred with forceps.

Process RNA

19. Add 55 μ l of 4 mg/ml proteinase K solution to each tube. Vortex and place at 37°C for 90 min. During the first 60 min, vortex periodically.

Repeated vortexing is especially important for the cytoplasmic samples.

20. Extract samples with 5:1 acid phenol/chloroform, pH 4.5.

To ensure that the interface is completely avoided, it is advisable to carry forward only 90% of the sample after extraction.

21. Ethanol precipitate RNA (APPENDIX 3A). Include a carrier such as 20 to 50 μ g glycogen or seeDNA, especially in the nuclear fractions.

**SUPPORT
PROTOCOL 1**

22. Air dry pellets and resuspend in RNA sample buffer (generally, 10 µl per oocyte).

RNA in sample buffer can be stored for several weeks, but again since it is radioactive, it is advised to analyze it within a few days if possible or up to 2 weeks.

23. After heating the sample 5 min at 68°C and vortexing, analyze RNAs by electrophoresis on a denaturing polyacrylamide gel (UNIT 6.1).

It is highly recommended to quantitate these results using a phosphorimager.

PREPARATION OF *XENOPUS* OOCYTES FOR MICROINJECTION

The ovary lies spread beneath the ventral surface of the female frog. The ovary consists of a mixed population of oocytes, representing various stages of maturation, that are held in loose proximity--much like a cluster of grapes. A small section, or lobe, of the ovary can easily be retrieved through a small incision towards the side of the abdomen. The desired amount of ovarian tissue is then clipped off the ovary and the incision sutured. The clump of oocytes is next teased apart and the immature oocytes are sorted away from the larger, mature oocytes.

Materials

Female *Xenopus laevis* (e.g., Nasco, Xenopus I, Xenopus Express)
0.05% (w/v) benzocaine (Sigma) in H₂O from 10% (w/v) stock in ethanol, stored at 4°C.
MBS (see recipe), 4°C
Toothed and Dumont no. 3 forceps (Fine Science Tools)
Surgical scissors
Sutures, Vicryl 4.0 (Ethicon) with attached needle
Hemostat
Recovery tank with lid
1-ml syringes
18-G needles bent into triangular loops
Disposable transfer pipets (e.g., Fisher)
18°C incubator

Make surgical incisions

1. Wearing gloves, place a female *Xenopus laevis* in a 4-liter beaker with 1 liter of 0.05% benzocaine for 10 min to anesthetize. Check efficacy of anesthetic by pinching the frog's toes.

The frog should remain flaccid.

2. Place frog on its back on a paper towel soaked in 0.05% benzocaine and spread on top of ice chips.
3. Make a small (~1 cm) incision with the corner of a razor blade in the skin towards the lower side of the abdomen. To facilitate the incision, grip the skin with a pair of toothed forceps and then start the incision between the prongs of the forceps.

A small amount of bleeding sometimes occurs and can simply be blotted with a tissue. At this point, it is usually possible to see the oocytes underlying the next layer of abdominal wall.

4. Using a pair of Dumont no. 3 forceps, grab the inner abdominal muscle wall, lift it up, and cut it with a pair of surgical scissors (so that this incision aligns with the initial one through the skin).

Again, this can be a small incision (slightly <1 cm).

Harvest ovarian lobe

5. Reach through the incision with the forceps. Retrieve a lobe of the ovary by closing the forceps and gently pulling them out through the incision.

Retrieving a lobe should be possible even if the view of the oocytes is somewhat obscured.

6. Clip off the desired amount of ovary with scissors and place in ice-cold MBS. Gently ease the remainder of the lobe back in through the incision.

At this point, the benzocaine-soaked towel can be removed.

Suture incisions

7. To suture the inner incision, draw a needle and Vicryl 4.0 suture through the layers of the wall on each side of the incision using a hemostat. Pull until there is only a short (~2 cm) piece of thread remaining on the opposite side.

8. Using the hemostat, loosely wrap the long piece of thread twice around the pair of forceps. Grab the short end of the thread with the forceps. Pull on thread wrapped around the forceps and slip it off the forceps to make a knot. Cut the ends of the thread close to this knot.

9. Repeat steps 7 and 8 one to two more times to suture the inner incision. Then repeat the same steps to suture the incision in the skin.

Alternatively, it is possible to suture both layers with the same stitches.

Because the skin can be more difficult to push the needle through, it helps to brace against the force with forceps that are spread such that the needle pushes between the two arms of the forceps.

10. Rinse frog in frog tank water and move it to a recovery tank with a shallow layer of water. Initially prop its head up out of the water.

Usually, the anesthetic wears off after 20 to 60 min and the frog can then be returned to a normal tank. It is advisable to monitor the frog an additional 12 hr or so before returning it to the colony.

A lid with air holes should be kept on the recovery tank as some frogs will try to jump out as soon as they recover.

Collect and sort oocytes

11. Using two pairs of Dumont no. 3 forceps, grab two parts of the outer layer of tissue that encloses the ovary. Pull forceps apart to partially invert the ovary and expose the oocytes.

12. Using the forceps, pull the ovary apart into smaller pieces (~50 oocytes each). Rinse chunks of ovary by swishing through ice-cold MBS and place in a clean petri dish with ice-cold MBS.

These clumps can be stored for a couple of days. In some cases they can be used for up to a week following the surgery; in this case, the buffer should be changed daily.

13. Using a 1-ml syringe attached to an 18-G needle bent into a triangular loop, press down on a section of stalk that attaches one oocyte to the rest of the ovary. Grab an adjacent piece of this connective tissue with no. 3 forceps and pull gently, detaching the oocyte from the ovary. Repeat until the desired number of oocytes are freed from the ovary.

14. Sort the oocytes, keeping only those that are large (stage V and VI) and in perfect condition. Use a disposable transfer pipet to place these in a new petri dish with fresh, ice-cold MBS and store overnight in an 18°C incubator.

Smith et al. (1991) provides illustrations of oocyte stages. Oocytes at earlier stages of maturation are also used in some types of experiments (Feldherr et al., 1998).

Oocytes with mottled pigmentation or any scarring where the stalk was detached should be discarded. Sometimes a small tear in the follicle layer will cause a slight bulge in the oocyte at this point. There is nothing wrong with these oocytes per se, but these bulges make the oocytes prone to breaking when they are blotted dry, so they are best discarded. Also, oocytes that appear to be softening (sometimes this is not obvious until dissection) should be discarded.

Some groups remove the layer of follicle cells that encase each oocyte. This can either be done manually or by collagenase treatment (Terns and Goldfarb, 1998). Removal of the follicle layer decreases resistance to the needle during injection. It is also useful for some procedures, such as when endogenous material is being examined. However, defolliculated oocytes tend to be more difficult to handle during the dissection.

SUPPORT PROTOCOL 2

PREPARATION OF RNA FOR INJECTION

RNA export cargo is most easily monitored by the injection of radioactively labeled RNA. Such RNA can be generated in vitro following standard procedures for in vitro transcription driven by bacteriophage polymerases (UNIT 11.6). In some cases, it is important to include a nucleotide cap analog in order to mimic the bona fide 5' end of a typical polymerase II transcript. During the analysis, this structural feature will contribute to RNA stability as well as to transport. When choosing which RNAs to monitor for export, an important point to keep in mind is that some steps in RNA biogenesis--such as splicing and export--are coupled (Luo and Reed, 1999). This may impact the decision of how an RNA should be produced for a particular experiment (i.e., with or without an intron). It should also be noted that as an alternative to injecting RNA cargo directly, oocytes can be injected with plasmids in conjunction with radioactive nucleotide. In this case, the plasmid template should contain a gene under the control of a eukaryotic RNA polymerase promoter. It is also possible to monitor the export of endogenous RNAs labeled in the presence of radioactive nucleotide. This procedure has been done to monitor the export of ribosomal RNAs (Powers et al., 1997). Although there are specific circumstances when these alternatives would be desirable, they do involve the injection of more radioactive material than in the case of the labeled RNAs.

Table 11.14.1 Reaction Mixture for RNA Labeling^a

Ingredient	Amount in one reaction	Stock concentration
ATP, CTP, UTP	0.5 μ l	5 mM each
DEPC-treated H ₂ O	to 10 μ l total	—
DNA template, linearized ^b	1-2 μ l	250-500 ng/ μ l
GTP	0.5 μ l	1 mM
m ⁷ GpppG ^c	1 μ l	5 mM
[³² P]GTP	3 μ l	3000 Ci/mmol
RNA polymerase buffer	1 μ l	10 \times
RNase inhibitor	0.25 μ l	40 U/ μ l
SP6 or T7 RNA polymerase ^d	0.5 μ l	20 U/ μ l

^aTo transcribe multiple RNAs, make a mix of common components and distribute this pool to tubes containing reaction-specific ingredients, such as DNA template.

^bTemplate DNA can be either a linearized plasmid or a PCR product (where the 5' oligo used to generate the PCR product contains the appropriate promoter sequence).

^cInclude only for capped RNA.

^dThe choice of polymerase is dependent on the type of promoter in the particular construct being used.

Materials

5 mM each ATP, CTP, UTP
20 U/μl SP6 or T7 RNA polymerase and 10× buffer
DEPC-treated H₂O (APPENDIX 2A)
250 to 500 ng/μl DNA template, linearized (APPENDIX 3A)
1 mM GTP
5 mM m⁷GpppG (for capped analogs only)
3000 Ci/mmol [³²P]GTP
40 U/μl RNase inhibitor (e.g., RNasin, Promega)
5 M ammonium acetate
20 mg/ml glycogen or seeDNA (Amersham Pharmacia Biotech)
70% and 100% (v/v) ethanol
Blue dextran (avg. mol. wt. 2,000 kDa; e.g., Sigma-Aldrich) or rhodamine B dextran (mol. wt. 70 kDa; e.g., Molecular Probes)

1. Set up reaction mixture in a microcentrifuge tube to include components listed in Table 11.14.1. Mix well and incubate 60 min at 37°C.
2. Add the following and mix well:

16 μl 5 M ammonium acetate
60 μl DEPC-treated H₂O
1 μl 20 mg/ml glycogen or seeDNA
240 μl 100% ethanol.

Again, when processing multiple samples it is easiest to make a master mix of these reagents.

Some protocols include DNase treatment and phenol/chloroform extraction (APPENDIX 3A) before precipitation.

3. Chill on ice ≥15 min. Pellet in a microcentrifuge ~30 min at maximum speed, 4°C.
4. Wash twice with 70% ethanol at room temperature.
5. Resuspend in 15 μl DEPC-treated water.
6. To analyze RNA, make a 1:15 (v/v) dilution in DEPC-treated water and quantitate the radioactivity in a 2-μl aliquot using a scintillation counter.

It is also useful to electrophorese 2 μl of the dilution on a 6% denaturing gel (UNIT 6.1). After a quick exposure to film (~30 min), the RNA can be assessed for size and uniformity.

7. Prepare the mixture of RNAs and dyes to be injected. For nuclear injections, add blue dextran or rhodamine B dextran to a final concentration of ~6 mg/ml or ~4 mg/ml, respectively.

Generally, 0.5 to 2 × 10⁶ cpm/μl of an RNA is used for an injection. A quick phosphorimager scan of a gel with the newly-synthesized RNAs can be used to generate the desired ratio of RNAs when injecting a mixed population. This avoids the problem of counting residual free nucleotide, which can be variable. In terms of absolute levels of RNA, typically total levels of RNAs should be in the low femtomole range to prevent saturation of an export pathway.

A high-molecular-weight dye can be mixed into the sample to monitor the injection accuracy (see Basic Protocol introduction). There are alternative markers for a nuclear hit, such as colloidal gold (Bataille et al., 1990).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Modified Barth's solution (MBS; Sive et al., 2000)

88 mM NaCl
1 mM KCl
2.4 mM NaHCO₃
15 mM HEPES
0.74 mM CaCl₂
0.82 mM MgSO₄

Autoclave and store up to 6 months at room temperature or 4°C

Add penicillin/streptomycin (Life Technologies) to 100 U/ml before use

HEPES is not included in all MBS recipes. The original recipe had a mix of CaCl₂ and Ca(NO₃)₂.

RNA harvest buffer

100 mM Tris-Cl, pH 7.6 (APPENDIX 2A)
10 mM EDTA, pH 8.0
300 mM NaCl
2% (w/v) SDS
Store up to 6 months at room temperature

RNA sample buffer

1× TBE buffer (APPENDIX 2A) containing:
10 M urea
0.25% (w/v) bromphenol blue
0.25% (w/v) xylene cyanol
Store at room temperature

COMMENTARY

Background Information

Oocyte microinjection studies have laid much of the groundwork for our understanding of the nuclear pore complex. Among the many important functional parameters that have been elucidated by this approach are the size limits of both diffusion and active transport through the pore (Bonner, 1975; Feldherr et al., 1984) and the identification of steps in the transport of different cargo that are either unique or shared with other pathways through the pore (Jarmolowski et al., 1994; Pokrywka and Goldfarb, 1995). Injection experiments have also been extremely useful in delineating both the *cis* signals within RNA and the *trans*-acting factors that are required for efficient export (e.g., Guddat et al., 1990; Fischer et al., 1995). More recently, microinjection studies have been used to address the specific roles of particular pore proteins (Stutz et al., 1996; Powers et al., 1997; Ullman et al., 1999).

The experimental strategies made possible by oocyte microinjection take advantage of the ability to survey a wide variety of transport

cargo simultaneously in an intact cell. The function of individual components of the transport machinery can be probed with reagents ranging from antibodies to dominant-negative recombinant proteins.

As both the nuclear pore and the soluble accessory factors involved in transport become understood at a molecular level, the oocyte will continue to be an indispensable tool in the field of nucleocytoplasmic transport. Of course, this method is most powerful when complemented with other approaches. For instance, examining a specific interaction biochemically *in vitro* provides critical substantiating evidence for a functional relationship that is identified through microinjection analysis. Similarly, structural and biochemical studies of the pore and the soluble factors involved in transport provide important clues to function that can then be tested in an *in vivo* setting. Thus, in combination with other techniques, further analysis of transport in the oocyte will be used to gain insight into how the transport machinery

coordinates the multitude of trafficking events that occur each minute through each pore.

Critical Parameters

One critical parameter for oocyte microinjection is simply the quality of the oocytes. There is a certain amount of variability from frog to frog and, however frustrating this may be, it is well worth demanding a high standard at the very first step of oocyte isolation. In other words, if the oocytes cannot be recovered from the ovary without damage or if they have any abnormalities in their pigment distribution in the first place, try a new frog! Once you find a frog that has good oocytes, this is usually a reproducible attribute, and the frog can be operated on several times (with a week or so of rest in between).

Some problems with the oocytes are not immediately evident. This is one reason why it is beneficial to store the oocytes overnight before use and cull those that do not remain stable during this time. Finally, it pays to inject more oocytes per experimental point than you actually want to harvest. Then, at harvest time, you can once again select only those oocytes that have remained stable through the treatment.

Another important parameter to consider is the amount of RNA that you inject. Export pathways are saturable. Thus, too much of any one RNA will result in a much slower apparent export rate. It is important to note that this problem can actually be used to an advantage. Saturation of a particular pathway with unlabeled RNA has been used to identify steps in transport that are shared by more than one substrate (Jarmolowski et al., 1994; Pokrywka and Goldfarb, 1995). This strategy has also been employed to purposefully create a situation where a particular factor is limiting, allowing an add-back experiment to be performed where a candidate factor is tested for its ability to overcome the saturation (Gruter et al., 1998; Kutay et al., 1998).

Finally, it is important to strictly control the drop size and needle tip size. Given that the volume of an oocyte nucleus is approximately equivalent to 40 nl, it is best if the volume of the injected material does not exceed 10 to 12 nl. This is a somewhat arbitrary cutoff, and some experiments have been done successfully with larger volumes; however, erring on the side of caution in terms of maintaining nuclear integrity is advisable. The same can be said for needle size. The authors have found that a 10-nl volume generated by a burst of ~100 msec with ~15 PSI pressure translates into a needle that is

fine enough for these experiments. The question of whether both drop size and needle size are within acceptable parameters can be assessed empirically by including appropriate controls for injection and dissection.

Troubleshooting

The needle becomes clogged during the injection procedure. There could be particulate matter in the injection material. Clear the solution with a high-speed spin prior to loading the needle. Sometimes the needle clogs simply because the sample at the tip has dried. To circumvent this, minimize the amount of time that the tip of the needle is out of the buffer solution. When the needle has clogged due to a dry tip, it can often be cleared by advancing and retracting the needle in and out of the buffer system.

The needle won't penetrate into the oocyte. The layer of follicle cells can sometimes make the oocyte somewhat resistant to injection. In most cases, this is easily overcome simply by slightly squeezing the oocyte with forceps to counter the pressure of the needle. If this does not work, make a slightly larger needle tip. If this is still a general problem, either bevel the needle or defolliculate the oocytes (Terns and Goldfarb, 1998). The latter procedure may lead to more difficulty in handling the oocyte at later steps.

U6 snRNA (or U3 snRNA) is entirely or partially in the cytoplasmic fraction. These two RNAs do not get exported and therefore can serve to confirm that the nucleus was both selectively injected and cleanly dissected. If these RNAs do not appear exclusively in the nuclear fraction, either the injection or dissection was not clean. Looking at the distribution of pre-mRNA and the splice products can help narrow the problem. Whether due to injection or dissection, the solution is more practice!

Pre-mRNA is in the cytoplasmic fraction. These two RNAs do not get exported and therefore can serve to confirm that the nucleus was both selectively injected and cleanly dissected. If these RNAs do not appear exclusively in the nuclear fraction, RNA was injected into the cytoplasm. Practice making nuclear injections with blue dextran dye. It is possible to get some export of pre-mRNA when there is a large excess of RNA injected. Injecting less pre-mRNA (that has a greater specific activity) may help. Also note that pre-mRNA containing a specific *cis* element (such as the constitutive transport element from type D retroviruses) can in fact be actively exported. Make sure that

there has not been an error in the templates used for generating injection RNAs.

The intron is in the cytoplasmic fraction. This indicates that there was a problem in the dissection, because the intron is generated only in the nucleus and is not exported. Again, more practice is necessary. Watch carefully for the entire nucleus to extrude followed by a small amount of cytoplasm. Also see comment about *cis* elements above.

After the injections, the oocytes seem mushy. These oocytes may not have been of good enough quality from the beginning. Sorting the oocytes very carefully is important. Switching frogs might also overcome the problem. Alternatively, the needle tip used in the injection may have been too large. Try breaking needles closer to the tip to create a finer opening and adjusting the pressure and time of the pico-injector to compensate. It also might help to divide the injection volume into two drops (or more for cytoplasmic injections).

RNA harvested from oocytes has a smeary appearance on gels. If the injected RNA looks good (eliminating an early problem with degradation), then this indicates that the RNA is being degraded at some point in the procedure. If this seems specific to mRNA and U1 RNA, the cap analog may not have been incorporated. Double-check concentrations used; try fresh stock if nothing else looks incorrect. Otherwise, there may be a general problem with degradation during the procedure. Try making clean, fresh solutions.

An oocyte has protruding bulges. This indicates that there is a rupture in the follicle layer. Be extremely gentle when pulling each oocyte off of the stalk that holds it in the ovary. In some cases, the oocytes are particularly difficult to isolate without damage. If this is the case, the simplest solution is to try another frog.

Anticipated Results

Once this procedure is up and running, it should yield a very definitive answer to the question of where different RNA export cargos localize under conditions of interest. By harvesting the oocytes at multiple time points, it is also possible to assess the kinetics of export, which may prove to be an important parameter. By altering the sequence of the RNA itself, the *cis* signals that contribute to the export of an RNA can be readily assessed. Just as importantly, mutational analysis of the RNA can be used to delineate which features contribute to the retention of certain RNAs.

Time Considerations

The entire procedure typically takes a minimum of 3 days: (1) a day to synthesize and double-check the RNA cargo (surgery can be performed the same day); (2) a day to perform the microinjections and harvest the oocytes (time points will range from minutes to a few hours); (3) a day to prepare and analyze the RNA distribution. It often makes sense to do >1 day of injections in between day 1 and day 3, because ultimately this is more time efficient than having to do a second operation to get more oocytes for a second injection.

It is also important to consider the amount of time it takes to learn this technique. It will take a few to several weeks to develop the eye-hand coordination and the experience needed to reproducibly inject the nucleus (and note that when using oocytes that have not been spun, the hit rate can vary from 70% to 90% even with experience). It also takes some practice to dissect the oocytes--mostly this is the experience it takes to know how much to squeeze the oocytes to get the nucleus to extrude as well as how big a hole to puncture in the first place. It is advisable to start by injecting blue dextran as a way to practice both the injection and the dissection.

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Key References

Lund and Paine, 1990. See above.

Describes the specific steps and advantages of dissecting oocytes under oil.

Terns and Goldfarb, 1998. See above.

Covers many technical aspects of oocyte microinjections, including a detailed description and illustration of suggested equipment.

Internet Resources

<http://oacu.od.nih.gov/ARAC/oocyte.htm>

*This site contains Animal Research Advisory Committee guidelines for oocyte harvest from *Xenopus laevis*.*

<http://froglab.biology.utah.edu/Oogenesis/oogenesis.html>

This site contains a summary of the different stages of oocyte maturation.

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In Vitro Analysis of Peroxisomal Protein Import

This unit describes a quantitative in vitro assay for peroxisomal protein import. The assay (outlined in Fig. 11.15.1) is ELISA-based and employs semi-permeabilized human cells and a biotinylated import substrate (see Basic Protocol). In the Basic Protocol, import is assessed directly in cells; in the Alternate Protocol, import is quantitated after isolation of cellular organelles/peroxisomes. A method for preparing biotinylated luciferase is also described (see Support Protocol).

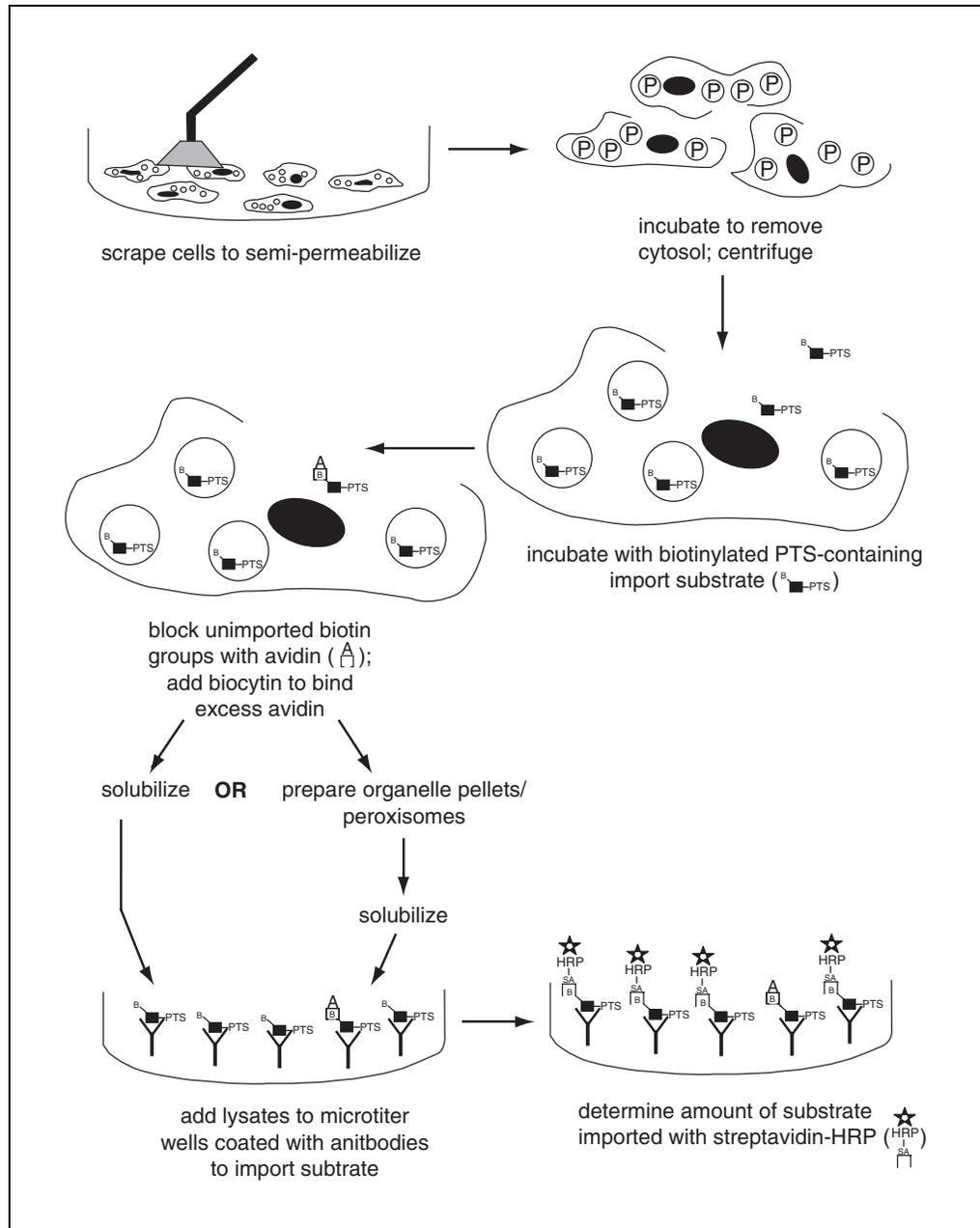


Figure 11.15.1 Quantitative in vitro assay for peroxisomal protein import. A, avidin; B, biotin; HRP, horseradish peroxidase; P, peroxisome; PTS, peroxisomal targeting signal; SA, streptavidin.

PEROXISOMAL PROTEIN IMPORT IN VITRO

Semi-permeabilized cells are incubated with import substrate, biotinylated luciferase. After the import reaction, cells are isolated and biotin groups on unimported substrate blocked. Cells are then lysed and import quantified by ELISA.

Semi-permeabilized cells are incubated with import substrate, biotinylated luciferase, and then unimported substrate is blocked. The extent of import is quantified by ELISA.

Materials

Rabbit polyclonal *APPENDIX 2A* anti-luciferase antibodies (*UNIT 16.2*)
ELISA coating solution: 50 mM sodium carbonate, pH 9.0
ELISA blocking solution (EBS; see recipe), pH 7.4
Human epidermoid carcinoma (A431; ATCC CRL-1555) cells or diploid lung (IMR90; ATCC CCL-186) fibroblasts growing in tissue culture
Hanks balanced salt solution (HBSS, Life Technologies; *APPENDIX 2A*)
Dulbecco's minimum essential medium containing 10% (defined) fetal bovine serum (FBS) (DMEM-10)
Import reaction buffer (IRB, see recipe), pH 7.4
ATP and regenerating system (ATP, see recipe)
800 μ M ZnCl in IRB
Biotinylated luciferase (B-Luc, see Support Protocol)
IRB containing 1.25% or 0.2% (w/v) bovine serum albumin (IRB/1.25% BSA or IRB/0.2% BSA, respectively)
Avidin (Calbiochem)
Biocytin (Calbiochem-Novabiochem)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), pH 7.4
Streptavidin horseradish peroxidase (Roche Diagnostics)
30% (v/v) hydrogen peroxide (H₂O₂)
o-Phenylenediamine
ELISA development buffer: 27 mM citric acid/51 mM sodium phosphate, pH 5.0
4 N sulfuric acid (H₂SO₄)

ELISA microtiter well strips (Maxisorp-Immunomodule, Nunc)
15-cm cell culture dishes (Corning)
Rubber policeman
50-ml conical centrifuge tubes (Corning)
Platform rocker
1.5-ml microcentrifuge tubes (Eppendorf)
37°C water bath
Microtiter plate washer (optional)
Microtiter plate reader

Prepare semi-permeabilized cells

1. Coat microtiter wells with antibody by adding 200 μ l of a 1:500 dilution of rabbit polyclonal anti-luciferase antibodies in ELISA coating solution to ELISA microtiter well strips and incubating overnight at 4°C.

Determine optimal concentration of coating antibody (and biotinylated ligand) added by serial dilution titration analysis. It is critically important that the amount of biotinylated ligand added to a given microtiter well not exceed the binding capacity of the coated antibody. Ideally, the amount of biotinylated ligand added should approach the upper range of where capture is still linear. This analysis must be performed for each ligand/antibody combination.

2. Wash ELISA microtiter well strips two times with 200 μ l PBS and block residual binding capacity with 200 μ l EBS. Incubate overnight at 4°C.

Coated ELISA microtiter well strips may be stored in EBS until use—typically within 2 days of preparation.

3. Wash cells near, but not at, confluence, two times with 25 ml HBSS and add 25 ml fresh Dulbecco's minimum essential medium containing 10% FBS for 30 min prior to permeabilizing.

Growing cells on 15-cm tissue culture plates works well for these procedures. These plates will contain $\sim 5\text{--}6 \times 10^6$ cells (sufficient for ~ 24 import reactions) when nearly confluent. The following steps describe procedures for treatment of each 15-cm plate.

Pool cells from different plates at the end of the permeabilization procedure.

4. Wash cells three times with 25 ml IRB and harvest in a small residual volume (~ 5 ml) by scraping firmly with a stiff rubber policeman.

Scraping in this manner removes portions of the plasma membrane and thus semi-permeabilizes cells.

5. Repeat scraping with an additional 5 to 10 ml IRB to harvest remaining cells.
6. Bring volume to 50 ml with IRB in a 50-ml conical centrifuge tube and rock 15 min at 4°C to remove cytosol.
7. After 15 min, centrifuge cells 5 min at $800 \times g$, 4°C. Aspirate supernatant.

Properly semi-permeabilized cells will not exclude trypan blue or contain more than $\sim 15\%$ of their cytosolic contents. Lactate dehydrogenase and Hsc70 are convenient cytosolic marker proteins that may be examined by enzyme assay or immunoblotting, respectively.

8. Carefully resuspend cells in ~ 275 μ l IRB and keep on ice until use.

Once prepared, the cells should be used as quickly as possible. Therefore, step 7 should be initiated prior to completion of cell preparation.

Prepare and incubate import reactions

9. Add the following import reaction components, excluding cells, to labeled 1.5-ml microcentrifuge tubes on ice (final reaction volume is 40 μ l, which includes 10 μ l of cells):

2 μ l ATP
5 μ l zinc chloride
5 μ l 80 μ g/ml B-Luc diluted in IRB/1.25% BSA
18 μ l IRB

The 18 μ l IRB addition may be reduced to accommodate such components as cytosol, (inhibitory) antibodies, chelators, and combinations or fractions thereof.

Consolidating pipetting steps by pooling components improves reproducibility. Minimally, each condition should be performed in duplicate.

10. Initiate import reaction by adding 10 μ l semi-permeabilized cells to microcentrifuge tubes containing import reaction mixture. Gently mix the contents and incubate 45 min at 37°C. Tap the microcentrifuge tubes gently every 5 min to ensure an even distribution.

For zero-time incubations, add cells to import reaction components at the end of the incubation, i.e., at 45 min.

11. Terminate import reaction by microcentrifuging tubes for 1 min at 13,000 rpm, 4°C. Aspirate supernatant.

The vast majority of unimported B-Luc is removed in this step. The small amount remaining in the pellet is “inactivated” in the following steps.

Block unimported B-Luc

12. Add 100 µl of 75 µg/ml avidin diluted in IRB/0.2% BSA and resuspend cell pellet. Incubate for 1 hr at 4°C on a platform rocker.

Avidin blocks biotin groups on unimported B-Luc.

13. Add 10 µl of 750 µg/ml biocytin diluted in IRB/0.2% BSA and incubate for an additional 15 min at 4°C.

Biocytin binds excess avidin.

14. Solubilize by adding 100 µl EBS and vortexing. Incubate 30 min at 4°C.

Unmodified B-Luc, the hallmark of import, is now available for capture and detection in microtiter wells coated with anti-luciferase antibodies.

Capture B-Luc

15. Just prior to plating lysates, wash ELISA microtiter well strips two times with 200 µl PBS. Add 200 µl of cell (or organelle) lysates to appropriate microtiter wells and incubate overnight at 4°C.

Develop plates

16. Wash ELISA microtiter well strips three times with 200 µl PBS and add 200 µl EBS for 5 min. During this time, dilute the streptavidin horseradish peroxidase 1:5000 in EBS.

17. After 5 min, wash ELISA microtiter well strips three times with 200 µl PBS, and add 200 µl of the streptavidin horseradish peroxidase/EBS mixture. Incubate for 1 hr at room temperature.

18. After 1 hr, wash ELISA microtiter well strips three times with 200 µl PBS and add 200 µl EBS for 5 min. During this 5-min period, prepare the development solution by adding 10 µl of 30% H₂O₂ and 10 mg *o*-phenylenediamine to 25 ml ELISA development buffer.

The development solution should not be prepared ahead of time.

CAUTION: o-Phenylenediamine is a carcinogen and contact should be avoided. All o-phenylenediamine-containing solutions should be discarded appropriately.

19. After 5 min, wash ELISA microtiter well strips three times with 200 µl PBS, and add 200 µl of H₂O₂/*o*-phenylenediamine/ELISA development buffer mixture. Allow development to proceed for 1 to 2 min.

20. Stop development with 50 µl of 4 N H₂SO₄ and, in a microtiter plate reader, measure absorbance at 490 nm.

IMPORT INTO ORGANELLE PELLETS/PEROXISOMES

**ALTERNATE
PROTOCOL**

In this protocol, import is carried out in semi-permeabilized cells. Then the cells are homogenized and fractionated, and import assessed in the resultant organelles.

Additional Materials (also see Basic Protocol)

- Antibody-coated microtiter well strips (Basic Protocol, steps 1 and 2)
- Homogenization buffer (see recipe), pH 7.8
- 15-ml conical centrifuge tubes (Corning)
- 27-G needle
- 1-ml syringe
- 2-ml glass Dounce tissue grinder, with loose- and tight-fitting pestles (Kontes)

Prepare semi-permeabilized cells

1. Wash cells near, but not at confluence, two times with 25 ml HBSS and add 25 ml fresh medium for 30 min prior to permeabilizing.

For this protocol, two nearly confluent 15-cm tissue culture plates will provide sufficient cells for ~6 import reactions. The following steps describe procedures for treatment of two 15-cm plates.

2. Wash cells three times with 25 ml IRB and harvest in a small residual volume (~5 ml) by scraping firmly with a stiff rubber policeman.
3. Repeat scraping with an additional 5 to 10 ml IRB added to harvest remaining cells. Pool cells from two plates at this step.
4. Bring volume to 50 ml with IRB in a 50-ml conical centrifuge tube and centrifuge cells 5 min at $800 \times g$, 4°C. Carefully aspirate supernatant.

Avoid touching cell pellet or it too may be aspirated. Leave a small amount of supernatant if necessary.

5. Carefully resuspend cells in ~12 ml IRB and dispense ~2-ml aliquots into each of six 15-ml conical centrifuge tubes on ice.

Repeated gentle pipetting with a 5-ml pipet helps reduce clumping of the cells. It is critically important that each 15-ml conical centrifuge tube receive an equal volume/number of cells.

6. Centrifuge the cells 5 min at $800 \times g$, 4°C. Carefully aspirate supernatant.

Once prepared, the cells should be used as quickly as possible. Therefore, the import reaction components should be prepared and pooled such that they may be added to cells in a one-step addition immediately after the supernatant is aspirated.

Prepare import reactions/incubations

7. Prepare a 187.5- μ l import reaction mix with the following components:

- 12.5 μ l ATP
- 31.3 μ l zinc chloride
- 31.3 μ l 80 μ g/ml B-Luc diluted in IRB/1.25% BSA
- 112.4 μ l IRB

The 112.4 μ l IRB addition may be reduced to accommodate such components as cytosol, (inhibitory) antibodies, chelators, and combinations or fractions thereof.

8. Gently resuspend the cells in the import reaction mix and immediately transfer the contents to a 1.5-ml microcentrifuge tube. Incubate for 45 min at 37°C. Tap the microcentrifuge tubes every 5 min to ensure an even distribution.

The cell volume is ~60 μ l, therefore the final reaction volume is ~250 μ l. For zero-time incubations, add import reaction components to cells at the end of the incubation, i.e., at 45 min.

**In Vitro
Reconstitution**

11.15.5

9. Terminate import reaction by microcentrifuging tubes 1 min at 13,000 rpm, 4°C. Aspirate supernatant.

Block unimported B-Luc

10. Add 250 µl of 187.5 µg/ml avidin diluted in homogenization buffer and resuspend cell pellet. Incubate for 1 hr at 4°C with rocking.

Avidin blocks biotin groups on unimported B-Luc.

11. Add 25 µl of 1.875 mg/ml biocytin diluted in homogenization buffer and incubate for an additional 15 min at 4°C.

Biocytin binds excess avidin.

Prepare organelle pellets/peroxisomes

12. Homogenize cells on ice by passing through 27-G needle attached to 1-ml syringe five times, followed by ten strokes with a loose-fitting pestle and ten strokes with a tight-fitting pestle in a 2-ml Dounce homogenizer.

13. Centrifuge 10 min at 1000 × g at 4°C to remove nuclei and unbroken cells.

14. Transfer supernatant to a fresh 1.5-ml microcentrifuge tube and microcentrifuge 20 min at 17,000 × g, 4°C, to prepare organelle pellet/peroxisomes. Aspirate supernatant.

15. Solubilize pellet by adding 200 µl EBS and vortexing. Incubate for 30 min at 4°C.

Unmodified B-Luc, the hallmark of import, is now available for capture and detection in microtiter wells coated with anti-luciferase antibodies in the ELISA assay (see Basic Protocol, steps 1, 2 and 15 to 20). Alternatively, proteins in the organelle pellet are separated by SDS-PAGE (UNIT 6.1), transferred to nitrocellulose, and probed with streptavidin alkaline-phosphatase (see Terlecky et al., 2001; also see UNIT 6.2).

SUPPORT PROTOCOL

LUCIFERASE BIOTINYLYATION

Biotinylated luciferase is used as the import substrate for the assays described in this unit. The protein contains a peroxisomal targeting signal and is readily available commercially.

Materials

Firefly (*Photinus pyralis*) luciferase (Sigma-Aldrich)
Phosphate-buffered saline (PBS; APPENDIX 2A) pH 7.4
8.25 mM 6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoic acid,
succinimidylester (Molecular Probes) in dimethylsulfoxide (B-XX-SE)
PBS containing 0.2% (w/v) BSA (PBS/0.2% BSA)
Streptavidin alkaline-phosphatase (KPL)
Rabbit polyclonal anti-luciferase antibodies (UNIT 16.2)
Micro Bio-Spin P-6 chromatography column (spin column, Bio-Rad)

1. Dissolve 2 mg of firefly luciferase in 200 µl PBS.
2. Add 20 µl of 8.25 mM B-XX-SE (about five times molar excess over luciferase). Incubate 1 hr at room temperature.
3. During 1-hr incubation, wash four spin chromatography columns first three times with 500 µl PBS, then one time with 500 µl PBS/0.2% BSA, followed by a 500-µl PBS wash. Centrifuge each wash for 1 min at 1000 × g, room temperature.
4. After 1 hr, add 55 µl of biotinylation reaction (step 2) to each column and centrifuge for 4 min at 1000 × g. Collect and pool filtrates.

This step effectively separates the modified protein from unincorporated labeling reagents.

5. Adjust volume of biotinylated luciferase (B-Luc) with PBS such that final protein concentration is ~2 mg/ml. Dispense into 10- μ l aliquots and store at -80°C .
6. Confirm biotinylation by blotting with streptavidin alkaline-phosphatase and rabbit polyclonal anti-luciferase antibodies.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

ATP and regenerating system (ATP)

20 mM adenosine 5'-triphosphate (disodium salt, Amersham Biosciences)
100 mM creatine phosphate (disodium salt, Calbiochem-Novabiochem)
8.0 IU creatine phosphokinase (Sigma-Aldrich)
Store up to 6 months at -80°C

ELISA blocking solution (EBS)

0.2% (w/v) BSA
1 mM EDTA (disodium salt)
50 mM sodium chloride
0.1% (w/v) sodium dodecylsulfate salt (SDS)
10 mM Tris·Cl
1.0% (v/v) Triton X-100
Adjust pH to 7.4 with HCl
Store up to 1 month at 4°C

Homogenization buffer

10 mM acetic acid
0.2% (w/v) BSA
1 mM EDTA (disodium salt)
0.1% (v/v) ethanol
0.25 M sucrose
10 mM triethanolamine
Adjust pH to 7.8 with NaOH
Store up to 1 month at 4°C

Import reaction buffer (IRB)

40 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane-sulfonic acid (HEPES)
2 mM magnesium acetate
100 mM potassium acetate
85 mM sucrose
Adjust pH to 7.4 with KOH
Store up to 1 month at 4°C

COMMENTARY

Background Information

Peroxisomes

Peroxisomes are critically important, ubiquitous subcellular organelles whose biology impacts human health, disease, and aging. In recent years, considerable efforts have been expended to try to understand how these single-membrane bounded multifunctional organelles arise. Powerful genetic approaches in a

number of diverse organisms have identified 23 proteins, called peroxins, which participate in various aspects of peroxisome biogenesis. Biochemical approaches have also contributed significantly to an understanding of how peroxisomes are formed from a mechanistic standpoint.

Peroxisome biogenesis appears to involve three steps: (1) synthesis and assembly of membrane proteins and lipids, (2) signal-mediated

post-translational import of proteins from the cytosol, and (3) growth and division of the organelle (reviewed in Terlecky and Fransen, 2000). It is the processes associated with protein import into the organelle, i.e., step 2, that are reconstituted in the assay described here. An important question at this point is what sets this *in vitro* system apart from others currently available?

In vitro assays

Three other *in vitro* assays for peroxisomal protein import are in use. The first involves isolating organelles from rat liver or yeast cells and incubating them with import substrates (Lazarow et al., 1991). Protease protection is used as the hallmark of import. The second involves microinjecting intact cells with import substrates and examining their redistribution to peroxisomes over time (Walton et al., 1992). Import is detected by immunofluorescence. The third involves bacterial cytolysin semi-permeabilizing cells and incubating them with import substrates (Wendland, 1994). Once again, import is detected by immunofluorescence. All three have contributed and will continue to contribute to the field. The assay described here is the most recently developed for peroxisomal protein import. Based on a cell-free system first designed to quantitate receptor-mediated endocytosis (Smythe et al., 1992), it employs mechanically disrupted cells and biotinylated import substrates. The hallmark of import is the accumulation of avidin-inaccessible biotin groups inside the peroxisome. The assay is a reliable and robust multisample system designed to permit examination of a number of parameters within a single experiment. It faithfully recapitulates the import process, is easy to use and reproducible, and it should greatly facilitate the search for novel biochemical factors and mechanisms fundamental to peroxisomal protein import.

Quantitative assay specifics/advantages

Nearly all peroxisomal proteins are directed to the organelle by virtue of a peroxisomal targeting signal (PTS); specifically, PTS1 or PTS2. Although the protocols described here employ the PTS1-containing protein luciferase, the assay may, in principle, be applied to other PTS1- and PTS2-containing substrates. Interestingly, PTSs exist as compositional motifs, and not as absolutely conserved sequences. The assay could be used to test the relative strength of signal variants; a powerful advantage over nonquantitative immunofluores-

cence-based assays that cannot discriminate between potentially subtle differences in import efficiencies.

The assay does not detect significant import in cells from patients who suffer from the fatal peroxisomal disorder Zellweger syndrome (Terlecky et al., 2001). In these particular cells, the basis of the import deficiency is known—they lack a functional PTS1 import receptor, called Pex5p. Perhaps in other patient cell lines, the system could be used as a “biochemical complementation assay” to identify missing or defective factors.

The assay is presented in two forms; as a Basic Protocol and as an Alternate Protocol. The two may be used interchangeably. The advantage of the (“cell”) Basic Protocol is that it is easier to use and is less time-consuming. It is the approach of choice in most circumstances. The (“organelle pellet/peroxisome”) Alternate Protocol is especially effective when trying to avoid problems associated with cell clumping in small volumes. Human fibroblasts, especially if over-confluent, can clump after being semi-permeabilized and concentrated. This can make dispensing equal (10 μ l) volume aliquots problematic. In the Alternate Protocol, cells are (equally) divided while in a larger and more easily divided (12 ml) volume.

Critical Parameters and Troubleshooting

Biotinylation

A procedure is detailed for the biotinylation of luciferase (see Support Protocol). Clearly, there are other methods for the biotinylation of this enzyme—some involve isolation of the biotinylated-species on (monomeric) avidin columns. The latter is an effective approach but can be problematic if elution involves more stringent conditions (e.g., acid). Under such circumstances, care should be taken to ensure that the renaturation/refolding steps result in a protein that is still recognized by antibodies and captured in antibody-coated microtiter wells, and remains a substrate for the peroxisomal protein import pathway.

Import assays

Peroxisomal protein import is blocked at low temperatures (e.g., 4°C). Therefore, in addition to a zero-time incubation, (complete) import reactions should also be carried out at 4°C. Typically, import will be reduced to <10% of the 37°C value.

Factors that modulate import should be determined empirically for each ligand. For the PTS1-containing protein, B-Luc, import is stimulated some six-fold by zinc ions (100 μM) and an additional two- to three-fold by cytosol (10 $\mu\text{g/ml}$; Terlecky et al., 2001). For other substrates, the concentrations of these and other factors should be titrated to maximize the import signal. It should be noted that the cytosol effect on B-Luc import is variable, depending on the source, the concentration, the amount of endogenous cytosol removed from cells, and other factors (Terlecky et al., 2001). As such, it is recommended that each cytosol preparation be individually evaluated for activity with a given substrate.

Lastly, the assay described in the (“cell”) Basic Protocol is performed in a final volume of 40 μl . It is, therefore, exquisitely sensitive to pipetting errors. Where possible, reduce or eliminate the potential for variance by pooling components.

ELISA quantitation

It is extremely important that the signal-to-noise ratio of the assay be high. Contributing to background noise (i.e., the absorbance detected in an import reaction at time-zero or at time 45 min at 4°C) is (1) impure or poor quality

development reagents, (2) ineffective blocking of microtiter wells, and (3) incomplete washing of microtiter wells between steps. Contributing to reduced signals (i.e., the absorbance detected in an import reaction at 45 min at 37°C) is (1) using poorly permeabilized cells, (2) exceeding the binding capacity of the coated antibody, and (3) inactivation of the import substrate.

Suggested remedies for these problems are as follows. For high background noise: (1) use the reagents and sources suggested in the protocols, (2) modify the blocking buffer to include higher concentrations of BSA or switch to gelatin-, casein-, or non-fat milk-based blocking buffers (however, be sure not to interfere with antibody-capture of the biotinylated-import substrate), and (3) wash thoroughly (a plate washer helps increase reproducibility and simplifies the process). For low signals: (1) scrape the cells more vigorously, or consider growing the cells on fibronectin or other attachment factor, (2) reduce the amount of cell or organelle lysates loaded (from the Basic Protocol or Alternate Protocol, respectively), or, alternatively, reduce the amount of substrate added to the import reaction, and (3) biotinylate the import substrate in another way. With respect to the latter point, it is possible that biotinylation of the import substrate results in a

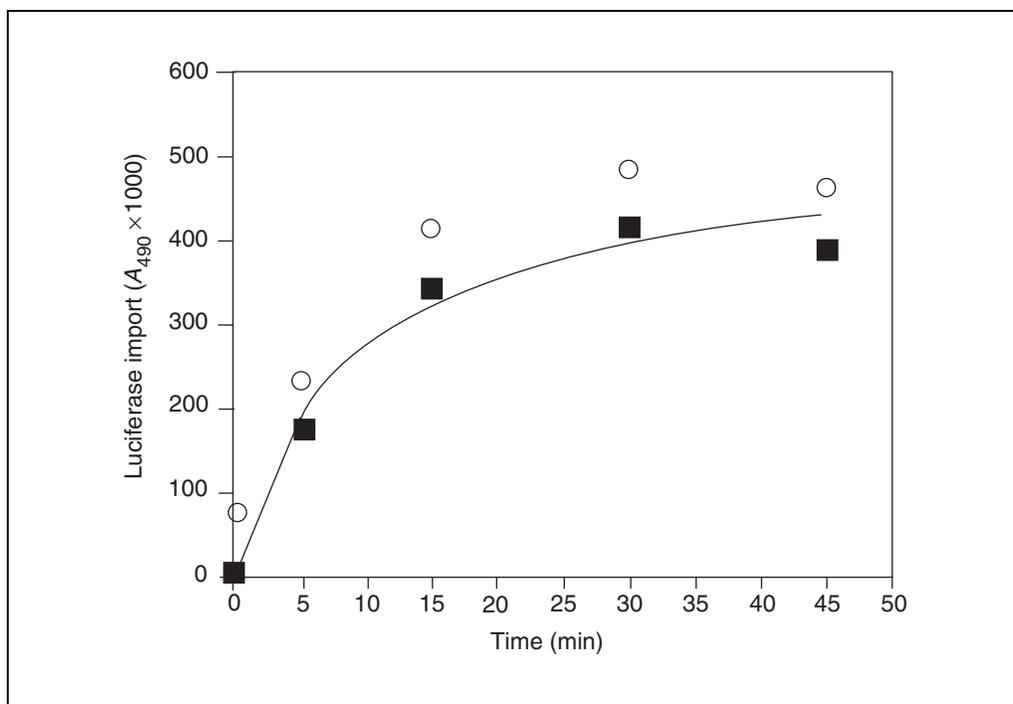


Figure 11.15.2 Peroxisomal import kinetics. Semi-permeabilized human A431 cells were incubated with biotinylated luciferase in the in vitro peroxisomal import reaction described in the Alternate Protocol. At the times indicated, the extent of import was quantified by ELISA. The raw absorbance units ($\times 10^2$) obtained at each time point (open circle); adjusted absorbance values reflecting subtraction of the time 0 value (filled square and solid line).

protein that is still recognized by antibodies and therefore captured in antibody-coated microtiter wells, but has had its PTS inactivated or shielded. Under such circumstances, consider using a different biotinylation method, or reducing the number of biotin groups coupled per protein molecule. If necessary, employ the immunofluorescence-based semi-permeabilized cell import assay (Wendland, 1994; Terlecky et al., 2001) to confirm import competence of the biotinylated substrate.

Anticipated Results

The protocols outlined here will result in a measurable absorbance at 490 nm. For example, Figure 11.15.2 illustrates results of an import time course. The challenge is to ensure that the signal is obtained under appropriate conditions (e.g., import conducted for 45 min at 37°C) and not inappropriate ones (e.g., import conducted at 4°C or for zero-time). Furthermore, to permit valid comparisons between experiments, every effort should be made to keep assay parameters as consistent as possible. This includes the number of cells and their treatment, the concentrations and preparations of assay components, and the conditions employed. Clearly, if the positive and negative controls are similar in experiments conducted on different days, then the results obtained for “experimental” conditions may be usefully and accurately compared.

Time Considerations

The biotinylation reaction described in the Support Protocol will require ~2 hr to complete. This excludes the time required for blotting the resultant biotinylated-species. Clearly, the Support Protocol must be completed before initiating import assays.

A typical import assay will require 30 min and an overnight incubation 2 days before the assay for coating microtiter wells with antibodies, and 30 min and an overnight incubation 1 day prior to the assay for blocking of wells. On the day of the import experiment, ~4.5 hr will be required for the “cell” Basic Protocol, and 6 hr for the “organelle pellet/peroxisome” Alternate Protocol. An overnight incubation is once again required, and on day 2 of the experiment, the assay is developed. This step requires ~2 hr

to complete. Every effort should be made to design the experiment, prepare reagents, label tubes, and check on the availability of required equipment well in advance of beginning the assay. The only other time consideration is that of optimizing the ELISA assay by serial dilution titration analysis. Performed after biotinylation of the ligand, it will require the 2-day preparation of antibody-coated and blocked microtiter wells, and ~2 hr to add appropriate amounts of the biotinylated-ligand. After an overnight incubation, once again ~2 hr is required to develop the assay.

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Key References

- Smythe et al., 1992. See above.
- Highly recommended first description of an ELISA-based in vitro internalization assay using biotinylated substrates and semi-intact cells. This method is the basis for the assay described in this unit.*
- Terlecky et al., 2001. See above.
- First description of the use of the system outlined in the previous reference for assaying peroxisomal protein import.*

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In Vitro Analysis of Chloroplast Protein Import

UNIT 11.16

This unit describes methods for isolating intact chloroplasts from pea (*Pisum sativum*; see Support Protocol 1) and *Arabidopsis thaliana* (see Support Protocol 2) for the study of the import of nuclear-encoded plastid precursor proteins. Chloroplasts from both preparations are competent for the in vitro import of recombinant preproteins synthesized using in vitro translation systems derived from reticulocyte lysates or wheat germ lysates (see UNIT 11.2 and Support Protocol 3). Preproteins expressed in *E. coli* also have been used for import studies using isolated pea chloroplasts (Pilon et al., 1990; Schnell and Blobel, 1993). These assays can be used to test whether a particular protein is targeted to chloroplasts, for analyzing the suborganellar location of newly imported preproteins, or to study the mechanism of import itself. The in vitro chloroplast protein import assay is described in the Basic Protocol, and methods for fractionating the chloroplasts and analyzing the results of import are provided in the Alternate Protocol.

IN VITRO CHLOROPLAST PROTEIN IMPORT ASSAY

Intact chloroplasts isolated from either pea or *Arabidopsis* using the methods described in Support Protocols 1 and 2 can be used for in vitro chloroplast protein import assays. These assays are often used to confirm the ability of proteins to be imported into chloroplasts, as chloroplast targeting prediction programs (Emanuelsson et al., 1999, 2000) are not 100% accurate, or to study the mechanism of import itself.

Generally, the intact chloroplasts are incubated with in vitro—translated, radiolabeled chloroplast preproteins in the presence of energy in the form of ATP (Pain and Blobel, 1987; Theg et al., 1989). The chloroplasts are re-isolated to remove any precursor not imported during the assay and analyzed using a PhosphorImager or by fluorography (UNIT 6.3) following SDS-PAGE (UNIT 6.1) to detect imported protein. The majority of chloroplast proteins are synthesized with a cleavable *N*-terminal transit peptide that is removed following import to the stroma (Bauer et al., 2001). This cleavage results in a shift in molecular weight, which provides a convenient indication that import has occurred. Chloroplasts are treated with exogenous protease (e.g., thermolysin), following the import assay, to digest any excluded preprotein and confirm that the processed preprotein has been imported, and thereby, protected from protease digestion (Cline et al., 1984). For proteins targeted to the outer envelope membrane, many of which do not have cleavable transit peptides, resistance to alkali extraction or the generation of a characteristic pattern of protease resistance can confirm integration into the membrane (Muckel and Soll, 1996; Froehlich et al., 2001).

Materials

- Freshly isolated, intact chloroplasts (see Support Protocols 1 and 2)
- HEPES-sorbitol buffer, pH 7.5 (if using pea chloroplasts, see recipe) or pH 8.0 (if using *Arabidopsis* chloroplasts, see recipe)
- Chloroplast import master mix (see recipe)
- 0.1 M dithiothreitol (DTT)
- 0.1 M ATP
- In vitro—translated [³⁵S]methionine-labeled chloroplast protein (see Support Protocol 3 or UNIT 11.2)
- 40% (v/v) Percoll (see recipe)
- 2 mg/ml thermolysin (see recipe)
- 0.5 M EDTA
- SDS-PAGE sample buffer (see recipe)
- Destain solution (see recipe)

**BASIC
PROTOCOL**

**In Vitro
Reconstitution**

26°C water bath
1.5-ml microcentrifuge tubes
Refrigerated microcentrifuge
Enlightening fluorography enhancer (NEN)
Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and fluorography or phosphorimager (UNIT 6.3)

Perform in vitro import assay

1. Adjust the chlorophyll concentration of freshly prepared chloroplasts (see Support Protocols 1 and 2) to 1 mg chlorophyll/ml using the appropriate HEPES-sorbitol buffer.

Use the equivalent of 50 µg chlorophyll in a standard 100-µl assay.

The amount of chlorophyll can be increased to 100 µg chlorophyll/100 µl assay if the sensitivity of import (i.e., the import efficiency of a particular substrate) is low.

2. Mix the following components on ice:

50 µl 1 mg/ml chloroplasts (50 µg chlorophyll)
10 µl chloroplast import master mix
1 µl 0.1 M DTT
2 µl 0.1 M ATP
62 µl appropriate HEPES-sorbitol buffer

Pre-incubate for 5 min at 26°C in the dark.

3. To begin import, add 1 to 5 µl [³⁵S]methionine-labeled in vitro translation product (equivalent to at least 10,000 cpm). Incubate 20 to 30 min at 26°C in the dark.

The amount of in vitro–translated import substrate added to each reaction can vary considerably depending on the efficiency of import of the substrate. A model import substrate, such as the precursor to the small subunit of rubisco (preSSU), that is synthesized in a reticulocyte lysate system and radiolabeled with [³⁵S]methionine is imported with efficiencies of up to 65% of added substrate. However, the authors recommend that investigators assume an import efficiency of ~20% for previously untested preproteins. With this in mind, the amount (cpms) of substrate should be increased proportionately to ensure sensitivity of detection with film or a PhosphorImager. Also, be aware that transit peptide processing might remove [³⁵S]methionine residues, thereby, decreasing sensitivity.

4. Stop import by diluting the reaction into 0.5 ml of ice-cold HEPES-sorbitol buffer and hold on ice.

Re-isolate the chloroplasts

5. Layer the diluted chloroplasts over a 0.5- to 1-ml cushion of ice-cold 40% Percoll in a 1.5-ml microcentrifuge tube and microcentrifuge for 5 min at 2000 × g, 4°C.
6. Carefully aspirate the supernatant and 40% Percoll, containing any broken chloroplasts and nonimported translation product.

It is important to remove all of the supernatant without disturbing the chloroplast pellet in order to minimize contamination of the chloroplasts with excess precursor.

Treat chloroplasts with thermolysin

7. Resuspend the chloroplasts in 100 µl of ice-cold HEPES-sorbitol buffer and transfer to a fresh 1.5-ml microcentrifuge tube. Add 5 µl of 2 mg/ml thermolysin, mix gently, and incubate for 30 min on ice.

A concentration of 100 µg thermolysin/ml during the protease treatment is standard for the analysis of proteins imported to the inner envelope, stroma, or thylakoid. For integral outer

envelope proteins that will be susceptible to proteolysis, it may be desirable to titrate the level of thermolysin to generate a digestion pattern that is characteristic of the integrated protein (Muckel and Soll, 1996; Tu and Li, 2000; Froehlich et al., 2001). The final thermolysin concentration can be varied from 10 to 200 µg/ml depending on the substrate being studied and the susceptibility to degradation. The optimal concentration to be used for each substrate should be determined empirically.

8. Stop the reaction by adding 0.5 M EDTA to a final concentration of 10 mM. Re-isolate the chloroplasts by centrifugation through a 40% Percoll cushion, containing 5 mM EDTA, as described in step 5.

Analyze imported proteins

9. Resuspend the chloroplast pellet (step 6 or step 8) directly in 25 µl SDS-PAGE sample buffer and analyze by SDS-PAGE (UNIT 6.1) and fluorography or phosphorimager (UNIT 6.3).

As an alternative to analyzing the chloroplast pellet directly by SDS-PAGE, the chloroplasts may first be fractionated to determine the precise suborganellar location of the imported protein (see Alternate Protocol).

10. Analyze the results of import by SDS-PAGE using a 10% or 12% polyacrylamide gel (UNIT 6.1).

Samples from each step of the import assay and subsequent treatments should be analyzed. For example, chloroplasts reisolated directly following the import assay (step 6) and following protease treatment (step 8) should be analyzed along with samples from alkaline extraction (see Alternate Protocol, steps 9 and 12) and each fraction from chloroplast subfractionation (Alternate Protocol, steps 18 and 23). A small aliquot of the in vitro translation product used in the import assay, usually equivalent to 1/10 of the amount added to the reaction, should be included on the gel. This will serve as a molecular weight reference, to confirm the transit peptide has been removed, and as a reference for the amount of radioactive substrate added to the reaction (keeping in mind that the processed protein might have fewer labeled methionine residues than the precursor protein with transit peptide). This will provide a complete and quantitative analysis of the import experiment.

11. Following electrophoresis, fix the gel in destain solution for 30 to 60 min. For exposure using a PhosphorImager, dry the gel under vacuum, wrap in plastic and expose to the phosphor storage screen overnight. For fluorography using film, soak the gel in Enlightening fluorography enhancer for 30 to 60 min, dry the gel under vacuum at 65°C for 1.5 to 2 hr, and expose to X-ray film overnight at -80°C.

FRACTIONATION OF RE-ISOLATED CHLOROPLASTS FOLLOWING IMPORT

It may be desirable to subfractionate chloroplasts following an import reaction to determine the suborganellar localization of the newly imported preprotein. To assess stromal versus membrane localization, chloroplasts can simply be lysed and separated into stromal versus membrane fractions by differential centrifugation. To confirm that a suspected membrane protein is integrated, the membrane fraction can be subjected to alkaline extraction. Alternatively, the chloroplasts can be fractionated into outer and inner envelope membranes, thylakoids, and stroma if the precise suborganellar compartment must be determined (Keegstra and Yousif, 1986). Subfractionation into the three membrane components should not be attempted with chloroplast samples corresponding to <0.5 mg chlorophyll because of the low yield of envelope membrane. The inner and outer envelope membranes constitute ≤1% of the total chloroplast membrane fraction.

ALTERNATE PROTOCOL

In Vitro Reconstitution

11.16.3

Materials

Chloroplast pellet from the import reaction (see Basic Protocol)
HEPES-sorbitol buffer (see recipe), ice cold
2 mM EDTA, ice cold
4 M NaCl
0.5% and 100% (w/v) trichloroacetic acid (TCA; see recipe)
SDS-PAGE sample buffer (see recipe)
0.1 M NaCO₃, pH 11.5, ice cold
TE/DTT buffer (see recipe) containing 0.6 M sucrose and buffer containing 0.2 M sucrose
TE/DTT buffer (see recipe)
1 M, 0.8 M, and 0.46 M sucrose

3-ml Beckman polyallomer thick-walled ultracentrifuge tubes
Ultracentrifuge and rotor (Beckman TL 100 and TLA 100.3 rotor, or equivalent)
Dounce homogenizer or Potter homogenizer and pestle
Swinging-bucket rotor (e.g., Beckman SW 50.1)
5-ml polyallomer tubes (e.g., for Beckman SW 50.1)
1-ml micropipettor
Gradient former (e.g., Buchler DensiFlow gradient former)
Gradient fractionator (e.g., ISCO gradient fractionator)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and fluorography or phosphorimagery (*UNIT 6.3*)

Lyse chloroplasts for stromal versus membrane association

1. Resuspend the chloroplast pellet from the import reaction (see Basic Protocol, step 6 or 8) in 150 μ l of ice-cold HEPES-sorbitol buffer.
2. Dilute the suspension with 800 μ l of ice-cold 2 mM EDTA. Vortex vigorously and let stand for 10 min on ice to lyse the chloroplasts. Add 50 μ l of 4 M NaCl, mix, and microcentrifuge the lysed chloroplasts for 30 min at 15,000 \times g, 4°C, to pellet both the thylakoid and envelope membranes.
3. Carefully transfer the supernatant (stroma) to a fresh 3-ml polyallomer thick-walled tube and precipitate the protein by adding 100% TCA to a final concentration of 12%. Incubate for 1 hr on ice. Resuspend the membrane pellet directly in 25 μ l SDS-PAGE sample buffer.
4. Collect the TCA precipitate by centrifugation for 30 min at 15,000 \times g, 4°C. Remove and discard the supernatant.
5. Wash the pellet with 500 μ l 0.5% TCA and resuspend the pellet of precipitated stromal proteins directly in 25 μ l SDS-PAGE sample buffer.

Prepare chloroplasts for alkaline extraction

6. Resuspend and lyse the chloroplast pellet from the in vitro import assay (see Basic Protocol, step 6 or 8) in 100 μ l of ice-cold 0.1 M NaCO₃, pH 11.5, by pipetting the pellet into solution. Transfer to a fresh microcentrifuge tube.
7. Add another 900 μ l of ice-cold 0.1 M NaCO₃, pH 11.5, and transfer the solution to a 3-ml polyallomer thick-walled ultracentrifuge tube. Incubate for 10 min on ice.
8. Ultracentrifuge the lysed chloroplasts for 30 min at 40,000 \times g, 4°C, to pellet the membranes.

- Carefully collect the supernatant containing the stroma and transfer to a fresh 3-ml ultracentrifuge tube. Resuspend the membrane pellet in 25 μ l SDS-PAGE sample buffer.

Precipitate the alkaline extract and chloroplast stroma

- Precipitate the alkaline supernatant by adding 100% TCA to a final concentration of 12%. Incubate for 1 hr on ice.
- Collect the TCA precipitate by centrifuging for 30 min at 15,000 \times g, 4°C.
- Wash the pellet with 500 μ l of 0.5% TCA and resuspend the pellet directly in 25 μ l SDS-PAGE sample buffer.
- Analyze all fractions by SDS-PAGE (UNIT 6.1) and fluorography or phosphorimager (UNIT 6.3).

Fractionate chloroplasts

- Resuspend the chloroplast pellet from the in vitro import assay (see Basic Protocol, step 6 or 8) in TE/DTT buffer containing 0.6 M sucrose to a concentration of 1 to 2 mg/ml chlorophyll. Let stand for 10 min on ice.

If the protein import assay was performed with <0.5 mg chlorophyll, the chloroplasts from the import assay should be mixed with a nonradioactive chloroplast fraction to bring the chlorophyll content >0.5 mg. Fractionation of samples containing 1 to 2 mg chlorophyll by this procedure is ideal.

- Freeze the chloroplast suspension for 1 to 2 hr at -20°C.

It is essential to lyse the chloroplasts under hypertonic conditions to ensure maximum separation of outer and inner envelope membranes in the subsequent sucrose gradient fractionation (Keegstra and Yousif, 1986). The sample may be transferred to -80°C at this stage and stored indefinitely.

- Thaw the suspension and dilute with 3 vol of TE/DTT buffer. Homogenize with 20 strokes in a Dounce or Potter homogenizer with a tight pestle.
- Ultracentrifuge the lysed chloroplasts in a swinging-bucket rotor for 1 hr at 40,000 \times g, 4°C.
- Remove the brownish supernatant containing the stromal contents using a pipet and store at -20°C.

The supernatant also might appear yellowish. This color indicates the presence of high levels of plastoglobules in the stroma. These low-density stromal lipid bodies are high in carotenoids.

Fractionate the chloroplast membranes

- Resuspend the membrane pellet in TE/DTT buffer containing 0.2 M sucrose to a concentration of 1 to 2 mg chlorophyll/ml with 20 strokes in a Dounce or Potter homogenizer.

The total chloroplast membranes may be stored at -80°C for at least 6 months or separated into thylakoid and envelope fractions, as described in subsequent steps.

- Set up a sucrose step gradient consisting of 2 ml of 1 M sucrose, 1 ml of 0.8 M sucrose, and 1 ml of 0.46 M sucrose in 5-ml polyallomer tubes. Layer the membrane suspension onto the top.

The gradients can be made during the centrifugation at step 17.

21. Apply 1 ml of membrane suspension (not >2 mg chlorophyll content) on each gradient. Ultracentrifuge the samples for 1.5 hr at $270,000 \times g$, 4°C , in a Beckman SW 50.1 rotor using low acceleration and deceleration rates.

Alternatively, the membranes can be separated using a TLS-55 rotor in a Beckman TL 100 or equivalent centrifuge. The volumes of the steps for this rotor are 0.75 ml of 1 M sucrose, 0.5 ml of 0.8 M sucrose, and 0.5 ml of 0.46 M sucrose. The membrane suspension should not exceed 0.5 ml in volume and 1 mg chlorophyll content. Centrifuge the samples for 1.5 hr at 55,000 rpm at low acceleration and deceleration rates.

22. Collect the interface from each step of the gradient (~0.6 ml from each interface) with a 1-ml micropipettor.

The upper (0.2:0.46 M sucrose) interface contains residual stroma and plastoglobules. The middle (0.46:0.8 M sucrose) interface is highly enriched in outer envelope membranes. The lower (0.8:1 M sucrose) interface is enriched in inner envelope membranes. The thylakoid membranes form a tight pellet at the bottom of the tube.

23. To collect the membranes, dilute each fraction with 3 to 5 vol TE/DTT buffer and centrifuge in a swinging-bucket rotor (e.g., Beckman SW-40) for 1.5 hr at $270,000 \times g$, 4°C . Remove the supernatant with a pipet and discard. Resuspend the pellets in a small volume of SDS-PAGE sample buffer for analysis.

SUPPORT PROTOCOL 1

ISOLATION OF INTACT CHLOROPLASTS FROM PEA

Intact chloroplasts capable of importing nuclear-encoded plastid proteins can be isolated from pea seedlings grown on soil (Chua and Schmidt, 1978; Cline, 1986; Pain and Blobel, 1987; Bruce et al., 1994). Peas have been used as the model system for chloroplast protein import because large amounts of material can be grown quickly and easily. Plants are typically grown in standard potting trays, in either greenhouse conditions or in growth chambers under long-day (12 to 16 hr) conditions. However, peas are easily propagated without growth chambers or greenhouses and can be grown on a well-lit windowsill. Plants should be watered regularly, and 10- to 14-day-old seedlings will yield ample import-competent chloroplasts.

Prior to beginning the isolation procedure, all buffers should be prepared and chilled on ice. All procedures should be performed on ice or in a cold room, and all centrifuges and rotors should be precooled to 4°C . Maintaining the temperature of the extract at or below 4°C throughout the procedure is essential for preventing degradation of chloroplast proteins by proteases. Proceeding as quickly as possible will also limit proteolysis. To limit degradation of chloroplast components that are particularly susceptible to proteolysis, a protease inhibitor cocktail can be included in all buffers throughout the procedure.

This procedure can also be used for the isolation of intact chloroplasts from other species, most commonly spinach (Joyard et al., 1982). Fresh young spinach leaves can be obtained from a local grocer for use with the procedure. However, the yield and import capacity of chloroplasts obtained using this source is unpredictable.

Materials

- Pea seeds (*Pisum sativum* Green Arrow, Jung Seed)
- Potting soil (e.g., Premier Brand Pro-Mix BX or Scotts Brand Metro Mix)
- 85% and 40% (v/v) PBF-Percoll (see recipe)
- Grinding buffer containing 0.25% (w/v) bovine serum albumin (BSA) and 0.1% (w/v) ascorbic acid (see recipe), ice cold
- Protease inhibitor cocktail (optional, e.g., Sigma-Aldrich, UNIT 3.4)
- HEPES-sorbitol, pH 7.5 buffer (see recipe)
- 80% acetone
- 40% (v/v) DMSO (see recipe), ice cold
- Liquid nitrogen

Standard potting trays (21.5 × 11 × 2.5-in.)
30-ml glass centrifuge tubes (Corex)
Scissors
2-liter beakers
Rotary homogenizer (Polytron or comparable with a 30-mm saw-tooth generator)
50-cm Miracloth squares
Large funnel (180-mm diameter)
250-ml centrifuge bottles with screw-cap lids
Superspeed centrifuge (e.g., Sorvall RC-5B)
Large rotor capable of holding 250-ml bottles (e.g., Sorvall GSA)
Swinging-bucket rotor capable of holding 50-ml tubes (e.g., Sorvall HB-4)
Large-bore pipet
Cryotubes

Prepare seeds and grow plants

1. Weigh 120 to 150 g of dry pea seeds and imbibe by submerging the seeds in water for ≥5 hr or overnight at room temperature.
2. Fill two standard potting trays 2/3-full with potting soil, and spread the seeds evenly over the surface of the soil. Cover seeds with a thin layer of soil, and water thoroughly, until soil is saturated.
3. Transfer the tray to a greenhouse or growth chamber, and grow plants under 12- to 16-hr days for 10 to 14 days at 20° to 25°C.

The light intensity is not critical. Plants can be grown on a sunlit windowsill if growth facilities are not available. Peas prefer a moderate growth temperature not exceeding 30°C.

Prepare pea tissue

4. Prepare three PBF-Percoll step gradients by gently overlaying 7 ml of 40% PBF-Percoll onto a 5 ml layer of 85% PBF Percoll in 30-ml glass centrifuge tubes. Hold tubes on ice until needed (see step 12).
5. Harvest the aerial portions of 10- to 14 day-old pea seedlings from two flats (~100 to 140 g of tissue) by clipping the seedlings at the base of the first leaves with sharp scissors. Place the tissue in a 2-liter beaker placed on ice and chill for 10 min. Avoid transferring soil with the seedlings.

The amount of tissue harvested may be scaled up or down depending on requirements; however, the ratio of tissue to buffer (see step 6) should be kept constant.

6. Add 1 liter of ice-cold grinding buffer containing 0.25% BSA and 0.1% ascorbic acid to the harvested tissue.

(Optional) Add a protease inhibitor cocktail to the grinding buffer to a final concentration of 0.05% (v/v) and mix well immediately prior to adding to the tissue (Chen et al., 2000).

Homogenize pea tissue

7. Grind the pea leaves for 30 sec to 1 min using a rotary homogenizer at 80 rpm.

Take care not to over aerate the solution with the generator. Grind until all seedlings have been homogenized and continue for 15 sec to adequately disrupt the tissue.

8. Pass the homogenate through two layers of 50-cm Miracloth squares held in place by a 180-mm diameter funnel, into another 2-liter beaker that has been pre-chilled on ice. Use two filtration systems to facilitate rapid filtration of the homogenate, passing equal volumes of the homogenate through each filter.

9. Transfer the filtrate immediately into four pre-cooled 250-ml centrifuge bottles and centrifuge in a pre-cooled rotor for 2 min at $1500 \times g$, 4°C , with the brake on and stop immediately.

Isolate chloroplasts

10. Carefully decant and discard the majority of the supernatant, leaving ~5 ml in each bottle.

The chloroplasts will be in the pellet, which is very loose. Therefore, use caution when decanting the supernatant, so as not to disturb and lose the pellet. Do not be alarmed if the supernatant remains dark green after the centrifugation step. These are broken chloroplasts. Do not leave >5 ml of supernatant on the chloroplast pellet. If too much supernatant is decanted, simply add back several ml of grinding buffer to aid in resuspending the chloroplast pellet.

11. Carefully resuspend the chloroplast pellets in the remaining 5 ml of grinding buffer by gently swirling the bottles and then combine all the pellets into a single bottle.

Resuspend the pellet with a gentle rotary motion. This step might require 5 min. Do not shake vigorously or resuspend by pipetting.

12. Transfer the chloroplast suspension equally onto the top of three previously prepared PBF-Percoll step gradients (see step 4). Centrifuge in a pre-cooled swinging-bucket rotor for 10 min at $1500 \times g$, 4°C , with the brake off.

The PBF-Percoll step gradients should be prepared prior to beginning the preparation (step 4) in order to proceed as quickly as possible and limit proteolysis. The protease inhibitor cocktail may also be added to the gradient to prevent degradation of particularly susceptible chloroplast components. Transfer the chloroplasts using a large-bore pipet and avoid rapid pipetting.

13. Following centrifugation, observe two green bands. The upper band at the 40% PBF-Percoll/grinding buffer interface contains broken chloroplasts and cellular debris. The lower band located at the interface between the 40% and 85% PBF-Percoll layers contains the intact chloroplasts. Aspirate the grinding buffer and most of the 40% PBF-Percoll layers, leaving the 40%:85% PBF Percoll interface and the band of intact chloroplasts.

It might be difficult to distinguish the two bands at the interfaces of the PBF Percoll gradients if the yield of chloroplasts is high. Aspirate the upper layers within 1 cm of the 40% and 85% PBF-Percoll interface.

14. Carefully remove the bands of intact chloroplasts from the three gradients with a large-bore pipet and transfer equal volumes into two 30-ml glass centrifuge tubes that each contain 20 ml of HEPES-sorbitol buffer, pH 7.5. Centrifuge for 2 min at $4000 \times g$, 4°C , with the brake on.

The band of intact chloroplasts forms a tight, viscous layer in the PBF-Percoll gradient. Remove the layer and the chloroplasts that adhere to the side of the tube, being careful to transfer as little PBF-Percoll as possible.

15. Carefully decant and discard the supernatant as the pellet is very loose. Gently resuspend the pellet of intact chloroplasts in a small volume (1 to 2 ml) of HEPES-sorbitol buffer, pH 7.5, by swirling.

Quantitate chloroplast yield

16. Measure chlorophyll content (Arnon, 1949). Dilute 5 μl of the chloroplast resuspension into 1 ml of 80% acetone. Mix vigorously and microcentrifuge the extract for 2 min at maximum speed to remove the protein precipitate. Remove the supernatant to a clean tube.

The supernatant should be bright green and the pellet completely white. If the pellet retains some pigment, the sample should be mixed and microcentrifuged again before proceeding.

17. Measure the A_{652} of the sample against an 80% acetone blank. Calculate the chlorophyll concentration (mg/ml) by:

$$(A_{652}/36) \times 200$$

to compensate for the dilution factor.

The typical yield of chloroplasts from two trays of 10- to 14-day-old seedlings (100 to 140 g tissue) is 25 to 40 mg chlorophyll.

The chloroplasts also can be inspected visually with a bright-field microscope to assess intactness. Intact chloroplasts appear as smooth spherical bodies with enclosed green thylakoids. Chloroplasts from this procedure should be ~90% intact. Counting the chloroplasts using a hemacytometer will allow the investigator to establish the conversion of chlorophyll content to chloroplast number (i.e., chloroplasts/mg chlorophyll). This unit can be used for absolute quantitation of molecules of substrate imported per chloroplast in the in vitro import assays.

The yield of chloroplasts from two trays of peas is greater than typically needed for a protein import assay with controls. However, the larger-scale preparation is provided because the chloroplasts from pea can be cryopreserved (see below) and used for subsequent import assays. Chloroplasts corresponding to 2 mg chlorophyll will provide sufficient material for a standard import assay with controls. As a result, the pea chloroplast isolation procedure can be scaled down if desired.

Cryopreserve chloroplasts

Intact chloroplasts isolated from pea seedlings can be stored in liquid N₂ and can be subsequently used for in vitro import assays (Yuan et al, 1991). Cryopreservation will lead to the loss of some intact chloroplasts (typically 40% to 50% loss). It is preferable to use freshly prepared chloroplasts whenever possible.

18. Gently resuspend the chloroplast pellet (step 15) in HEPES-sorbitol buffer, pH 7.5, to a concentration of 5 mg chlorophyll/ml by swirling on ice.
19. Measure the volume with a pipet. Add an equal volume of ice-cold 40% DMSO buffer drop-wise to the chloroplast suspension while gently swirling to give a final concentration of 20% DMSO. Allow the chloroplast mixture to equilibrate for 5 min on ice. Immediately pipet 0.5-ml aliquots into cryotubes on ice and snap freeze in liquid nitrogen.
20. Store the tubes under liquid nitrogen.

Chloroplasts can be stored under these conditions for up to 3 months.

21. When ready to use, thaw the chloroplast suspension on ice and quickly dilute with an equal volume of HEPES-sorbitol buffer, pH 7.5. Layer over a cushion of 40% Percoll and centrifuge for 10 min at 3000 × g, 4°C.
22. Remove the buffer and 40% Percoll layers by aspiration.

The yield of chloroplasts from the 40% Percoll step following cryopreservation is ~50% to 60%.

23. Gently resuspend the chloroplast pellet in the appropriate volume of ice-cold HEPES-sorbitol buffer, pH 7.5.

The chloroplasts are ready to be used for an import assay (see Basic Protocol).

**ISOLATION OF INTACT CHLOROPLASTS FROM *ARABIDOPSIS
THALIANA***

Intact chloroplasts can be produced from *Arabidopsis* plants grown either on soil or on agar plates (Fitzpatrick and Keegstra, 2001). The growth conditions required to produce intact chloroplasts from soil-grown plants are not critical. A 12- to 16-hr day, at 20° to 25°C with a light intensity of 80 to 200 $\mu\text{Em}^{-2}\text{sec}^{-1}$ will suffice. Plants can be grown on trays or in pots. However, for a high yield, it is important to prevent the plants from experiencing stress, particularly from watering too much or too little. Yields may be improved slightly by destarching these plants by incubating for 12 to 24 hr in the dark before harvesting. Soil-grown plants that are between 3- and 5-weeks-old typically give the best yield of intact chloroplasts. However, chloroplasts isolated from plants grown on soil do not import precursor proteins efficiently *in vitro*. Therefore, to obtain import-competent chloroplasts from *Arabidopsis*, plants should be grown on agar plates.

The following method is geared to produce chloroplasts from petri plate-grown plants that are capable of importing precursor proteins. However, if a capacity for importing proteins is not required, the method also works for soil-grown plants, observing the modifications noted in the text.

The aerial portions of the plants are incubated with cell-wall-degrading enzymes to release protoplasts. These are collected, washed, and then ruptured. The chloroplasts that are released are purified on a Percoll gradient. Harvesting of protoplasts and chloroplasts, should be done as quickly as possible to ensure a good yield of high-quality chloroplasts. In *Arabidopsis*, this method has the advantage over the traditional method described in Support Protocol 1, as it produces a higher yield of chloroplasts (up to ten times greater), which import precursor proteins well. This method uses two petri plates containing 4-week-old plants (~10 g total of tissue), and yields 0.5 to 1 mg chlorophyll of intact chloroplasts. This method can be scaled up if more chloroplasts are desired, but caution is advised as yields can suffer because of the longer time needed to harvest protoplasts and chloroplasts from large amounts of tissue.

Materials

- Murashige and Skoog growth medium (see recipe)
- Seed sterilizing solution (see recipe)
- Arabidopsis thaliana* seeds, 30 to 40 mg per plate
- Sterile, autoclaved water
- 0.1% (w/v) agarose (autoclaved, sterile)
- Digestion buffer (see recipe)
- Digestion enzyme solution (see recipe)
- 40% and 85% (v/v) AT Percoll (see recipe)
- Protoplast resuspension buffer (see recipe)
- Protoplast breakage buffer (see recipe)
- HEPES-sorbitol buffer, pH 8.0 (see recipe)

- Plastic petri plates, 20-25 mm \times 150-mm diameter, sterile
- Sterile laminar-flow hood
- 1.5-ml microcentrifuge tubes or 15-ml tubes
- Platform shaker
- Parafilm
- Growth room or chamber (16-hr day, 70 to 120 $\mu\text{Em}^{-2}\text{sec}^{-1}$, 20° to 25°C)
- Single-edge razor blades
- 100-mm petri dishes
- 500-ml beakers (optional)

Plastic wrap (optional)
60 W light source (optional)
30-ml glass centrifuge tubes (e.g., Corex)
200- μ m nylon mesh (e.g., Sefar America), 100- to 120-mm squares, fashioned into a cone and stapled to hold its shape
Small funnel
50-ml centrifuge tubes
Tabletop centrifuge with swinging-bucket rotor, capable of slow acceleration and deceleration
Protoplast rupturing device (see recipe)
Superspeed centrifuge capable of $39,000 \times g$, with a 50-ml tube swing-out rotor
23-cm Pasteur pipet

Prepare plates

1. After autoclaving, allow the Murashige and Skoog growth medium to cool before pouring into the 150-mm diameter plates. Pour 50 ml of growth medium per plate in a sterile laminar-flow hood and allow to set.

Plates may be used immediately, or stored in a sealed, sterile environment for up to 6 weeks at 4°C.

Plants from about two plates, yielding 0.5 to 1 mg chlorophyll intact chloroplasts, are used for each chloroplast isolation procedure.

Prepare seeds and grow plants

2. Add 1 or 12 ml of seed sterilizing solution to seeds in a microcentrifuge tube (if sowing two plates) or a sterile 15-ml tube (for sowing 3 to 12 plates), respectively. Rock for 20 min at room temperature on a platform shaker.

3. Wash seeds four times with sterile water.

Seeds may be centrifuged for 15 sec at $100 \times g$ to facilitate washing. The supernatant can be decanted in a laminar-flow hood to maintain sterility.

4. Resuspend seeds in 0.1% (w/v) sterile agarose and dispense ~ 1 ml per plate in the laminar-flow hood. Add 2 to 3 ml sterile water to each plate. Tip and shake the plates to evenly distribute the seeds. Allow the plates to dry with lids slightly askew in a laminar-flow hood.

Excess water can be removed from the plates by pipetting to speed the drying process.

5. When the plates are dry, seal with Parafilm and vernalize the seeds by incubating the plates at 4°C for 24 to 48 hr.

6. Transfer to a growth room (16-hr day, 70 to 120 $\mu\text{Em}^{-2}\text{sec}^{-1}$, 20° to 25°C).

After several days, condensation may accumulate on the lids. Plants remain small in these conditions, growing no larger than 15 mm in diameter. Import-competent chloroplasts can be obtained from plants between 2 and 6 weeks of age (assuming obvious senescence or death has not occurred by 6 weeks). Plants that are 4-weeks-old work well as they provide ample tissue without evidence of senescence. Destarching of plate-grown plants, by incubating them in the dark, is not necessary.

Harvest the tissue

7. Harvest the entire aerial portions of the plants with a razor blade, and place immediately in a 100-mm petri dish containing 15 ml digestion buffer on ice. When all the tissue has been harvested, place the petri dish on a firm surface and chop the tissue rapidly for up to 1 min using a fresh razor blade.

The tissue should separate from the agar easily, and be chopped such that it is no longer matted. If soil-grown tissue is used, leaves or the entire green portion of the plants may be harvested. The larger soil-grown leaves can be gently sliced such that each leaf is cut at least once.

8. Drain the tissue and wash with 10 to 20 ml digestion buffer. Allow the dish to sit on ice for several minutes and swirl to release cell contents released during the chopping. Pour off the digestion buffer and repeat the washing process until the discarded buffer is no longer, or only faintly, green.

The petri dish, with the lid held firmly on, may be held vertically over a beaker and allowed to drain, to facilitate washing. Typically, two to three washes are required.

Digest the tissue

9. Add 10 ml digestion enzyme solution to the drained tissue, swirl to mix, and distribute the tissue uniformly with fingertips.

The ratio of tissue to enzyme solution is high, but there should be sufficient liquid such that it is visible when the petri dish is tilted. A small amount of additional digestion buffer may be added if necessary, to achieve this.

The amount of digestion enzymes should be halved for soil-grown plants, such that the final concentrations are 2% cellulase, 0.4% macerozyme.

10. Place the petri dish by a window that receives indirect sunlight at ~ 50 to $100 \mu\text{Em}^{-2}\text{sec}^{-1}$ and a temperature of 21° to 25°C for 4 to 5 hr.

Alternatively, the digestion may be carried out in a 500-ml beaker, covered with plastic wrap, placed in a water bath at 23°C , and illuminated with a 60 W bulb.

The digestion time required for soil-grown tissue is ~ 3 to 3.5 hr.

11. When digestion nears completion, prepare a 40% (10 ml): 85% (7 ml) AT Percoll step gradient in a 30-ml glass centrifuge tube and hold on ice.

Alternatively, a 50% continuous AT Percoll gradient may be used. Prepare by mixing well 15 ml Percoll and 15 ml gradient buffer in a 50-ml centrifuge tube. Centrifuge for 30 min at $39,000 \times g$, 4°C , with the brake off. A swinging-bucket rotor is not essential at this step. Hold on ice.

12. When the tissue is sufficiently digested, it becomes a darker green and the bathing medium becomes green when swirled gently. Transfer the petri dish to ice.

Refrain from swirling the tissue until it is reasonably certain that digestion is complete. Protoplasts may be less stable once released into the medium and yields can suffer. In soil-grown tissue, tiny holes appear in the leaves when digestion nears completion.

Harvest protoplasts

13. Harvest the protoplasts by gently swirling the petri dish for 30 to 60 sec and filtering the medium through a 200- μm mesh cone, fitted inside a small funnel, into a 50-ml centrifuge tube on ice.

Using a 5-ml Gilson pipetman, with ~ 15 mm cut from the end of the tip, facilitates harvesting. Avoid splashing and dripping of the protoplast suspension by allowing it to run down the inside of the tube.

14. Transfer any tissue from the nylon mesh back into the petri dish and wash the tissue with 10 to 15 ml of fresh digestion buffer, filtering it into the same 50-ml centrifuge tube. Wash the tissue one or two times more until little green is released from the tissue.

Usually this coincides with the centrifuge tube becoming full.

15. Centrifuge the protoplasts for 5 min at $100 \times g$, 4°C , in a swinging-bucket rotor using a slow-accelerating tabletop centrifuge.
16. Remove and discard the green supernatant carefully with a pipet. Be careful not to disturb the protoplast pellet as it is very loose. Resuspend the pellet in 5 ml protoplast resuspension buffer by gently swirling the pellet while the buffer is dispensed down the side of the tube.

A few drops of the protoplast suspension may be removed at this stage for inspection under a light microscope.

17. Centrifuge the protoplasts for 2 min at $100 \times g$, 4°C , in a tabletop centrifuge.

Break the protoplasts

18. Remove the supernatant and resuspend the pellet in 5 ml protoplast breakage buffer using the technique described in step 16.

Before resuspending the pellet, ensure that the tools needed are at hand and the centrifuge is prepared. Once the protoplasts are broken, the goal is to load the released chloroplasts on the Percoll gradient as quickly as possible to minimize their exposure to proteases or other inhibitory compounds from either the digestion enzymes or from the ruptured protoplasts.

19. Immediately transfer the resuspended protoplast pellet into the barrel of the protoplast rupturing device (see recipe). Holding the end of the device over a 50-ml centrifuge tube on ice, carefully replace the plunger and gently and firmly force the suspension through the layers of mesh. Repeat this procedure.

A few drops of the suspension may be removed at this stage for inspection under a light microscope to ensure that all of the protoplasts have been ruptured. If this cannot be done quickly, it is prudent to assume that this has occurred so as not to delay loading the gradient.

Isolate chloroplasts

20. Quickly and carefully layer the broken protoplasts onto the AT Percoll step gradient (step 11). Centrifuge in a swing-out rotor for 10 min at $2500 \times g$, 4°C , with the brake off.

If using a continuous gradient, centrifuge in a swing-out rotor for 10 min at $8000 \times g$, 4°C , with the brake off.

21. Following centrifugation, visualize two green bands in the gradient: an upper band of broken chloroplasts at the protoplast breakage buffer/40% AT Percoll interface, and a lower band of intact chloroplasts at the 40%/85% AT Percoll interface. Remove the load zone and the upper band of broken chloroplasts by aspiration. Harvest the lower band using a 23-cm Pasteur pipet and transfer to a 50-ml centrifuge tube.

About 5 ml is typically harvested from the gradient.

22. Dilute with 40 to 45 ml HEPES-sorbitol buffer, pH 8.0, and centrifuge for 5 min at $700 \times g$, 4°C .

The authors routinely use an AT Percoll step gradient because of the ease of reproducibility of the gradient and the sharp resolution of chloroplasts at the step interfaces. The linear gradient also will yield two major bands: one containing broken chloroplasts near the top of the gradient, and a second band of intact chloroplasts near the bottom of the tube. Occasionally, a third band may be visible between the upper and lower bands towards the top of the Percoll gradient. It is composed of mostly broken chloroplasts and should be ignored.

- Carefully decant the supernatant and, without righting the tube, blot away all excess buffer. Resuspend the pellet in a small volume (200 to 300 μ l) of HEPES-sorbitol buffer, pH 8.0 and quantify the chlorophyll (see Support Protocol 1).

Occasionally, Arabidopsis chloroplasts have a tendency to clump together when being resuspended. To minimize this, swirl the pellet gently to loosen it, and continue to swirl while adding the HEPES-sorbitol buffer, pH 8.0, drop-wise. It may be necessary to gently pipet the suspension up and down to completely resuspend the pellet.

PRODUCTION OF [³⁵S]METHIONINE-LABELED IMPORT SUBSTRATE BY IN VITRO TRANSLATION

Typically, chloroplast-targeted proteins to be used as import substrates are synthesized as [³⁵S]methionine-labeled proteins using an in vitro translation system. These proteins can be made using the methods outlined in *UNIT 11.2* or using commercially available coupled transcription-translation systems (e.g., Promega). The coupled systems are relatively easy to use and generally produce enough substrate to be used in multiple import reactions. There has been some suggestion that wheat-germ extracts are preferable for producing import substrates to be used in chloroplast import assays, since they are plant-based. However, in practice, import substrates produced using rabbit reticulocyte lysates are efficiently imported into chloroplasts as well. Therefore, the method used to produce the import substrate will depend on which system is best suited for the individual protein being tested.

Materials

- Expression vector harboring the gene that encodes protein to be used as import substrate
- Plasmid isolation kit (e.g., Qiagen Plasmid Midi kit)
- In vitro translation kit (e.g., TNT Coupled Reticulocyte Lysate system, Promega)
- Destain solution (see recipe)
- Image acquisition software (e.g., Molecular Dynamics)
- Additional reagents and equipment for SDS-PAGE gel (*UNIT 6.1*)

In vitro translate [³⁵S]methionine-labeled import substrate using commercial reticulocyte lysate coupled transcription-translation system

- Isolate the expression vector harboring the gene that encodes the protein to be used as an import substrate from an overnight culture of *E. coli*.

Many commercial plasmid-isolation kits can be used for this purification. It is recommended that the isolated plasmid be extracted with phenol/chloroform prior to being used for in vitro transcription/translation. Novagen's pET series of vectors that use the T7 promoter work well for in vitro transcription/translation of proteins.

- Follow the manufacturer's instructions for synthesizing [³⁵S]methionine-labeled protein.

Promega's TNT Coupled Reticulocyte Lysate system produces ample, high-quality translation product that can be used for numerous import assays.

- Run a small aliquot (0.5- to 1- μ l) of the translation product on an SDS-PAGE gel (*UNIT 6.1*). Fix the gel in destain solution for 30 min and dry under a vacuum. Expose to a storage phosphor screen overnight (*UNIT 6.3*).
- View an image of the gel and quantify the radioactivity for each of the translation products using commercial software.

Adjust the amount of translation product used in the in vitro import assay to be at least 10,000 cpm (see Basic Protocol).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

AT Percoll, 40% (v/v)

40% (v/v) Percoll (Sigma-Aldrich)
50% (v/v) gradient buffer (see recipe)
Store up to 1 month at -20°C and thaw before use

Optional, for isolating Arabidopsis chloroplasts using a Percoll step gradient.

AT Percoll, 85% (v/v)

For 50 ml:
42.5 ml Percoll (Sigma-Aldrich; final 85%)
2.5 ml 1 M HEPES-KOH, pH 7.3 (final 50 mM)
3.01 g sorbitol (final 330 mM)
 H_2O to 50 ml
Store up to 1 month at -20°C and thaw before use

Optional, for isolating Arabidopsis chloroplasts using a Percoll step gradient.

Chloroplast import master mix, 50 ml

For 50 ml:
2.5 ml 1 M HEPES-KOH, pH 7.5 (final 50 mM)
3.01 g sorbitol (final 330 mM)
2.5 ml 1 M magnesium acetate (final 50 mM)
2.5 ml 5 M potassium acetate (final 250 mM)
 H_2O to 50 ml
Store up to 4 weeks at 4°C

DMSO, 40% (v/v)

For 10 ml:
250 μl 1 M HEPES-KOH, pH 7.5 (final 25 mM)
0.0613 g sorbitol (final 330 mM)
4 ml DMSO (final 40% v/v)
 H_2O to 10 ml
Store up to 2 weeks at 4°C

Destain solution

45% (v/v) methanol
10% (v/v) acetic acid
Store indefinitely at room temperature

Digestion buffer

For 400 ml:
8 ml 1 M MES-KOH, pH 5.2 (final 20 mM)
29.13 g sorbitol (final 400 mM)
200 μl 1 M CaCl_2 (final 0.5 mM)
 H_2O to 400 ml
Store up to 2 weeks at 4°C

Digestion enzyme solution

Dissolve 0.4 g cellulase “onozuka” R 10 (e.g., Yakult, Kanematsu) and 0.08 g macerozyme R 10 (e.g., Yakult, Kanematsu) in 10 ml digestion buffer (see recipe). Centrifuge for 5 min at $2000\times g$ to pellet insoluble materials. Use supernatant immediately.

The volume, 10 ml, is enough for two plates of Arabidopsis.

continued

**In Vitro
Reconstitution**

11.16.15

Gradient buffer

For 100 ml:

10 ml 1 M HEPES-KOH, pH 7.3 (final 100 mM)
12.03 g sorbitol (final 660 mM)
100 μ l 2 M MgCl₂ (final 2 mM)
200 μ l 1 M MnCl₂ (final 2 mM)
800 μ l 0.5 M EDTA (final 4 mM)
0.2 g BSA (final 0.2%)
H₂O to 100 ml
Store up to 2 weeks at 4°C

Grinding buffer

For 2 liters:

50 ml 1 M HEPES-KOH, pH 7.7 (final 25 mM)
120.25 g sorbitol (final 330 mM)
8 ml 0.5 M EDTA (final 2 mM)
1 ml 2 M MgCl₂ (final 1 mM)
2 ml 1 M MnCl₂ (final 1 mM)
H₂O to 2 liters
Store up to 2 weeks at 4°C
Just prior to use, add BSA to a final concentration of 0.25% (w/v) and sodium ascorbate to a final concentration of 5 mM (0.1% w/v).

HEPES-sorbitol buffer, pH 7.5

For 500 ml:

12.5 ml 1 M HEPES-KOH, pH 7.5 (final 25 mM)
30.06 g sorbitol (final 330 mM)
H₂O to 500 ml
Store up to 2 weeks at 4°C

HEPES-sorbitol buffer, pH 8.0

For 100 ml:

5 ml 1 M HEPES-KOH, pH 8.0 (final 50 mM)
6.01 g sorbitol (final 330 mM)
H₂O to 100 ml
Store up to 2 weeks at 4°C

Murashige and Skoog growth medium

1 liter for 20 plates:

4.4 g Murashige and Skoog salt and vitamin mix with buffer (e.g., Life Technologies)
10 g sucrose
H₂O to 800 ml
Adjust pH to 5.7 with 1 M KOH
H₂O to 1 liter
Add 8 g Phytagar (e.g., Life Technologies)
Autoclave
Allow to cool to touch
Pour into large, sterile petri dishes
Allow plates to set and dry
Store unused plates, sealed in a bag, up to 6 weeks at 4°C

If a Murashige and Skoog salt and vitamin mix without buffer is used, supplement with 50 mM MES-KOH, pH 5.7.

PBF-Percoll

For 170 ml:

5.1 g polyethylene glycol 4000
1.7 g BSA
1.7 g Ficoll
Percoll (Sigma-Aldrich) to 170 ml
Store up to 1 month at -20°C

PBF-Percoll, 40% (v/v)

For 50 ml:

20 ml PBF-Percoll (final 40%)
1.25 ml 1 M HEPES-KOH, pH 7.5 (final 25 mM)
3.01 g sorbitol (final 330 mM)
200 μl 0.5 M EDTA (final 2 mM)
25 μl 2 M MgCl_2 (final 1 mM)
0.0077 g glutathione (reduced; final 500 μM)
0.05 g sodium ascorbate (final 0.1%)
 H_2O to 50 ml
Store up to 1 month at -20°C , thaw immediately before use

PBF-Percoll, 85% (v/v)

For 50 ml:

42.5 ml PBF-Percoll (final 85%)
1.25 ml 1 M HEPES-KOH, pH 7.5 (final 25 mM)
3.01 g sorbitol (final 330 mM)
200 μl 0.5 M EDTA (final 2 mM)
25 μl 2 M MgCl_2 (final 1 mM)
0.0077 g glutathione (reduced; final 500 μM)
0.05 g sodium ascorbate (final 0.1%)
 H_2O up to 50 ml
Store up to 1 month at -20°C , thaw immediately before use

Percoll, 40% (v/v)

For 50 ml:

20 ml Percoll (Sigma-Aldrich; final 40%)
2.5 ml 1 M HEPES-KOH, pH 7.5 (final 50 mM)
3.01 g sorbitol (final 330 mM)
 H_2O to 50 ml
Store up to 1 month at -20°C , and thaw before using

Protoplast breakage buffer

For 50 ml:

1 ml 1 M tricine-KOH, pH 8.4 (final 20 mM)
2.733 g sorbitol (final 300 mM)
0.5 ml 0.5 M EDTA (final 5 mM)
0.5 ml 0.5 M EGTA (final 5 mM)
0.042 g NaHCO_3 (final 10 mM)
 H_2O to 50 ml
Store up to 2 weeks at 4°C
Immediately before use add 0.05 g BSA (final 0.1% w/v)

continued

**In Vitro
Reconstitution**

11.16.17

Protoplast resuspension buffer

For 50 ml:

8 ml 1 M MES-KOH, pH 6.0 (final 20 mM)

29.13 g sorbitol (final 400 mM)

200 μ l 1 M CaCl₂ (final 0.5 mM)

H₂O to 50 ml

Store up to 2 weeks at 4°C

Protoplast rupturing device

10-ml disposable syringe

10- μ m nylon mesh, 30- to 35-mm square

20- μ m nylon mesh, 30- to 35-mm square

Electrical tape, 15- to 20-mm wide

Cut off the end of the syringe barrel so that it resembles a hollow tube. Put the 10- μ m mesh on top of the 20- μ m mesh and place both over the cut end of the syringe such that the 10- μ m mesh faces the outside and the 20- μ m mesh is against the syringe barrel. Fix the mesh in place using the electrical tape to hold the two layers of mesh to the sides of the syringe leaving the mesh exposed at the end of the barrel.

This procedure is tricky. The meshes must be very firmly taped to the barrel or leaks will occur during use. Use a lot of electrical tape, wrapping it multiple times, overlapping the tape as far as half way down the syringe barrel and back up to the top. Constantly, and very firmly pull and stretch the tape to ensure a good seal. Both 20- and 10- μ m meshes are used to more gently rupture large protoplasts (with 20- μ m mesh), before more rigorously rupturing smaller protoplasts (with 10- μ m mesh).

Seed sterilizing solution

1.5% (w/v) sodium hypochlorite (dilute commercially available bleach accordingly)

0.02% (v/v) Triton-X 100

Store at room temperature until used

SDS-PAGE sample buffer

For 1 ml:

350 μ l 1 M Tris base (final 350 mM)

250 μ l 20% (w/v) SDS (final 5% (w/v))

80 μ l 1 M dithiothreitol (DTT; final 80 mM)

150 μ l 50% (v/v) glycerol (final 7.5%)

10 μ l saturated bromophenol blue

160 μ l H₂O

Store at -20°C until used

TE/DTT buffer

For 50 ml:

2.5 ml 1 M tricine-KOH, pH 7.5 (final 50 mM)

200 μ l 0.5 M EDTA (final 2 mM)

50 μ l 1 M dithiothreitol (DTT; final 1 mM)

47.25 ml H₂O

Store up to 2 weeks at 4°C

Thermolysin, 2 mg/ml

Dissolve 2 mg thermolysin in 1 ml HEPES-sorbitol buffer (see recipe). Divide into 50- μ l aliquots and store up to 6 months at -80°C. Do not freeze/thaw more than one time.

Trichloroacetic acid (TCA), 100% and 0.5%

To make 100% (w/v) TCA, add 227 ml of water directly to a bottle containing 500 g of solid TCA. Dissolve by shaking the closed container. Dilute part of the 100% solution to 0.5% (w/v) with an appropriate volume of water. Store indefinitely at room temperature.

COMMENTARY

Background Information

Traditionally, pea chloroplasts have been used as a model system for measuring chloroplast precursor protein import. The pea offers the advantages of the ability to obtain large amounts of material that can be grown quickly and easily, and rapid and simple isolation of intact import-competent chloroplasts. The basic mechanism of protein import into chloroplasts appears to be conserved across diverse plant species. In fact, isolated pea chloroplasts are capable of importing preproteins from species as far removed as the algae (Mishkind et al., 1985; Yu et al., 1988; Su and Boschetti, 1994). This makes the pea an ideal system for investigating protein targeting to chloroplasts, and in most cases, the demonstration of preprotein import and processing in pea chloroplasts is sufficient to establish chloroplast localization. However, the growing importance of *Arabidopsis thaliana* as a model system for the investigation of genetic, molecular, and cellular processes in plants makes it desirable to use a homologous biochemical system to analyze chloroplast targeting. In particular, the identification of gene families encoding components of the import apparatus in the complete *Arabidopsis* genome has focused considerable attention on *Arabidopsis* as a model for investigating the molecular mechanism of the import reaction (Jackson-Constan and Keegstra, 2001). The methods for isolating functional chloroplasts from *Arabidopsis* are less developed than those for pea, but experimental amounts of import-competent chloroplasts can now be isolated from *Arabidopsis* (Fitzpatrick and Keegstra, 2001).

Critical Parameters

The yield and import-competence of chloroplasts is directly related to the age and condition of the plant material. The plant material should be obtained from young seedlings. The protein import capacity of chloroplasts is developmentally regulated (Dahlin and Cline, 1991). The highest import capacities are observed in chloroplasts from young, rapidly growing tissues. Therefore, allowing plants to

grow larger to obtain more material or isolating chloroplasts from mature plants will result in low import yields.

Temperature is one of the most important parameters that will determine the quality of chloroplasts isolated by these protocols. As stated above, it is essential that all reagents for the isolation of chloroplasts from both species be kept ice-cold and that all procedures be performed on ice or in a cold room to ensure that the extracts never warm up during isolation. The degradation of components of the import machinery during chloroplast isolation has been documented, and although protease inhibitor mixtures can aid in preventing degradation, they are only moderately effective (Bolter et al., 1998; Chen et al., 2000). The best approach to minimize degradation is to work quickly and in the cold. In addition, it is preferable to work in moderate to low light conditions during the isolation procedure. This will prevent photooxidation-induced damage to the chloroplasts.

Freshly isolated chloroplasts can be stored on ice for up to 30 min before initiating the import assay. However, as stated for the isolation procedure, it is best to have all reagents for the import assay assembled and ready to proceed directly to the import assay following the isolation procedure.

Troubleshooting

The potency of the cell wall-degrading enzymes used to generate the *Arabidopsis* protoplasts can vary. Therefore, if yields of protoplasts or chloroplasts from *Arabidopsis* (see Support Protocol 2) seem low, it may be necessary to adjust the digestion time accordingly. Since the enzyme preparations are rather crude and may contain many other degradative enzymes that could decrease yield, use caution when extending digestion times. In addition, it is important not to agitate the tissue during the digestion but to swirl the tissue thoroughly to release the protoplasts only when digestion is complete.

Import efficiency of precursor proteins (see Basic Protocol) varies widely. Should effi-

ciency be <20% of the added import substrate or if it is difficult to detect the imported product, it may be necessary to increase the amount of translation product or chloroplasts used in the assay.

Some proteins, particularly those targeted to the chloroplast envelope, may be susceptible to proteolysis by proteases that co-purify with chloroplasts. It may be possible to limit this proteolysis by performing the import reaction at 20°C, rather than 26°C (see Basic Protocol).

Anticipated Results

The expected results from an import assay will vary depending on the preprotein being studied. All proteins targeted to the internal compartments of the chloroplast contain a cleavable *N*-terminal transit peptide that is removed in the stroma after translocation across the envelope. Therefore, a protein imported into the stroma will have a lower molecular weight than its precursor form prior to import. While the length of transit peptides varies greatly (Chen and Schnell, 1999; Keegstra and Cline, 1999), in most cases the reduction in molecular weight (3000 to 10,000 Da) should be large enough to be easily detected by SDS-PAGE analysis. In order to confirm that the lower molecular weight protein has been imported, chloroplasts are treated with the protease thermolysin following the import reaction. At least 70% of the lower-molecular-weight protein should be protected from proteolysis to confirm that import to the stroma has taken place. Often, both processed and unprocessed forms of the import substrate will be detected in chloroplasts before treatment with exogenous protease. The unprocessed form of the precursor protein represents protein that is nonspecifically associated with the outer chloroplast envelope or bound to components of the import apparatus at the early, binding stages of import. The unprocessed form should therefore be removed by treatment with thermolysin. Thermolysin treatment can also be used to confirm that outer envelope membrane proteins have been integrated. In this case, the thermolysin will degrade only those portions of the protein exposed to the exterior of the chloroplast, leaving a predictable portion of the protein protected from proteolysis. In addition, resistance to alkaline extraction can be used to confirm the integration of membrane proteins.

Time Considerations

Chloroplast isolation from pea should not take >1 hr from the time of tissue homogenization to isolation of the intact chloroplasts. The

entire time for the procedure, including gradient preparation and chlorophyll determination, may take ~2 hr. Isolation of chloroplasts from *Arabidopsis* takes considerably longer, as the tissue is digested with protoplast-forming enzymes for 4 to 5 hr. Isolation of intact chloroplasts from protoplasts takes an additional 45 to 60 min. The *in vitro* import assay requires 20 to 30 min of incubation time and additional time to re-isolate and process the chloroplasts following import. This time can vary from 2 to 8 hr depending on the method used to treat the chloroplasts and the degree of chloroplast sub-fractionation. A polyacrylamide gel takes 2 to 3 hr to run, 30 min to fix, and 30 to 60 min to dry. The gel is then exposed to a storage phosphorimaging screen or X-ray film overnight (16 hr) or longer, depending on the strength of signal and efficiency of import.

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In Vitro RNA Splicing in Mammalian Cell Extracts

Almost every eukaryotic pre-mRNA generated by RNA polymerase II transcription requires the removal of introns to create mRNA. The correct splicing of constitutive exons is thus critical for normal protein expression and function. Moreover, the removal of many introns by the spliceosome is controlled in a tissue-specific or developmentally specific manner. The differential skipping or inclusion of exons in the final mRNA is called alternative splicing, and often leads to changes in the final protein product. By this mechanism, a finite number of genes in the genome can code for a more complex proteome. Aberrant splicing can lead to a disease state by altering the protein products of a gene. Thus, understanding the regulation of many genes requires a study of the splicing of their RNA products.

In order to study RNA splicing at a biochemical level, it is necessary to employ an *in vitro*, or cell-free, system. Cell-free splicing systems require two main components: (1) an extract made from mammalian cell nuclei and (2) the introns and exons of the eukaryotic gene of interest, cloned downstream from a prokaryotic phage promoter, such as T7 or SP6. This minigene construct allows the synthesis of sufficient quantities of pre-mRNA substrates *in vitro*, which are then incubated in the nuclear extract and analyzed for splicing. Nuclear extracts, first developed for studying transcription *in vitro* (UNIT 11.6), were subsequently modified for splicing (Support Protocol 1). Extracts are commonly generated from the HeLa cell line, but other cell lines and tissues can be used (Support Protocol 2). This is important when studying the regulation of an exon that is alternatively spliced in different cell types. These extracts can be used for functional studies of splicing and for isolation and characterization of splicing factors. This unit describes how to set up an *in vitro* splicing reaction (see Basic Protocol 1) using a mammalian nuclear extract derived from either cell line or tissue, and how to analyze the splicing reaction products (see Basic Protocol 2).

CREATING A MINIGENE CONSTRUCT FOR TESTING A NOVEL SUBSTRATE

To study the splicing of a gene of interest, its exons and introns must be cloned downstream of a bacteriophage promoter to create a minigene construct. Minigenes are subcloned from genomic DNA. It is difficult to analyze the splicing of introns larger than 1000 nucleotides *in vitro*. This is because lariat intermediates containing long introns cannot be resolved in a single denaturing acrylamide gel with smaller splicing intermediates and products (see Basic Protocols 1 and 2). Since mammalian introns can be very long (much longer than 1000 nucleotides), it is often not possible to test a full-length intron in an *in vitro* splicing reaction. This problem is overcome by deleting stretches of intron sequence that are not critical for splicing regulation.

Before testing the splicing of the original genomic clone and deletion mutants *in vitro*, it is necessary to test them *in vivo*. This will confirm that any shortened minigene version of the gene shows proper splicing and regulation. To test the splicing substrates *in vivo*, the equivalent minigene can be constructed in a mammalian expression vector and transfected into the appropriate cell line(s). After transfection and the appropriate incubation period, RNA is harvested from the cells and the percentage of inclusion or skipping of the exon in the mRNA is assayed by primer extension, RT-PCR, and ribonuclease protection assays. Further deletions can be made based on the initial *in vivo* results. One

way to guess which sequences are important in splicing regulation is through comparing the same intronic sequences of different species. Phylogenetically conserved intronic sequences are likely to be more important in splicing regulation than those sequences that are divergent. The goal is to create a minigene with short introns while maintaining the regulation of splicing of the exon. Once this has been achieved, the minigene can then be subcloned downstream of a bacteriophage promoter and used in the in vitro system.

NOTE: All reagents for these protocols should be high-quality, molecular-biology grade, and RNase- and DNase-free. All glass and plasticware should be RNase-free. Care should be taken to avoid RNase contamination (also see *APPENDIX 2A* for considerations when working with RNA).

IN VITRO SPLICING OF PRE-MESSENGER RNA

Methods for in vitro splicing of pre-mRNA in nuclear extract derived from animal cell lines or tissues have been described for many years (Hernandez and Keller, 1983; Padgett et al., 1983; Hardy et al., 1984; Krainer et al., 1984; Rio, 1988; Kramer and Keller, 1990; Black, 1992; and Eperon and Krainer, 1994). In these protocols, a uniformly radiolabeled pre-mRNA is incubated for several hours in nuclear extract supplemented with salts and other required cofactors. The RNA is then extracted from the nuclear extract, subjected to denaturing polyacrylamide gel electrophoresis, and visualized by autoradiography of the dried gel on film or a phosphor imager screen. The pre-mRNA and the reaction products and intermediates are identified as bands on the autoradiogram (see Basic Protocol 2).

Materials

- Buffer DG (see recipe)
- 10 mM ATP
- 0.5 M creatine phosphate
- 55 mM MgCl₂
- 20% (w/v) polyethylene glycol 3350 (PEG; mol.wt. 3350; Sigma)
- Nuclear extract (Support Protocols 1 and 2)
- 20 U/μl RNase inhibitor (RNA guard; Amersham Pharmacia Biotech; Ribonuclease Inhibitor, porcine)
- 5 to 20 fmol/μl uniformly radiolabeled pre-mRNA splicing substrate (Support Protocol 3)
- 20 mg/ml proteinase K (Amersham Pharmacia Biotech)
- 10% (w/v) sodium dodecyl sulfate (SDS; *APPENDIX 2A*)
- 1.1× PCA buffer (see recipe)
- 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (PCA)
- 20 mg/ml glycogen (Roche)
- 100% ethanol, ice-cold
- RNA loading buffer (see recipe)
- 30°C and 80°C water baths or heating blocks
- Whatman 3MM filter paper
- Kodak XAR X-ray film (standard 14 × 17-in.) with intensifying screen, or phosphor imager screen and imager (e.g., PhosphorImager from Molecular Dynamics or equivalent; see *UNIT 6.3*)
- Additional reagents and equipment for denaturing PAGE of nucleic acids (*APPENDIX 3A*) and autoradiography (*UNIT 6.3*)

Prepare splicing reaction

1. Place microcentrifuge tube(s) on ice and add in the order indicated:

- ≤15 μl buffer DG (for total of 15 μl buffer DG and nuclear extract)
- 1 μl 10 mM ATP
- 1 μl 0.5 M creatine phosphate
- 1 μl 55 mM MgCl₂ (2.2 mM final)
- 2.5 μl 20% PEG 3350
- ≤15 μl nuclear extract (for total of 15 μl buffer DG and nuclear extract)
- H₂O to bring total volume of reaction to 24 μl
- 0.5 μl 20 U/μl RNase inhibitor.

The sum of the volumes of buffer DG and the nuclear extract should equal 15 μl, or 60% of the total reaction volume. The splicing activity of the nuclear extract will vary with each nuclear extract preparation. Also, different pre-mRNAs will splice with different efficiencies in each nuclear extract preparation; thus the amount of extract used in a splicing reaction should be titrated with respect to each pre-mRNA being tested. In addition, the splicing efficiencies of different pre-mRNAs can be affected by the concentration of MgCl₂ (see Critical Parameters). Therefore, the amount of MgCl₂ should also be titrated to optimize the splicing reaction. The use of PEG 3350 and RNase inhibitors is not always necessary. For notes on RNase contamination, see Critical Parameters in the Commentary section.

The total reaction volume can be scaled up if needed. Other protocols have been used in which the total reaction volume can vary from 10 to 50 μl.

2. Remove the reaction mixture(s) from the ice, mix by gently flicking the tube, and incubate in a 30°C water bath or heating block for 8 to 10 min.
3. Add 1 μl of a solution containing 5 to 20 fmol radiolabeled pre-mRNA to bring the total reaction volume to 25 μl. Mix the reagents by gently flicking the microcentrifuge tube. Centrifuge briefly to collect any droplets on the side of the tube and continue incubating at 30°C for 2 to 4 hr.

The duration of the incubation is dependent on the rate of splicing for a particular pre-mRNA substrate, i.e., the percent conversion to intermediates and products as a function of time. The rate and overall splicing efficiency of a particular substrate is determined by doing a time-course experiment. Doing a time course can also help in identifying the splicing reaction products (see Basic Protocol 2). For some pre-mRNAs, splicing is readily detectable within 2 hr or less, but weaker substrates may take up to 4 hr to accumulate sufficient intermediates and products for analysis.

4. Terminate the splicing reaction by adding 3 μl of 20 mg/ml proteinase K and 2 μl of 10% SDS to the reaction. Continue to incubate at 30°C for 20 to 30 min.

Extract and separate RNA products

5. Add 370 μl 1.1× PCA buffer and 200 μl of 25:24:1 PCA. Vortex well. Microcentrifuge 1 min at maximum speed, room temperature.

Adding 370 μl of 1.1× PCA buffer to the splicing reaction (25 μl) will bring the final concentration of PCA buffer to ~1×.

6. Transfer the upper, aqueous layer to a new microcentrifuge tube.
7. Add 1 μl of 20 mg/ml glycogen (to act as a carrier for the RNA) and 1.0 ml of ice-cold 100% ethanol. Mix well. Incubate on dry ice for 10 min or overnight at –20°C. Microcentrifuge 10 min at maximum speed, 4°C, to collect the pellet of RNA at the bottom. Carefully remove as much supernatant as possible, being careful not to disturb the pellet.

A drawn-out Pasteur pipet can aid in removing the last droplets.

8. Dry the pellet in a Speedvac evaporator for 1 min.

If a Speedvac evaporator is not available, the pellet may be dried on the benchtop for 10 min.

9. Pour the appropriate percentage denaturing polyacrylamide gel (APPENDIX 3A).

The gel should be poured using a large gel-running apparatus (30 × 40-cm gel or approximate equivalent), and it should contain 8 M urea, 0.5× or 1× TBE, 19:1 acrylamide:bisacrylamide, and 4% to 12% total acrylamide. The lariat intermediates formed during splicing will have anomalous electrophoretic mobility when run in a denaturing gel. This, and the sizes of the pre-mRNA and free exon, should be taken into account when deciding what percentage polyacrylamide gel to use and how far to run it. For pre-mRNAs between 350 nucleotides to 550 nucleotides, 6% to 8% polyacrylamide gels are standard. See Basic Protocol 2 for additional considerations regarding this denaturing PAGE step.

10. Resuspend the pellet in 8 to 10 μl RNA loading buffer by vortexing, heat to 80°C for 5 min, load on the denaturing polyacrylamide gel, and separate the RNA by denaturing PAGE (APPENDIX 3A).

11. Dry the gel on Whatman 3MM filter paper on a gel dryer. Visualize the RNA by autoradiography on 14 × 17-in. Kodak film or a large phosphor imager screen.

Both of these visualization techniques are described in UNIT 6.3

BASIC PROTOCOL 2

ANALYSIS OF SPLICING REACTION RNA PRODUCTS

During the first step of splicing, the RNA is cleaved at the 5' splice site between the first exon and the intron, and this intron 5' end forms a 2'-5' phosphodiester bond at the branch-site adenosine (Fig. 11.17.1). This generates a free 5' exon and lariat-3' exon intermediate. During the second step of splicing, the 3' splice site is cleaved and the two

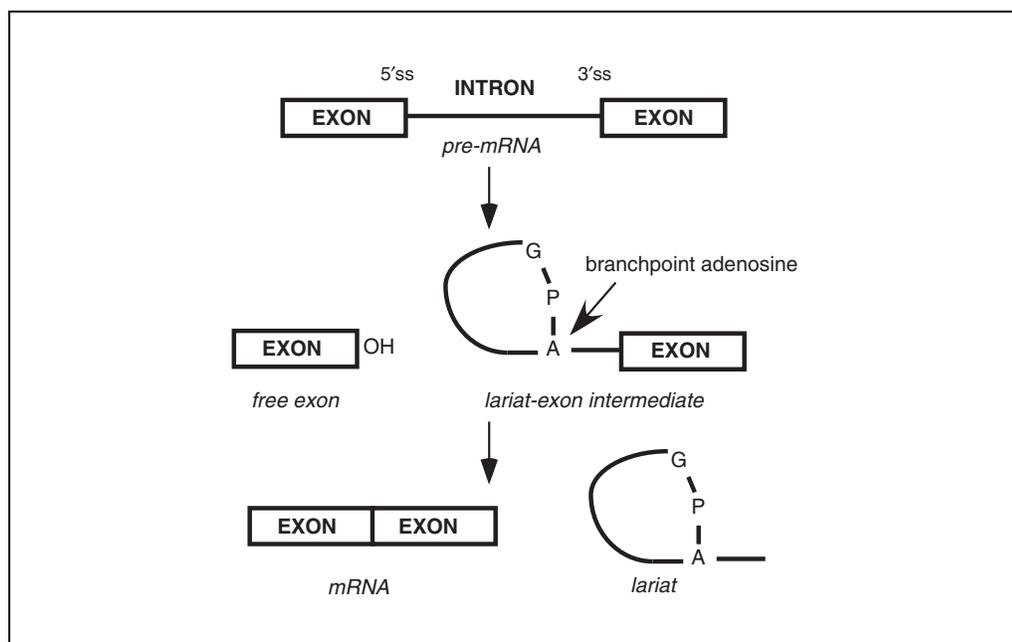


Figure 11.17.1 The splicing pathway of a two-exon, one-intron pre-mRNA. In the first step of splicing, the 2' hydroxyl of the branchpoint adenosine attacks the 5' end of the intron, creating a 2'-5' phosphodiester bond. Two intermediates are generated, a free exon and a lariat-exon intermediate. In the second step, the 3' hydroxyl of the free exon attacks the phosphate at the 3' splice site. The lariat is released, and the two exons are ligated together, generating the final mRNA.

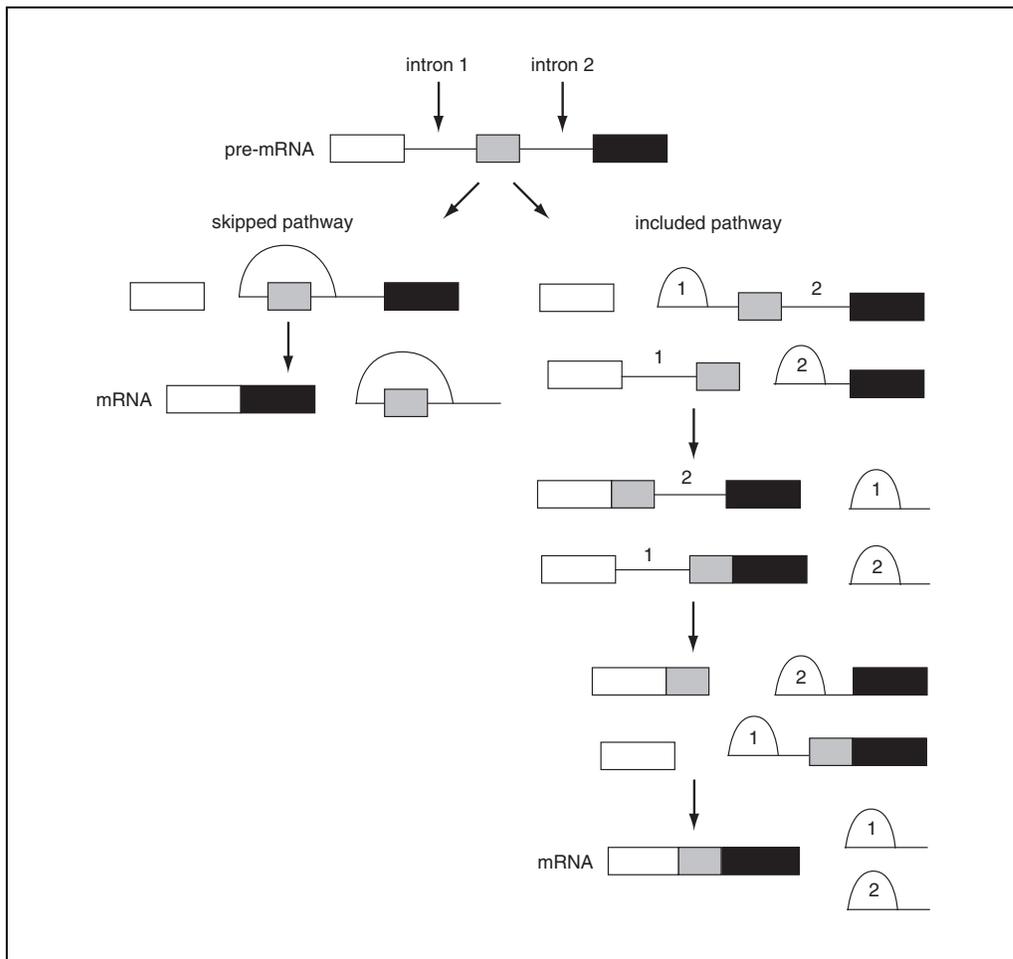


Figure 11.17.2 The splicing pathway of a three-exon, two-intron pre-mRNA. A pre-mRNA with three exons and two introns can proceed through two pathways, where the middle exon is either skipped or is included. In the included pathway, either the upstream or downstream intron can be spliced out first. Illustrated are the intermediates and products in both the skipped and included pathways.

exons are joined together, generating the final linear mRNA and the excised lariat intron. The pre-mRNA, lariat intermediate, free exon intermediate, lariat product, and final mRNA product will have distinct mobilities on a denaturing polyacrylamide gel, creating a pattern of bands. These bands will appear differentially over time. For example, the bands for the lariat intermediate and the 5' exon intermediate will appear earlier than the final mRNA and lariat intron bands.

The splicing pattern of a two-exon, single-intron pre-mRNA is relatively simple. However, when studying regulated exons it may be useful to utilize a three-exon, two-intron pre-mRNA. The complexity of products resulting from pre-mRNAs that undergo multiple splicing patterns makes the identification of bands and the interpretation of the results more difficult. Figure 11.17.2 illustrates the intermediates and products expected in the splicing pathway of a three-exon, two-intron pre-mRNA. Figure 11.17.3 is a gel image for such a pre-mRNA, which was spliced *in vitro* in HeLa nuclear extract. The latter figure demonstrates the type of splicing pattern expected with a more complex substrate. Note that, in addition to the more prominent darker bands corresponding to splicing intermediates and products, a number of other bands may appear. These are most often caused by RNA degradation in the extract, as well as by radiolysis, and are often unavoidable.

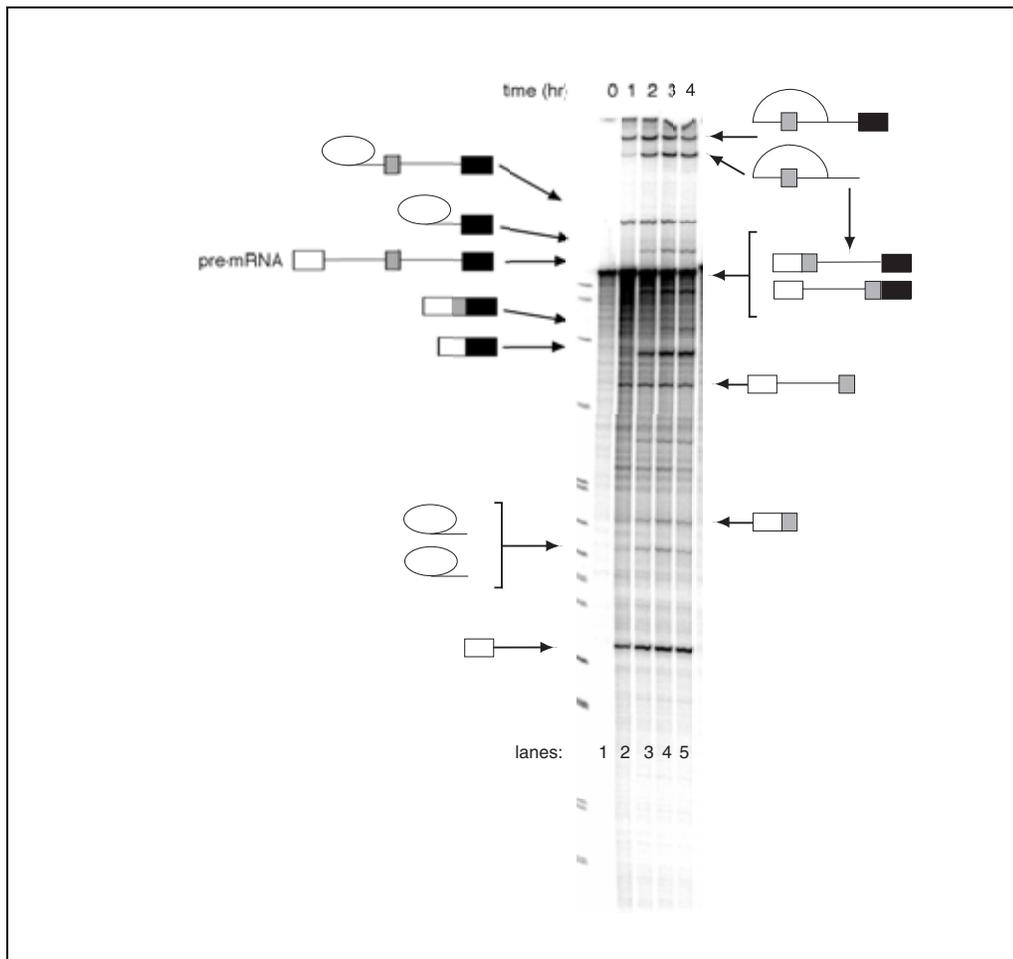


Figure 11.17.3 Autoradiogram of a splicing reaction with a three-exon, two-intron pre-mRNA. Splicing reactions containing the pre-mRNA were incubated in HeLa nuclear extract under the conditions described in Basic Protocol 1. A time course was conducted by terminating the reactions at 1-hr intervals after the initial start of incubation. The pre-mRNA used in this experiment has equal-sized introns, somewhat simplifying the pattern of splicing. It is possible to see the appearance of intermediates during the first hour of splicing, but the final products do not appear until the second hr or later (compare lanes 2 and 3).

Determining if an RNA Contains a Lariat

Determining which bands on the gel correspond to the different RNAs generated during the splicing reaction can be laborious. The linear pre-mRNA product and 5' exon intermediate can be tentatively identified by their gel mobility. Their time of appearance in a time-course experiment is also diagnostic, in that the 5' exon should appear earlier. Conclusive demonstration of the mRNA comes from RT/PCR amplification of the suspected band and sequencing of the product. This also confirms the position of the splice sites. Lariat molecules will also show diagnostic appearance times in the time course. The lariat intermediate should appear with the 5' exon and the lariat product with the mRNA. The anomalous gel mobility of the looped RNA lariat molecules is also used to determine which bands correspond to the lariats. As the percentage of polyacrylamide increases, the mobility of lariat molecules decreases dramatically relative to linear molecules. A splicing reaction can be analyzed in two denaturing gels, one of low-percentage acrylamide (4% to 5%) and one of high-percentage acrylamide (8% to 10%), and the mobility of the different bands compared. RNA lariats will migrate with a marked difference between the two gels relative to linear markers. Often, in a high-percentage gel, the lariats will

shift above the precursor RNA and be the only bands in this region of the gel. This method is further described in Grabowski (1994).

Another approach to identifying a lariat is by debranching using the lariat debranching enzyme. RNA extracted from a band of interest is incubated with cytoplasmic S100 extract (see Support Protocol 1, step 8) or recombinant debranching enzyme (Ruskin and Green, 1990; Ooi et al., 2001). The S100 extract contains the lariat debranching enzyme, which specifically recognizes and cleaves the 2'-5' phosphodiester bond. Samples of the RNA before and after debranching are separated on a denaturing acrylamide gel and their mobilities compared. The debranched lariat will migrate faster on a high-percentage gel than the intact lariat, at a position diagnostic of the linearized molecule.

Finally, it is possible to identify splicing intermediates and products by comparing related substrates. For example, increasing the length of the 3' exon in a particular substrate will cause the intermediates containing this exon to migrate more slowly in a denaturing gel. Thus, comparison of the mobilities of the intermediates and products of the initial substrate versus the substrate with a longer 3' exon can aid in the identification of bands.

Mapping the Branchpoint

Once an RNA is demonstrated to contain a lariat, it is possible to determine the site of the branchpoint. Primer extensions are used to map the site of the branchpoint. During the primer extension reaction, the 2'-5' phosphodiester bond formed at the branchpoint partially blocks extension by reverse transcriptase at the base prior to the branch. The branchpoint location is determined by finding the primer extension stop. Primer extension on a debranched or linear RNA is used as a control to determine the positions of natural stops in the RNA. These reactions are run next to a sequencing ladder to identify the position of the stops. The most widely used protocol for debranching and primer extension to determine lariat intermediates and branchpoint location is that of Ruskin and Green (1990).

PREPARATION OF NUCLEAR EXTRACT FROM TISSUE CULTURE CELLS

HeLa cells are the standard source for a nuclear extract that is active in pre-mRNA splicing. This method was originally developed to prepare nuclear extracts competent for RNA polymerase II transcription, and was later adapted to study pre-mRNA splicing (Dignam et al., 1983 and *UNIT 11.6*). Subsequent revisions of this protocol have improved the splicing activity on some pre-mRNA substrates (Krainer et al., 1984; Reichert and Moore, 2000). Nuclear extracts from other tissue-culture cells have also been successfully prepared that reflect alternative splicing phenotypes present in vivo (Noble et al., 1987; Rio, 1988; Black, 1992; Guo and Kawamoto, 2000). Briefly, the cells are swelled in a hypotonic buffer and lysed by Dounce homogenization. After centrifugation of the lysed cells, the pelleted nuclei are recovered and resuspended in a high-salt buffer. During gentle mixing, this buffer extracts RNA processing factors, but does not lyse the nuclei. After extraction, the nuclei are pelleted by centrifugation and the supernatant is the nuclear extract. After dialysis into a low-salt buffer, the extracts are ready for RNA processing reactions.

This extract protocol may be scaled down for smaller numbers of cells. Monolayer cell cultures may also be used, but should be grown on a scale that will produce at least 1 ml of packed cell volume and thus make the use of a small Dounce homogenizer feasible. Other extraction protocols have been described for preparing extracts from smaller cell numbers (Lee et al., 1988).

NOTE: This protocol uses HeLa cells, but the general technique can be used for other cell types.

SUPPORT PROTOCOL 1

In Vitro Reconstitution

11.17.7

Materials

~5–8 × 10⁹ HeLa cells (~12 liters of suspension culture grown in spinner flasks; roller bottles are utilized for some cell types) *or* other mammalian cell of interest, in suspension or monolayer culture

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Phosphate-buffered saline (PBS; *APPENDIX 2A*) with 1 mM EDTA

Buffer A (see recipe)

Buffer C420 (see recipe)

Buffer DG (see recipe)

Beckman centrifuge with JS-4.2 and JA-20 rotors (or equivalents) and appropriate centrifuge tubes/bottles

50-ml (or other appropriate size) conical tubes, graduated

Rubber policeman or cell scraper

40-ml Dounce homogenizer with tight (type A) and loose (optional) pestle (Wheaton)

Dialysis tubing (10,000 to 14,000 MWCO), prepared as described in *APPENDIX 3C*

Conductivity meter (optional)

Liquid nitrogen

Additional reagents and equipment for trypan blue staining (*UNIT 1.1*), dialysis (*APPENDIX 3C*), and Bradford protein assays (*APPENDIX 3H*)

NOTE: Perform all steps of this procedure at 0° to 4°C. All buffers, vessels, and centrifuge rotors should be precooled to this temperature.

Isolate cell nuclei

For suspension cultures

- 1a. Grow cells to log phase. Transfer a quantity of suspension containing a total of ~5–8 × 10⁹ log-phase cells to 1-liter centrifuge bottles and centrifuge for 20 min at 1800 × g in a Beckman JS-4.2 rotor, 4°C. Decant and discard the supernatants.

Overgrowth of cells can result in low splicing activity in the final extract.

- 2a. Resuspend the cells in 25 ml of PBS for each liter of suspension culture volume and transfer to graduated 50-ml graduated conical tubes. Consolidate the cells into one tube by recentrifuging, resuspending in a smaller volume of PBS, and transferring all pellets to on 50-ml tube.

For monolayer cultures

- 1b. Grow cells on ten 100- to 150-mm dishes to ~80% to 90% confluency. Remove the medium from the cells and wash the monolayer once with PBS. After washing, add fresh PBS containing 1 mM EDTA to cover the cells, then scrape the cells from the dish with a rubber policeman or cell scraper. Pool cells into conical tubes of the appropriate size for the volume of cells.

Overgrowth of cells can result in low splicing activity in the final extract.

- 2b. Centrifuge cells 10 min at 1800 × g in a JS-4.2 rotor, 4°C. Decant the supernatant and discard. If necessary, resuspend cells in a small volume of PBS, combine, and centrifuge again at 1800 × g to consolidate cells into a single conical tube.

3. Using the graduations on the conical tube, estimate the packed cell volume (pcv).

This starting pcv is utilized to estimate the volume of each buffer that is used in the preparation of the extract.

4. Gently resuspend the cells in a volume of buffer A that is ~5 times the pcv. Centrifuge cells 5 min at $1800 \times g$ in a JS-4.2 rotor, 4°C , and discard the supernatant.

This step washes residual PBS from the cells so that efficient osmotic swelling can occur in the subsequent step. Some swelling may occur during this first buffer A wash, so perform this step quickly.

5. Resuspend the cell pellet in a volume of buffer A that is ~3 times the starting pcv (from step 3). Incubate on ice for 10 min.
6. Transfer the cells to a precooled Dounce homogenizer. Homogenize using 5 to 15 slow strokes with a tight pestle.

Homogenize slowly and use special care during the down stroke to avoid loss of the sample. Lysis can be monitored by staining a small aliquot of the cells with trypan blue (UNIT 1.1). Lysis should be ~90% before proceeding. Different cell types may require different amounts of homogenization to achieve cell lysis.

7. Transfer the lysed cells to appropriate centrifuge tubes and pellet the nuclei by centrifuging 15 min at $3300 \times g$ in a JA-20 rotor, 4°C .
8. Discard supernatant or save it to prepare a cytoplasmic S100 extract. Estimate the packed nuclear volume (pnv) using the tube graduations.

A cytoplasmic extract can be prepared by slow addition of 0.11 volumes of $10\times$ cytoplasmic extract buffer (see recipe) to the above supernatant. After centrifugation for 1 hr at $100,000 \times g$, the supernatant is the S100 extract. This should be dialysed and subsequently cleared of precipitates in the same manner as a nuclear extract (see steps 11 to 15, below).

Preparation of nuclear extract

9. Resuspend the nuclear pellet in 1 pnv of buffer C420. If clumps of nuclei are difficult to resuspend, homogenize the sample in a Dounce with a loose pestle. Transfer to a small precooled glass beaker containing a small magnetic stir bar.

The salt concentration in buffer C is one parameter that can be optimized during extract preparation. Active splicing extracts from HeLa cell nuclei are usually obtained by extraction in 0.4 to 0.6 M KCl. Lysis of the nuclei can sometimes be reduced by resuspending the cells in 0.5 pcv of buffer C100 and then adding 0.5 pcv of buffer C (high salt) dropwise to yield the appropriate final concentration.

10. Extract the nuclei by stirring very slowly for 30 min.
11. Transfer the extract to a centrifuge tube and centrifuge 30 min at $25,000 \times g$ in a JA-20 rotor, 4°C .

The supernatant after this spin is the nuclear extract.

12. If conductivity will be used to monitor the dialysis, save a small sample before transferring the extract into dialysis tubing.
13. Dialyze the nuclear extract against 2 liters of buffer DG for 2.5 hr using 10,000 to 14,000 MWCO dialysis tubing (APPENDIX 3C). Change the dialysis buffer to new buffer DG and dialyze for another 2 hr. If desired, check the conductivity to monitor the dialysis.

Conductivity is checked by diluting 10 μl of the extract into 1 ml of distilled water. An equivalent dilution of the dialysis buffer (buffer DG) is used for comparison. Diluted buffer DG should read ~16 to 18 $\mu\text{S}/\text{cm}$. Dialysis is complete when the conductivity of the extract matches that of buffer DG. These dialysis times and volumes are based upon a large extract preparation. Smaller volumes can be dialyzed in less time. Overnight dialysis can result in a decrease in RNA splicing activity.

14. Remove the extract from the dialysis tubing and transfer to a centrifuge tube. Centrifuge 20 min at $12,000 \times g$ in a JA-20 rotor, 4°C . Keep the supernatant, which is the completed nuclear extract, and discard the pellet.

This step removes protein and nucleic acid precipitates that form during dialysis. The precipitates are normal and result from the drop in salt concentration.

15. Remove a small aliquot of the supernatant and use it to determine the protein concentration by the Bradford method (*APPENDIX 3H*). Divide the remaining nuclear extract into aliquots in separate tubes and quick-freeze in liquid nitrogen. Store the extracts at -80°C .

Aliquots of 200 or 500 μl are convenient for in vitro splicing experiments.

Nuclear extracts vary widely in final protein concentration (2 to 20 mg/ml), and the concentration does not always correlate with the splicing activity. All extracts should be tested for splicing on constitutive splicing substrates as a positive control, as well as any regulated splicing substrate that will be studied. The most common constitutive substrates are derived from the adenovirus major late first intron (Grabowski et al. 1984) and the β -globin first intron (Krainer et al. 1984).

SUPPORT PROTOCOL 2

PREPARATION OF NUCLEAR EXTRACT FROM PRIMARY TISSUE

In this protocol, the nuclear extract is prepared from a tissue that is isolated from an animal immediately after euthanasia. Like the extract protocol for cultured cells (Support Protocol 1; Dignam et al., 1983), this protocol was initially used to isolate extracts that display tissue-specific RNA polymerase II promoter activity (Sierra et al., 1993). The Grabowski laboratory adapted this protocol to study neuron-specific splicing and demonstrated that splicing-competent nuclear extracts could be generated from whole rat brain (Ashiya and Grabowski, 1997). They have further developed this procedure to produce rat cerebellum extracts that have higher activity for certain neuron-specific exons (Zhang et al., 1999). The protocol that follows is for rat brain and will require optimization for other tissues. Specifically, the salt concentration used for nuclear extraction is a critical parameter and may be changed to maximize the extraction of the nuclei and to minimize nuclear lysis. Note that some tissues (e.g., pancreas) have very high RNase content and may not generate usable extracts.

Briefly, the protocol involves homogenization of primary tissue to break open the cells. The nuclei are subsequently fractionated away from cellular debris by centrifugation through a sucrose cushion. The nuclei are then extracted and the resulting nuclear extract is processed in a manner similar to the standard Dignam extract (Support Protocol 1).

Materials

- Sucrose cushion buffer (see recipe)
- Dissected whole brains from six 28-day Sprague-Dawley rats
- Phosphate-buffered saline (PBS; *APPENDIX 2A*)
- Homogenization buffer (see recipe)
- Buffer C230 (see recipe)
- Buffer DG (see recipe)
- Dissection tools
- Small Petri dish or tissue-culture plate
- Beckman SW 28 rotor (or equivalent) and polyallomer ultracentrifuge tubes
- Motor-driven Teflon-glass homogenizer (e.g., Wheaton Overhead Stirrer; Wheaton Science Products)
- Small dialysis cassette (0.1 to 0.5 ml Pierce “Slide-a-lyzer” or equivalent), 10,000 MWCO (also see *APPENDIX 3C*)

Additional reagents and equipment for dialysis (*APPENDIX 3C*), trypan blue staining (*UNIT 1.1*), and Bradford protein assays (*APPENDIX 3H*)

NOTE: Chill homogenization buffer and glass-Teflon homogenization apparatus overnight at -20°C before the procedure and perform all steps at 0° to 4°C . All other buffers, vessels and centrifuge rotors should be precooled. The volumes in this procedure assume a starting material of six whole brains from 28-day rats, ~ 15 grams of tissue. For other tissues, this protocol should be scaled according to the mass of starting material.

Isolate nuclei

1. Transfer 10 ml of sucrose cushion buffer into each of two ultracentrifuge tubes. Chill at -20°C .
2. Dissect the tissue and transfer the material of interest to a Petri dish on ice. Rinse tissue in cold PBS. Remove as much blood as possible by gently dabbing with a Kimwipe. Mince the washed tissue with a razor blade or sterile scissors.
3. Add 5 ml of homogenization buffer (containing DTT and protease inhibitors added immediately before use) to the homogenizer. Transfer the minced tissue to the homogenizer vessel and add another 25 ml of homogenization buffer with DTT and protease inhibitors.

As a rule of thumb, the tissue should not constitute more than 10% to 15% of the total volume at the homogenization stage. However, the final homogenate must be less dense than the sucrose cushion on which it is layered.

4. Turn the homogenizer motor on at ~ 200 rpm. Begin homogenization by slowly raising the glass homogenizer over the rotating Teflon “pestle” to bring the pestle into contact with the homogenate. Set the motor to ~ 400 to 500 rpm. Raise the tube until the pestle reaches the bottom of the glass vessel. Slowly move it back out of the homogenate. Repeat this process one or two times until nuclei and not intact cells are observed when monitoring the homogenization by light microscopy and trypan blue staining (*UNIT 1.1*).

It requires significant pressure to raise and lower the tube over the pestle. Glass homogenizers can break if the pestle does not seat properly, so safety glasses should be worn during this step. Plastic-coated homogenizer tubes reduce the shattering danger and are recommended. Keep the homogenizer dipped in an ice water bath to maintain maximum cooling during the procedure. Upon monitoring of the homogenate by light microscopy, the majority of the cells should be broken, but the nuclei should be intact.

5. Dilute the homogenate to 50 ml with homogenization buffer. Carefully layer 25 ml of the homogenate onto each 10-ml sucrose cushion.
6. Centrifuge 1 hr at $75,000 \times g$ in SW 28 rotor, 0° to 4°C .
7. Remove any floating material and carefully aspirate and drain the solution without disturbing the nuclear pellet. Cut off the tube above the pellet with a clean razor blade or scissors.
8. Fill a 10-ml syringe with cold Milli-Q water and attach an 18-G needle. Holding the centrifuge tube upside down, rinse the tube walls with the syringe. Rinse the pellet twice with water and then twice with buffer C230 using a pipet. Dry the tube with a Kimwipe without touching the pellet.

Prepare nuclear extract

9. Resuspend the two pellets in a total volume of 600 μ l of Buffer C230 using a micropipettor with a 1-ml tip that has had the end cut off to generate a 3-mm aperture. Transfer to a microcentrifuge tube and incubate on ice for 30 min.

Brain nuclei are very sensitive to lysis and thus require the lower-salt (C230) buffer rather than the C420 buffer used for the tissue culture cells (Support Protocol 1). The salt concentration in buffer C is one parameter that can be optimized during extract preparation. Lysis of the nuclei can sometimes be reduced by resuspending the cells in 0.5 pcv of buffer C100 and then adding 0.5 pcv of buffer C (higher-salt) dropwise to yield the appropriate final concentration. Nuclei from different tissue types may require altered conditions. The buffer C100 suspension can be divided into several aliquots and various buffer Cs, differing in salt concentration, can be added to the aliquots in parallel to optimize the procedure.

10. Microcentrifuge 10 min at maximum speed, 0° to 4°C. Keep the resulting supernatant, which is the nuclear extract.
11. Load the supernatant into a small dialysis cassette (“Slide-a-lyzer”) with a 1-ml syringe and 18-G needle (APPENDIX 3C).
12. Dialyze for 1 hr against 1 liter of buffer DG. Change to fresh buffer DG and dialyze for an additional 1 hr.

The small volume of the extract and the high relative surface area of the Slide-a-lyzer assures that the dialysis is rapid. Monitoring the dialysis by conductivity is recommended for larger preparations (see Support Protocol 1).

13. Remove the extract from the dialysis cassette with a 1-ml syringe (18-G needle). Transfer to a microcentrifuge tube and microcentrifuge 15 min at 16,000 \times g, to clear any precipitates. Retain the supernatant.
14. Determine the protein concentration by the Bradford assay (APPENDIX 3H). Divide the the extract into aliquots in microcentrifuge tubes and quick-freeze in liquid nitrogen. Store the nuclear extracts at -80°C .

See the comments in Support Protocol 1 on testing nuclear extracts. Some tissue-derived nuclear extracts benefit from concentrating the extract (e.g., using Amicon centrifugal concentrators). This is a possible option if initial splicing assays are unsuccessful with a particular batch of extract.

SUPPORT PROTOCOL 3

IN VITRO TRANSCRIPTION OF CAPPED, RADIOLABELED RNA

The substrate for in vitro splicing reactions is usually produced by in vitro transcription of a DNA template by a bacteriophage RNA polymerase (e.g., T3, T7, SP6) in the presence of an $[\alpha\text{-}^{32}\text{P}]\text{NTP}$. The resulting radiolabeled RNAs are of high specific activity and possess a 5' mRNA cap structure (diguanosine 5'-5' triphosphate). The cap structure stabilizes the RNA against exonuclease activity and may bind proteins that are important for RNA processing efficiency. The relative importance of the cap-binding complex to RNA splicing varies by substrate, but the cap is usually necessary to reduce nuclease digestion and thus produce cleaner results.

The DNA template can be derived by two methods. Commonly, the pre-mRNA sequence is cloned into a plasmid downstream of a bacteriophage promoter, and the resulting plasmid can be linearized after the pre-mRNA sequence with a restriction endonuclease to generate the template. Alternatively, the corresponding pre-mRNA sequence can be amplified by PCR with a special “tailed” sense primer. The primer (5' to 3') consists of the top strand sequence of a bacteriophage promoter followed by at least one guanosine

followed by the region that complements the pre-mRNA 5' end. The antisense primer is designed at the 3' end of the pre-mRNA sequence to specify the point where the polymerase "runs off." The resulting PCR product can be purified by agarose gel electrophoresis, cleaned up by standard methods (Beulsey et al., 1991; e.g., "freeze and squeeze" or kit), and used as transcription template. Any DNA used for transcription should be free of nuclease activity and thoroughly desalted.

NOTE: All water and microcentrifuge tubes used in this protocol should be RNase-free (see *APPENDIX 2A*).

CAUTION: All proper guidelines for radioisotopes should be followed. Use care when handling transcription reactions to avoid radioactive contamination. Monitor all work spaces, pipets, and the microcentrifuge for counts. Dispose of waste as specified by the local radiation safety officer (also see *APPENDIX 1D*).

Materials

RNase-free water (*APPENDIX 2A*)
10× transcription buffer (provided with polymerase, or see recipe)
0.1 M DTT
NTP mix (see recipe)
Cap analog solution (see recipe)
12.5 μM [α -³²P]UTP (800 Ci/mmol)
0.1 μg/μl DNA template (linearized plasmid or PCR product) in 10 mM Tris·Cl, pH 8.1 (see *APPENDIX 2A* for Tris buffer)
10 U/μl bacteriophage RNA polymerase (T3, T7, or SP6)
RNase-free DNase I (if PCR product is used as template)
Formamide loading buffer (see recipe)
RNA elution buffer (see recipe)
70% and 100% ethanol, -20°C
TE-acetate buffer (see recipe)
85°C heating block
Scintillation counter and vials appropriate for Cerenkov counting
Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis (*APPENDIX 3A*) and autoradiography (*UNIT 6.3*)

1. Bring all solutions to room temperature. Assemble the 25-μl reaction in the following order:

6.5 μl RNase-free water
2.5 μl 10× transcription buffer
2 μl 0.1 M DTT
2 μl NTP mix
3 μl cap analog solution
5 μl 12.5 μM [α -³²P]UTP (800 Ci/mmol)
2 μl DNA template
2 μl 10 U/μl bacteriophage RNA polymerase.

The final concentrations of the components are: 40 mM Tris·Cl, pH 7.9; 6 mM MgCl₂; 2 mM spermidine; 8 mM DTT; 1.2 mM cap analog; 0.2 mM each of GTP, ATP, and CTP; 0.01 mM cold UTP; 2.5 μM [α -³²P]UTP; ~20 μM DNA template; 0.8 U/μl bacteriophage RNA polymerase.

The order of addition and the assembly at room temperature is critical. This order prevents precipitation of the DNA from contact with high concentrations of spermidine.

2. Incubate at 37°C for 1 to 2 hr. While the mix is incubating, prepare a 0.8-mm-thick analytical-size 4% 1× TBE urea-acylamide gel with wells capable of holding 50 µl. Pre-run the gel.

APPENDIX 3A provides references for preparing and running denaturing PAGE gels.

3. If a plasmid serves as the template, proceed to step 4. If a PCR product is being used, add 2.5 U of RNase-free DNase I and incubate at 37°C for 10 min.

PCR templates and the transcript RNA are so similar in size that they run in nearly the same position on a denaturing gel. Degrading the DNA after transcription resolves this problem.

4. Stop the reactions by addition of 25 µl of formamide loading buffer.
5. Incubate the stopped reactions at 85°C for 5 min.
6. Load 50 µl per well of each reaction directly onto the pre-run 4% 1× TBE urea-acylamide gel. Electrophorese at 25 V/cm for 10 min and then 40 V/cm until the bromphenol blue reaches the bottom of the gel.
7. Disassemble the gel so as to leave the radioactive gel slab on one of the glass plates. Cover in plastic wrap.

CAUTION: The bottom buffer chamber will be radioactive due to the unincorporated α -³²P-UTP. Dispose of the radioactive buffer appropriately (also see APPENDIX 1D).

8. Visualize the transcript by brief autoradiography (UNIT 6.3).

Exposure of the gel for 15 sec is usually adequate.

9. Align the film with the gel to locate the position of the radiolabeled RNA. Cut out the band using a razor blade and transfer to a microcentrifuge tube.

Shredding the gel does not usually improve the RNA yield significantly and makes a clean transfer (step 11) more difficult.

10. Incubate overnight in 400 µl of RNA elution buffer.

For large RNAs (> 600 nt), incubation at 37°C can generate more complete elution. For small RNAs (<300 nt), SDS can be omitted from the elution buffer and thus the second precipitation is not necessary.

11. Transfer the elution buffer to a new microcentrifuge tube.

Elution efficiency can be monitored with a Geiger counter by comparing the elution buffer and gel slice counts.

12. Add 1 ml of 100% ethanol. Mix well and incubate on dry ice for 10 min.
13. Microcentrifuge 10 min at maximum speed, 0° to 4°C. Carefully dispose of supernatant as radioactive waste.
14. Rinse the pellet with 1 ml of cold 70% ethanol.
15. Resuspend pellet in 400 µl TE/acetate buffer and repeat the ethanol precipitation (steps 12 to 14).

This step removes any residual SDS contamination from the first precipitation. Gel filtration over a disposable G-50 spin column (Amersham Pharmacia Biotech) can also be utilized.

16. Resuspend the pellet in 50 µl of RNase-free water and assay 1 µl by Cerenkov counting.

Radiolysis occurs relatively rapidly with transcripts of high specific activity. The RNA should be used within 48 hr of completing this procedure, to assure the highest quality RNA.

REAGENTS AND SOLUTIONS

Use RNase-free (**APPENDIX 2A**) high-purity deionized (Milli-Q or Nanopure) water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.

Buffer A

10 mM HEPES, pH adjusted to 7.9 with KOH (at 25°C)

1.5 mM MgCl₂

10 mM KCl

Store buffer with above components up to 3 months at 4°C

Add the following immediately before use, with rapid mixing:

0.2 mM PMSF (from 100 mM stock in ethanol)

1 mM DTT

1 µg/ml pepstatin A (from 1 mg/ml stock in ethanol)

1 µg/ml leupeptin (from 10 mg/ml stock in H₂O)

1 µg/ml aprotinin (from 10 mg/ml stock in 10 mM HEPES, pH 8.0)

Prepare fresh buffer with DTT and protease inhibitors for each extract preparation

Buffer C20

20 mM HEPES, pH adjusted to 7.9 with KOH (at 25°C)

25% (v/v) glycerol

1.5 mM MgCl₂

0.2 mM EDTA

20 mM KCl

Store buffer with above components up to 3 months at 4°C

Add the following immediately before use, with rapid mixing:

0.2 mM PMSF (from 100 mM stock in ethanol)

1 mM DTT

1 µg/ml pepstatin A (from 1 mg/ml stock in ethanol)

1 µg/ml leupeptin (from 10 mg/ml stock in H₂O)

1 µg/ml aprotinin (from 10 mg/ml stock in 10 mM HEPES, pH 8.0)

Prepare fresh buffer with DTT and protease inhibitors for each extract preparation

Buffers C230 and C420

Prepare as for Buffer C20 (see recipe) but use 230 mM and 420 mM KCl, respectively.

Buffer DG

20 mM HEPES, pH adjusted to 7.9 with KOH (at 25°C)

20% (v/v) glycerol

80 mM monopotassium glutamate

0.2 mM EDTA

Store buffer with above components up to 3 months at 4°C

Add the following immediately before use, with rapid mixing:

0.2 mM PMSF (from 100 mM stock in ethanol)

1 mM DTT

1 µg/ml pepstatin A (from 1 mg/ml stock in ethanol)

1 µg/ml leupeptin (from 10 mg/ml stock in H₂O)

1 µg/ml aprotinin (from 10 mg/ml stock in 10 mM HEPES, pH 8.0)

Prepare fresh buffer with DTT and protease inhibitors for each extract preparation.

Many in vitro splicing protocols use buffer D, which contains 100 mM KCl instead of potassium glutamate. These can sometimes work as well as the buffer DG used here. However, the use of glutamate as a potassium counterion instead of chloride improves in vitro splicing efficiency of some substrates and lowers RNA degradation (Black, 1992; Reichert and Moore, 2000; also see Commentary).

Cap analog solution

Dissolve 25 A₂₆₀ units of dry diguanosine 5'-5' triphosphate dinucleotide (New England Biolabs or Amersham Pharmacia-Biotech) in 130 µl of water to obtain a final concentration of 10 mM diguanosine 5'-5' triphosphate. Store up to one year at -20°C.

Cytoplasmic extract buffer, 10×

0.3 M HEPES, pH adjusted to 7.9 with KOH (at 25°C)
1.4 M KCl
30 mM MgCl₂
Prepare fresh for each extract preparation

Formamide loading buffer

95% (v/v) formamide
15 mM EDTA pH 8.0 (APPENDIX 2A)
0.05% (w/v) bromphenol blue
Store in aliquots for up to 3 months at -20°C

Homogenization buffer

10 mM HEPES, pH adjusted to 7.9 with KOH (at 25°C)
15 mM KCl
1 mM EDTA
2.2 M sucrose
5% (v/v) glycerol
0.15 mM spermine
0.5 mM spermidine
Store buffer with above components up to 3 months at 4°C
Add the following immediately before use, with rapid mixing:
0.2 mM PMSF
1 mM DTT

Prepare fresh buffer with DTT and protease inhibitor for each extract preparation

NTP mix

2.5 mM GTP
2.5 mM ATP
2.5 mM CTP
0.125 mM UTP
Store up to one year at -20°C

Ribonucleotide triphosphates can be purchased as 100 mM stock solutions at neutral pH. Alternatively, the dry NTP salts can be dissolved in water and titrated to pH 8.0 with Tris base.

PCA Buffer, 1.1×

0.33 M sodium acetate
0.05 M Tris-Cl, pH 8.0 (APPENDIX 2A)
0.005 M EDTA, pH 8.0 (APPENDIX 2A)
0.2% (w/v) SDS
Store up to 1 year at room temperature

RNA elution buffer

20 mM Tris-Cl, pH 8.0 (APPENDIX 2A)
2 mM EDTA
0.5 M ammonium acetate
0.4% (w/v) SDS
Store for up to 1 year at room temperature

RNA gel loading buffer

0.1× TBE buffer (*APPENDIX 2A*)
8 M urea
0.025% (w/v) xylene cyanol
0.025% (w/v) bromphenol blue
Store up to one year at room temperature

Sucrose cushion buffer

10 mM HEPES, pH adjusted to 7.9 with KOH (at 25°C)
15 mM KCl
1 mM EDTA
2.0 M sucrose
10% (v/v) glycerol
0.15 mM spermine
0.5 mM spermidine
Store buffer with above components up to 3 months at 4°C
Add the following immediately before use, with rapid mixing:
0.2 mM PMSF
1 mM DTT
1 µg/ml pepstatin A
1 µg/ml leupeptin
1 µg/ml aprotinin
Prepare fresh solution with DTT and protease inhibitors for each extract preparation and filter sterilize

Transcription buffer, 10×

400 mM Tris·Cl, pH 7.9 (*APPENDIX 2A*)
60 mM MgCl₂
20 mM spermidine
Store for up to 1 year at -20°C

TE-acetate buffer

10 mM Tris·Cl pH 8.0 (*APPENDIX 2A*)
1 mM EDTA
0.5 M ammonium acetate
Store for up to 1 year at room temperature

COMMENTARY

Background information

Nuclear extracts from cultured mammalian cells were developed for analysis of transcription and were later adapted to the study of pre-mRNA splicing (Dignam et al., 1983; Krainer et al., 1984). Nuclear extracts that splice pre-mRNAs into mRNAs in vitro have become an indispensable tool for the analysis of splice site choice and splicing factors. In particular, the preparation of nuclear extract from various cell lines or tissues has enabled the study of regulated exons. Nuclear extracts produced by these methods are also used to study other RNA processing events such as 3' cleavage and polyadenylation (Moore and Sharp, 1985; Takagaki et al., 1988; Christofori

and Keller, 1988). Recently, nuclear extracts were shown to possess factors involved in mRNA export (Le Hir et al., 2000 and Zhou, et al. 2001). Laboratories are also now using nuclear extracts as a tool to investigate the coupling of the processes of transcription, splicing, 3' end processing, and export.

Critical Parameters

The original splicing extract procedure and many subsequent adaptations utilized a final dialysis step into a buffer containing 100 mM KCl. Most laboratories still utilize these conditions. Several laboratories have reported an increase in splicing efficiency for some substrates by replacing potassium chloride (KCl)

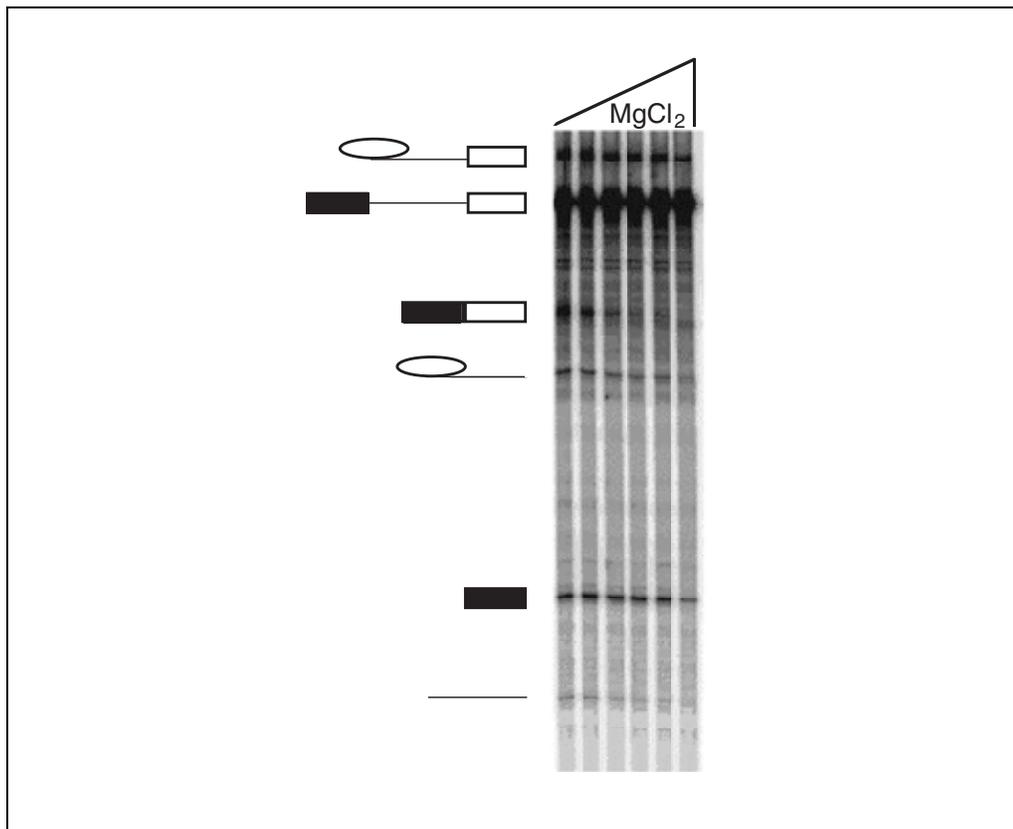


Figure 11.17.4 Magnesium titration on the β -globin first intron splicing substrate, HB Δ 6 (Krainer et al., 1984). The final MgCl_2 concentration was titrated in 0.5 mM steps from 2.5 mM to 5 mM. Both the stability of the mRNA product and the efficiency of the second catalytic step of splicing are reduced as Mg is increased above 3 mM, while the efficiency of the first catalytic step is relatively constant over a wider Mg range.

with potassium acetate or potassium glutamate in the preparation of the nuclear extract (Black, 1992; Reichert and Moore, 2000). Moreover, some introns that are inactive in KCl are spliced in potassium glutamate. It is found that most constitutive and regulated substrates splice as well, if not better, in glutamate. This anion effect is seen in other processes involving nucleic acid binding proteins (Ha et al., 1992). The protocols presented in this unit all use glutamate.

Consistent preparation of the nuclear extract is also important to allow the technique to be optimized to yield the highest splicing activity. The condition of the starting material, whether cells or tissue, should be carefully monitored. Overgrowth of tissue culture cells will result in a loss of splicing activity in the prepared extract. The time that it takes to perform each step of the extract protocol, as well as any errors, should be noted so as to improve future preparations. For extracts made from tissues, it is important to minimize the time between isolation of the tissue from the animal and the homogenization stage.

Several parameters can be changed during optimization of the nuclear extraction. Extraction of the nuclear contents takes place in buffer C, and the KCl concentration of this extraction can affect the activity. Too low a salt concentration will result in a poor protein yield, while too high a salt concentration will result in lysis of the nuclei. This can be optimized by dividing the nuclei into several batches to be extracted under different salt conditions. Nuclei from various tissues or cell types are likely to require different conditions.

Extracts vary widely in concentration, and there is not always a direct correlation between extract concentration and splicing activity. All new extracts should be tested on constitutive and regulated substrates. It is recommended that a simple two-exon/single-intron constitutive pre-mRNA be used when optimizing the reactions, because the splicing efficiency of these substrates is usually greater than a multiple intron substrate. As mentioned earlier, new extracts may require reoptimization of the splicing reaction (e.g., magnesium concentration). An extract that is dilute may show activity

on an efficiently spliced substrate (e.g., adenovirus first intron), but have no activity on a weaker splicing substrate.

Splicing conditions may need to be reoptimized for different pre-mRNA substrates. Pre-mRNA substrates have different optimal magnesium and monovalent salt concentrations, different requirements for macromolecular crowding reagents (e.g. PEG), and different optimal extract concentrations. For an example of a magnesium titration, see Figure 11.17.4.

As is standard when working with RNA, it is important to avoid contamination with RNases. Should contamination occur, the problem is usually most easily solved by discarding all solutions and preparing new ones. The bench area in which the experiment is being performed should be cleaned as well. This is true not only for the splicing reaction but also for the nuclear extract preparation. It is extremely important that all of the reagents used in the preparation be RNase-free and of the highest available purity. Filter sterilize all solutions and use only highly pure deionized (Milli-Q or Nanopure) water.

Anticipated Results

Using this protocol, it is possible to produce extracts between 4 to 20 mg protein/ml that will splice a uniformly labeled pre-mRNA generated by in vitro transcription

For most pre-mRNAs, the splicing reaction is more robust in vivo than in vitro. A standard in vitro splicing reaction does not usually convert all of the pre-mRNA to products during a standard reaction time of 1 to 4 hr. Much of the pre-mRNA input is still intact at the end of the incubation. With weak splicing substrates, the pre-mRNA is usually the most intense band on the splicing gel. Usually the linear molecules of the splicing reaction are identified by their migration relative to a radiolabelled DNA marker set (e.g., end labelled pBR322 *MspI* digest). Linear RNA polynucleotides will migrate slightly slower through the urea-PAGE than the same length DNA. Branched products are identified by their retarded mobility in high percentage (8% to 12%) gels and by their susceptibility to debranching. Time course experiments run on the same splicing gel can often elucidate which products are from Steps 1 and 2 of the reaction. Normally, a splicing gel shows a number of radiolabeled RNA bands that are splicing-independent and splicing-dependent. Splicing-independent bands are often sites in the RNA with a high propensity for hydrolysis or radiolysis. Splicing-dependent bands will

require the energy source ARP and creatine phosphate for their appearance, so this can be used as criteria for assigning band identities.

Time Considerations

Nuclear extract preparation takes 9 to 12 hr, including the final dialysis step. The extract preparation prior to dialysis requires 3 to 5 hr. Additional time for dissection is necessary for extracts from tissues and will depend upon the complexity of the tissue isolation. The entire procedure should be completed in 1 day. In vitro transcription and gel purification can be performed in ~4 hr, and the RNA is eluted overnight and used for splicing the next day. The in vitro splicing reaction requires ~3 to 5 hr. The preparation of the polyacrylamide gel and electrophoresis of the splicing reaction requires 4 to 5 hr.

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CHAPTER 12

Cell Motility

INTRODUCTION

The ability of eukaryotic cells to move and respond to their environment is critical for morphogenesis, and underlies the invasiveness of tumor cells. The majority of cells have the capacity to actively migrate toward a chemoattractant. For a cell to move in this manner, it must be able to detect the direction of a chemical gradient, orient toward the gradient, and move directionally toward it. The cellular processes that mediate this response are numerous, including extension of a pseudopod, attachment of the cell to the substratum, and breakage of existing attachments. Chapter 12 describes methods for analyzing these processes.

UNIT 12.1 describes basic assays for studying directed cell movement along a chemical gradient. These are useful for identifying new chemoattractants and studying the cellular processes required for such movement. Among the assays described are those that monitor a population of cells after exposure to a chemoattractant in a steep gradient across a thin porous filter or over short distances through a gel of extracellular matrix or agarose. In these assays, the gradient is applied gradually or suddenly depending on the purpose of the experiment. The latter approach is useful for biochemical analysis of chemoattractant signal transduction, where a rapid change in chemoattractant concentration is required to generate a signal. Cell migration in these assays is detected using radioactive, fluorescent, or enzymatic cell markers, or cytochemistry. Direct observation of an individual cell's behavior in response to a chemoattractant is an alternative visual approach described in several assays in this unit. These assays can be used to dissect the complex processes involved in the chemotactic response; the questions of how a cell detects a chemical gradient and determines its direction, orients its movement toward the signal, and moves along the gradient are emphasized in this unit. Selection of an appropriate assay is important because cells move at vastly different rates in response to a chemotactic signal (e.g., neutrophils and *Dictyostelium* amoebae move about ten times faster than fibroblasts and neurons) and require different time scales and conditions for monitoring their movements.

UNIT 12.2 describes a protocol for assessing the invasiveness of cells through a basement-membrane matrix. Cells are placed in the upper well of a Boyden migration chamber that is coated with basement-membrane matrix, and a chemoattractant is applied in the lower well. After a short incubation period, cells that have migrated through the matrix-coated filter are counted and can be recovered for further analysis. This type of assay can be used to identify compounds that either promote or inhibit invasion of cells across the matrix-coated filter, and is easily adapted for large-scale screening of compounds. In addition, because cells that have moved to the lower chamber can be recovered, subpopulations that have increased invasiveness can be isolated and studied. A useful application of the assay is for analysis of metastatic tumor cells that invade somatic tissues through blood vessel basement membrane and underlying connective tissue.

UNIT 12.3 describes protocols for monitoring the traction forces exerted by migrating cells as they move along a substratum. These assays provide important information relevant to understanding subcellular events occurring during migration, including breakage of adhesive contacts with the extracellular matrix, cytoskeletal rearrangements involved

in forward motion, and the formation of new contacts. The least expensive and easiest method described for studying cell traction uses a silicone sheet to measure traction forces under the entire cell. The orientation of wrinkles generated in the substrate by locomoting cells is used to describe the global orientation of the forces under the cell. A newer, more expensive technique that is also included measures subcellular traction forces by employing a system of deformable levers with variable stiffnesses to measure forces directly under regions of the cell as small as $4 \mu\text{m}^2$.

Many cells reside in environments where they frequently experience disruptions in membrane integrity. When such tearing or wounding of the cell surface occurs, an active and complex resealing mechanism rapidly repairs the membrane disruptions. *UNIT 12.4* describes methods for studying the processes of cell wounding and resealing. The methods are based on the finding that wounded cells will take up tracer macromolecules to which they are normally impermeable; upon resealing, the tracers are trapped in the cell. Because dead cells do not reseal, they do not trap the tracers and are thereby distinguishable from live wounded cells. Protocols in this unit describe how to detect wounded cells using fluorescent dextrans or immunostaining for rat serum albumin added to the medium. Also covered are electron microscopic analysis of wounded cells and quantification of wounded cells by image analysis and flow cytometry. Because plasma membrane disruptions provide a route of entry for otherwise impermeable macromolecules that an experimentalist might want to introduce into the cytosol of cells (including enzymes, antibodies, and expression vectors), understanding how plasma membrane disruptions arise and reseal is of considerable practical utility.

A powerful system for studying cell dynamics is *Dictyostelium discoideum* amoebae, which grow as separate independent cells, but upon starvation actively cluster together to form multicellular structures. During this process the amoebae undergo a range of different behaviors, including chemotaxis, signal transduction, plasma membrane ruffling, phagocytosis, and cytokinesis. *UNIT 12.5* describes how to grow and image *D. discoideum* amoebae in order to study motile and chemotactic behavior in wild-type and genetically modified strains. Protocols are described for growing and maintaining the cells and how to transform them with GFP fusion plasmids. Methods are also provided for characterizing the functionality of GFP fusion proteins expressed in the amoebae. The unit also includes methods for imaging GFP fusion proteins in chemotaxing cells, aggregates, mounds, and slugs.

UNIT 12.7 presents assays relevant for studying actin-based motility within cells. The assays are based on the propulsion of the *Listeria* and *Shigella* or functionalized microspheres reconstituted from five proteins, including profilin, actin-depolymerizing factor, gelsolin, neural Wiskott-Aldrich syndrome protein (N-WASP) and the actin-related protein, Arp2/3 complex (all of whose purification protocols are described). By quantifying the dependence of motility parameters on the concentration and/or combination of these components, these assays provide an approach for characterizing the molecular processes underlying actin-based motile behavior of cells.

Jennifer Lippincott-Schwartz

Chemotaxis is defined as a bias in the direction of cell movement along a chemical gradient. Chemotaxis contributes to morphogenetic movements and to cell accumulation at sites of inflammation. In recent years, interest in the field of chemotaxis has focused on identifying new chemoattractants, especially the chemokines, which attract various leukocytes (Baggiolini et al., 1997; Baggiolini, 1998), and the netrins and semaphorins/collapsins, which attract or repel neuronal growth cones (McKenna and Raper, 1988; Tessier-Lavigne and Goodman, 1996; Song et al., 1997). Much attention is also focused on the cellular and molecular basis of chemotaxis (Devreotes, 1994; Schleicher et al., 1995; Van Haastert, 1995). Assays appropriate for identifying new chemoattractants often differ from those appropriate for investigating the mechanisms of chemotaxis. This unit describes both types of assays in general terms, provides detailed instructions for performing various assays, and considers issues specific to neutrophils, lymphocytes, fibroblasts, and *Dictyostelium discoideum* amoebae.

Chemotaxis is most clearly documented by observing individual cells moving up a gradient, e.g., toward a pipet or a well containing a chemoattractant. However, to assay a number of test materials in this manner can be time-consuming. Thus, assays have been developed that, although less definitively detecting chemotaxis, allow an efficient search for potential chemoattractants. Most of these assays monitor the redistribution of a population of cells after exposure to a test material established in a steep gradient (change in concentration $\geq 2\%$ per $10\ \mu\text{m}$) across a thin porous filter (filter assay; see Basic Protocol 1), or over short distances (several mm) through a gel of extracellular matrix or agarose (under-agarose and small population assays; see Basic Protocols 2 and 3, respectively). The rigid matrix allows a gradient to form by diffusion, while protected from disruption due to fluid flow. Cell migration toward the test material, detected microscopically or through radioactive, fluorescent, or enzymatic cell markers, is quantified after a set incubation time. Such assays allow a large number of materials and concentrations to be tested in parallel. Increased migration toward a test material in these assays suggests that the material might be a chemoattractant. However, increased migration might also be seen if the material merely enhances the number of cells migrating and/or their rate of migration (chemokinesis; see Background Information). A protocol is also included for distinguishing enhanced migration from chemotaxis (see Support Protocol 1).

For a cell to exhibit chemotaxis, it must detect the direction of a chemical gradient, orient its translocation in that direction, and then move along the gradient. The signaling pathways that mediate this complex response are being investigated. Often it is helpful to consider different aspects of the response. For example, a particular cellular component (i.e., a mutated gene or a pharmacologically altered enzyme) might be required for a cell to extend a pseudopod, direct the pseudopod up the gradient, limit pseudopods from extending in other directions, stimulate an attachment to the substratum, or break attachments. To investigate the responsible mechanism, assays are used that give detailed information about an individual cell's behavior through direct observation of the cell during the response. Such assays include observation of cells in established gradients (under-agarose, bridge assays; see Basic Protocols 2 and 4, respectively), video recording of cells presented with a pipet containing attractant (pipet assay; see Basic Protocol 5), and observation of cells exposed to a sudden increase or decrease in concentration of chemoattractant (upshift assay; see Basic Protocol 6). These methods can be modified to allow monitoring by fluorescence microscopy to follow the time course of redistribution of GFP-tagged proteins (Maniak et al., 1995; Moores et al., 1996; Westphal et al., 1997). Image analysis systems may also be used to analyze results (see Support Protocol 2).

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STRATEGIC PLANNING

In all assays, the accuracy of cell orientation in a gradient of chemoattractant (Fig. 12.1.1) depends on both the steepness of the gradient and the mean concentration of the chemoattractant. To exhibit chemotaxis, a cell must detect a change in the concentration of chemoattractant over time and/or distance. A cell detects the change in concentration by a change in the occupancy of its chemoattractant receptors. The change in occupancy is greatest when the gradient is steepest and when the concentration of the attractant is well below that which saturates the receptors. In linear gradients of a constant steepness (e.g., a 10-fold increase in concentration over 1 mm), a concentration of chemoattractant somewhat less than the K_d of the receptor (i.e., the concentration that results in occupancy of half of the receptors) gives optimal cell orientation. Because the geometry of the gradient varies between assays, the concentration needed to achieve a steep gradient in the appropriate concentration range also varies. The gradient needs to have an appropriate concentration and steepness throughout the time the cells are migrating.

Selection of an appropriate assay is influenced by four principal factors. (1) The choice of assay depends on the rate of locomotion of the cell under study. Neutrophils and *Dictyostelium* amoebae move about ten times faster than fibroblasts and neurons ($\sim 10 \mu\text{m}/\text{min}$ versus $\sim 1 \mu\text{m}/\text{min}$). Since the gradient in most assays is both established and then dissipated by diffusion, assays of slower moving cells may require a special means of renewing or maintaining a gradient (Fisher et al., 1989). Filter assays are useful for most cell types because the filter is so thin, a steep gradient is established, and even slowly moving cells can move across a thin filter before the gradient is lost. For specific information about different cell types and media considerations, see Critical Parameters.

The choice of assay also depends upon (2) the purpose of the assay (i.e., whether to determine if a factor is chemotactic or to assess the mechanism of chemotaxis) and (3)

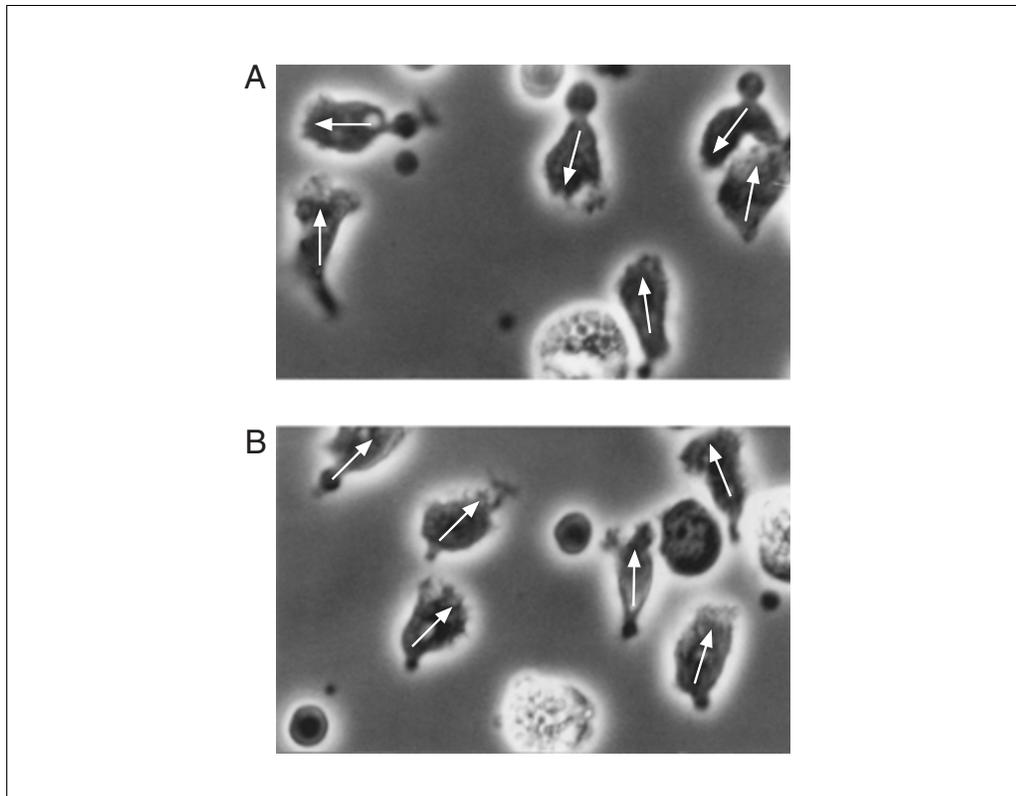


Figure 12.1.1 Cell orientation in (A) random migration (chemokinesis) versus (B) chemotaxis. Arrows indicate direction of individual cell movement; direction of chemotaxis in (B) is to the top.

Table 12.1.1 Appropriate Chemotaxis Assays for Desired Information

Cell type	Assay to detect a response	Assay to determine mechanism of response
Leukocyte	Filter, under-agarose	Under-agarose, bridge, pipet, upshift
<i>Dictyostelium</i>	Small population, pipet, aggregation ^a	Bridge, pipet, upshift
MTLn3/fibroblast	Filter	Filter, pipet, upshift

^aNormal aggregation of *Dictyostelium* is mediated in part by chemotaxis. For assay details see Soll (1987).

Table 12.1.2 Experimental Requirements for Chemotaxis Assays

Assay type	Cells per assay	Chemoattractant		Replicates
		Concentration ($\times K_d$)	Minimum volume (μ l)	No. of simultaneous assays
Multiwell filter	10^4 - 10^5	0.1-10	25 ^a	12-48 per chamber
Culture insert	0.5 - 5×10^6	0.1-10	10	24 per chamber
Under-agarose	10^5	10-1000	10	10-20 per chamber
Small population	10^3	0.1-10	0.3 ^b	~8 per chamber
Bridge	10^3	0.1-10	50 ^a	5
Pipet	1-50	10^3 - 10^4	5	1

^aVolumes indicated for 48-well multiwell chambers and for glass bridge chambers. Other multiwell chambers and Plexiglas bridge chambers require more chemoattractant.

^bFrom three sequential 0.1- μ l applications.

the time available to perform the assay. Assays vary in their time commitment and in the type of information that they can provide (Table 12.1.1 and Table 12.1.2). “Survey assays” are those that can be performed on many samples simultaneously and scored at a later time, either automatically or at least at one’s leisure. These are useful for screening for potential chemoattractants. These assays would need to be modified to examine directional neurite extension from neurons. Information about how a particular agent affects cell behavior is often best obtained from “visual assays,” which allow direct monitoring of cell movement and behavior. However, direct visualization (and video recording) must usually be performed by altering one parameter at a time. Finally, the choice of assay is dependent upon (4) the availability of cells and chemoattractants, because assays vary in the number of cells and the total amount of chemoattractant required (Table 12.1.2).

FILTER ASSAY FOR CHEMOTAXIS

In this protocol, cells suspended in buffer are placed on top of a porous filter while chemoattractant is placed below the filter. After incubation, cell migration into or through the filter is measured (Boyden, 1962). Prior to performing the assay, it is necessary to select the appropriate (1) chamber (e.g., blind-well or multiwell chambers from Neuro Probe, Fig. 12.1.2, or 24-well tissue culture plates with filter inserts), (2) filter type (thick cellulose nitrate or thin polycarbonate), (3) filter pore size, and (4) filter coating for the cell type being assayed (see Critical Parameters). Migration is quantified by measuring (1) the distance cells have migrated into the filter, (2) the number of cells attached to the bottom of the filter, and/or (3) the number of cells that have migrated through the filter and fallen into the lower chamber. It is surprising that both of the latter measures have been used, because they would be expected to shift in opposite directions if the test factor

BASIC PROTOCOL 1

Cell Motility

12.1.3

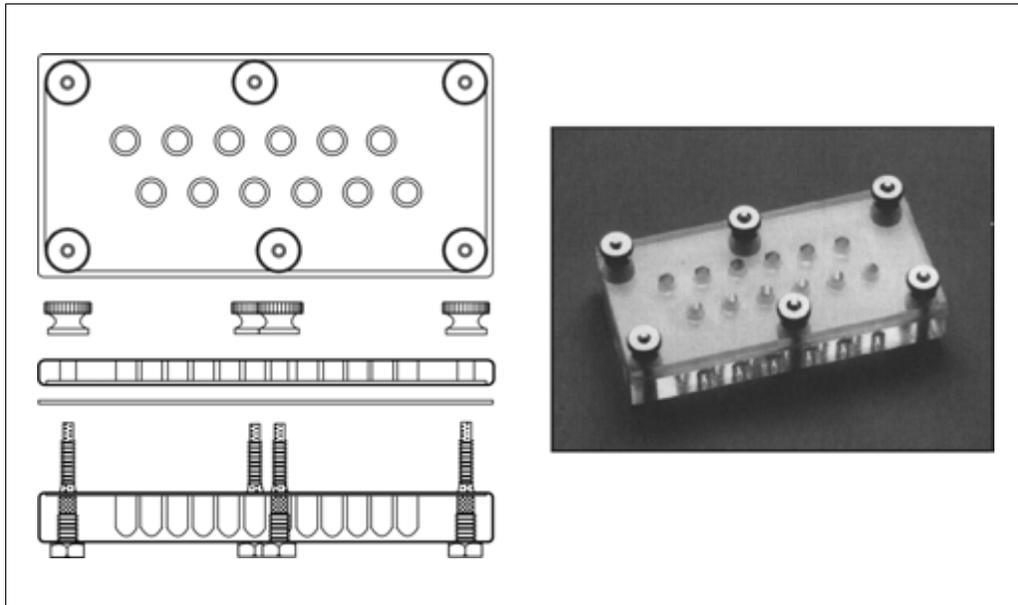


Figure 12.1.2 A standard 12-well multiwell chemotaxis chamber from Neuro Probe is made of acrylic and has 4.75-mm-diameter wells that expose 18 mm² of filter. Volumes of upper and lower wells are 100 and 150 μ l, respectively. A 48-well multiwell chamber is also available (upper and lower well volumes 50 and 25 μ l, respectively). A blind-well chamber, which is a nonstandard special-order chamber, is also available with upper and lower well volumes of 200 and 75 μ l, respectively. Figure redrawn with permission from Neuro Probe.

alters all adhesivity. However, each measure has been used successfully with particular cell types. In general, fibroblasts have been quantified on the lower surface while lymphocytes, which are less adhesive, have been quantified in the lower chamber. Neutrophils are frequently in both places (Jungi, 1975); thus, it is advisable to initially monitor the number of cells both on the filter bottom and in the lower chamber.

If a factor induces an increase in cell migration, additional tests are required to determine whether this is due to an increase in the rate of random migration (chemokinesis) or in a directional movement (chemotaxis) toward the test material, or both (Zigmond and Hirsch, 1973). One can examine the effects on chemokinesis by measuring migration in a series of homogeneous (isotropic) concentrations of test material placed both above and below the filter. The results from these assays are compared to those from cells migrating in a gradient, to determine whether migration in a gradient exceeds that expected from random locomotion alone. Alternatively one can use a different assay in which directional migration is assayed directly (e.g., small population assay, see Basic Protocol 3; bridge assay, see Basic Protocol 4; pipet assay, see Basic Protocol 5). For thick filter assays, the migration distance expected in the absence of chemotaxis (see Support Protocol 1) can be used to determine if chemotaxis is present.

This assay is described for neutrophils; adaptations for other cell types are indicated in annotations.

Materials

- Buffer for upper and lower wells (see Critical Parameters discussion on media)
- Chemoattractant in buffer
- Cells of interest
- 3.7% (w/v) formaldehyde in PBS (*APPENDIX 2A*)
- Giemsa stain (see recipe) or Diff-Quick (EM Science) for neutrophils;
hematoxylin for lymphocytes

70% and 100% isopropanol
 1:1 (v/v) isopropanol/Americlear
 Americlear (Baxter Biotech)
 Permout (EM Science)
 15- μ m polystyrene beads (Polysciences)
 Chemotaxis chamber: blind-well or multiwell chamber (Fig. 12.1.2; Neuro Probe)
 with appropriate filters, or 24-well tissue culture plate with filter inserts (see
 Critical Parameters)
 Filter scraper (Neuro Probe; optional)
 Microscope with 40 \times objective and micrometer (16 \times objective optional)
 Additional reagents and equipment for counting cells using a hemacytometer
 (UNIT 1.1) or cell sorter

Perform assay

1. Fill the lower wells of a chemotaxis chamber with buffer or with chemoattractant in buffer. Use sufficient volume to slightly overfill the well, to prevent air bubbles from being trapped when the filter is lowered (see Table 12.1.3).

The cells in the middle of the filter will be exposed to the mean of the concentrations of chemoattractant present above and below the filter. Thus, when the upper well contains no chemoattractant, the optimal chemoattractant concentration in the lower well is approximately that of the dissociation constant, K_d , of the receptor. Initially, if the K_d is known, one might test concentrations from one tenth to ten times K_d (Table 12.1.2). If K_d is not known, one could test 10-fold dilutions between 0.1 nM and 10 μ M. Most leukocyte chemokines are active at nM concentrations.

2. Lower a filter smoothly onto the chamber, checking that the liquid in each well makes full contact with the filter (i.e., that there are no air bubbles). Assemble rest of chamber and tighten screws. Before adding cells, aspirate off any fluid from the lower well that came through the filter.

When using multiwell chambers, prewetting the filter with buffer helps prevent wicking of fluid out of wells. Avoid moving the filter after it is lowered so that chemoattractant concentrations in different wells are not mixed.

When using a 24-well tissue culture plate, place a tissue culture insert containing the appropriate pore size into each well, just prior to adding the cells.

3. Dilute cells into the upper-well buffer such that the correct number of cells is added when the chamber is filled (Table 12.1.3).

The number of cells is based on the exposed filter area. The cells will rapidly settle by gravity onto the upper surface of the filter.

4. Incubate chamber in a humid environment at 37°C.

The duration of incubation depends of the cell type, filter, and parameter assayed (see Table 12.1.4).

Table 12.1.3 Characteristics of Assay Chambers Used for Filter Assay

Assay chamber	Lower well volume (μ l) ^b	Filter surface area	Upper well volume (μ l)	Number of cells
Blind-well ^a	200	18 mm ²	200	50,000
12-well multiwell ^a	150 ^b	18 mm ²	100	48,000
48-well multiwell ^a	25	3.2 mm ²	50	15,000
24-well tissue culture plate	600	0.3 cm ²	100	1,000,000

^aAvailable from Neuro Probe.

^bIt is best to load a small excess to prevent air bubbles.

Fix and stain cells

5. Drain off top wells with a pipet. Disassemble chamber and remove filter carefully. Immediately put empty chambers in water to soak. If a thin filter is used, scrape the upper side with a filter scraper.

It is easier to clean chambers if they are not allowed to dry.

It is difficult to separate the focal planes at the top and bottom of thin filters. Most investigators, therefore, wipe the cells off the upper surface before attempting to count those on the lower surface (Harvath et al., 1980). As an alternative to a filter scraper, a clip can be attached to either end of the filter, and a moist Q tip can be used to scrape the cells from the top side of the filter while the filter hangs from one clip. It is important to work quickly before the filter dries.

6. Place the filter in 10 ml of 3.7% formaldehyde in a small Coplin jar and incubate 30 min to overnight at room temperature.

Alternatively, a thin filter can be fixed in methanol and air dried for staining with Diff-Quick (Harvath et al., 1980).

7. Stain the cells on the filter ≥ 1 hr at room temperature (see Table 12.1.4). Make sure multiple filters are separated from one another so that stain readily penetrates each filter.

For Diff-Quick and hematoxylin, follow manufacturer's instructions.

8. Destain and clear the filter by incubating 5 min each as follows: twice in water, once in 70% isopropanol, twice in 100% isopropanol, once in 1:1 isopropanol/Americlear, and three times in Americlear. Incubate the filter 30 min in Permount and mount on a microscope slide.

Large slides (e.g., 75 \times 50-mm) are convenient for mounting and viewing the larger filters (e.g., 48-well filter).

Score cell migration

- 9a. *To score distance moved in a thick filter:* Under a 40 \times objective, use the micrometer on the fine focus knob of the microscope to measure the distance from the top of the filter to the furthest two cells in a single focal plane (or some other convenient measure). Repeat in 5 separate fields across the filter.

In some instances, investigators have measured the population distribution in the filter (Zigmond and Hirsch, 1973; Buettner et al., 1989; Table 12.1.5).

Table 12.1.4 Incubation Times and Staining Procedures for Filter Assays

Cell type	Filter type	Pore size (μm)	Duration of incubation	Stain
Neutrophils	Thick	3.0	0.5-2.0 hr for migration into filter; 3-4 hr for migration through filter	Formaldehyde fixed: freshly prepared Giemsa, 1 hr to overnight
Neutrophils	Thin	3.0	20 min	Methanol-fixed, air dried: Diff-Quick
Lymphocytes	Thin	3.0-5.0	4 hr	Hematoxylin, overnight
Fibroblasts	Thin	8.0	3-4 hr	Hematoxylin, 1 hr to overnight

Table 12.1.5 Response of Neutrophils to *f*-Met-Met-Met: Mean Distance From the Top of the Filter to the Front PMNs^a

Concentration above the filter (M)	Distance to front PMNs (μm) at concentration below the filter (M)			
	10^{-9}	10^{-8}	10^{-7}	10^{-6}
10^{-9}	27	30 [27]	57 [30]	69 [35]
10^{-8}	27 [30]	30	47 [30]	62 [35]
10^{-7}	34 [33]	36 [34]	34	60 [38]
10^{-6}	38 [52]	34 [52]	48 [52]	56

^aNumbers in brackets are the distances to the front two PMNs calculated for a hypothetical population undergoing random migration (see Support Protocol 1), based on PMN migration observed in a uniform concentration gradient (numbers along the diagonal of the table). Data from Showell et al. (1976); reproduced with permission from Rockefeller University Press. *f*-Met-Met-Met, *N*-formyl-methionyl-methionyl-methionine; PMN, polymorphonuclear leukocyte.

- 9b. *To score number of cells on lower surface of a thin filter:* Using a 16 \times or 40 \times objective, count the number of cells per field that are on the under side of the filter for each well. Repeat for at least 5 fields.

When using a new cell type, it is important to check that cells are not falling off the filter into the bottom well.

- 9c. *To count cells recovered from lower chamber:* Collect fluid from the lower well, pipetting fluid up and down to remove cells stuck to bottom and sides of the well. Use a hemacytometer or cell sorter to count the number of cells in the cell suspension. To quantify by cell sorting, mix half of the cell suspension with a known number of 15- μm polystyrene beads. Run the suspension through the sorter and acquire a plot of forward scatter versus side scatter. Determine the ratio of beads to cells, and thereby calculate the number of cells in the cell suspension.

Since they have different light-scattering properties, beads and cells will appear as two discrete populations, allowing quantitation of cells from the known number of beads (Campbell et al., 1997b).

Differentiate chemotaxis from chemokinesis

10. If enhanced migration is observed at a particular concentration in the lower well, test the effects of that concentration on migration (both rate and number of migrating cells) when it is present both above and below the filter. Also test $\frac{1}{2}$, $\frac{1}{4}$, and $\frac{1}{8}$ of that concentration.

This approach allows a rough evaluation of whether the results observed under gradient conditions are due to chemotaxis or to effects on the rate of migration or on the number of migrating cells (Wilkinson, 1982). It makes the reasonable assumptions that the gradient through the filter is linear, and that cells moving from one concentration to another rapidly adjust their rate of locomotion to that observed when cells are incubated exclusively at that concentration. Simple mathematical analysis of chemokinesis versus chemotaxis can also be applied (see Support Protocol 1).

CALCULATING THE DISTANCE CELLS ARE EXPECTED TO MOVE IN THICK FILTERS IN THE ABSENCE OF CHEMOTAXIS

This calculation, which comes from Zigmond and Hirsch (1973), can be used to roughly estimate the contribution of unstimulated migration and chemokinesis to the extent of migration into a thick filter (see Basic Protocol 1). More sophisticated calculations are found in Buettner et al. (1989). Migration beyond this value can be considered a chemotactic response. Calculation of migration in the absence of chemotaxis is based on a number of assumptions: (1) a linear gradient exists across the filter, (2) acceleration of the cells between two known velocities is constant, and (3) movement of the leading two cells of the population is equal to that of a single particle moving with a velocity that is a function of the concentration of stimulatory material.

For this sample calculation, migration of cells was initially examined in a gradient of 10% to 50% serum across a 130- μm -thick filter. To test migration in the absence of chemotaxis, the velocity of migration was determined at three homogeneous concentrations (i.e., same above and below the filter): 10%, 30%, and 50%. The duration of the assay (30 min, or 1 time unit) is the same in both gradient and homogeneous assays. The following assay parameters/results are used. (1) The velocity (V) in a given concentration (both above and below the filter) is the distance from the top of the filter to the front two cells after an incubation of 1 time unit. In this example, velocities were determined to be: $V_0 = 84 \mu\text{m}/\text{unit time}$ (10% serum), $V_1 = 73 \mu\text{m}/\text{unit time}$ (30% serum), and $V_2 = 60 \mu\text{m}/\text{unit time}$ (50% serum). (2) The distance from the top of the filter to the first concentration for which a velocity is determined is d_1 , the distance from the first concentration to the second is d_2 , and so on. Since this example calculated velocities for three evenly distributed concentrations over a linear gradient, d_1 is the distance from the top of the filter (10%) to the middle of the filter (30%), and d_2 is the distance from the middle to the bottom of the filter (50%). Thus, $d_1 = d_2 = 65 \mu\text{m}$.

Since acceleration is constant, the mean velocity over distance d_1 is $\bar{V}_1 = (V_0 + V_1)/2 = 78.5 \mu\text{m}/\text{unit time}$. Thus, the time required to move from the top of the filter over distance d_1 is $T_1 = (d_1)/(\bar{V}_1) = 0.828 \text{ unit time}$. Similarly, the mean velocity over distance d_2 is $\bar{V}_2 = (V_1 + V_2)/2 = 66.5 \mu\text{m}/\text{unit time}$, and the time to move over d_2 is $T_2 = (d_2)/(\bar{V}_2) = 0.977 \text{ unit time}$.

Since $T_1 + T_2 > 1 \text{ unit time}$, the front two cells will not get all the way across $d_1 + d_2$ in 1 unit time. To calculate the distance the cells will move into d_2 in one unit time, acceleration over d_2 is used, where $a_2 = (V_2 - V_1)/T_2 = -13.3 \mu\text{m}/(\text{unit time})^2$. The time remaining after the cells have crossed d_1 (i.e., the time they migrate through d_2) is $T_f = 1 - T_1 = 0.172 \text{ unit time}$. The distance migrated into d_2 is

$$V_1(T_f) + \frac{a_2(T_f)^2}{2}$$

Thus, the total distance the front cells would be expected to move is

$$d_t = d_1 + V_1(T_f) + \frac{a_2(T_f)^2}{2}$$

$$d_t = 65 \mu\text{m} + (73 \mu\text{m} / \text{unit time} \times 0.172 \text{ unit time}) +$$

$$\frac{[-13.3 \mu\text{m} / (\text{unit time})^2] \times (0.172 \text{ unit time})^2}{2} = 77 \mu\text{m}$$

Table 12.1.6 Sample Data for Cell Response to Serum^a

Concentration above the filter (%)	Distance migrated (μm) at concentration below the filter (%)		
	10	30	50
10	84		[77]
30		73	
50			60

^aUnbracketed numbers are measured values at homogenous concentrations. Bracketed number is the calculated value for the 10% to 50% gradient.

The measured distances at homogeneous concentrations and the calculated distance for this gradient are presented as for a checkerboard assay in Table 12.1.6. To determine whether chemotaxis occurs with these cells in a 10% to 50% serum gradient, the measured distance for migration in the gradient is then compared to this calculated value. Movement greater than 77 μm would suggest that chemotaxis occurs.

UNDER-AGAROSE CHEMOTAXIS ASSAY

In this assay (Nelson et al., 1975), a tissue culture dish is filled with a mixture of agarose and cell culture medium. Wells are cut in the hardened agarose, and chemoattractant added to one well forms an approximately exponential gradient as it diffuses into the agarose (Foxman et al., 1997). Cells are placed in a nearby well, from which they migrate in the small space between the agarose layer and the culture dish. The cell distribution, in the presence and absence of chemoattractant, is determined after an incubation period (Fig. 12.1.3). The assay is useful for monitoring migration of neutrophils, monocytes, and possibly lymphocytes (Nelson et al., 1975; Laroche et al., 1983), as well as more adherent cells (Orredson et al., 1983; Stokes et al., 1990).

This protocol describes the analysis of cells fixed after a set migration time. Alternatively, the behavior of migrating cells can be monitored under the agarose by video microscopy using an inverted microscope fitted with a charge-coupled device (CCD) camera. It is important to determine the best parameters (e.g., magnification, contrast) to observe the behavior of interest. To maintain the proper environment, a stage warmer should be used to keep the cells at 37°C, a humidified chamber should be used to prevent drying, and HEPES-buffered agarose and migration medium should be used to maintain pH.

Materials

- Agarose plate mixture (see recipe)
- Migration medium (see recipe)
- Cell suspension ($10^7/\text{ml}$) in migration medium
- Chemoattractant solution in migration medium
- Absolute methanol
- 37% (w/v) formaldehyde
- 0.5% (w/v) Fields stain B (Gallard-Schlesinger)
- 2.5% (w/v) Fields stain A (Gallard-Schlesinger)
- 35-mm tissue culture dishes
- 3-mm hole cutter (e.g., steel punch or plastic pipet tip), sterile, with a vacuum line and a catch flask
- Template for cutting 3-mm wells separated by 2 mm in a single line
- Counting grid (optional)

**BASIC
PROTOCOL 2**

Cell Motility

12.1.9

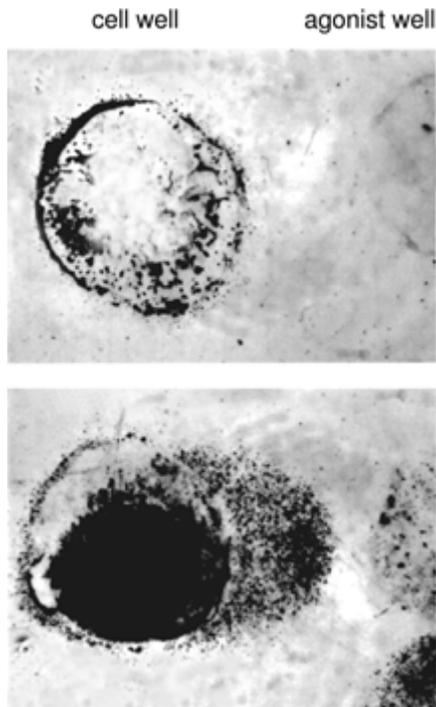


Figure 12.1.3 Under-agarose assay: response of neutrophils to interleukin 8 (IL-8). (A) Neutrophils exposed to a well containing no IL-8. (B) Neutrophils exposed to a well containing 1 pmole IL-8. Reproduced from Foxman et al. (1997) with permission from Rockefeller University Press.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

Prepare plates

1. Add 3 ml agarose/plate mixture to each 35-mm tissue culture dish and allow to cool and solidify at room temperature. Transfer plates to a humidified 37°C, 5% (v/v) CO₂ incubator, and incubate several hours to overnight.
2. Attach a sterile 3-mm hole cutter to a vacuum line with a catch flask. Use a template to make five 3-mm wells in a straight line, separated by 2 mm. Push the implement through the agarose layer until it reaches the plastic plate, using the vacuum to remove the agarose plug.

If using a steel punch, avoid scratching the plastic; scratches become barriers to cell migration.

The agarose plugs collect in the catch flask. To prevent the tubing from clogging, flush with water after cutting holes.

Cut wells no more than several hours before the assay. If a well is cut too long before it is filled with fluid, the agarose around the well dries and sticks to the plastic, preventing the cells from crawling underneath.

A good template is a piece of metal containing precisely positioned holes (Nelson et al., 1975) and constructed to fit over a 35-mm dish. Alternatively, an actual-size diagram of the desired well pattern can be placed underneath the dish and used as a guide for cutting wells.

The pattern of wells can be varied. Linear and parallel cell and attractant wells can be cut, yielding a steeper gradient of attractant, with a linear rather than radial distribution (Tranquillo et al., 1988). A triangular placement of wells allows analysis of migration in the presence of two chemoattractant sources (Foxman et al., 1997).

Prepare cells and chemoattractants

3. Suspend chemoattractants at the desired upper concentrations in migration medium. Suspend cells at 10^7 cells/ml in migration medium.

To form an effective gradient, the chemoattractant concentration should be ~10 to 1000 times the chemoattractant receptor K_d (Table 12.1.2).

Perform assay

4. At the start of the assay, fill the two most peripheral wells with 10 μ l migration medium. Next, place 10 μ l cell suspension (10^5 cells) in each of the two intermediate wells. Finally, place 10 μ l chemoattractant in the central well.
5. Return the plate to the 37°C, 5% CO₂ incubator for 2 hr (neutrophils) or longer (other cell types).

Fix and stain cells

6. Flood each plate with 1 ml absolute methanol. Allow cells to fix 30 min at room temperature, or overnight at 4°C.
7. Pour off methanol and flood each plate with 1 ml of 37% formaldehyde. Allow cells to fix 30 min at room temperature (longer if cold).
8. Remove agarose, using a Pasteur pipet to pry the edge loose, if necessary.

The cells should now be fixed to the plastic and thus remain on the dish.
9. Add 1 ml of 0.5% Fields stain B followed by 1 ml of 2.5% Fields stain A, and allow to stain for 1 to 2 min. Rinse plates with water and allow to dry.

Measure cell migration

- 10a. *Measure leading edge distance:* Along a line running through the center of the five wells, measure the distance from the edge of the cell well to the edge of the migrating cell front. Compare the distance cells have migrated toward the chemoattractant well to the distance they have migrated toward a well containing only medium (i.e., the peripheral wells).

A magnifier with reticles (Fisher Scientific) is a useful tool for performing these measurements.

- 10b. *Determine number of migrating cells:* Use a counting grid to count the number of cells that have migrated into squares at different distances from the cell well.

This method allows comparison between the number of cells migrating in one assay versus another. A plot of cell density at different distances from the starting well provides more information about cell migratory behavior than an estimate of the leading front distance alone (Lauffenburger et al., 1983).

11. Evaluate chemokinesis and chemotaxis coefficients.

Because most chemoattractants stimulate cell motility in a dose-dependent manner, a careful analysis is required to distinguish chemoattractant-stimulated motility (chemokinesis) from directional migration (chemotaxis). One approach is to measure chemoattractant-stimulated motility by observing the effect of a uniform field of chemoattractant (i.e., incorporated into the agarose) on cell migration, at a variety of different concentrations. Using these measurements, a chemokinesis coefficient can be determined, which can be used to calculate the relative contribution of chemokinesis and chemotaxis to the cell migration pattern in a chemoattractant gradient (Tranquillo et al., 1988).

The orientation and directionality of migration of individual cells can be observed by viewing migrating cells using video microscopy. In fixed cells, some investigators have used nuclear orientation to determine if cells are oriented toward the chemoattractant source (Palmlad et al., 1982; Krauss et al., 1994).

SMALL POPULATION CHEMOTAXIS ASSAY

This assay utilizes a hydrophobic agar surface to allow the placement of two small drops of liquid extremely close to each other (Konijn, 1970; Konijn and Van Haastert, 1987; Fig. 12.1.4). Diffusion through the agar generates a transient gradient, which allows a qualitative (yes or no) judgment as to whether there is a chemotactic response. This assay has not been used for any cell types other than *Dictyostelium*.

Materials

Purified agar (e.g., Noble agar, Difco)
Bonner's salts (see recipe)
Dictyostelium cells at $\leq 5 \times 10^6$ cells/ml
17 mM Sorensen's phosphate buffer, pH 6.2 (see recipe)
Chemoattractant solution in Sorensen's phosphate buffer
10-cm petri dishes

1. Prepare 0.5% to 1% (w/v) purified agar in Bonner's salts.

Purified agar can be replaced by less-expensive bacterial agar (e.g., Bacto agar, Difco) if it is extensively washed. A 20-g aliquot should be washed five times with 1 liter distilled water, followed by ten to fifteen times with an equal volume of deionized water, using no. 1 Whatman filter paper and a funnel to remove each wash (Konijn and Raper, 1961).

The correct hydrophobicity and agar density is reached when 0.1- μ l drops of cell suspension form half spheres and do not spread out from their original deposition site, and when the cells are unable to move out of the area covered by the original drop.

2. Pour 10 ml agar/salt solution in 10-cm petri dishes and allow to solidify.

Plates can be stored 1 day at room temperature followed by up to one week at 4°C.

3. Wash *Dictyostelium* cells twice in ice-cold 17 mM Sorensen's phosphate buffer, pH 6.2, and resuspend at 10^7 cells/ml. Shake suspension 1 hr (for measuring responses to folate) or 6 to 8 hr (for cAMP) at 150 rpm, 21° to 23°C.

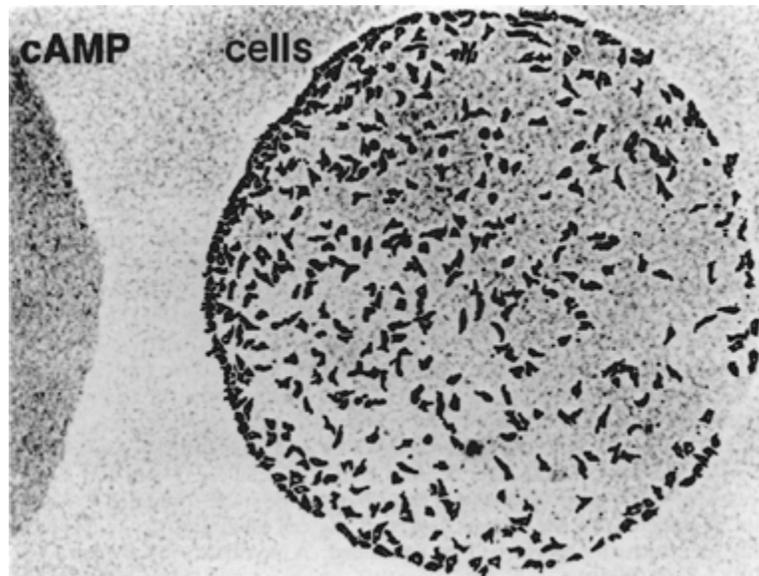


Figure 12.1.4 Small population assay. A single drop containing 500 to 1000 cells was exposed to cAMP (applied on the left in three 0.1- μ l drops containing 10 nM cAMP) and was photographed 5 min after stimulus. Note the higher density of cells on the side nearest the stimulus. Reproduced from Konijn (1970) with permission from Birkhauser Verlag AG.

It is important that starting cells be grown at or below 5×10^6 cells/ml so that they are in log growth phase. For information on preparing Dictyostelium, consult Devreotes et al. (1987). Grow axenic strains in axenic media such as HL5 (Watts and Ashworth, 1970). Grow nonaxenic strains in association with bacteria (Sussman, 1987).

4. Wash cells once more and resuspend at 10^7 /ml in 17 mM Sorensen's phosphate buffer. Place 0.1- μ l drops (10^3 cells) at regular intervals on the agar surface.

The small drops can be generated by using a Pasteur pipet with the tip drawn out by hand over a flame, and broken off (once cooled) to provide a small bore (40- to 70- μ m inside diameter). Fine pressure control can be achieved by using rubber tubing and either mouth pipetting or using a pipet bulb. Uniform speed of deposition of the drops is important to minimize variation in cell density. The pipet should be refilled after every ten to twenty drops to prevent cells from settling in the pipet and ensure uniformity of cell density. It is useful to set up rows of 10 replicate drops, with each row stimulated by a different concentration of chemoattractant.

5. Add 0.1- μ l drops of chemoattractant \sim 100 μ m away from the cell drops. Repeat at 5-min intervals for a total of three applications.

The concentration of chemoattractant should span a range between one tenth and ten times K_d (Table 12.1.2).

6. Incubate \sim 30 min at 21° to 23°C.

The plates should be checked every 10 min, since the precise details of when the gradient forms and cells respond will vary slightly from assay to assay, and the gradient may be relatively transient.

7. Examine the distribution of cells within each drop to determine whether more cells are pressing against the side of the drop that is next to the stimulus (Fig. 12.1.4). Quantify the response by calculating the percent of responding drops, with 100% showing a strong response.

See Konijn and Van Haastert (1987) for images of responding populations.

BRIDGE CHEMOTAXIS ASSAY

Cells are prepared on coverslips and are then inverted and observed on a 1-mm-wide bridge that connects two wells via a thin layer of fluid (Fig. 12.1.5). When different concentrations of chemoattractant are placed in the two wells, an approximately linear gradient develops across the bridge. The orientation or movement of cells toward one or the other well is monitored.

Materials

Cells: whole blood, neutrophils in suspension, or *Dictyostelium discoideum*
0.9% (w/v) NaCl

HEPES-buffered HBSS (prepare as for HBSS in APPENDIX 2A, but replace bicarbonate with 10 mM HEPES acid) containing 0.2% (w/v) BSA (for neutrophils in suspension)

17 mM Sorensen's phosphate buffer, pH 6.2 (for *Dictyostelium*; see recipe)

Chemoattractant in HEPES-buffered HBSS/1% (w/v) gelatin

HEPES-buffered HBSS/1% (w/v) gelatin

20 \times 40-mm coverslips, unwashed (neutrophils) or acid-washed (*Dictyostelium*; see recipe)

Glass (Neuro Probe) or Plexiglas bridge chamber (Fig. 12.1.5; see Critical Parameters)

Microscope with 40 \times phase objective

BASIC PROTOCOL 4

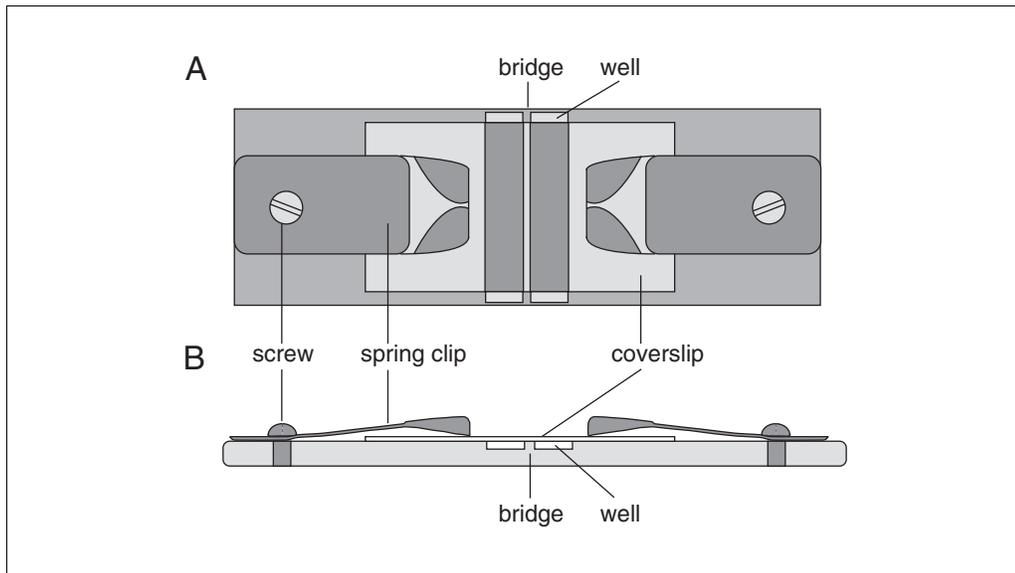


Figure 12.1.5 Apparatus for bridge assay. **(A)** Top view; **(B)** side view. A Plexiglas (or glass) base (3 mm high \times 2.5 cm wide) contains two wells (4 to 5 mm wide \times 1 mm deep) separated by a 1-mm-wide bridge. A 20 \times 40-mm coverslip is placed across the wells and secured by two spring clips, so that a thin gap (e.g., 5 μ m) is made between the coverslip and the bridge. Cells on the underside of the coverslip are flattened between the coverslip and bridge, and the direction of migration (to one or the other wells) is monitored.

Prepare cells on coverslips

For neutrophils from whole blood:

- 1a. Place \sim 0.1 ml human blood (e.g., from finger prick) across the center of a 20 \times 40-mm coverslip.

Enough blood must be placed on each coverslip to allow it to clot and partially retract without drying.

- 2a. Place the coverslip in a moist chamber (e.g., a petri dish containing a wet piece of filter paper) at 37°C (with or without 5% to 10% CO₂, depending on whether the medium is buffered with bicarbonate). Incubate \sim 45 min, until the blood has clotted and is beginning to retract so that fluid is visible around the edges.

- 3a. Gently rinse the clot and red blood cells off with 0.9% NaCl.

A monolayer of cells, mostly neutrophils, remains on the coverslip. Care must be taken not to let the cells dry.

For neutrophils in suspension:

- 1b. Prepare a suspension of human or rabbit cells at 3×10^5 cells/ml in HEPES-buffered HBSS containing 0.2% BSA.

Neutrophils do not stick well in gelatin, which is used during the assay.

- 2b. Apply 0.1 ml cell suspension in a \sim 200-mm² strip across the center of a 20 \times 40-mm coverslip. Allow to settle for \sim 5 min at room temperature.

- 3b. Rinse the cell layer with a few drops of incubation medium immediately before placing coverslip on bridge chamber (step 4).

For Dictyostelium:

- 1c. Prepare a single-cell suspension of *Dictyostelium* at 5×10^4 cells/ml in 17 mM Sorensen's phosphate buffer, pH 6.2.

- 2c. Place 50 μl cell suspension in a thin strip across the center of an acid-washed $20 \times 40\text{-mm}$ coverslip.

Dictyostelium adhere best to acid-washed coverslips (Soll, 1988; Segall, 1992). The coverslips can also be coated with 2 mg/ml BSA.

- 3c. Allow cells to settle and attach for 5 to 10 min at room temperature.

Assemble bridge chamber

4. Quickly remove most of the fluid over the cells by tipping the coverslip and allowing fluid to flow onto a Kimwipe.
5. Invert the coverslip onto a clean chamber so that cells lie over the bridge. Lower the coverslip slowly, allowing one edge to touch first.

A clean chamber is essential to successful chemoattractant-induced orientation of cells (see Critical Parameters). Touching one side of the coverslip to the bridge and then lowering the other side helps eliminate air bubbles.

6. Secure the clips on each side without moving the coverslip.

Any movement of the coverslip will cause enough shear force to lyse the cells on the bridge.

Chamber assembly requires practice, as short distances between the coverslip and the bridge are required for optimal cell orientation (see Critical Parameters). The chamber thickness can be measured by the difference in focal plane between the top of the bridge and the bottom of the coverslip, using the micrometer on the fine focus knob of the microscope.

Perform assay

7. Fill the two wells by capillary action with ~ 0.1 ml HEPES-buffered HBSS or chemoattractant both containing 1% gelatin.

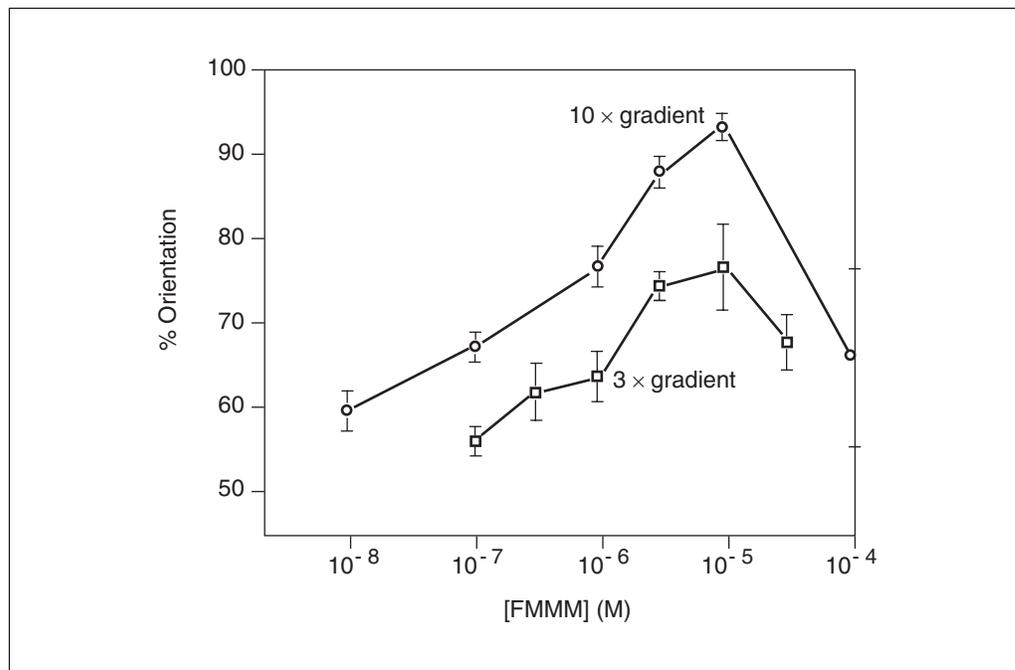


Figure 12.1.6 Results from bridge assay expressed as percent oriented cells versus chemoattractant concentration. The two curves represent different gradient steepnesses. The x axis corresponds to the high-concentration well. The other well contained one-tenth (circles) or one-third (squares) this value. fMMM, *N*-formyl-methionyl-methionyl-methionine. Reproduced from Zigmond (1977) with permission from Rockefeller University Press.

The chemoattractant concentration in the middle of the bridge will be approximately the mean of the two concentrations in the wells. Thus, the concentration of chemoattractant should range from one tenth to ten times the K_d of the receptor (Table 12.1.2).

A gradient of formyl peptide is ~90% established within 10 min and is roughly stable for 1 hr (Lauffenburger and Zigmond, 1981).

8. Incubate chambers 10 to 20 min at 37°C (or room temperature for *Dictyostelium*) to allow the cells to respond.
9. View cells at the middle of the bridge with a 40× phase objective. Determine the direction of cell migration for 100 cells based on morphological criteria, and plot percent cells oriented toward chemoattractant versus chemoattractant concentration (Fig. 12.1.6).

The front of a locomoting cell has a broad lamellipodium, while the tail is thinner and can be knob-like or drawn out into retraction fibers. The polarized morphology is clearest when the cells are moving well. When cooled, even to room temperature, neutrophils can round up and are difficult to score. The direction of movement can also be evaluated by video recording.

BASIC PROTOCOL 5

PIPET CHEMOTAXIS ASSAY

A pipet containing chemoattractant is brought up to a cell or group of cells, and the behavior of the cells is observed directly or by video microscopy.

Materials

Chemoattractant solution in assay buffer

Assay buffer: 17 mM Sorensen's phosphate buffer, pH 6.2 (see recipe), with or without CaCl_2 and MgCl_2 (for *Dictyostelium*), or DPBS (APPENDIX 2); JRH Biosciences; for MTLn3 cells)

Cells of interest

Omega dot tubing (4-in. glass capillary tubing, 1-mm o.d. × 0.58-mm i.d.; A-M Stevens)

Pipet puller for microinjection or neurobiology, capable of producing ~0.1- μm tip diameters (e.g., David Kopf Instruments, Narishige, Sutter Instruments)

Syringe with fine-bore needle (e.g., 3-in., 30-G)

Micromanipulator that can be attached to a microscope stage (e.g., Leitz, Narishige)

1. Prepare pipets (~0.1- μm tip diameter) using omega dot tubing and a pipet puller.

The thin tip allows a steep concentration gradient to be set up outside the pipet by diffusion, rather than by bulk flow. Omega dot tubing is used because it provides an internal filament to allow backfilling via surface tension (capillary action).

Alternatively, microloader micropipet tips are available from Eppendorf. To evaluate the suitability of the pipet, trial pipets can be filled with a high concentration (0.1 to 1 mM) of fluorescein or other fluorescent dye, and then viewed with a fluorescence microscope. If the tip is too large, a polarized stream of fluorescent material will be seen coming out of the tip. With the correct size tip, a spherically symmetric ball of fluorescence will be seen, brightest near the pipet tip, and getting dimmer with distance from the pipet, reflecting the spatial gradient generated by diffusion. Appropriate tips will be too small to resolve well with a 40× long-working distance objective.

2. Backfill the pipet by inverting and placing a drop of chemoattractant solution on the base. When the tip is full, fill the shank using a syringe with a fine-bore needle.

For expensive protein chemoattractants, load 5 μl directly to the tip with a microloader (Eppendorf).

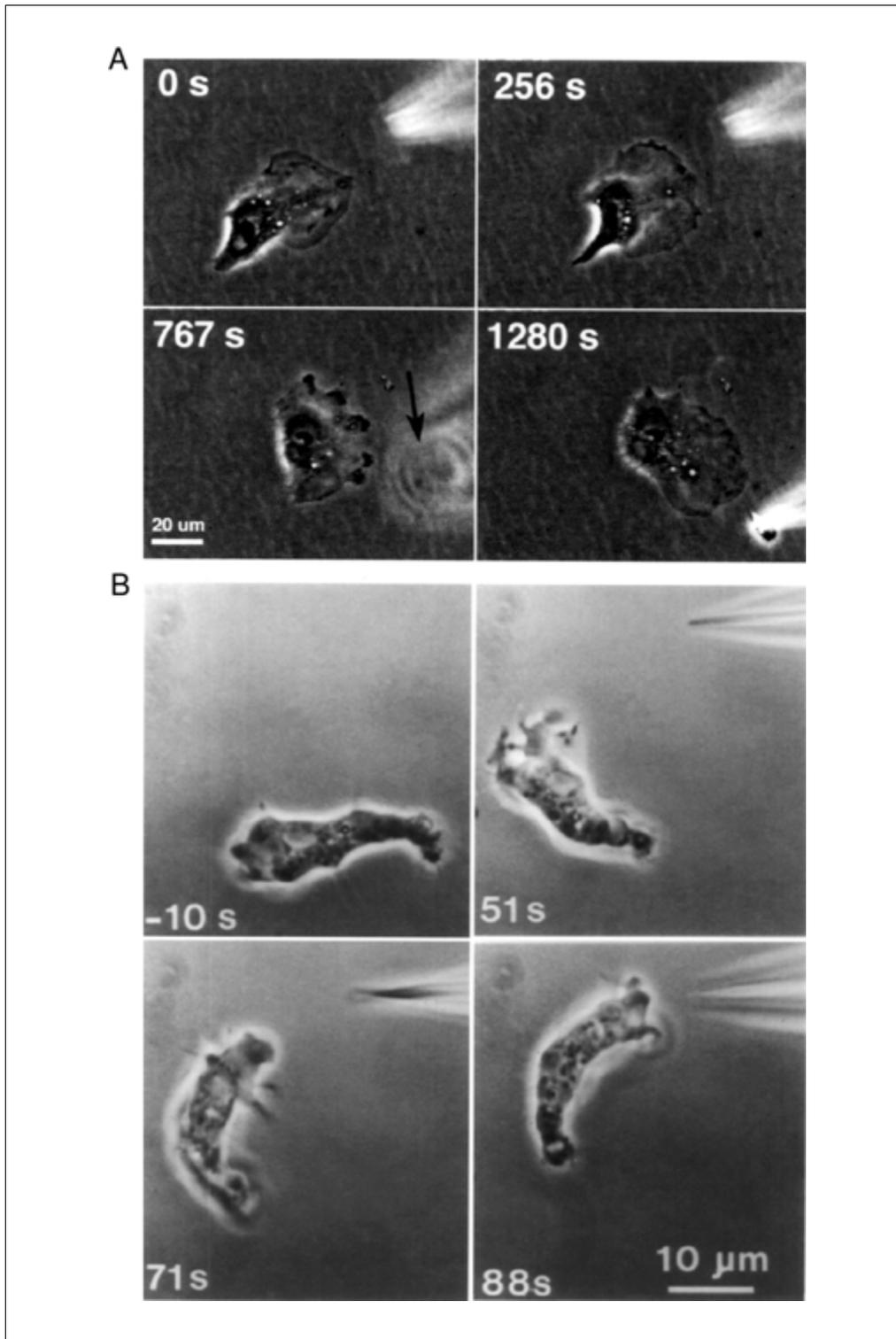


Figure 12.1.7 Pipet assay. **(A)** Response of mammary adenocarcinoma cell to a micropipet filled with 50 μM epidermal growth factor. The cell moved toward the micropipet (0 and 256 sec) and then reoriented when the pipet was moved (767 and 1280 sec). Arrow indicates movement of pipet. Reproduced from Bailly et al. (1998) with permission from Academic Press. **(B)** Response of *Dictyostelium discoideum* cell to a micropipet containing 0.1 mM cAMP. At -10 sec, the cell is moving to the left. At 0 sec, the pipet was placed in the upper right corner of the image field and the cell reoriented rapidly, moving toward the pipet. Reproduced from Segall and Gerisch (1989) with permission from Current Biology Limited.

A high concentration of chemoattractant (10^3 to 10^4 times K_d of the receptor; Table 12.1.2) is required inside the pipet. For example, in *Dictyostelium*, with a receptor K_d of 20 to 100 nM, 0.1 to 1 mM cAMP in the pipet provides a strong stimulus (>1000 times K_d). For MTLn3 tumor cells, with receptor K_d of 1 to 10 nM, 50 μ M EGF in the pipet is necessary.

3. Place cells in a petri dish and cover with a thin layer of assay buffer.

If temperature control is important, the buffer can be covered by mineral oil to minimize evaporation.

4. Place the micropipet in the micromanipulator and carefully bring the tip of the pipet to within 2 to 5 μ m of the surface of the dish. If necessary, use positive pressure to increase the ejection rate.

Note, however, that fluid flow generated by positive pressure may perturb a gradient that is generated purely by diffusion.

Depending on the concentration of chemoattractant in the pipet and the chemotactic abilities of the cells, the tip may need to be within 10 μ m of the cell, or it may be better at 50 μ m away (for pipets containing high concentrations of chemoattractant). However, it is important to note that the gradient becomes shallower as the pipet is moved away from the cell, so that the steepest gradients are produced by pipets near the cell but perhaps utilizing a lower chemoattractant concentration so as to avoid saturating the receptors.

5. Incubate for the appropriate time.

For Dictyostelium, responses can occur within seconds to minutes, while for slower-moving cells (e.g., MTLn3 cells), reorientation and movement may take 10 to 20 min.

6. Score the response of the cells (Fig. 12.1.7) by measuring (1) the percent of cells that move toward the pipet, (2) the accuracy of redirection (cosine of the angle between the direction of cell movement and the direction of the pipet), or (3) the speed of cell movement toward the pipet.

Typically, a 40 \times long-working distance objective provides adequate resolution. Lower magnification objectives may be useful in order to visualize the responses of multiple cells.

BASIC PROTOCOL 6

UPSHIFT CHEMOTAXIS ASSAY

In this assay, cell behavior is observed after a sudden homogeneous increase (or decrease) in concentration of chemoattractant. This assay is useful for biochemical analysis of chemotactic signal transduction, which almost always relies upon bath application of chemoattractant to cells followed by lysis and biochemical assays. In such studies, there is little or no significant spatial gradient. Rather, a sudden temporal change in chemoattractant concentration is the stimulus.

Although this is different from the stimulus experienced by cells in a spatial gradient, it provides useful information about the kinetics of various biochemical responses. A similar evaluation of behavioral responses to such a stimulus is also possible, allowing the correlation of biochemical changes with specific behaviors. In its simplest form, the upshift assay can be performed by microscopic evaluation of cells moving in a petri dish. By adding an equal volume of chemoattractant solution, a relatively efficient concentration increase is effected and cell behavior can be observed. An example of this approach is shown in Figure 12.1.8, and a detailed protocol is provided in Cammer et al. (1997). Alternatively, a number of specialized chambers or procedures can be utilized to allow a more precise control of the concentration (Varnum-Finney et al., 1988; Segall, 1992) or of the rate of increase in concentration (Omann and Sklar, 1988).

USING IMAGE ANALYSIS PROGRAMS TO ASSESS CHEMOTAXIS

In many chemotaxis assays (e.g., under-agarose, bridge, micropipet, and upshift assays), the area, shape, speed, and direction of movement of individual cells must be measured. Although it is possible to trace video or still images on the monitor screen, a number of image processing programs that aid in subsequent quantification are available. DIAS (Dynamic Image Analysis System) is specifically designed for such analysis (Soll, 1995). Depending on the image itself, the program can directly determine cell borders (for slightly defocused bright-field images). Alternatively, it allows a user to trace high-resolution differential-interference-contrast (DIC) or phase-contrast images. The program then calculates the center of mass, area, roundness, speed, and direction of movement of each cell in the field. A number of other parameters can be calculated as well. Other programs, such as NIH Image (Cammer et al., 1997) and Metamorph (Universal Imaging), can also be adapted to provide a limited subset of similar measurements.

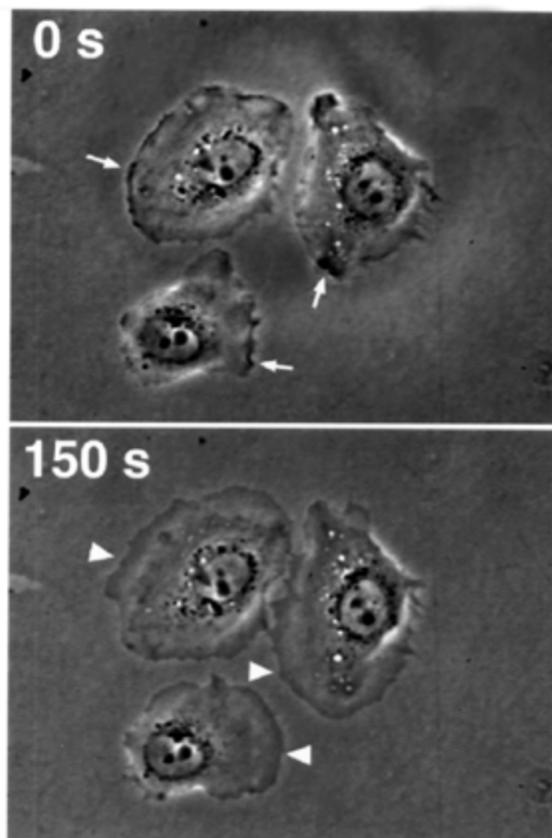


Figure 12.1.8 Upshift assay. MTLn3 cells were grown 24 hr on tissue culture plastic in growth medium in a 37°C CO₂ incubator. They were transferred to a microscope with a 37°C incubator box. The lid of the dish was removed and the medium was covered with a layer of prewarmed mineral oil (Sigma). After tracking for 5 to 10 min, an equal volume of 10 nM epidermal growth factor (EGF) in 37°C growth medium was added under oil using a sterile pipet (final 5 nM stimulus). The top panel shows cell morphology immediately after addition of EGF. The phase-dark areas at the edges of the cells are vertical ruffles (arrows). The bottom panel shows cell morphology 150 sec after addition of EGF. The ruffles have disappeared and flat hyaline lamellipodia have extended (arrowheads), increasing the total area of the cells. Reproduced from Segall et al. (1996) with permission from Kluwer Academic Publishers.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Acid-washed coverslips

Soak coverslips 10 min in 2.7% HCl. Rinse extensively in water and dry in a clean area or a laminar-flow hood. Store several days to a week at room temperature in a clean, sealed container to reduce the rate at which the surfaces become hydrophobic.

Agarose plate mixture

For bicarbonate-buffered under-agarose plates:

Boil agarose in HBSS (APPENDIX 2A) at a final concentration of 2.4% (w/v). Make a separate solution of 20% (v/v) heat-inactivated bovine calf serum (BCS) in bicarbonate-buffered RPMI 1640. When agarose solution is cooled to 50°C, mix solutions 1:1 (final 1.2% agarose, 10% BCS, 0.5× RPMI, 0.5× HBSS) and use immediately.

For HEPES-buffered under-agarose plates:

Boil 2.4% (w/v) agarose in sterile water. In a separate container, dilute 1 M HEPES (Life Technologies) to 20 mM in 2× RPMI 1640 without bicarbonate (from low-endotoxin 10× RPMI 1640 stock; Sigma). Add 20% (v/v) heat-inactivated BCS to RPMI/HEPES and adjust to pH 7.2. When agarose solution is cooled to 50°C, mix solutions 1:1 (final 1.2% agarose, 10% BCS, 1× RPMI) and use immediately.

For both types of plates, the agarose must be boiled and cooled before addition of protein solution, to avoid denaturing the protein. For serum-free assays, replace 20% BCS with 1% (w/v) BSA or 0.25% gelatin (see Critical Parameters).

Media with serum/protein (but no agarose) can be filter sterilized and stored up to 6 months at 4°C.

Bonner's salts

10 mM NaCl
10 mM KCl
2.7 mM CaCl₂
Store at 4°C (stable at least 1 month)

Giemsa stain

4 ml 95% (v/v) ethanol
3 ml Giemsa stock (Ricca Chemical)
43 ml 15 mM sodium or potassium phosphate buffer, pH 6.8
Prepare fresh before use

Migration medium

For bicarbonate-buffered under-agarose plates (see agarose plate mixture above), use 1× bicarbonate-buffered RPMI 1640 with 10% (v/v) heat-inactivated BCS. For HEPES-buffered under-agarose plates, use 1× RPMI without bicarbonate (from low-endotoxin 10× RPMI 1640 stock; Sigma) containing 10 mM HEPES and 10% (v/v) heat-inactivated BCS. Filter sterilize and store up to 6 months at 4°C.

For either medium, 10% BCS can be replaced with 0.5% (w/v) BSA (see Critical Parameters).

Sorensen's phosphate buffer, 17 mM, pH 6.2

For 10× stock solution:

20.6 g/liter KH₂PO₄
5.05 g/liter Na₂HPO₄·7H₂O
Store at room temperature or 4°C (stable at least 1 month)
Before use, dilute to 1× and supplement with 1 mM MgCl₂ and 0.2 mM CaCl₂

COMMENTARY

Background Information

Distinguishing chemotaxis from chemokinesis

Chemotaxis assays are designed to determine if a biological compound has the ability to induce directed migration of cells. To demonstrate that a factor elicits chemotaxis, it is important to show that a gradient of the factor biases the direction of locomotion rather than simply stimulating locomotion in random directions (chemokinesis). Unfortunately, merely demonstrating that cell migration is not stimulated when the gradient is reversed does not prove that the test substance is a chemoattractant. This is because many factors, including many chemoattractants, stimulate the rate of migration in a concentration-dependent manner, and because very high concentrations of some substances can be less stimulatory than lower concentrations (Harvath et al., 1980; Tranquillo et al., 1988).

To determine if chemotaxis is present in a spatial gradient, one must show that the enhanced migration cannot be accounted for quantitatively by enhanced rates of random migration. To do this one needs to first define the effects on random migration, by quantifying migration in a series of homogeneous concentrations of the test material (a checkerboard assay as in Tables 12.1.5 and 12.1.6). Once the effects of different concentrations on random migration are known, it is possible to determine if they account fully for the migration observed in a gradient. Migration in a positive gradient that exceeds that expected from random migration is evidence for chemotaxis. This analysis does not correct for the possibility that migration is stimulated as cells are exposed to higher concentrations of attractant and inhibited as the concentrations decrease (Rhodes, 1982). Such effects on migration rates have been observed, but their magnitude is probably too small to appreciably alter the results. It is also possible to determine if a factor testing positive in an initial assay is truly chemotactic by testing it in an assay that directly monitors the movement of individual cells.

When and why should one determine if a factor is really a chemoattractant?

In some cases the investigator may not care if the response is due to enhanced migration or chemotaxis. If documentation of chemotaxis is not shown, a valid way to report the results is

that the factor “stimulated (or enhanced) cell migration.” However, understanding the cellular basis for the increased migration is important for predicting how the factor will function in a given situation. Consider a test factor that merely enhances migration in a concentration-dependent manner. A purely chemokinetic factor can appear to attract cells in one assay but repel them in a different assay. If, in one assay, cells are placed in a well and exposed to a gradient of this factor emanating from an adjacent well, cells exposed to the higher concentration will move faster and spread further toward the well than cells exposed to the lower concentration. However if, in a second assay, the cells are distributed evenly throughout a gradient, again emanating from an adjacent well, the cells exposed to the higher concentration will again move the fastest, but now the movement results in net dispersion of cells away from the well containing the factor. Thus, the factor could appear to be a chemoattractant in one assay and a repellent in a second. In reality, it simply stimulates migration in a concentration-dependent manner.

Chemotaxis when the gradient is attached to the substratum

There are many situations in which the chemoattractant is not in solution but rather is attached to the substratum. Cells can respond to a chemoattractant gradient even when the chemoattractant is attached in a fixed gradient (Wilkinson and Allan, 1978). In vivo, it is likely that binding of chemoattractant to the extracellular matrix allows a gradient to be maintained in the midst of water flux through the extracellular space (McCormick et al., 1993, 1995; Webb et al., 1995). In other cases, the chemoattractant acts directly through adhesive receptors that signal to the cell interior to stimulate spreading and process outgrowth (Aznavorian et al., 1996; Condic and Letourneau, 1997). Finally, adhesivity itself can result in cell accumulation in regions of high adhesivity, a phenomenon initially termed haptotaxis (Carter, 1965). This cell accumulation can result merely from the trapping of cells in regions of very high adhesivity, not from directed movement (Harris, 1973).

Variations in the basic assays

The assays presented in this unit represent basic starting procedures. They can be modified

for a variety of specific purposes. Some examples of such modifications are described here.

Filter assays. Cell migration assays performed with filters can be automated through the use of radiolabeled or fluorescently tagged cells. In addition, instead of filters, cell migration can be assayed across epithelial cell monolayers (Nash et al., 1987), endothelial cell monolayers (Huang et al., 1988), or gels of extracellular matrix components (Loike et al., 1995).

Under-agarose assays. An interesting variation has been developed by Tranquillo and colleagues (Moghe et al., 1995). These investigators initiate the assay with neutrophils evenly distributed throughout a fibrin gel rather than concentrated in a well. This geometry allows analysis of cell movements that are uncomplicated by the effects of high cell concentration and gradients of cell concentration.

Bridge assays. The chemoattractant can be added after the cells have had time to equilibrate in the chamber. With *Dictyostelium* amoebae, where the thickness of the fluid layer does not appear to be so critical, it is possible to first fill the wells with buffer and incubate the bridge chamber in a humidified chamber for 20 min at room temperature to allow the cells to equilibrate. Unstimulated cell behavior can be measured under these conditions. One well can then be flushed with 10 nM to 2 μ M cAMP, while the other is flushed with just buffer. After allowing 10 min for gradient formation, cell behavior over the next 20 to 30 min will demonstrate the chemotactic response. Cells move at ~ 10 μ m/min with a strong bias toward the well filled with cAMP (cosine of direction of movement relative to the direction of the gradient is typically 0.3 to 0.6). With neutrophils, also, it is possible to remove the fluid from a well and replace it with a different solution. However, care must be taken not to move the coverslip, which will shear the cells on the bridge.

For slower moving cells such as fibroblasts or epithelial cells, a chamber that may have a more stable, longer-lasting gradient has been designed (Zicha et al., 1997) and is available from Weber Scientific. An alternate approach to generating a stable spatial gradient utilizes hollow fibers embedded in an agarose gel that is continuously supplied with new medium. This method provides the longest-lasting gradients, but is quite delicate and is not commercially available. Details on construction of the chamber are described in Fisher et al. (1989).

Pipet assay. A two-pipet assay has been used to analyze the dynamics of leukocyte pseudopod extension (Zhelev et al., 1996).

Critical Parameters

Equipment options for filter assays

For filter assays (see Basic Protocol 1), there are a number of chamber and filter options to select from (see Tables 12.1.3. and 12.1.4).

Chamber design. Filter assays can be performed in individual, blind-well chambers (Fig. 12.2.1), or in multiwell chambers (Fig. 12.1.2) containing 12 or 48 wells. These are available from Neuro Probe. Alternatively, cells can be induced to migrate through porous tissue culture inserts (Collaborative Biomedical Products, Corning Costar) that fit in standard 24-well tissue culture dishes.

Filters. The original Boyden filter assay used thick cellulose acetate or nitrate filters (Boyden, 1962). They are ~ 100 μ m thick, and have torturous pores (Millipore or Neuro Probe). Because these filters are thick, measurable parameters include: the distance of migration into the filter (Zigmond and Hirsch, 1973; see Support Protocol 1), the number of cells that have moved a given distance into the filter, and the number of cells that have migrated all the way through the filter. These filters may be used with blind-well or multiwell Neuro Probe chambers.

Thin polycarbonate filters (Nucleopore) are only ~ 10 μ m thick and have pores that are straight channels through the filter. These filters can be used to count the number (or fraction) of cells that have migrated through the filter to the lower surface (Harvath et al., 1980) and to count the number of cells that have fallen off into the lower chamber. Since the filters are only one cell diameter thick, it is impossible to further define the distance migrated. Polycarbonate membranes are available for blind-well and multiwell Neuro Probe chambers. In addition, two types of thin filters are available as tissue culture inserts for standard 24-well tissue culture plates, including polyethylene terephthalate (PET) BIOCOAT control inserts (Collaborative Biomedical Products) and polycarbonate Transwell inserts (Corning Costar). It appears that chemokinetic effects are less pronounced in thin polycarbonate filters than in thick cellulose nitrate filters (Bignold, 1988).

Pore size. Both filter types come in different mean pore sizes. The appropriate pore size depends on the cell type being studied (see

Table 12.1.4). Monocytes can be assayed using filters with 5.0- to 8.0- μm pores, and mesenchymal cells using filters with 8.0- μm pores. Neutrophils are usually assayed with 3.0- μm pores. They can migrate into very small pores (1 μm) when induced by a gradient of chemoattractant, but not when exposed to a homogeneous concentration of attractant (Campbell et al., 1997a). Thus, a small pore size can be chosen to minimize the contribution of chemokinesis.

Filter coating. Usually the clean filters are toxic and some protein coat must be included. Albumin can be added to the assay medium, or the filter can be precoated with an extracellular matrix protein. For example, for MTLn3 cells or fibroblasts it is helpful to coat the filter 2 hr with type I collagen (27 $\mu\text{g}/\text{ml}$ rat tail collagen) in Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium (JRH Biosciences; also see DPBS and CMF-DPBS in APPENDIX 2A). The filter is then rinsed in DPBS before use. Depending on the cell type, better responses may be obtained by drying the matrix molecules onto the filter.

Equipment considerations for bridge assays

Glass chambers are available from Neuro Probe. The glass chamber is thin and provides excellent optics. Plexiglas chambers can usually be cut locally. The shop should be advised that the Plexiglas must be completely flat (i.e., not warped). After cutting the wells, the burrs on the edge of the wells must be removed, but the top of the chamber should not be polished, as this lowers the height of the bridge.

The distance between the bridge and coverslip must be precise for proper cell orientation. For instance, optimal neutrophil orientation occurs when the distance between the coverslip and bridge is $\sim 5 \mu\text{m}$. Improved orientation when the fluid layer is thin probably reflects the fact that the gradient across the bridge is formed by diffusion. The thin space creates resistance to fluid flow between compartments, which disturbs the gradient. The thin space, and resultant high cell concentration, probably allows the cells to steepen the gradient by removing/degrading the chemoattractant.

The chamber thickness can be measured by the difference in focal plane between the top of the bridge and the bottom of the coverslip, using the micrometer on the fine focus knob of the microscope. When the distance is 5 μm , the cells are somewhat flattened. To obtain a fluid layer in the 5- to 10- μm range requires a clean

chamber and sufficient force applied by the clips. Chambers exposed to room air rapidly absorb "junk." Therefore, chambers should be washed just before use in warm water with tissue culture detergent, rinsed well with distilled water, and wiped dry. The bridge can also be wiped with 70% or 95% ethanol. To obtain the proper fluid layer, it also helps to use a minimal amount of fluid when making the cell preparation. It is usually necessary to prepare extra chambers and then incubate and score only those of appropriate thickness.

Cell-specific issues

Neutrophils like to be squeezed and move rapidly through filters, under agarose, and when compressed between two surfaces as in the bridge assay. When merely placed on a surface, neutrophils show a variable ability to translocate, and are frequently described as being "stuck by their tails". These "stuck" cells extend processes into the medium but appear unable to form effective new attachments. This behavior is remedied by altering the extracellular matrix protein present (Marks et al., 1991; Maxfield, 1993), and is rarely seen when cells are in contact with (squeezed between) two or more surfaces, such as in a filter, bridge, or under-agarose assay.

Neutrophils respond to various chemoattractants, including bacterial-derived peptides (*N*-formyl peptides), a cleavage product of complement factor 5 (C5a), lipids such as leukotriene B₄ (LTB₄), proteolytic fragments of the extracellular matrix (Senior et al., 1980), and many of the C-X-C family of chemokines (Baggiolini et al., 1997).

With neutrophils, the method of cell isolation and the time between drawing the blood and starting the assay can have important effects. Different labs use different protocols. Endotoxin-free neutrophils can be stored at room temperature for several hours without activity. Note that some investigators have demonstrated that neutrophils change phenotype when assayed >2 hr after cell preparation (>5 hr after blood drawing; Seligmann et al., 1981). Rabbit peritoneal neutrophils can be stored overnight at 4°C, as long as they are kept in the peritoneal fluid.

Monocytes move more slowly than neutrophils but are readily assayed in filter assays. They respond to many of the CC family of chemokines as well as to *N*-formyl peptides.

Lymphocytes (T and B cells) respond chemotactically to a variety of chemokines. Lymphocytes migrate well through thin poly-

carbonate or polyethylene terephthalate membranes.

Dictyostelium amoebae respond well in filter, under-agarose, small population, bridge, and micropipet assays. *Dictyostelium* amoebae do not typically show tight attachment to surfaces and do not require surfaces coated with specific adhesion molecules. The sensitivity of *Dictyostelium* cells to specific chemoattractants can vary with cell state. Cells taken directly from growth medium are chemotactic to folate. As cells are starved, they undergo a developmental program that involves formation of aggregates of cells by chemotaxis to extracellular cAMP. During morphogenesis in aggregates to form pseudoplasmodia or fruiting bodies, responsiveness to cAMP may be reduced and responses to monapterin derivatives develop. Thus far, the best characterized chemotactic responses have been those to extracellular cAMP by cells starved for 6 to 8 hr.

Mammalian fibroblasts and epithelial cells (including many tumor cell types) typically move slowly (0.2 to 2 $\mu\text{m}/\text{min}$). This may reflect the slow detachment of focal contacts to surfaces coated with typical extracellular matrix molecules such as collagen, laminin, vitronectin, or fibronectin. Chemotactic responses of such cells have been characterized using filter assays. Identified chemoattractants for these cells include ligands for G protein-coupled receptors—such as autocrine motility factor (AMF)—and growth factors that bind to receptor tyrosine kinases—such as fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin, platelet-derived growth factor (PDGF), and hepatocyte growth factor/scatter factor (HGF/SF). The methods described in this unit have been developed for a chemotactic tumor cell line (termed MTLn3 cells), but similar conditions should work for most fibroblast and epithelial cells. Extracellular matrix molecules can be used instead of chemoattractants to evaluate haptotactic responses.

Neurons have shown a variety of motile responses. One distinctive feature of neurons is the formation of a growth cone, which shows polarized movement while the main cell body may remain stationary. Growth factors, neurotransmitters, and adhesion molecules have been shown to attract or repel neuronal growth cones. The assays typically utilized for growth cone responses are micropipet or tissue fragment assays. Thus far, the focus has mainly been on identifying chemoattractants for various neuronal types. Future studies will begin to focus on mechanistic questions, given the recent ad-

vances in characterizing chemoattractants for neurons.

Media

Since most neutrophil assays are short, the cells can be assayed in Hanks' balanced salt solution (HBSS). Alternatively, a full medium such as RPMI can be used. Neutrophils move best in slightly acidic (pH 7.1 to 7.2) and slightly hypotonic conditions. Bicarbonate-buffered tissue culture medium can be used when assays are incubated in 5% to 10% CO_2 . For incubations in room air, both RPMI and HBSS can be purchased without bicarbonate, and then supplemented with 10 to 20 mM HEPES. All solutions should be kept sterile and as endotoxin-free as possible, since endotoxin activates neutrophils.

All assays require some protein to allow migration and prevent toxicity from glass, filters, and agarose. The protein can be albumin, gelatin, or serum proteins. Most commonly, bovine serum albumin is added to a final concentration of 0.1% to 1% (w/v). Although albumin itself can stimulate migration, this effect is diminished through use of fatty acid-free albumin. Gelatin is dissolved fully in boiling water as a 10% (w/v) stock, which is then warmed to remelt the gelatin and diluted tenfold (final 1%) into medium. Gelatin is non-stimulatory (Chenoweth et al., 1979) and minimizes neutrophil adhesion to various surfaces. Gelatin enhances migration in the bridge assay but does not provide enough traction to allow cells to enter pores of the Millipore filter. However, once cells have entered the pores, substitution of gelatin for BSA stimulates subsequent migration (Zigmond, unpub. observ.). Heat-inactivated serum ($\leq 10\%$) may also be used as a protein source. Serum is heat-inactivated by warming to 56°C for 25 min. However, even after heat inactivation, serum contains chemoattractants.

The optimal medium for studies with *Dictyostelium* is 17 mM Sorensen's phosphate buffer, pH 6.2, with 1 mM MgCl_2 and 0.2 mM CaCl_2 .

For MTLn3 cells/fibroblasts, α -MEM with 12 mM HEPES, pH 7.4, can be used. BSA can be included at 0.35% (w/v) to provide an osmotic strength similar to 5% BCS. Optimal responses to EGF are obtained by always growing and passing cells at $<80\%$ confluency, and by serum starving the cells in HEPES-buffered MEM with 0.35% BSA for 3 to 24 hr just before stimulation.

Decreased orientation in the bridge assay

The level of orientation in the bridge assay is reduced when competing chemoattractants are present. Neutrophils adjacent to air bubbles or cell clumps produce chemoattractants, and thus become foci for attracting adjacent cells. These foci reduce the level of orientation induced by the chemoattractant gradient across the bridge. When chemoattractant is present in both wells, cells near the edge of the well containing the lower concentration are sometimes observed to orient toward this edge. This “edge effect” appears to be due to a high concentration of cells on the bridge degrading chemoattractant until there is a lower concentration at the center of the bridge than in the well containing the lower concentration.

Controls

Under all conditions, the most important control is using medium without chemoattractant. Cell responses under these conditions provide a baseline for comparison with medium containing chemoattractant. Distinguishing between chemokinesis and chemotaxis can then be done, if desired, by evaluating cell responses when the same concentration of chemoattractant is present in all solutions.

Anticipated Results

Filter assay

With a thick cellulose nitrate filter, unstimulated cells typically move 10 to 15 μm into the filter and stimulated cells move 80 to 90 μm into the filter. When measuring the distance to the cell front, it is important to stop the incubation before cells have moved all the way through the filter (~ 100 μm after staining and clearing; Zigmond and Hirsch, 1973). When measuring the number of cells on the bottom of the filter, between 20% and 50% of the cells move to the bottom following stimulation (Harvath et al., 1980).

For thin-filter assays, with neutrophils migrating through tissue culture inserts with 1- μm pores, typically 5% to 20% of the cells added to the top well traverse the filter and can be counted in the bottom well in response to an optimal gradient. Without stimulation, <1% of cells appear in the bottom well (Campbell et al., 1997a).

The percentage of lymphocytes/lymphoid cells that migrate through 3- or 5- μm pores varies widely (from 1% to 50%) with cell type and chemoattractant used. However, when the optimal pore size is used, the background mi-

gration without chemoattractant is typically very low (<1% of cells added).

Under-agarose assay

Neutrophils migrating in response to an optimal chemoattractant source can migrate up to 1.6 mm toward the attractant during the 2-hr migration period. Neutrophils in this assay tend to migrate farther toward some chemoattractants than others, e.g., migrating more toward interleukin 8 or *N*-formyl-methionyl-leucyl-phenylalanine (*f*MLP) than C5a or LTB_4 (Foxman et al., 1997). The differences in migration may reflect differences in gradient formation, difference in cellular responsiveness to chemoattractants, or both. Neutrophils in the migrating cell population are densely packed. In the absence of stimulation, individual neutrophils can be observed to migrate as far as 0.5 mm away from the starting well, but the cells are much more sparsely distributed (Fig. 12.1.3).

Small population assay

This assay is designed to score rapid movement of *Dictyostelium* cells toward a chemoattractant. As the chemoattractant concentration is increased, the fraction of responding cells will go from 0% to a maximum of near 100%, followed by a decrease when the concentration becomes too high. The optimal concentration range may be very narrow.

Bridge assay

Random migration results in $\sim 50\%$ of the cells moving toward each well. In an optimal gradient of a potent chemoattractant, it is possible to observe 100% of the cells with polarized morphology, all oriented toward the attractant. Often, only between 50% and 80% have clear enough polarity to be scored, and of these, between 80% and 90% are oriented toward the chemoattractant (Zigmond, 1977). In video studies, up to 100% of the cells are observed to move toward the chemoattractant, although the accuracy of the orientation toward the attractant varies.

Pipet assay

For *Dictyostelium* or neutrophils, changes in cell shape and extensions are seen within seconds after placement of the pipet, with net movement toward the pipet observed within 1 min. For epithelial or mesenchymal cells, such as MTLn3 cells or fibroblasts, a slow response involves extension of projections followed by net cell movement, which may take up to 20 to

30 min. In the most extreme cases, such as orientation of *Saccharomyces cerevisiae* cells in a gradient of mating factor, responses may take hours (Segall, 1993). In all cases, too high of a concentration of chemoattractant in the pipet will result in responses similar to those seen in the upshift assay (see below), with no oriented movement toward the pipet.

Upshift assay

Within 20 min a series of shape changes should occur. The specific changes and their kinetics vary with cell type. For *Dictyostelium*, cells stop and round up within 30 sec, and extend lamellipodia at 1 to 2 min, followed by at least a partial restoration of motility (Varnum-Finney et al., 1988; Segall, 1992). Restoration can be observed within 10 min, especially at low chemoattractant concentrations. For neutrophils, dramatic lamellipodial extension occurs 1 min after stimulation, followed by motility at 3 to 5 min (Zigmond and Sullivan, 1979). For MTLn3 cells, maximal lamellipodial extension is seen at 3 to 5 min (Segall et al., 1996). For mammalian cells, restoration may take 30 min, or a permanent change (either increase or decrease) in rate of movement may be seen.

Time Considerations

Filter assay

Isolating cells from blood or a peritoneal exudate takes 3 to 6 hr, respectively. It takes 30 min to prepare medium and 10 min to prepare a 12-well multiwell chamber. The assay itself requires 30 min to 3 hr for incubation (depending on cell and filter type), 2 to 3 hr for staining and clearing the filter (staining can be performed overnight), and 5 to 15 min per filter for mounting and scoring.

When the filter assay is performed using a 24-well plate with inserts, ~30 min are required to prepare the medium, but it may be prepared the night before and equilibrated overnight in a tissue culture incubator. It takes ~30 min to set up the chamber with filter and medium. The amount of time required to prepare the cells for the assay depends on the cells, on average from a few minutes to 2 to 3 hr or more. Incubation time for the assay is 1.5 hr for neutrophils and 3 to 4 hr for lymphocytes or lymphoid cell lines. Scoring the assay requires 5 min per well.

Under-agarose assay

It takes ~1 hr to prepare the medium and pour the plates, and this can be done the night

before the assay. On the day of the assay, it takes ~1 min per plate to cut the wells. The time required to prepare cells for the assay ranges from a few minutes for cultured cells to 2 to 3 hr for cells isolated from blood. It takes ~1 min to set up each plate, 2 hr to incubate neutrophils (longer for other cells), 1.5 hr to fix and stain cells, and ~2 min per plate to score migration distance.

Small population assay

It takes 1 to 2 days to prepare the agar dishes for this assay. Practice is required to prepare Pasteur pipets of the appropriate tip diameter. Cell preparation time will vary depending on the cell type. For example, for optimal chemotactic response to cAMP, *Dictyostelium* requires starvation for 4 to 8 hr. Performance of the assay itself requires 15 to 20 min to place the various drops, and 30 min to record the responses.

Bridge assay

The time to perform an individual assay includes 3 to 6 hr to obtain the cells from blood or peritoneal exudate, or 45 min from a finger prick sample; ~30 min to prepare the medium, which can be done during cell preparation; 1 to 3 min to prepare each chamber; 20 min to incubate; and 5 to 10 min to score orientation or migration on a video recording.

Pipet assay

For rapidly moving cells such as *Dictyostelium* or neutrophils, the responses of individual cells can be determined within 2 to 4 min. For slowly moving cells such as fibroblasts or epithelial cells, responses occur after 10 to 20 min. The time required to quantitate the results depends upon the number of cells and images analyzed. On average, using NIH Image or DIAS, 10 to 30 min per cell are required to quantitate area, speed, and direction of motion (Cammer et al., 1997; Bailly et al., 1998; Fig. 12.1.7).

Upshift assay

Preparing and warming solutions requires 20 to 30 min. Recording of cell responses in a microscope requires 20 min per upshift compartment. Quantitation of results depends on the method used. For NIH Image or DIAS, roughly 10 min are required to trace out the perimeter and calculate the area for one cell if one image per min is recorded.

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The basement membrane is a thin extracellular matrix that underlies epithelial and endothelial cells and separates these tissues from stroma. Tumor cells must cross the vessel basement membrane and penetrate the underlying stroma in order to invade tissue and form distant metastases. They do this by producing proteases that degrade the extracellular matrix. Several *in vitro* invasion assays have been developed using various extracellular matrix barriers including amnion, type I collagen gels, and a reconstituted basement membrane termed Matrigel (see *UNITS 10.2 & 10.3*). Matrigel-based invasion assays (see Basic Protocol) are the most reliable, reproducible, and representative of *in vivo* events, and they are the assays most frequently used. Porous filters are coated with a thin layer of Matrigel and placed in a Boyden migration chamber with a chemoattractant in the lower well and tumor cells in the upper well (Fig. 12.2.1). The entire chamber is then incubated for ~3 to 10 hr, depending on the tumor cells used. After incubation, the filter is removed, fixed, and stained, and the cells on the lower surface of the filter are quantitated. This assay is advantageous because it is quick, reliable, quantitative, and in most cases does not require sterility. Compounds that either promote or inhibit invasion can be assayed. In addition, at the end of the assay, the invasive cells can be recovered and used for further study. The invasion assay can be used to screen for a large variety of compounds in 48-well chambers, with the advantages that smaller amounts of test material and fewer cells are needed. Commercial kits are also available. This protocol is provided for those investigators who seek to test a large number of samples or to modify the basic protocol (for example, by increasing or decreasing the amount of Matrigel coated on to accommodate cells with high or low invasive activity, respectively).

MEASURING INVASION THROUGH A MATRIX

This protocol describes a method for assessing tumor cell invasion through a basement membrane matrix *in vitro*. Tumor cells are placed in the upper well of a Boyden migration chamber, which is separated from the lower well by a porous filter coated with basement membrane matrix (Matrigel). A chemoattractant is placed in the lower well to facilitate cell migration. After a short incubation, invasive cells are counted or recovered from the lower surface of the filter.

BASIC PROTOCOL

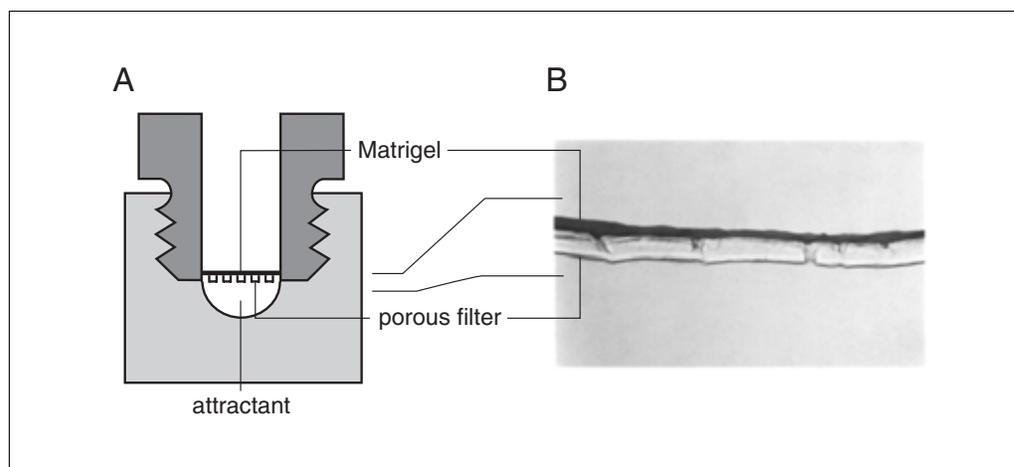


Figure 12.2.1 Configuration of chamber used for invasion assay. (A) Schematic representation of the Boyden chamber assembly. (B) Photomicrograph of matrix barrier cross-section, consisting of a porous polycarbonate filter coated with Matrigel. Reproduced with permission from the American Association for Cancer Research (Albini et al., 1987).

Materials

3T3-conditioned medium (see recipe) or selected chemoattractant or inhibitor
Tumor cells in culture
0.5 M EDTA, pH 8.0 (*APPENDIX 2A*)
Culture medium containing 0.1% (w/v) BSA (varies by cell type)
Diff-Quik fixative and stains (Baxter)

Boyden chambers (Neuro Probe; available as single or 48-well chambers)
Polyvinylpyrrolidone (PVP)-free polycarbonate membranes, 8- or 12- μ m pore size (e.g., Nucleopore filters, Neuro Probe), sized to fit Boyden chambers (13-mm for single chambers and 25 \times 80-mm for 48-well chambers) coated with Matrigel (see Support Protocol)

1. Add 3T3-conditioned medium or other chemoattractant to be tested to lower wells of Boyden chambers until a small meniscus appears.

Each assay data point is generally based on triplicate filters/wells. Always include three negative control wells that lack the migration factor in the lower well. If using 48-well chambers, each filter should include three positive- and three negative-control wells (i.e., medium alone). Generally nano- or microgram amounts of chemoattractants are used. It is best to test several different concentrations.

2. Place a Matrigel-coated filter over the lower well of each Boyden chamber and secure the filter with a gasket.

For 48-well chambers, start putting the filter down from the middle of the chamber to the edges to avoid bubbles. Never move the filter after it has been placed or there will be a possibility of cross-contamination of the wells. Do not screw the gasket too tight.

3. Release tumor cells from their culture vessel by first removing the culture medium and then adding enough 0.5 M EDTA to fully cover the cell layer in the culture dish. Centrifuge the cell suspension 5 min at 170 \times g in a tabletop centrifuge, room temperature. Decant the supernatant and resuspend the pellet in serum-free medium at 5 \times 10⁵ cells/ml (1 \times 10⁶ cells/ml for 48-well chambers).

The cells should not be confluent. Best results are obtained when the cells are split 24 hr before the assay and always seeded at the same density. It is very important to use EDTA rather than trypsin to release the cells from the plate, as trypsin will degrade the Matrigel.

4. Add cells to the upper wells of the chambers.

Generally 100,000 to 200,000 cells per 0.3-ml well are placed in the upper chamber. When using the smaller 48-well chambers, 50,000 cells per 0.05-ml well are placed in each upper chamber well.

5. Incubate 3 to 6 hr in a humidified 5% CO₂, 37°C incubator.

The time of incubation is dependent on the tumor cells used; 3 to 6 hr is the most frequently used range of incubation times. The optimal time can be determined in an initial test assay with and without attractant in the lower well, looking for the greatest difference between the two conditions. Usually <2% of the added cells migrate in the presence of the attractant.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

6. Stop the incubation by removing and fixing the filters using the Diff-Quik system. Remove the filters, dip in fixative for 10 sec, and place in solutions I and II for 2 min each. Wash in water for a few seconds.

Small weigh boats or Coplin jars may be used for these steps and the solutions can be reused. The cells will appear dark blue with a pale blue to white background.

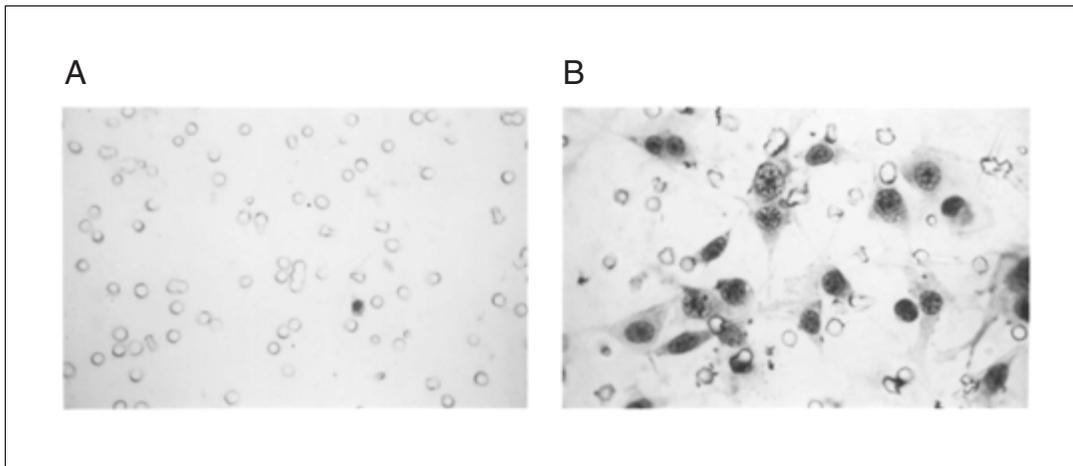


Figure 12.2.2 Photomicrographs showing results of an invasion assay: filter underside (attractant side) after incubation with (A) noninvasive and (B) invasive cells. Reproduced with permission from the American Association for Cancer Research (Albini et al., 1987).

7. Wipe cells from the upper surface with a cotton-tipped swab and mount filter on a slide with the lower side down.

This step removes the cells that have attached to the upper surface of the filter and makes counting easier. The migrated cells will be on the underside of the filter facing the glass slide.

8. Count the cells.

This can be done with a computer system or by eye (see Fig. 12.2.2). Be sure to survey the entire filter, as the distribution of migrated cells may be uneven. Count the most representative areas. Counting with a 10 \times magnification using a grid has proven to be reliable and reproducible.

PREPARATION OF MATRIGEL-COATED FILTERS

Coating the filter with Matrigel provides a surface similar to that encountered by cells migrating in vivo.

Materials

PVP-free polycarbonate membranes, 8- or 12- μ m pore size (e.g., Nucleopore filters, Neuro Probe), sized to fit Boyden chambers (13-mm for single chambers; 25 \times 80-mm for 48-well chambers)

Matrigel (UNIT 10.2; Matrigel can also be obtained commercially from Becton Dickinson Labware or Sigma)

1. Number the filters on the dull side with a permanent ink pen.
- 2a. *If using single chambers:* Dilute Matrigel to 1 μ g/ μ l and pipet 25 μ l as a center spot on the dull side of the 13-mm-diameter filter. Air dry overnight.
- 2b. *If using 48-well chambers:* Dilute Matrigel to 0.5 μ g/ μ l in cold water. Place the filter shiny side down and cover the top with 1 ml of the diluted Matrigel. Incubate 15 to 20 min, then hang the filter to air dry for 5 to 10 min (a pin or needle stuck in one corner through the filter and into a board is convenient for drying). Cut the corner so that the orientation of the filter can be determined at the end of the assay.

SUPPORT PROTOCOL

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

3T3-conditioned medium

Grow 3T3 cells to confluence and replace medium with serum-free medium containing freshly prepared 50 µg/ml ascorbic acid. DMEM or RPMI 1640 can be used, but it is generally best to use the medium in which the invasive cells to be tested are normally cultured. Collect the conditioned medium after 24 hr and store in 1-ml aliquots at –20°C for up to 2 years.

COMMENTARY

Background Information

To metastasize, tumor cells invade through vessel basement membrane and the underlying connective tissue by attaching to, degrading, and migrating across the basement membrane matrix (Liotta, 1989). The *in vitro* assay described in this unit is performed to determine the invasive character of tumor cells, to test for factors that promote or inhibit invasion, and to select for malignant subpopulations, which are invasive (Kramer et al., 1986; Albini et al., 1987). The assay is rapid, quantitative, and proven to be highly reliable in a number of research laboratories.

Matrigel is a better barrier than amnion or other natural substrates, because it shows high reproducibility in its activity, and the assay time is much shorter (Hendrix et al., 1989). Many known inhibitors of tumor metastases, such as protease inhibitors, block invasion in this assay (Reich et al., 1988; Stahl and Mueller, 1994; Kobayashi et al., 1995). Likewise certain growth factors, such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), transforming growth factor α (TGF α), hepatocyte growth factor (HGF), and thyroid-stimulating hormone (TSH), have all been found to promote invasion (Nicolson et al., 1993; Hoelting et al., 1994; Rong et al., 1994; Sunitha et al., 1994; Chambers et al., 1995). Having an assay that is quick, reliable, and accurate can facilitate diagnosis and aid in the development of therapeutics. The assay can be used as a prescreen for a large number of test compounds.

Critical Parameters and Troubleshooting

The exact time of incubation varies depending on the cells used and the amount of coated Matrigel. Trials using different incubation time in the presence and absence of a chemoattractant such as 3T3-conditioned medium should

be performed initially to determine the optimum time of incubation. The optimum is the time at which there is the largest difference in the number of infiltrating cells between the control and positive attractant. In some cases, it may also be desirable to vary the number of cells added. For example, if the cells are highly invasive and too numerous to count, fewer cells can be used. In addition, because of the assay's sensitivity, if possible stimulators or inhibitors are being assessed, several different concentrations of the test substance should be tested.

Choosing an appropriate attractant is important. Many studies employ conditioned medium from 3T3 cells, which is collected for 24 hr with serum-free medium containing 50 µg/ml ascorbic acid. The conditioned medium is generally used directly without preconcentration. With cells that have not been previously tested, it may be desirable to test a range of concentrations. Several other chemoattractants have also been identified and include various growth factors, such as EGF (Hoelting et al., 1995), bFGF (Hasegawa et al., 1994), and HGF (Rong et al., 1994). Tissue extracts have been found to act as chemoattractants with some cell types (Hujanen and Terranova, 1985), and laminin as well as other matrix molecules can also promote invasion when placed in the lower well of the chamber (Koochepour et al., 1995). To determine the best chemoattractant, it is advisable to test 3T3-conditioned medium first and then various growth factors and matrix molecules if needed. Cells generally respond by migrating toward growth factors for which they have receptors. The attractant needs to be in the lower well of the chamber and should be present during the entire assay. It may be necessary to test various concentrations as cells usually migrate to factors at concentrations approximately one-tenth of that needed for growth, and many chemoattractants are inhibitory for migration when used at high concen-

trations. Preincubation with the chemoattractant is not required.

It is important to include controls in each assay. Generally the negative control is medium alone (lacking attractant), whereas the positive control is either 3T3-conditioned medium or a medium containing a substance already identified as a chemoattractant for the cells being tested. The assay should be performed in triplicate for the best results.

Anticipated Results

Results are assessed quantitatively by comparing the cell counts for experimental and control wells or plates: for example, when testing an attractant, very few cells invade in its absence, whereas more cells (usually at least twice background) should be observed in its presence.

Time Considerations

The assay should be completed in 1 day. Filters can be coated the night before the assay. Once fixed, the filters are stable for months and can be easily stored in slide boxes.

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Traction forces are exerted by cells against their substrates during migration and can be measured under the entire cell or under subcellular regions. This unit describes several protocols for making silicone sheets to measure traction forces under the entire cell, as well as a protocol for developing a micromachined device to measure forces under subcellular regions.

The original technique used to measure tractions, which examined the orientation of wrinkles generated in the substrate by locomoting cells, is described first (see Basic Protocol 1). The technique is useful for providing a global orientation of the forces under cells such as fibroblasts, which typically exert large traction forces (10 nN/ μm of leading cell edge; Harris et al., 1980). Calibrated microneedles (see Support Protocol 1) are used to calibrate all of the substratum (see Basic Protocols 1 and 2 and Support Protocols 1 and 2).

Also described (see Alternate Protocol 1) is a modification of the original protocol that provides increased optical resolution as well as a simple method for quantitatively decreasing the stiffness of the substrate. This second substratum can be made with stiffnesses ranging from 0.26 to 50 nN/ μm (Burton and Taylor, 1997).

Another modification is described in Alternate Protocol 2. This method measures forces with enhanced spatial resolution by determining the displacement of fiduciary markers embedded within the substrate. This protocol can also produce substrates of varying stiffness, ranging from 5 to 20 nN/ μm (Lee et al., 1994; Oliver et al., 1995); however, the time constant for the beads to recover to their original position increases from ~ 1 to 15 sec (Lee et al., 1994; Oliver et al., 1995) between these two levels of stiffness. A modified airbrush apparatus (see Support Protocol 2) is used to coat the substrate with the fiduciary markers (Oliver et al., 1995).

Finally, a new technique is described for measuring subcellular traction forces using a micromachined substrate (see Basic Protocol 2). This substrate is based on a system of deformable levers that have stiffnesses ranging from 0.09 to 89 nN/ μm . It can measure forces under regions of the cell as small as 4 μm^2 in real time, but cannot measure forces under multiple regions of the cell simultaneously (Galbraith and Sheetz, 1997). A procedure for creating silanized coverslips is presented in Support Protocol 3. The assay requires polarized reflection microscopy for visualization (see Support Protocol 4).

The protocols are presented in order of increasing cost and complexity. All three of the silicone-substrate protocols can be implemented in a reasonable amount of time, are not particularly expensive, and can measure forces under the entire cell. The protocol using micromachined substrates is more costly, but it has the advantage of measuring forces under subcellular regions. The appropriate protocol should be selected according to the magnitude of the forces that a particular cell exerts, the time constant of the events to be measured, and the resources available for development of the substrate.

MEASURING CELL TRACTION ON WRINKLING SUBSTRATES

This protocol outlines the construction and calibration of wrinkling silicone sheets and the use of these sheets in measuring cell traction. The fabrication of the sheets follows the procedure developed by Harris et al. (1980), and the calibration follows the procedure developed by Burton and Taylor (1997). Sheets that monitor forces by observing the displacement of fiduciary markers are discussed in Alternate Protocol 2. For a given

experiment, sheets should be chosen so that their stiffness matches the forces expected to be generated by the cell type of interest (see Table 12.3.1).

Materials

- Cells of interest
- Culture medium specific for cells being used, phenol red-free and supplemented with 20 to 50 mM HEPES
- Dimethylpolysiloxane (60,000 or 30,000 centistokes; Sigma)
- Phenol red-free culture medium degassed using house vacuum
- Temperature-control system for microscope stage (or hair dryer and controller)
- Glass coverslips, acid washed
- Petri dishes slightly larger than the coverslips used
- Inverted microscope with long-working distance-objective *or* upright microscope with a multi-immersion-medium objective, equipped with video system
- Micromanipulator (adjustable in three dimensions; e.g., Nanishige) with needle holder (World Precision Instruments)
- Calibrated microneedles (see Support Protocol 1)
- Computer with a video-frame capture card and image-analysis software (optional; e.g., NIH Image, available by anonymous FTP from zippy.nimh.nih.gov)
- Macintosh computer with Scion frame grabber running
- Additional reagents and equipment for trypsinization of cells (*UNIT 1.1*)

Construct the wrinkling substrate

1. Spread a thin layer, 20 to 50 μm deep, of dimethylpolysiloxane fluid over the surface of a clean glass coverslip.

Table 12.3.1 Traction Forces Exerted by Various Cell Types

Traction assay	Cell type and method	Force	Reference
Microneedle (Basic Protocol 1)	Chick heart fibroblast, force exerted against particle attached to dorsal surface	50 pN/ μm^2	Felder and Elson, 1990
	Chick sensory neuron growth cone, force pulling neurite during growth cone advance	2 nN	Lamoureux et al., 1989
	Chick heart fibroblast, force required to stall cell per micron of cell leading edge	10 nN/ μm	Harris et al., 1980
	Fish keratocyte, force required to stall cell	60 nN	Oliver et al., 1995
Silicone substrates with fiduciary markers (Alternate Protocol 2)	Fish keratocyte, forces from local markers under cell pincer region	~45 nN	Dembo et al., 1996; Lee et al., 1994; Oliver et al., 1995
Wrinkling silicone substrates (Alternate Protocol 1)	3T3 fibroblast, forces at cleavage furrow during division	~75 nN	Burton and Taylor, 1997
	3T3 fibroblast, force of respreading after division	300-1200 nN	Burton and Taylor, 1997
Micromachined substrates (Basic Protocol 2)	Chick embryo fibroblast, forces under subcellular regions	0.9-4.0 nN/ μm^2	Galbraith and Sheetz, 1997

2. Set the gas on a Bunsen burner to its minimal level. Invert the coverslip and use forceps to pass it over the upper part of the flame for ~1.5 sec.

This method typically cross-links only the topmost layer (1 μm) of fluid. The silicone sheet will spontaneously wrinkle into many folds, making it opaque, but the sheets become transparent again within 5 to 20 sec as they cool. If the sheets are exposed to the flame for too long, permanently roughened surfaces which are less flexible will be made. These sheets contain an appreciable number of “prestress” wrinkles that will not diminish with time.

Prepare cells for traction assays

3. Set up a temperature-controlled environment for the cells on the stage of the microscope that will be used for making the measurements (if they are normally not cultured at room temperature).

This usually takes the form of a 37°C incubator that surrounds the microscope stage. Incubators can be purchased from most microscope manufacturers; alternatively, inexpensive incubators can be constructed by building a plastic enclosure around the microscope and then heating it with a hair dryer. The temperature can be regulated manually by adjusting a Variac rheostat connected to the hair dryer or by purchasing an independent controller. Controllers are available from a variety of sources (e.g., Fisher). It is important to pick a controller that is capable of supplying the current needed to run a hair dryer (a 1500-W hair dryer requires ~13 A).

4. Grow cells or isolate primary cultures in the phenol red–free formulation of the regular medium supplemented with 20 to 50 mM HEPES.
5. If cells can be passaged, remove cells from the culture vessel using a mild trypsin/EDTA treatment (UNIT 1.1), then inactivate the trypsin by adding an excess of degassed phenol red–free culture medium supplemented with HEPES. Resuspend cells in the same medium.

Cells to be used in traction assays should be removed from the culture vessel using as gentle a procedure as possible. The normal method of passaging any cell line or isolating any primary cell culture can be used.

The medium used to inactivate trypsin should contain serum or BSA.

Plate cells on substrate and measure wrinkling

6. Place the coverslip, fluid side up, into a petri dish just slightly larger than the coverslip. Flood the petri dish with degassed tissue culture medium to a depth of ≥ 0.5 cm. Incubate for 1 to 3 hr at normal culture temperature or until the cells resume their normal shape and migration speed.

Plate the cells onto the traction-assay substratum at densities that do not allow cells to contact each other but provide a sufficient number of cells on the surface for measurement. This number must be determined empirically for all of the assays. The density of cells will depend upon the stiffness of the substrate, since the wrinkles (Basic Protocol 1 and Alternate Protocol 1) and bead displacements (Alternate Protocol 2) generated by cells on very compliant substratum can interfere with each other. The density of cells will also depend upon the density of the measuring devices (see Basic Protocol 2).

There are a number of difficulties wetting the membrane that are due to the hydrophobic nature of the silicone surface. If there is insufficient medium in the petri dish, then the coverslip will float to the surface and the silicone sheet will “unwet,” killing the cells and possibly rupturing the surface. A rapid wetting from one end of the dish to the other usually works well, but expect to rupture a number of membranes while practicing.

Normal migration speed typically refers to the migration speed on glass coverslips that are coated with the same extracellular matrix (ECM) as the traction assay substrate.

7. Place cells on the temperature-controlled microscope stage and record the length of the wrinkles produced by the cells. Record a calibration scale for the video field and use this field to measure the length of the wrinkles either directly or with a computer equipped with a frame grabber and image-analysis software.

Calibrate the substrate

8. Mount the substrate in a petri dish containing cell culture medium on the stage of a microscope equipped with a video system. Position a micromanipulator equipped with a needle holder so that the tip of the needle can reach the center of the optical field.

Do not coat the membrane with cells if you are going to calibrate the membrane. Note that it is difficult to do the calibration under sterile conditions, so several duplicate membranes that will never be plated with cells should be calibrated instead of the actual "experimental" membrane.

The calibration technique described in Alternate Protocol 2 for nonwrinkling substrates is more accurate, but it requires that the substrate be larger (>10-fold) than the diameter of the needle and that the substrate be approximately parallel to the coverslip. However, the substrate is often concave with respect to the coverslip in this assay.

9. Mount a calibrated microneedle in the micromanipulator and use it to push laterally on the surface of the sheet until it produces wrinkles that are shorter and longer than those produced by cells.

An alternative method (Burton and Taylor, 1997) is to plate cells on the sheet and then fix the cells and sheets in 1% glutaraldehyde for 10 min. The fixed cells now act as "handles" that can be pushed by the microneedles.

10. Determine the displacement of the tip of the needle that is needed to produce each wrinkle length from a video recording of the calibration experiment. Record a calibration scale for the video field, and then use this field to calculate the displacement of the needle tip from its position before and after producing the wrinkle as described in step 7.
11. For each wrinkle length, calculate the force needed to generate the wrinkle as the product of the needle stiffness (see Support Protocol 1) and the needle deflection. This produces a curve of force versus wrinkle length.

Calculate traction force

12. Use the force versus wrinkle length curve generated in step 11 to calculate the force needed to produce the wrinkles measured in step 7.

SUPPORT PROTOCOL 1

CALIBRATING MICRONEEDLES

Calibrated microneedles can be used to measure the force of a locomoting cell by determining the amount of force required to stall the forward movement. The microneedles are also a necessary tool for measuring the stiffness of any of the devices whose construction is described in this unit.

Materials

- 25- μ m-diameter Chromel thermocouple wire (Omega Engineering)
- Pipet puller (David Kopf Instruments)
- Pyrex capillary glass of various diameters and wall thicknesses (Drummond Scientific)
- Small microscope mounted with its optical axis parallel to the bench
- Eyepiece reticule (i.e., a calibration scale that fits into the eyepiece of the microscope; Edmund Scientific)

1. Cut a long (~30-cm) piece of 25- μ m-diameter Chromel wire, weigh it, and carefully determine the length. Use the mass/length ratio to determine the mass of smaller pieces of Chromel wire that will be used in step 3.

2. Pull glass needles using a pipet puller.

As a guide, 0.86-mm-o.d., 0.51-mm-i.d. Pyrex glass pulled on a vertical puller (David Kopf Instruments) at heat 27 and pull 10 should produce a microneedle in a useful starting range. Make sure that the diameter of the needle is less than the width of the wrinkles and that it can fit within the micromachined substrate (Basic Protocol 2).

3. Calibrate the needle by mounting it on the stage of the horizontal microscope (microscope turned onto its back so that its optical axis is parallel to the bench) and hanging a series of different-mass pieces of Chromel wire off the end of the needles. Measure the deflection of the tip by each mass using the eyepiece reticule. Pieces of wire ranging from 0.02 to 1.0 mg should provide a useful starting range.

It is important to place the weights consistently on the very tip of the needle so that the deflection is consistent throughout the calibration.

If needles that are too flexible to be calibrated by hanging weights are desired, then calibrate the needles by measuring the relative stiffness of the more flexible needles against stiffer needles that are calibrated as described above (Nicklas, 1983).

As an alternative to the stiffer calibrated reference needles, 8- and 16-mm lengths of the Chromel wire can be used to make the "reference needles" (Dennerll et al., 1988). These lengths of wire can be glued into the center of capillary glass and tested with the same techniques as the needles. This should yield a stiffness (see Basic Protocol 2 for mathematical definition) of 2.43×10^{-2} N/m for the 8-mm length of wire. Note that the moment of inertia (I) for a circular beam is $1/4\pi r^4$.

4. Calculate the slope of the curve for force (mass of wire in g \times 980 cm/sec²) versus tip deflection to obtain the stiffness of the needle.

MEASURING CELL TRACTION ON WRINKLING SUBSTRATES WITH AN ALTERNATIVE POLYMER

ALTERNATE PROTOCOL 1

Deformable substrates can be formed with an alternative silicone polymer that has been described by Burton and Taylor (1997). Substrates formed with this polymer (phenylmethylpolysiloxane) have several advantages over those formed with the polymer described in Basic Protocol 1 (dimethylpolysiloxane)—i.e., they are suitable for differential interference microscopy and their stiffnesses can be decreased by exposure to ultraviolet light. This polymer can be cross-linked with the Bunsen burner technique described in Basic Protocol 1, the sputter-coater technique described in Alternate Protocol 2, or the tungsten-wire technique described by Burton and Taylor (1997). An example showing fibroblasts wrinkling a sheet that was made with this polymer cross-linked in a sputter coater is shown in Figure 12.3.1.

Additional Materials (also see Basic Protocol 1 or Alternate Protocol 2)

Phenylmethylpolysiloxane, trimethyl terminated (Dow Corning 710 fluid)
Ultraviolet lamp (UVGL-58 from UVP)

1. Prepare coated coverslips, replacing the dimethylpolysiloxane with trimethyl-terminated phenylmethylpolysiloxane. Perform the measurements in Basic Protocol 1 or Alternate Protocol 2.

The advantage of this silicone is that it has a high index of refraction (1.536), which is suitable for polarized (differential interference) and interference reflection microscopy. Additionally, the silicone absorbs ultraviolet light (peak absorbance $\lambda = 254$ nm), which

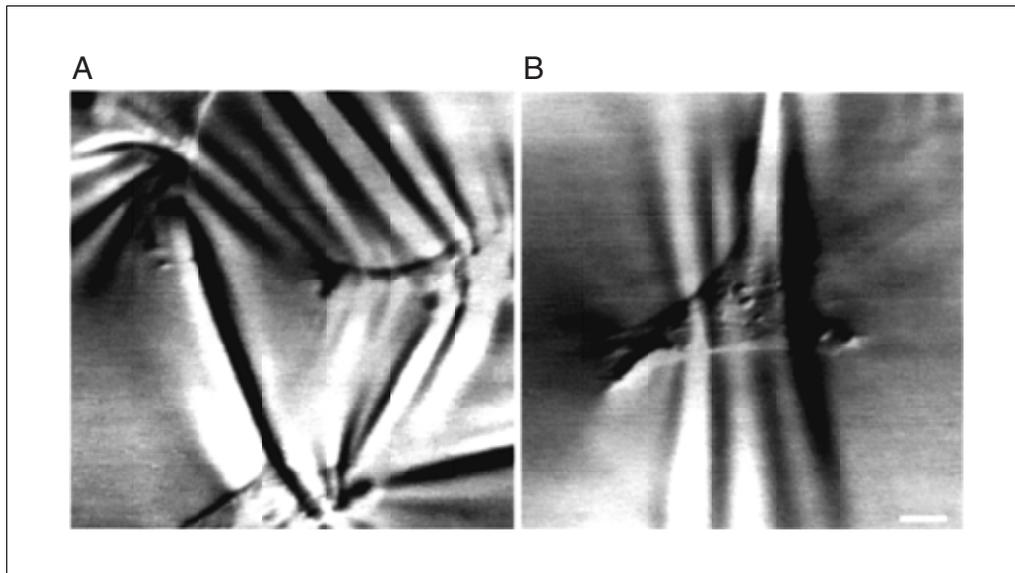


Figure 12.3.1 Chick embryo fibroblasts plated on a membrane made from phenylmethylpolysiloxane (J.A. Galbraith, unpub. observ.). The membrane was cross-linked in a sputter coater according to Alternate Protocol 2. Cells were plated in phenol red-free DMEM that was supplemented with 10% fetal bovine serum and antibiotics. Substrates were visualized with differential interference microscopy on a Zeiss inverted microscope using a 40×0.75 -NA long-working-distance objective. **(A)** A group of three fibroblasts generates wrinkles that are perpendicular to their long axis. **(B)** A single fibroblast that is moving from left to right also generates wrinkles perpendicular to its long axis and the direction of migration. Bar is 10 μm .

weakens the sheet, making it more distensible. The disadvantage of this polymer is that it is considerably more expensive than dimethylpolysiloxane.

2. If calibration experiments indicate that the sheets are too stiff, make the sheets more distensible by exposing them to ultraviolet irradiation (6 W at 15 to 20 mm above the sheet, $\lambda = 254$ nm, $300 \mu\text{Wcm}^2$ at 15 cm for the surface) using a UVGL-58 lamp. Repeat the measurements.

The curve for stiffness versus time of irradiation needs to be determined for each method of curing the silicone fluid. The curve given by Burton and Taylor (1997) for creating a sheet of any stiffness should be used as a starting point, but the actual time of exposure may depend upon the method of cross-linking.

ALTERNATE PROTOCOL 2

MEASURING CELL TRACTION ON NONWRINKLING SUBSTRATES

This protocol describes the use of nonwrinkling silicone sheets containing 1- μm diameter beads that act as fiduciary markers for measuring displacements and calculating force (Lee et al., 1994; Oliver et al., 1995).

Additional Materials (also see Basic Protocol 1)

- Vacuum grease or Valap (UNIT 13.1)
- Dimethylpolysiloxane (12,500 centipoise; Sigma)
- 1- μm liquid bead suspension (Polysciences)
- Phenol red-free formulation of culture medium
- Culture medium degassed using house vacuum
- 22 \times 8-mm Pyrex cylinders (Bellco Glass)
- Glass coverslips, acid washed
- Airbrush apparatus (see Support Protocol 2)
- Vacuum source
- Sputter coater (e.g., Electron Microscopy Sciences)

Construct nonwrinkling substrate

1. Make Rappaport chambers by sealing the Pyrex cylinders to acid-washed coverslips with vacuum grease or Valap.
2. Pour 60 mg dimethylpolysiloxane into each chamber and allow it to spread evenly.
3. Disperse 1- μm beads on the surface of the dimethylpolysiloxane at a density of $\sim 3 \times 10^4$ beads/ mm^2 , using a modified airbrush apparatus as described in Support Protocol 2.

Although this can be done with a bristle brush using freeze-dried beads, a modified airbrush apparatus (see Support Protocol 2) using a liquid suspension of beads produces fewer aggregates.

4. Allow air bubbles from the dimethylpolysiloxane to escape or outgas under vacuum.
5. Cross-link the top layer of gel using a sputter coater at a vacuum of 0.15 torr and a target height of 60 mm. Apply the voltage to maintain a constant current of ~ 2 mA for 2 sec.

The time, vacuum, and target height may need to be adjusted between different sputter coater models.

6. Add degassed culture medium to each cross-linked chamber (see Basic Protocol 1, step 6).

Degassing the medium prevents the nucleation of small air bubbles on the silicone surface which can pull and distort the membrane. The presence of serum seems to stabilize the surface (Oliver et al., 1995).

Calibrate the substrate

7. Place the chamber on the microscope stage. Mount two calibrated microneedles of the same stiffness in the micromanipulators and position them toward the center of the sheet.

A more accurate method of calibrating silicone sheets was introduced by Dembo et al. (1996). This method is similar to the one described in Basic Protocol 1, except that two identical calibrated needles and two manipulators are needed. The revised technique has the advantages of eliminating boundary effects due to the size of the Rappaport chamber and of satisfying global force balance during the calibration. See Oliver et al. (1998) for a more complete discussion.

8. Simultaneously move the needles toward each other, pinching the sheet between them.
9. Determine the stiffness of the sheet (force versus bead displacement curve) from the bending of the needles (see Oliver et al., 1998).

Measure cell traction

10. Add a suspension of cells to another cross-linked chamber, incubate for 1 to 3 hr, and measure the displacement of the beads (see Basic Protocol 1, steps 6 and 7).
11. Use the stiffness of the sheet determined in step 9 and the displacement of the beads by the cells determined in step 10 to calculate the force exerted by the cells.

PREPARING A MODIFIED AIRBRUSH APPARATUS

The airbrush is used to evenly disperse beads on the surface of polymer-coated coverslips (see Alternate Protocol 2). This device is illustrated in Oliver et al. (1995) and Figure 12.3.2. The details of this protocol were kindly supplied by Tim Oliver.

Materials

Nebulizer (DeVilbiss model 646)
Drying reservoir (16-liter container; Nalgene)
T-connector
Connecting tubing
Glass transfer pipet with tip drawn to 1-mm-i.d. opening
Impactors (37-mm Air Sampling Cassettes; model 4339, Gelman)

Assemble the apparatus

1. Connect one end of the nebulizer to a supply of filtered air.
2. Connect the other end of the nebulizer to the drying reservoir (16-liter Nalgene container).

This is the input side of the drying reservoir.

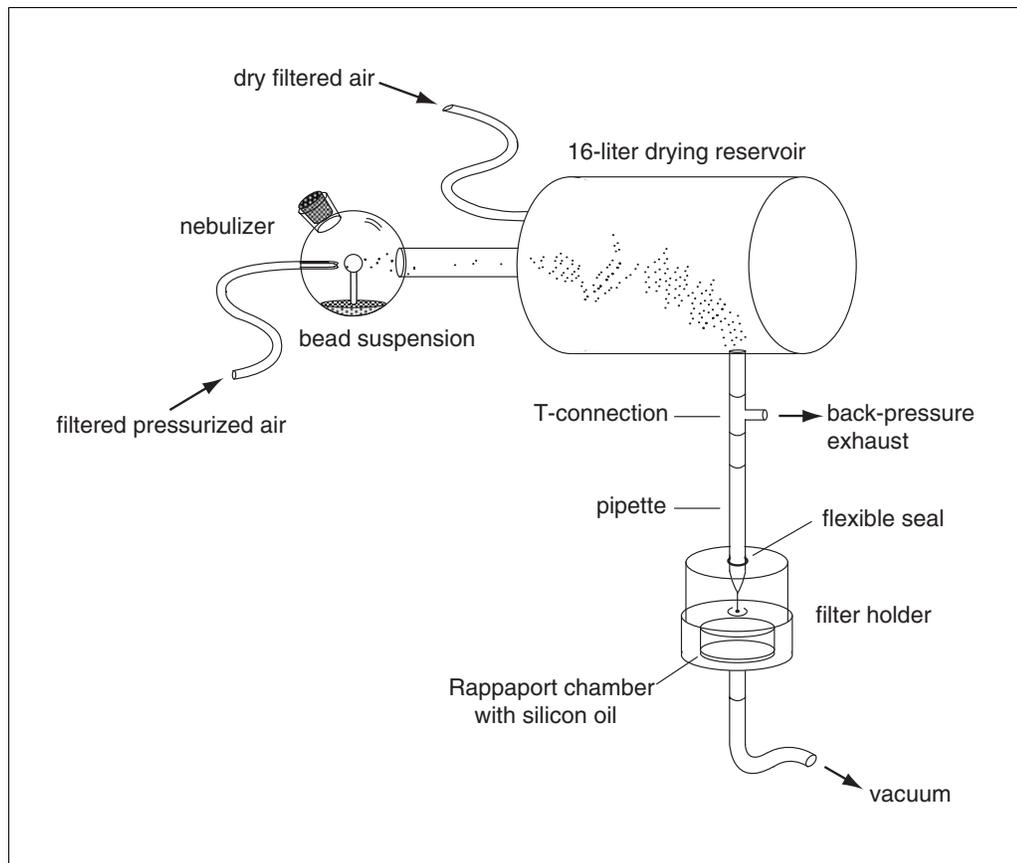


Figure 12.3.2 Illustration of a modified airbrush apparatus based on that of Oliver et al. (1995). This device separates a solution of beads into single dried particles. One end of the nebulizer is connected to a supply of filtered air. The other end of the nebulizer is connected to the input side of the drying reservoir. Another, independent filtered air supply is also connected to the input side of the reservoir. The output side of the reservoir is connected to a pipet with a 1-mm-i.d. tip. The pipet tip enters a modified impactor that contains the Rappaport chamber filled with silicone oil. The tip can be moved around to disperse the beads.

3. Supply the input side of the reservoir with dry filtered air, independent of the supply of air to the nebulizer.

A pressure of 5 psi is recommended, but the air flow should be adjusted so that beads remain in the reservoir for several seconds.

4. Attach a T-connector to the output side of the reservoir.

The perpendicular end of the T will be used as an exhaust.

5. Use a piece of tubing to connect the other end of the T to the drawn glass pipet.
6. Create an impactor thick enough to accommodate the Rappaport chambers by stacking the middle inserts of several cassettes between the top and bottom of one of them. Place a Rappaport chamber containing degassed silicone inside this thicker impactor.
7. Place the drawn pipet into the top of the impactor, and connect the two with a piece of flexible tubing.
8. Connect the bottom of the impactor to a vacuum.

Use airbrush apparatus to disperse beads (also see Alternate Protocol 2, step 3)

9. Add a suspension of the beads in ethanol to the nebulizer, then turn on the air and vacuum.
10. Move the pipet to disperse the beads over the top of the silicone.

A recommended concentration is 3×10^4 beads/mm².

MEASURING CELL TRACTION ON MICROMACHINED SUBSTRATES

This protocol describes a method for developing micromachined substrates to measure traction forces under subcellular regions. An example of this is shown in Figure 12.3.3 (Galbraith and Sheetz, 1997). This device is based on a system of 5904 cantilevers of different lengths and therefore different stiffnesses. The levers are buried beneath the surface of the device, but on the free end of the lever there is a small pad that is planar with the surface of the device. The pads range in area from 4 to 25 μm^2 and are surrounded by a square hole that provides a 2- μm space on each side of the pad to allow the lever to move when a cell pulls on the pad. The forces that the cells exert on the pads can be determined by measuring the displacement of the pads and calculating the product of the pad displacement and the stiffness of the cantilevered lever. This approach is conceptually similar to the use of calibrated microneedles to measure forces.

Materials

- 50% (v/v) hydrofluoric acid
- Appropriate extracellular matrix (ECM; e.g., 40 $\mu\text{g}/\text{ml}$ laminin)
- 25% (w/v) MgSO_4
- Phenol red-free culture medium
- Nanofabrication facility
- Computer-aided drawing (CAD) software package
- Polarized reflection cube (see Support Protocol 3)
- Fluorescence illumination for microscope
- Red filter (645 nm long-pass)
- Cloning cylinders
- Silanized coverslips (see Support Protocol 3)
- Sylgard 184 (Dow Corning)

***BASIC
PROTOCOL 2***

Cell Motility

12.3.9

1. Contact a nanofabrication facility that can perform the actual fabrication of the device.

MCNC (Web site, <http://www.mcnc.org>; email, pr@mcnc.org) is one facility that can design and fabricate devices.

Design cantilever-beam force-measuring device

2. Estimate the traction forces that will be generated by the cells (see Oliver et al., 1994, and Table 12.3.1). Be certain to provide over- and underestimates of force in addition to the best guess.
3. Determine the maximum deflection allowable for beam displacement.

A general guideline is several microns, but the actual number will depend upon the precision of the micromachining.

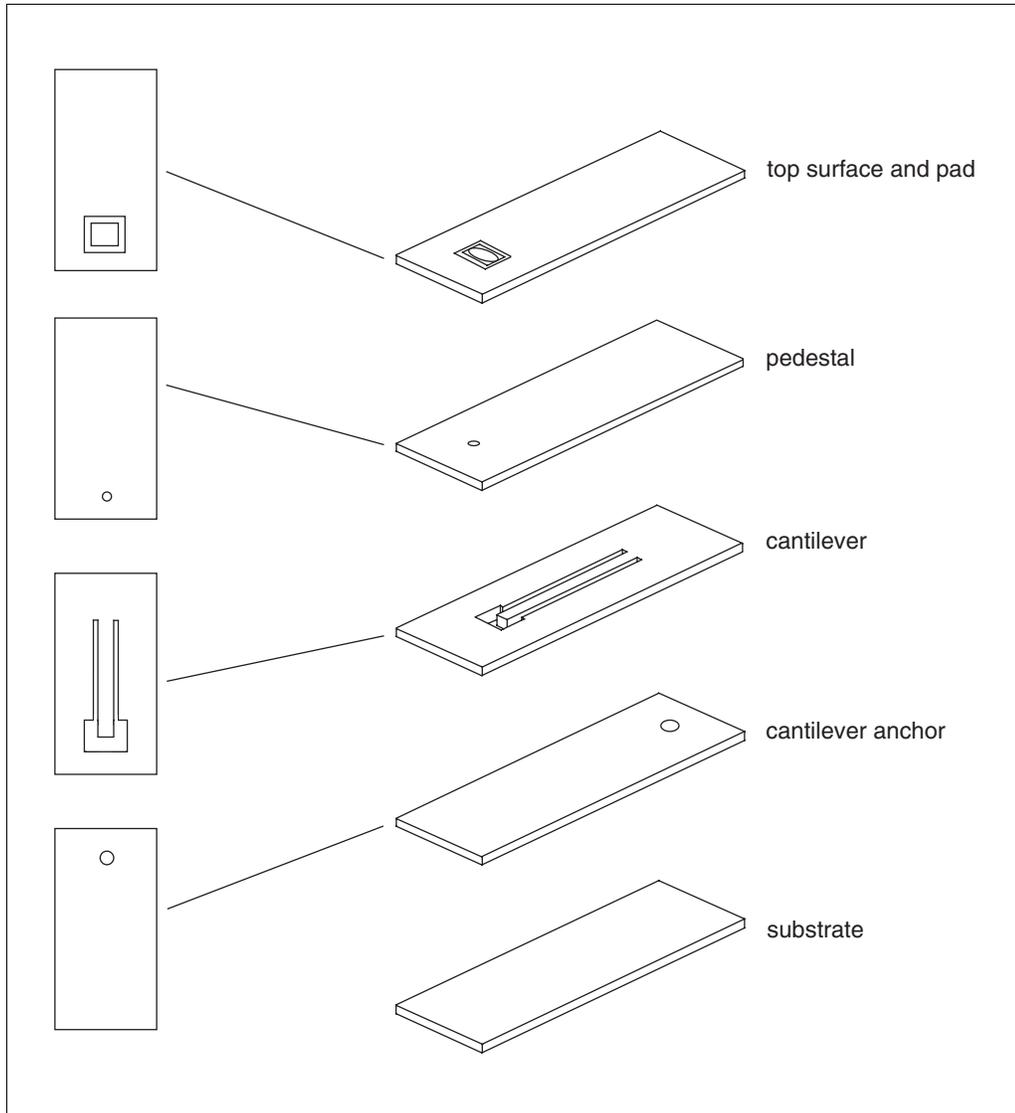


Figure 12.3.3 Masks and layers that are involved in making a single cantilever. A silicon wafer is used as substrate. Phosphosilicate glass (PSG) is placed on the silicon wafer, and a mask with holes is used to lithographically pattern the PSG to form anchors for the beams. Polycrystalline silicon is deposited over the PSG, and the mask with the beams and surrounding wells is used to pattern these structures. A layer of spin-on glass is added, and another mask is used to place a small hole at what will be the free end of the beam. The surface is then coated with a plasma-deposited amorphous silicon that fills the holes and forms a pedestal that anchors the pad to the beam. A final mask is used to etch the pad and square hole in the top layer.

4. Determine the appropriate beam stiffness by using the following equation:

$$\text{stiffness} = \frac{\text{traction force}}{\text{beam displacement}}$$

5. Calculate the appropriate geometric factors that will provide beams of this stiffness, using the following formulas, which give the relationship between the pertinent parameters for a square beam.

$$\text{stiffness} = \frac{3EI}{l}$$

$$I = \frac{h^4}{12}$$

In these equations, E is the Young's modulus of the silicone substrate, l is the length of the beam, I is the moment of inertia, and h is the height of the beam, which is equal to the width. Note that the height and width of the beam may be limited by constraints imposed by the fabrication facility. In the case of the authors' device, E is between 145 and 160 GPa, the width and height of the beams are 2 μm , and the lengths vary from 0.86 to 0.086 mm. These geometric parameters yield beams with stiffnesses ranging from 89 to 0.09 nN/ μm . Additionally, very long beams may have "stiction" problems that will cause them to adhere to the walls of the channels in which they reside. The authors believe that they have encountered this with the longest beams used, which often appear immovable.

6. Determine the spacing and orientation of the measurement sensors (beams).

This will determine the layout for each unit, which will be repeated to make up the device or chip. Numerous chips can then be fabricated from one wafer of silicon. A typical layout has 9 repeating units, and ~40 chips are fabricated on one wafer.

Fabricate the device

7. Using a CAD package, generate mechanical drawings of each mask that is needed to create the layers of the repeating unit.

This step can be done by the fabrication facility. Micromachining is similar to a multiple-step photolithography: silicon layers are grown, exposed, and etched, and the process is repeated. For each layer a separate mask must be generated to create the appropriate pattern for etching. Figure 12.3.4 illustrates the types of masks and layers that are involved in making a single cantilever.

8. Use the CAD drawings to generate the actual masks that will be used for machining each layer.
9. Have the nanofabrication facility manufacture wafers containing numerous chips, then cut the wafer into individual pieces and perform the final etch (using 50% hydrofluoric acid, HF) that frees the assembly.

Initial releases that are performed by the facility can cause many of the pads to be dissociated from the ends of the beams. Titrate the time of exposure to HF to obtain free beams that still have pads on their ends.

CAUTION: *HF is an aggressive reagent requiring appropriate protective clothing and appropriate tools such as Teflon containers and forceps.*

Different reagents are needed to release other substrates.

Prepare the device for cells

10. Remove any residual HF from the chip to ensure the biocompatibility of the chip. After release, soak the chips in deionized water for 1 hr and then 25% MgSO₄ for 2 hr.

This procedure seems to effectively sequester any free fluoride left trapped beneath the surface of the chip.

11. Prepare chambers for chips by attaching cloning cylinders to silanized coverslips with Sylgard gel.

Follow manufacturer's instructions for curing. Note that large batches of the 2-part gel can be mixed and frozen in aliquots at -80°C until use.

12. Place the chips in chambers and coat the chip with an appropriate extracellular matrix (ECM) in the same way that you would coat a glass coverslip. However, take care

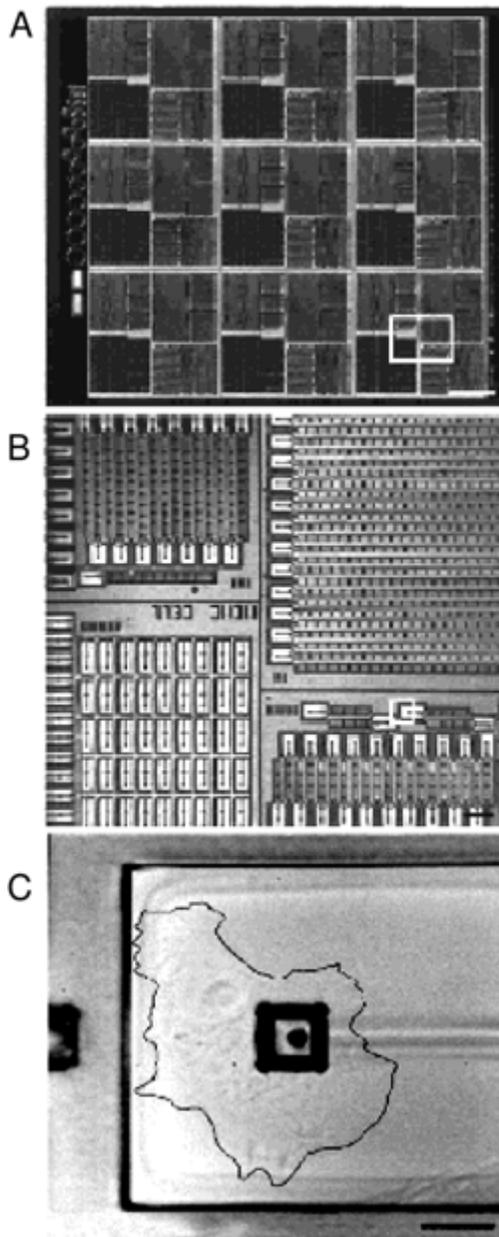


Figure 12.3.4 Micrographs of micromachined substrate. **(A)** Entire surface of a device that has nine repeats of a basic pattern containing four different lever lengths. Box indicates area shown in the next panel. Bar is 1 mm. **(B)** The four different lengths of beams. Box indicates area shown in the next panel. Bar is 0.1 mm. **(C)** A chick embryo fibroblast crossing a 25- μm^2 pad. Bar is 10 μm .

never to let the chip completely dry out, since changes in surface tension can remove the pads from the ends of the chips.

13. Plate cells on the chips by placing a suspension of cells in the cloning cylinder chambers. Follow step 6 of Basic Protocol 1 to determine concentration and incubation time. View the chips by polarized reflection microscopy, using a polarized reflection cube (see Support Protocol 3) and a microscope with fluorescence illumination, a 645-nm long-pass filter, and temperature control if needed.

Reflection microscopy must be used since the chips are opaque and do not transmit light. This viewing method produces high-contrast images of the chip, but low-contrast images of the cell.

14. Calibrate the movable pieces of the device with calibrated microneedles (see Basic Protocol 1, steps 8 to 11).

For this procedure, a long working distance water-immersion objective on an upright microscope is convenient.

15. Compare the calibrated stiffness with the theoretical calculations.

SILANIZING COVERSLEIPS

Silanized coverslips are used as a base for assays of cell traction using micromachined substrates (see Basic Protocol 2). This protocol describes a simple procedure for coating glass coverslips.

Materials

20% HNO₃
Acetone, reagent grade
1,1,1,3,3,3-Hexamethyldisilazane (HMDS; Aldrich)
70% ethanol

Glass coverslips and coverslip racks (Thomas Scientific)
Pyrex dish large enough to hold coverslip racks
Drying oven
Nitrogen gas cylinder with regulator
3-cc syringe with needle

1. Place coverslips on coverslip racks and soak in 20% HNO₃ for 1 hr.
2. Rinse coverslips in Milli-Q-purified water for 1 hr.
3. Soak in reagent-grade acetone for a few minutes. Pour off acetone and allow excess fluid to air dry.
4. Place coverslip racks in Pyrex dish and cover with aluminum foil. Bake 1 hr in 140°C oven.
5. Collect a plastic bag full of nitrogen gas. Puncture the bag with a needle attached to a 3-cc syringe. Withdraw 2.5 cc of nitrogen gas, insert the needle into the top of the HMDS container, and withdraw 2.5 cc HMDS.

The gas is used to equalize the pressure in the container without oxidizing the HMDS.

6. Dispense HMDS into the Pyrex dish through the aluminum foil, coating the coverslips.
7. Bake 20 min at 140°C.

SUPPORT PROTOCOL 3

8. Evacuate the oven for 5 min, then lift foil cover off the Pyrex dish and continue to evacuate for a further 15 min.
9. Continue baking for 1 hr at 140°C.
10. Cool coverslips and store in 70% ethanol.
11. Drain excess ethanol and allow coverslips to air dry before use.

PREPARING A POLARIZED REFLECTION CUBE

Reflection microscopy must be used with micromachined substrates (see Basic Protocol 2), because the chips are opaque. While commercial polarization microscopes may be used, this method provides a convenient way to modify existing laboratory microscopes for use in this technique.

Materials

Fluorescence slider or cube (available from microscope manufacturers)
50-50 mirror (Chroma)
Polarizers (Chroma)

1. Place a 50-50 mirror in the position of the fluorescent slider or cube that normally holds the dichroic mirror.
2. Place polarizers where the excitation and emission filters normally reside, and orient the polarizers to be perpendicular each other.

Chroma will provide polarizers backed with glass in the appropriate sizes and mounts.

COMMENTARY

Background Information

Migrating cells exert traction forces against their substrates. These tractions result from the interactions between the force-generating cytoskeleton, the focal adhesion complex, and the extracellular matrix (ECM). The interaction of the intracellular forces results in a net forward movement of the cell. However, the majority of the force acts to break the adhesive contacts with the ECM, since traction forces are several orders of magnitude larger than the force required to move a cell swimming in solution. Traction forces measured on the ventral surface of the cell are also much larger (Galbraith and Sheetz, 1997) than forces generated on the dorsal surface (Felder and Elson, 1990), and this may be due to differences in the cytoskeletal organization between the ventral and dorsal surfaces (Cramer et al., 1997). Given this difference between tractions on the two surfaces, it has been important to develop techniques and technologies that can measure many of the subcellular events of cell migration on the dorsal surface of the cell.

Most of the techniques for measuring traction forces have been developed around sheets of cross-linked silicone polymer (see Basic

Protocol 1). The original technique used to measure tractions examined the orientation of wrinkles generated in the sheets by locomoting cells. This technique has been useful for providing a global orientation of the forces under cells (Harris et al., 1980) and for comparing the relative forces of different cell types (Harris et al., 1981). These original experiments did not determine the magnitude of the forces generated underneath a cell, but they did establish the force required to stop a locomoting chicken heart fibroblast (~200 nN if the cell has a 20- μ m-long leading edge). These sheets also demonstrated that cells that migrated at the highest velocities generated the smallest traction forces, since fast-moving cells such as leukocytes and nerve growth cones were unable to wrinkle the sheets (Harris et al., 1981).

This assay was modified to produce non-wrinkling substrates that measure traction forces by examining the displacement of beads embedded within the sheet (Lee et al., 1994; see Alternate Protocol 2). These sheets were able to determine the small traction forces generated under different regions of rapidly moving keratocytes. The forces measured by these sheets were limited in temporal resolution,

since the half time for recovery was ~0.5 sec for the stiffest membranes. However, the forces were easier to quantitate than those measured by wrinkles, and the bead displacements have been used to provide detailed information about the orientation and location of traction forces (Oliver et al., 1995; Dembo et al., 1996).

A more recent modification of the original traction assay used a different polymer to generate another wrinkling sheet whose stiffness can be easily modified by exposure to ultraviolet light (Burton and Taylor, 1997; see Alternate Protocol 1). These less stiff sheets can be wrinkled by cells undergoing cytokinesis (Burton and Taylor, 1997) and can measure forces as small as 10 to 20 nN. All three of these silicone substrates are useful for measuring forces under the entire cell.

Another technique for measuring traction forces is based on micromachining technology (Galbraith and Sheetz, 1997; see Basic Protocol 2). The device is constructed with a system of movable levers that have stiffnesses ranging from 0.09 to 89 nN/ μm . This device can measure forces under regions of the cell as small as 4 μm^2 in real time, but it cannot measure forces under multiple regions of the cell simultaneously. Therefore, the protocols presented in this unit either provide detailed real-time subcellular information from a small number of adhesive contacts or extensive spatial information of slower events under the entire cell.

Critical Parameters

The time of cross-linking for the silicone sheets will critically affect their stiffness. It may be necessary to manufacture a number of sheets using an array of different conditions. This will provide a number of specimens, some of which will be suitable.

The other critical parameter in using the membranes is how they are wetted with the cell-containing medium. Rapid coating, starting from one side and moving to the other, produces the fewest membrane ruptures, but any coating method requires practice.

Troubleshooting

The traction forces generated by several different cell types have been reported in the literature. It is therefore important to compare the measurements obtained with values presented in the literature (Table 12.3.1; Oliver et al., 1994). If the measurements obtained are vastly different, recheck the calibration. Begin by recalibrating the 8-mm length of Chromel wire and comparing its stiffness with the value

obtained by Dennerll et al. (1988). If this is accurate, then check the calibration of the needles. Finally, recalibrate the film or micromachined device.

Anticipated Results

Typical traction forces generated by various cell types are listed in Table 12.3.1.

Time Considerations

The time required to manufacture any of the sheets is not extensive, however finding the conditions under which the sheets are produced with suitable stiffness may require some experimentation. In contrast, the time required to manufacture the micromachined devices may be considerable. Depending upon the schedule of the microfabrication facility and the number of process iterations required, the time from concept inception to product can easily consume 6 months to 1 year.

Additionally, the time required to collect data will vary greatly, depending upon the migration speed of the cells of interest. It should also be noted that the time required to analyze the video data is severalfold greater than the amount of time required to collect the data.

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It is often assumed that plasma membrane continuity is a constant feature of the life of the normal, healthy cell, and that when there is evidence of a disruption in continuity, one can conclude that cell death has occurred. Neither of these assumptions is correct. The plasma membrane, like other moving parts, is vulnerable to mechanically induced wear and tear. Many animal cells that reside in mechanically active tissue environments normally and frequently experience disruptions in plasma membrane integrity (McNeil, 1993; McNeil and Steinhardt, 1997; McNeil et al., 1981). Cells locomoting in culture periodically tear off small pieces of cytoplasm as their trailing end is drawn out into long retraction fibers that eventually break (Chen, 1981). Pathological levels of mechanical stress can, of course, exacerbate these constitutive levels of cell “wounding.” An active and complex Ca^{2+} -regulated resealing mechanism rapidly repairs disruptions, preventing influx of potential toxins and loss of vital cytosolic constituents (Steinhardt et al., 1994). Indeed, many cells survive surprisingly large membrane disruptions. Skeletal muscle cells and certain free-living amoebae, for example, recover after being cut in half, and sea urchin eggs can be fertilized and undergo cleavage after a $20 \times 40\text{-}\mu\text{m}^2$ patch of plasma membrane and underlying cortex is ripped from their surface (Terasaki et al., 1997).

The study of cell wounding, whether it has biological or practical aims (see Commentary), requires methods for identifying wounded cells, often present as a small minority in a culture or tissue. This unit describes such methods, which can also yield quantitative data concerning the frequency and magnitude of wounding. Wounding can be detected microscopically in cultures of adherent cells and in tissues using fluoresceinated dextrans (see Basic Protocol and Alternate Protocol 1, respectively) or using an animal’s endogenous serum albumin and enzyme immunohistochemistry (see Alternate Protocol 2). Electron microscopy of wounded cells or tissues (see Alternate Protocol 3) can be used to verify the cytoplasmic location of the tracer molecule. Wounding can be quantified by image analysis (see Support Protocol 1) or flow cytometry (see Support Protocol 2).

STRATEGIC PLANNING

The wounded cell is identified by virtue of cytosolic labeling with a normally impermeant tracer macromolecule (Fig. 12.4.1). An open plasma membrane disruption allows the tracer to enter the cytosol, and resealing traps it there. Dead cells, which do not reseal, are not labeled as long as all exogenous tracer is washed away after the disruption. There are several wound tracers to choose from. Fluoresceinated dextrans are relatively inexpensive and are available in a range of sizes (mol. wt. 5,000 to 2,000,000). If aldehyde-fixed specimens will be employed, dextrans containing conjugated lysine residues must be used. The advantage of an exogenously added tracer, such as fluoresceinated dextran, is that its time of addition and removal are known, and thus it can be used in pulse-chase labeling protocols to determine the timing of wound events. Albumin is an excellent endogenous wound tracer. For whole-animal studies it has the prominent advantage of normally bathing the cells of many tissues at high concentration.

Detection of a wound tracer by microscopy provides useful structural detail. For example, one can identify wounded cells on a cell-by-cell basis in a background of nonwounded cells, and determine which cell types in a tissue are vulnerable and at what location. Microscopy also allows one to determine whether the labeling pattern is cytosolic, the pattern expected of a wound tracer. Endocytosed tracer is recognizable as a punctate staining of the cytoplasm only. In contrast, tracer entering through a disruption results in

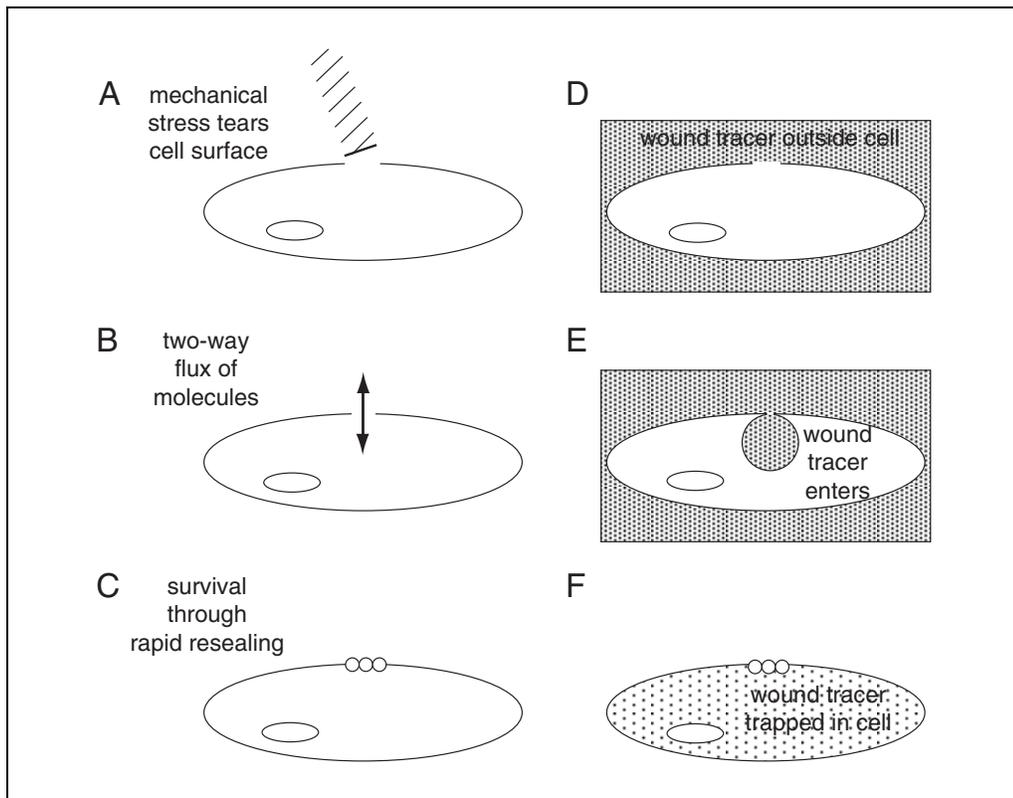


Figure 12.4.1 Cell wounding defined and identified. Mechanical stress creates a plasma membrane disruption (**A**) through which normally impermeant molecules may enter and leave the cell (**B**). Rapid resealing (<5 sec, mediated by Ca^{2+} -induced membrane mobilization and fusion events) allows the cell to survive the wound (**C**). When the disruption occurs in the presence of a normally impermeant wound tracer molecule (**D**), it can enter the cell (**E**), and become trapped there upon successful resealing (**F**). If the wound tracer is visible microscopically or fluorometrically, the cell can be identified as having been wounded.

a diffuse, even labeling of cytoplasm and nucleoplasm (if a probe <40,000 mol. wt. is employed). Often such patterns are discernible by fluorescence microscopy, but electron microscopic localization provides a more definitive indication of tracer location, especially in tissues.

BASIC PROTOCOL

WOUND DETECTION IN CULTURED MONOLAYERS USING FLUORESCHEIN DEXTRAN

A procedure for microscopical detection of wounded cells growing in adherent culture is described in this protocol, along with a useful positive control (mechanical injury of the monolayer; Swanson and McNeil, 1987). This procedure is written for human umbilical vein endothelial cells, but is applicable to many and perhaps most cell types commonly grown in the laboratory. The authors have applied it to HeLa, COS, fibroblast (NIH and Swiss 3T3), various endothelial, and smooth muscle cells.

Materials

Adherent cells of interest (e.g., human umbilical vein endothelial cells; HUVEC;

UNIT 2.3)

Cell culture medium (e.g., DMEM/10% FBS for HUVEC; Life Technologies)

Dulbecco's phosphate-buffered saline (DPBS), sterile (Life Technologies; APPENDIX 2A)

5 mg/ml FDxLys solution: lysine-fixable fluorescein dextran, 10,000 mol. wt. (Molecular Probes) in DPBS

3.7% (w/v) formaldehyde (Fisher) in DPBS

Antibleaching microscopic mounting medium (e.g., ProLong, Molecular Probes)

22 × 22 × 1-mm glass coverslips (Fisher), sterilized by flaming with ethanol (*UNIT 1.3*)

Sterile petri dishes (Falcon; e.g., 100 × 15-mm with four compartments)

1. Plate cells of interest onto flame-sterilized coverslips and culture in an appropriate cell culture medium until the desired degree of confluency is reached.

The wound protocol will work at any cell density, although higher densities induce a greater number of wounding events per coverslip.

2. Remove coverslip from its culture dish or well with tweezers and wash two times by dipping in two 50-ml beakers containing sterile DPBS. Touch the coverslip edge to a paper towel to wick off excess DPBS after each wash, but do not allow cells to dry out.

In this and all following manipulations, be sure to keep track of which side of the coverslip the cells are plated on.

3. Place the coverslip cell-side up in the bottom of a plastic petri dish that is large enough to comfortably accommodate the coverslip. Pipet 100 μ l of 5 mg/ml FDxLys solution onto and off of the coverslip several times.

This ensures that the cells, which reside in an “unstirred” layer, are thoroughly and uniformly exposed to the tracer solution.

4. Initiate experimental conditions suspected of causing wounding. Leave negative controls undisturbed.

Examples of mechanical stress that might be suspected of causing wounding are shear stress due to medium flow over or around cells and tensile stresses due to compression or stretching of the cell or its substratum. Negative controls receive FDxLys but are not mechanically disturbed.

Time in the dye should be minimized after wounding has been completed.

5. As a positive control, scratch the coverslip approximately ten times using a single-edge razor blade. View under phase-contrast optics to ensure that zones of cells (2 to 4 cells wide) have been denuded from the substratum by this maneuver.
6. Wash all coverslips as in step 2 and return to normal culturing conditions (add culture medium, return to incubator) for the desired interval.
7. Wash each coverslip three times in DPBS and then immerse in 3.7% formaldehyde for 10 to 30 min.

The investigator may need to determine the appropriate fixative for other fluorescent wound tracers.

8. Wash the coverslip twice in distilled water and then mount in an antibleaching microscopic mounting medium according to manufacturer’s instructions. View microscopically using a standard fluorescein filter set.

An example of FDxLys-labeled cells is shown in Figure 12.4.2.

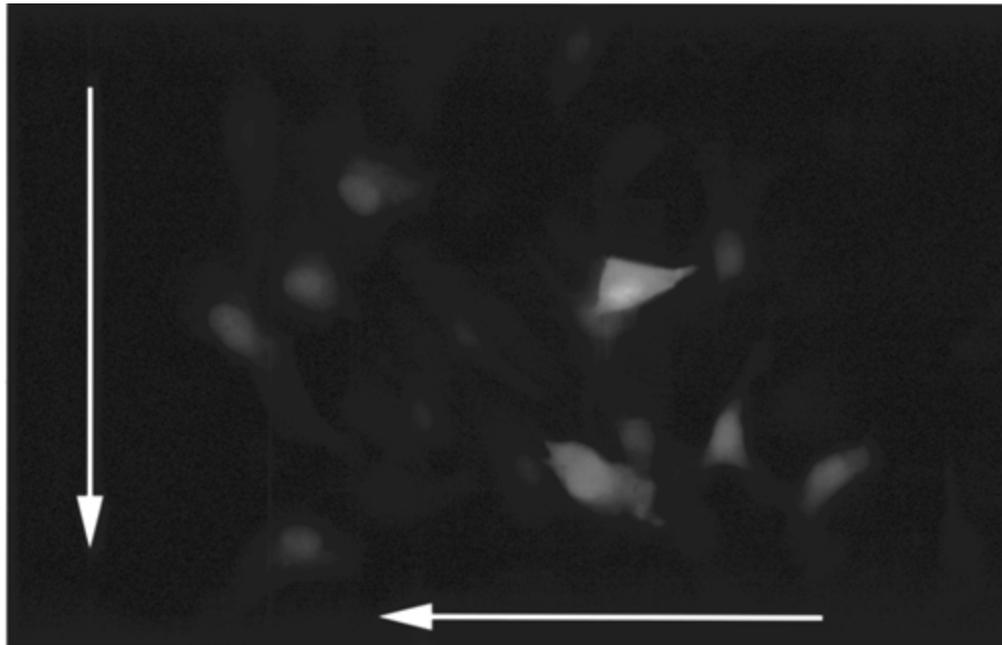


Figure 12.4.2 Example of detection of mechanically induced cell wounding *in vitro*. A monolayer of bovine aortic endothelial cells growing on a glass coverslip was scratched with a syringe needle in two directions (arrows indicate scratch tracks) in the presence of the wound tracer FDxLys (1 mg/ml). The culture was fixed in 4% formaldehyde 3 hr later. Cells that incurred plasma membrane disruptions that were successfully resealed are labeled with FDxLys (green). Cells that were not labeled are present in the upper right-hand corner of the micrograph. In the absence of this wound tracer, it would have been impossible to tell which of the cells in this field had been wounded, since morphologically they are indistinguishable from undisturbed neighbors. **See color figure.**

**ALTERNATE
PROTOCOL 1**

**WOUND DETECTION IN MAMMALIAN TISSUES USING FLUORESCEIN
DEXTRAN**

This procedure describes detection of wounded cells when the wound is induced in an intact animal by physiological or pathological mechanical stress. As with the *in vitro* procedure (Basic Protocol), wounded cells are detected by virtue of labeling with an impermeant marker. However, experimental access to the extracellular environment of cells in tissues is severely restricted compared to that available in the monolayer tissue culture situation. *In vivo*, tracer addition and removal and cell fixation are most efficiently accomplished with minimal tissue perturbation by delivering reagents (tracer, wash solution, fixative) through the blood vascular system (Clarke et al., 1995; McNeil and Khakee, 1992). Hence, the technique of vascular perfusion is described in detail in this protocol. The technique presented here focuses on the triceps skeletal muscle of the rat front leg. It should provide adequate perfusion for detecting cell wounding in most other mammalian tissues/organs, but may not be adequate for all. For example, it is not suitable for the heart, because the heart is purposefully injured in gaining access to the blood vascular system and its circulation is then bypassed during the perfusion.

In this procedure, as in the Basic Protocol, wounded cells are labeled by virtue of uptake of fluorescein dextran. Alternatively, tissue sections can be immunostained for rat serum albumin (see Alternate Protocol 2).

CAUTION: Due to the highly reactive and volatile nature of freshly prepared formaldehyde solution, the perfusion apparatus and the animal being perfused should be contained in a fume hood.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Male Sprague-Dawley rats (250 g)
Lysine-fixable fluorescein dextran (FDxLys; Molecular Probes; 10,000 mol. wt.)
Dulbecco's phosphate-buffered saline (DPBS), sterile (Life Technologies; *APPENDIX 2A*)
1% (w/v) procaine solution in DPBS, 37°C
4% and 8% (w/v) freshly prepared formaldehyde solution in DPBS
10%, 20%, and 30% (w/v) sucrose solutions in DPBS
Tissue-Tek OCT compound (Fisher)
OCT/sucrose solution: DPBS containing 50% (v/v) OCT compound and 30% (w/v) sucrose
Isopentane
Liquid nitrogen
100 mM Tris·Cl (*APPENDIX 2A*; but adjust to pH 7.0)
Antibleaching microscope mounting medium (e.g., ProLong; Molecular Probes)
500-ml reservoirs
0.3-meter lengths of 0.2-mm-i.d. plastic tubing
1.5-meter length of 0.1-mm-i.d. plastic tubing
Three-way valve
5-cm-long, 1- to 2-mm-o.d. blunt cannula
18-G needles
1-ml syringes
Cryostat microtome
Superfrost plus microscope slides (Fisher) coated with the appropriate adhesion factor (*UNITS 2.3 & 4.3*)

Set up perfusion apparatus

1. In a fume hood, set up two 500-ml reservoirs connected by two separate 0.3-meter lengths of 0.2-mm-i.d. plastic tubing to a three-way valve. At the third end of the valve, connect a single 1.5-meter length of 0.1-mm-i.d. plastic tubing to a 5-cm-long, 1- to 2-mm-o.d. blunt cannula. Elevate the solution reservoirs ≥ 1 meter above the animal to produce an adequate perfusion pressure.

This setup, illustrated in Figure 12.4.3, allows perfusion of the animal first with procaine solution and then with formaldehyde solution.

Introduce tracer into blood vascular system

2. Grasp a male Sprague-Dawley rat firmly by the skin over its neck/shoulder region. With the free hand, administer 0.25 ml sterile DPBS containing ~400 mg FDxLys/kg body weight by i.p. injection with an 18-G needle and 1-ml syringe.

FDxLys is rapidly distributed throughout the vascular system and extracellular spaces within 2 min of successful injection into the peritoneal cavity. This can be confirmed by illuminating the animal's ear with a hand-held UV lamp; it should rapidly become fluorescent.

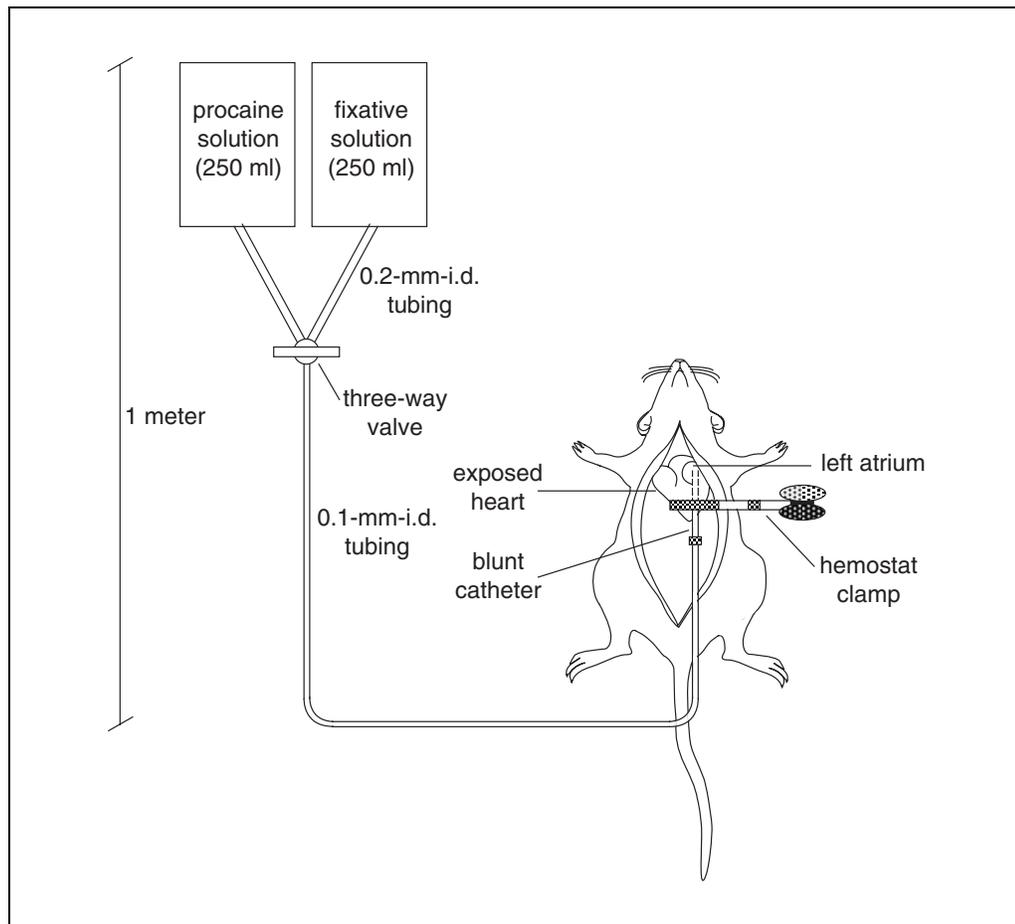


Figure 12.4.3 Perfusion setup for use after wounding in situ. Procaine and fixative solutions are elevated ≥ 1 meter above the subject and are administered sequentially to the left atrium via a three-way valve and a 5-cm-long cannula.

Initiate experimental conditions suspected of causing wounding

3. Expose the rat to the experimental condition of interest (e.g., exercise or administration of positively inotropic agents).

Negative controls are not exposed to these conditions; positive controls are exposed to pathological levels of mechanical stress (e.g., a needle-puncture injury; Fig. 12.4.4).

Perfuse blood vascular system to remove exogenous tracer

4. Deeply anesthetize rat by administering an i.p. injection of 200 mg/kg pentobarbital.
5. Place the animal on a dissection board and immobilize the front limbs with surgical tape so that the triceps muscles are in an unstretched, uncontracted state (Fig. 12.4.3).
6. With the three-way valve in the closed position, fill one reservoir of the perfusion apparatus with 250 ml warm (37°C) 1% procaine solution and the other with 250 ml of 8% formaldehyde solution. Displace any air bubbles trapped in the plastic tubing.
7. Make a small horizontal incision through the abdominal wall of the rat. Using this incision to gain access to the abdominal cavity, expose the abdominal organs by making a longitudinal incision in the abdominal wall along the full length of the animal. Expose the ribcage, puncture the diaphragm using a small pair of surgical scissors, and, working quickly, dissect out the diaphragm to expose the heart.

Be careful not to damage any of the major blood vessels, heart, or lungs within the chest cavity.

8. Make a small incision (0.2 mm) in the apex of the left ventricle. Turn the three-way valve of the perfusion apparatus so that procaine solution is flowing, and insert the blunt cannula through the small incision, positioning the end of the cannula so that it rests in the left atrium close to the aortic arch. Clamp the cannula in place with a hemostat so that the perfusion solutions cannot exit the heart except through the aorta.
9. Begin perfusing the animal with procaine solution.
10. A few seconds after initiating perfusion with procaine, make an incision in the right atrium to allow the perfusion solution to exit the vasculature. Perfuse all 250 ml procaine.

Adequate whole-body perfusion (with the exception of the heart) has been achieved if the liver is clear of blood (i.e., has changed from a deep red to a light brown color).

11. Without allowing air to enter the three-way valve, switch to the formaldehyde perfusion solution and perfuse the animal with 250 ml formaldehyde.

Fixation is rapid and can be checked by gently pressing on the liver, which should become hard to the touch.

12. Fill the body cavity with absorbent pads to contain the fixative solution and leave the animal for 1 hr in the fume hood.

Prepare tissue for microscopy

13. Carefully dissect out the tissue or organ of interest and place it in fresh 8% formaldehyde solution overnight.
14. Wash tissue once quickly with DPBS and cut into pieces of a suitable size for mounting for frozen sectioning.
15. Infiltrate tissue sequentially with 10%, 20%, and 30% sucrose solutions and with OCT/sucrose solution for a minimum of 24 hr per solution.
16. Immerse infiltrated tissue in OCT compound for 3 hr and slowly freeze the tissue onto a sectioning stub using liquid nitrogen-cooled isopentane.
17. Cut 10- μ m-thick frozen sections on a cryostat microtome and collect onto adhesion factor-coated Superfrost plus microscope slides.
18. Postfix sections in 4% formaldehyde solution for 10 min at room temperature.
19. Wash sections in 100 mM Tris·Cl, pH 7.0, for 5 min. Mount in antibleaching microscope mounting medium and coverslip.
20. View sections directly using a fluorescence microscope equipped with a standard fluorescein filter set.

Cells that have suffered a membrane wound during the exercise period will contain the green FDxLys fluorescence signal (Fig. 12.4.4A).

WOUND DETECTION USING ALBUMIN AS A WOUND TRACER

Albumin, a prominent protein constituent of blood (~10 mg/ml; ~60,000 mol. wt.), is present in most mammalian extracellular fluids. Hence, it can be used as an endogenous wound tracer in whole animal experiments (Clarke et al., 1995; McNeil and Khakee, 1992). Cultured cells can be labeled with albumin as described in the Basic Protocol. Wounded cells are labeled with an anti-rat serum albumin antibody directly conjugated with horseradish peroxidase, and are then detected enzymatically. The protocol can be

**ALTERNATE
PROTOCOL 2**

Cell Motility

12.4.7

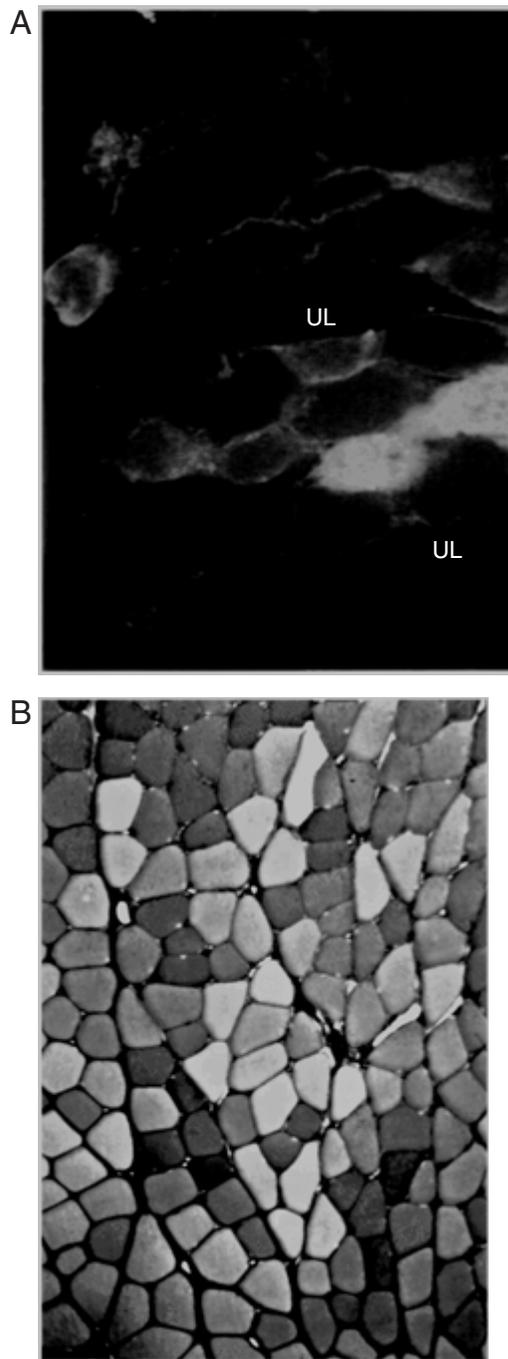


Figure 12.4.4 Example of wounded cells in skeletal muscle detected using FDxLys (**A**) or albumin (**B**) as the wound tracer. Mouse quadriceps muscles were injured by penetration with a narrow-gauge needle. Wounded cells (myofibers) are labeled with FDxLys as described in Alternate Protocol 1 (UL, unlabeled cells) or with rat serum albumin as described in Alternate Protocol 2. **See color figure.**

modified for use with alkaline phosphatase–conjugated antibodies or for secondary antibody amplification of the albumin signal.

CAUTION: Diaminobenzidine (DAB) is carcinogenic. Wear gloves and work in a fume hood when handling. Treat all DAB-containing solutions and contaminated supplies with bleach, and dispose according to institutional guidelines for hazardous chemicals.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Additional Materials (also see *Basic Protocol and Alternate Protocol 1*)

- 10 mM ammonium chloride solution in DPBS
- 0.1% (v/v) Triton X-100 solution in DPBS
- Wash buffer: 0.05% (v/v) Triton X-100 in DPBS
- H₂O₂/NaN₃/DPBS: 0.3% (v/v) hydrogen peroxide and 2% (w/v) sodium azide in DPBS (add stock H₂O₂ to azide buffer immediately before use)
- Heat-inactivated sheep serum (Life Technologies)
- Horseradish peroxidase (HRP)–conjugated sheep anti–rat serum albumin (RSA) polyclonal antibody (Organon Teknika Cappel)
- 100 mM Tris–Cl, pH 7.0 (*APPENDIX 2A*; but adjust pH to 7.0)
- HRP substrate kit (Vector Laboratories) with 3,3′-diaminobenzidine (DAB)
- 50%, 75%, 90%, 95%, 99%, and 100% (v/v) EM-grade ethanol
- EM-grade xylene
- Permanent mounting medium (e.g., Cytoseal 60, Stephen’s Scientific)

NOTE: All staining procedures are carried out at room temperature unless otherwise specified.

- 1a. *For cultured monolayers:* Prepare experimental and control cultured cells as described (see *Basic Protocol*, steps 1 to 7), but replace 5 mg/ml FDxLys with 5 mg/ml rat serum albumin (step 3).
- 1b. *For tissues:* Prepare experimental and control tissue sections as described (see *Alternate Protocol 1*, steps 1 to 18), but omit the FDxLys injection.
2. Wash the specimen twice with DPBS and incubate in 10 mM ammonium chloride solution for 30 min to inactivate reactive aldehyde groups.
3. To permeabilize tissue sections, incubate 5 min in 0.1% Triton X-100 solution, followed by 5 min in wash buffer.
Use gentle agitation for these and all subsequent incubations and washes.
4. To block endogenous peroxidase activity, incubate 30 min in H₂O₂/NaN₃/DPBS followed by 5 min in wash buffer.
5. To block nonspecific protein binding, incubate tissue section 1 hr in wash buffer containing 4% (v/v) heat-inactivated sheep serum, followed by 5 min in wash buffer alone.
6. Incubate overnight at 4°C with HRP-conjugated sheep anti-RSA polyclonal antibody made up in wash buffer/1% sheep serum.

Due to batch-to-batch variations, titration of primary antibody concentration will be required to achieve optimal staining intensity of wounded cells; a range of 1:100 to 1:1000 is recommended as a starting point.

7. Wash five times with wash buffer and two times with 100 mM Tris buffer.
8. Prepare fresh HRP substrate buffer per manufacturer's instructions and incubate tissue section in substrate buffer for 30 min in the dark.
9. Wash section in distilled water for 5 min and dehydrate in a graded ethanol series: 10 min each in 50%, 75%, 90%, 95%, 99%, and 100% ethanol.
10. Incubate in fresh xylene with two to three changes over a total of 10 min. Mount in permanent mounting medium and view by light microscopy.

Cells that have suffered a membrane wound within the 24 to 48 hr prior to animal sacrifice will be positively stained for serum albumin in their cytoplasm (Fig. 12.4.4B).

**ALTERNATE
PROTOCOL 3**

ELECTRON MICROSCOPE VISUALIZATION OF WOUND TRACERS

It is often desirable to use electron microscopical (EM) localization techniques to demonstrate that the wound tracer is present in cytosol and not in a membrane-bounded cytoplasmic compartment. This resolves the issue of whether the tracer has entered directly into the cytosol after crossing the plasma membrane boundary (the localization predicted if entry occurred through a disruption) or gained access to the cell interior by an endocytotic process. In this procedure, cells that have wounded in tissues in the presence of albumin are prepared for EM as well as preliminary light microscopy. The procedure requires expertise in preparing and handling ultrathin sections.

CAUTION: Osmium tetroxide and uranyl acetate are hazardous. Wear gloves when handling. Perform steps in a fume hood to avoid vapors. Dispose of used osmium and uranyl acetate according to institutional guidelines for hazardous chemicals.

Additional Materials (also see *Alternate Protocols 1 and 2*)

- 70% (v/v) EM-grade ethanol
- LR-White acrylic resin, hard (EM Science)
- Goat anti-rat serum albumin (RSA) primary antibody (Oregon Teknika Cappel)
- Biotinylated rabbit anti-goat IgG secondary antibody (Oregon Teknika Cappel)
- Streptavidin-gold (10- μ m particles; Auroprobe EM kit; Amersham)
- Intense Silver Enhancement kit (Amersham) containing silver salt (solution A), initiator (solution B), and sodium thiosulfate solution
- Immersion oil
- 100 mM sodium cacodylate, pH 7.4/1% (w/v) osmium tetroxide
- 60°C oven
- Formvar-coated nickel grids (Monsanto)
- Additional reagents and equipment for preparing and handling ultrathin sections and for uranyl acetate/lead citrate staining (e.g., Glauert, 1975)

Prepare sample

1. Wound tissues in the presence of albumin and perfuse/dissect tissues as described (see *Alternate Protocol 1*, steps 1 to 13).
2. Wash tissue in DPBS and cut into 5-mm³ or smaller pieces.
3. Dehydrate in 70% ethanol with constant agitation for 2 hr.

Prepare thin sections

4. Transfer samples from ethanol directly into LR-White acrylic resin and incubate overnight with constant specimen agitation.

5. Polymerize at 60°C for 2 hr according to manufacturer's instructions.
6. Prepare 1- μ m-thick sections according to standard ultramicrotomy methods.

Stain sections for preliminary light microscopy

7. Perform immunostaining for RSA as described (see Alternate Protocol 2, steps 5 to 7) but use an unlabeled goat anti-RSA primary antibody.

These and all subsequent incubations and washes should be done with gentle agitation.

8. Wash thoroughly (5 min) with wash buffer and incubate 2 hr in biotinylated rabbit anti-goat IgG secondary antibody at 1:200 in wash buffer/1% sheep serum.
9. Wash thoroughly (5 min) with wash buffer and incubate 2 hr in streptavidin-gold according to manufacturer's instructions.
10. Mix silver salt (solution A) and initiator (solution B) 1:1 (v/v) immediately before use and float a gold-labeled section on a drop of this solution. Monitor the intensity of staining to determine the optimal staining time (typically 5 to 10 min). Rinse the stained section with distilled water.
11. Incubate section for 2 to 3 min in sodium thiosulfate solution to stop the reaction, and rinse again with water.
12. Dry section thoroughly, mount in immersion oil, and view by transmitted-light microscopy (see example in Fig. 12.4.5A).

Prepare and stain ultrathin sections

13. Prepare 70-nm ultrathin sections from the same block according to standard ultramicrotomy techniques and collect on a formvar-coated nickel grid.
14. Perform immunostaining as described above (steps 7 to 9). Do not use silver enhancement (step 10).
15. Incubate overnight in 100 mM sodium cacodylate, pH 7.4/1% osmium tetroxide at room temperature.
16. Stain with uranyl acetate and lead citrate and view by standard transmission EM (for example see Fig. 12.4.5B).

QUANTIFICATION OF WOUNDING FREQUENCY AND INTENSITY: IMAGE ANALYSIS

Because the details of the following procedure (Clarke et al., 1995; McNeil and Khakee, 1992; McNeil et al., 1981) are computer and software dependent, the following protocol is written in generic form and refers to common image analysis software capabilities. Universal Imaging (<http://www.image1.com>) sells appropriate software for Windows-based PCs. For Macintosh, NIH Image software can be downloaded free of charge at <http://www.rsb.info.nih.gov>.

1. Acquire a digitized 256–grey scale image of the specimen in a blinded fashion or using a predetermined pattern of image acquisition from the microscopic specimen. Acquire all images that are to be compared with one another under identical conditions with regard to magnification, specimen illumination, camera sensitivity and gain settings, and image acquisition time.
2. Import the digitized images into an image analysis program.

**SUPPORT
PROTOCOL 1**

Cell Motility

12.4.11

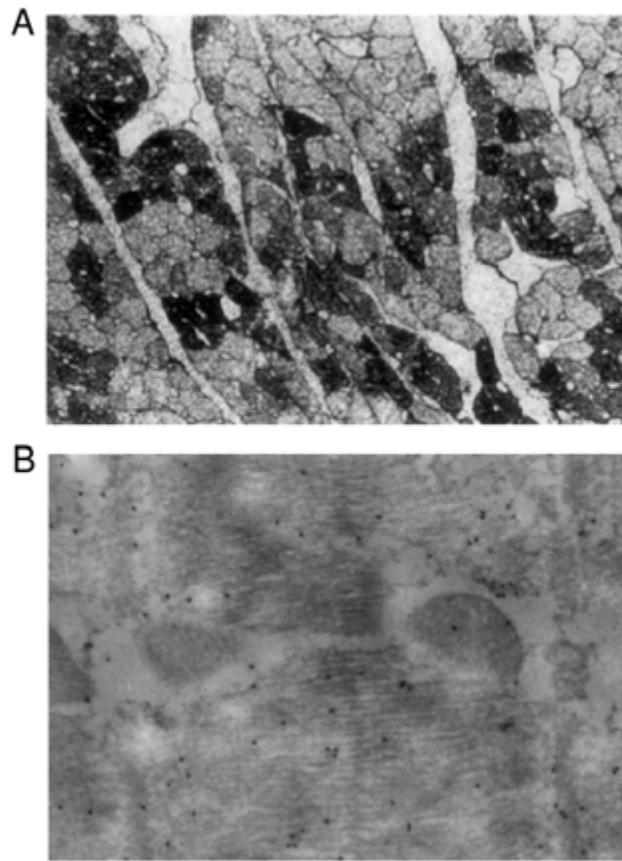


Figure 12.4.5 An example of EM localization of a wound tracer (albumin). **(A)** Light microscope image of an $\sim 1\text{-}\mu\text{m}$ -thick plastic section of rat cardiac muscle stained with an antibody to rat serum albumin, a biotinylated secondary antibody, and streptavidin-gold according to Alternate Protocol 3. The gold is visualized by intensification with silver, resulting in dark labeling of the albumin-positive (wounded) cells. **(B)** Electron microscope image of the same specimen showing that the gold label, and therefore the wound tracer, is present in the cytosol, not in an organellar compartment. This rules out uptake of the rat serum albumin by endocytosis or through this cell's T-tubule system.

- Utilizing the densitometry option of the software (or functional equivalent), create a measurement “window” that comfortably fits within the on-screen image of the cell or portion of a cell from which a measurement will be made.

This can be repeated for as many cells/areas as are present on the screen. By varying the size of the measurement window to suit the cell type being studied, membrane wounding in a variety of different tissues/in vitro specimens can be analyzed using this protocol.

- Instruct the image analysis software to measure the average pixel intensity in each measurement window, and to record the data in a file for later analysis.
- Export these values to a spreadsheet software package.
- Determine a control, nonwounded level of labeling by measuring the average pixel intensities of cells that clearly contain no wound tracer.
- Calculate an average threshold value for designating wounded cells by calculating the average pixel value of the control data pool obtained in step 6.

- Using this threshold value, calculate the percentage of wounded cells with the spread sheet.
- To compare two different experimental conditions, plot data as a population histogram or calculate mean population intensities.

QUANTIFICATION OF WOUNDING FREQUENCY AND INTENSITY: FLOW CYTOFLUOROMETRY

**SUPPORT
PROTOCOL 2**

This protocol is written with the assumption that the flow cytometer will be operated by a skilled technician, and that the goal is to assess the degree of wounding in a cell population (Clarke and McNeil, 1994; McNeil et al., 1984). The compatibility of potential wound tracers with the available flow apparatus should be determined in advance by consultation with its operator or other local expert. This procedure requires additional reagents and equipment for trypsinization (*UNIT 1.1*) and flow cytometry (Shapiro, 1995; Robinson et al., 1999).

Prepare single-cell suspensions

- Wound and label adherent cells (see Basic Protocol, steps 1 to 6). For controls, include cells that were exposed to the wound tracer but not to wound-inducing conditions (pinocytosis control) and wounded cells that were not exposed to the tracer (autofluorescence control).
- Wash twice with DPBS.
- Trypsinize adherent cells from substratum (*UNIT 1.1*) or otherwise manipulate cells or tissues to obtain a single-cell suspension.
- Resuspend in 0.25 to 1 ml DPBS or similar saline suitable for flow equipment.

If the cells tend to form clumps, filter through a nylon mesh (20-mm mesh Nitex, Tetko) before use.

For most efficient flow analysis (important when paying per unit time of instrument usage), the density of the suspension should be 10^6 cells/ml or higher. Discuss this, and the exact method employed locally for filtering out cell clumps, in advance with the flow operator.

Perform flow analysis

- Acquire 20,000 events from the autofluorescence control, measuring both forward scatter and fluorescence.

Excitation and emission settings, filters, and so on will depend on the instrument and wound tracer used.

- Use this data to set a low threshold for the fluorescence channel acquisition.

Living cell and dead cell populations, as well as cell doublets, should be discernible from the forward scatter data. If not, adjust instrument accordingly.

- Acquire 20,000 events from the experimental population. Use this data to adjust the high end of parameters such as gain and laser intensities so that the entire range of fluorescence intensities in the population is captured.

Fluorescence intensities may range over several log values.

- Acquire 20,000 events from the pinocytosis control population. Use the fluorescence profile of this population to set a threshold allowing discrimination between this population and that subset of the experimental population whose fluorescence intensities fall above this threshold.

Cell Motility

12.4.13

9. Calculate the percentage of cells in the experimental population that are above the level derived from the pinocytosis control and display the distribution of fluorescent cells within this population.

This displays wound-induced uptake of the tracer.

COMMENTARY

Background Information

Why is survivable plasma membrane disruption, referred to here as cell wounding, of interest to the cell and molecular biologist? There are number of reasons. First, such disruptions provide a route out of cytosol for numerous impermeant molecules that are not capable of being exported by the classical secretory pathway but nevertheless are present outside cells and/or have an extracellular function that can only be understood in terms of a release mechanism (Smallheiser, 1996). An example is basic fibroblast growth factor (bFGF). This polypeptide lacks a classical signal sequence for import into the endoplasmic reticulum, but is a potent extracellular cell growth-promoting signal. One route of release of bFGF is through disruptions. Characterization of cell wounding using the methods described here has demonstrated how this mode of release may have both physiological and pathological relevance *in vivo* (McNeil, 1993; McNeil and Steinhardt, 1997).

Second, disruptions provide a route into the cytosol for potentially potent second messengers, such as Ca^{2+} . When a cell suffers a plasma membrane disruption, Ca^{2+} can enter down a 10,000-fold concentration gradient until resealing intervenes. This entry of a potent, broadly acting intracellular second messenger might have profound consequences on subsequent cell functioning, both in the short term through the activation of Ca^{2+} -sensitive proteins and in the long-term through changes in gene expression. For example, Ca^{2+} entry is now known to evoke massive intracellular vesicle-vesicle fusion (Terasaki et al., 1997) and vesicle transport to and docking and fusion with the plasma membrane (Bi et al., 1995; Miyake and McNeil, 1995; Steinhardt et al., 1994). Ca^{2+} entry also may evoke *cfos* and other gene expression events (Grembowicz et al., 1999).

Third, plasma membrane disruptions can be of considerable practical utility to the cell and molecular biologist. They provide a route of entry for otherwise impermeant macromolecules that an experimenter might wish to introduce into cytosol. Enzymes, antibodies, oligonucleotides, and expression vectors are all ex-

amples of macromolecules that can be loaded into living cells through disruptions (McNeil, 1989; McNeil et al., 1981). Skeletal muscle cells can be transfected with naked DNA simply by injecting it through a syringe needle into a muscle (Wolff et al., 1990). It is clear from studies using the methods described in this unit that the injected DNA enters muscle cells through plasma membrane disruptions, which are prominently induced by the needle puncture.

Critical Parameters

Certain artifacts must be avoided. First, artifactual membrane wounding induced by animal handling must be kept to a minimum prior to removal of wound tracers and fixation. For example, early studies indicated that relatively low levels of mechanical force applied to skin caused significant membrane wounding of the epithelial cells (McNeil and Ito, 1990). Second, as membrane wound quantification relies on densitometry, section thickness is extremely important. The authors routinely mount two or more specimens for quantitative comparisons on the same stub to ensure equivalency of section thickness. However, any such potential problems associated with differing section thickness can be avoided if the experimenter has access to a confocal microscope, which allows the volumetric quantification of fluorescent wound tracer within the specimen in optical sections of identical thickness.

For additional critical parameters related to immunofluorescence and immunoperoxidase staining procedures, see *UNITS 4.3 & 4.6*, respectively.

Troubleshooting

Poor staining of wounded cells in positive control experiments could be due to insufficient wound tracer. A higher concentration of the wound tracer should be used, and its access to the extracellular environment of interest verified. Artificially high labeling in negative control experiments not subject to mechanical stress prior to fixation is most likely due to probe entry during and/or after cell fixation. The cells/tissue must be more thoroughly

washed to remove all exogenous tracer prior to fixation.

Anticipated Results

In all mechanically active tissues/culture environments tested by the authors, evidence of plasma membrane disruption events has been obtained. However, the percentage of cells affected and the range of labeling intensities varies tremendously (<1% up to 80%). It is therefore impossible to generalize as to expected results.

Time Considerations

Most of these protocols can be completed in a day or less. Exceptions are the EM immunostaining and image analysis quantitation protocols, which may take several days to complete.

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Dictyostelium discoideum is a powerful, genetically accessible system that can be used to study fundamental cellular processes, including cytokinesis, motility, phagocytosis, chemotaxis, signal transduction, and aspects of development such as cell sorting, pattern formation, and cell-type determination (Maeda et al., 1997; Dauderer et al., 1999; Kay and Williams, 1999). *D. discoideum* amoebae grow as separate, independent cells but, upon starvation, interact to form multicellular structures (see Commentary). Among the great strengths of this model system is the capacity to track the dynamic behaviors of individual cells. Most importantly, phenotypic complementation of null mutants with green fluorescent protein (GFP) fusion proteins is providing an extremely useful tool for cell biology.

This unit describes how to grow and image *D. discoideum* amoebae and focuses primarily on cell motility and chemotaxis processes. Specific step-by-step procedures are described for imaging GFP-labeled proteins in live amoebae (see Basic Protocol 1), aggregation stream and mound preparations (see Basic Protocol 2), and slug preparations (see Basic Protocol 3). Alternate Protocols 1 and 2 describe the imaging of GFP-labeled proteins in the presence of a chemoattractant. Support Protocols 1 and 2 give an in-depth description of how the cells are cultured and maintained. Support Protocol 3 describes the transformation of *D. discoideum* with a GFP fusion plasmid. Support Protocols 4 and 5 describe two different ways to characterize the functionality of the fusion protein generated by the complementation of null mutants or by overexpression in wild-type backgrounds.

IMAGING GFP-LABELED PROTEINS IN LIVE SINGLE CELLS

Once the functionality of a GFP fusion protein expressed in *Dictyostelium* amoebae is established (see Support Protocols 4 and 5), studying its cellular localization can be performed by examining single cells (as described here) or by analyzing the behavior of cells as they come together to form aggregates or mounds (see Basic Protocol 2) and slugs (see Basic Protocol 3). The methods described in this protocol are divided in two sections. First, a brief overview of the imaging arrangement is presented. Second, a detailed step-by-step description of how samples are prepared for live microscopy is provided.

Materials

- Transformed *D. discoideum* cells (see Support Protocol 3) expressing desired green fluorescent protein (GFP) fusion protein
- Phosphate buffer (PB; see recipe)
- Developmental buffer (DB; see recipe)
- Stock solution of adenosine 3',5'-cyclic monophosphate (cAMP, sodium salt) to give appropriate final concentration, for developed cells only
- Inverted microscope equipped with a charge-coupled device (CCD) camera, mercury light source, and appropriate filter sets for GFP imaging
- Computer with either IPLab-Spectrum (Scanalytics) or Openlab (Improvision) acquisition software
- Chambered coverglass (e.g., Lab-Tek II, Nalge Nunc International)
- Brass holder designed to fit in a standard mechanical stage holder
- Peristaltic pump connected to timer (for developed cells only)
- Additional materials and reagents for growing *D. discoideum* cells (see Support Protocols 1 and 2) and for counting cells (UNIT 1.1)

BASIC PROTOCOL 1

Cell Motility

12.5.1

Contributed by Carole A. Parent

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Supplement 9

Set up imaging hardware

1. Set up an inverted microscope and its associated hardware.

The CCD camera must be capable of acquiring images rapidly and with high sensitivity. Good examples of this type are the Photometrics PXL (Roper Scientific) and the Hamamatsu Orca (Hamamatsu Photonic Systems) cameras.

For double-labeling experiments, a filter wheel is required to ensure rapid changes in the filters while the cells are moving.

2. Set up a computer with IPLab-Spectrum or Openlab acquisition software to control image acquisition.

These software programs handle image acquisition, processing, measurement, and analysis for a variety of applications. Since the amoebae are light sensitive, the exposure time and the intensity of the fluorescence excitation light should be kept at a minimum using neutral-density and UV filters and shutters. Of course, the exposure time will be highly dependent on the level of expression of the GFP fusion protein, and will therefore have to be empirically determined.

3. Mount the chambered coverglass on a standard stage holder.

Alternatively, a 35-mm coverslip can be positioned on an Attofluor cell chamber (Molecular Probes) and directly mounted in a standard 35-mm-diameter stage holder.

Visualize cells

For growing cells:

- 4a. Grow transformed *D. discoideum* cells to log phase in liquid medium (see Support Protocol 1) or on bacterial lawns (see Support Protocol 2).
- 5a. Count cells (*UNIT 1.1*), dilute in PB to $\sim 1 \times 10^6$ cells/ml, and deposit 4 μ l (4000 cells) onto a mounted chambered coverglass.
- 6a. Let cells adhere 5 min.
- 7a. Gently add PB to cover the surface of the coverglass, and examine for GFP.

For developed cells:

- 4b. Grow transformed *D. discoideum* cells to log phase in liquid medium (see Support Protocol 1) and dispense 50-ml aliquots into conical tubes.
- 5b. Centrifuge 4 min at $1500 \times g$, 22°C, and wash cells in DB.
- 6b. Centrifuge again as in step 5b. Pool and count cells (*UNIT 1.1*), and resuspend in a flask at 2×10^7 cells/ml in DB.
- 7b. Shake 1 hr at 100 rpm at 22°C.
- 8b. To optimize development, while shaking at 100 rpm for 4 to 5 hr add cAMP stock solution at a final concentration of 75 nM to the cells every 6 min using a peristaltic pump connected to a timer (see Commentary).
- 9b. Remove 1 ml cells, centrifuge 4 min at $1500 \times g$, 22°C, and resuspend in 1 ml PB.
- 10b. Vortex to break up cell aggregates.
- 11b. Dilute 20 fold in PB and deposit 4 μ l onto a mounted chambered coverglass.
- 12b. Let cells adhere 5 min.
- 13b. Gently add PB to cover the surface of the coverglass, and examine for GFP.

IMAGING GFP-LABELED PROTEINS FOLLOWING A UNIFORM INCREASE IN CHEMOATTRACTANT

ALTERNATE PROTOCOL 1

The distribution of the GFP fusion protein can be studied under basal, nonstimulated conditions where processes such as cytokinesis and phagocytosis (for growth stage cells) as well as random motility can be measured. In addition, the effect of receptor stimulation on the localization of the labeled protein can be easily assessed on developed cells. This can be performed in two ways: in the presence of a uniform concentration of chemoattractant (Figure 12.5.1A) or a chemoattractant gradient (Figure 12.5.1B,C; see Alternate Protocol 2).

Materials

Transformed *D. discoideum* cells (see Support Protocol 3) expressing desired green fluorescent protein (GFP) fusion protein
Developmental buffer (DB; see recipe)
Adenosine 3',5'-cyclic monophosphate (cAMP, sodium salt) to give appropriate final concentration

Phosphate buffer (PB; see recipe)

Inverted microscope equipped with a charge-coupled device (CCD) camera, mercury light source, and appropriate filter sets for GFP imaging
Computer with either IPLab-Spectrum (Scanalytics) or Openlab (Improvision) acquisition software

Eight-chambered coverglass (Lab-Tek II, Nalge Nunc International)

Peristaltic pump connected to timer

Additional materials and reagents for growing *D. discoideum* cells to log phase in liquid medium (see Support Protocol 1) and for counting cells (UNIT 1.1)

Set up imaging hardware

1. Set up an inverted microscope and its associated hardware.

The CCD camera must be capable of acquiring images rapidly and with high sensitivity. Good examples of this type are the Photometrics PXL (Roper Scientific) and the Hamamatsu Orca (Hamamatsu Photonic Systems) cameras.

For double-labeling experiments, a filter wheel is required to ensure rapid changes in the filters while the cells are moving.

2. Set up a computer with IPLab-Spectrum or Openlab acquisition software to control image acquisition.

These software programs handle image acquisition, processing, measurement, and analysis for a variety of applications. Since the amoebae are light sensitive, the exposure time and the intensity of the fluorescence excitation light should be kept at a minimum using neutral-density and UV filters and shutters. Of course, the exposure time will be highly dependent on the level of expression of the GFP fusion protein, and will therefore have to be empirically determined.

3. Mount an eight-chambered coverglass on a standard stage holder.

Prepare developed *D. discoideum* cells

4. Grow transformed *D. discoideum* cells to log phase in liquid medium (see Support Protocol 1) and dispense 50-ml aliquots into conical tubes.
5. Centrifuge 4 min at $1500 \times g$, 22°C , and wash cells in DB.
6. Centrifuge again, pool and count cells (UNIT 1.1), and resuspend in a flask at 2×10^7 cells/ml in DB.

Cell Motility

12.5.3

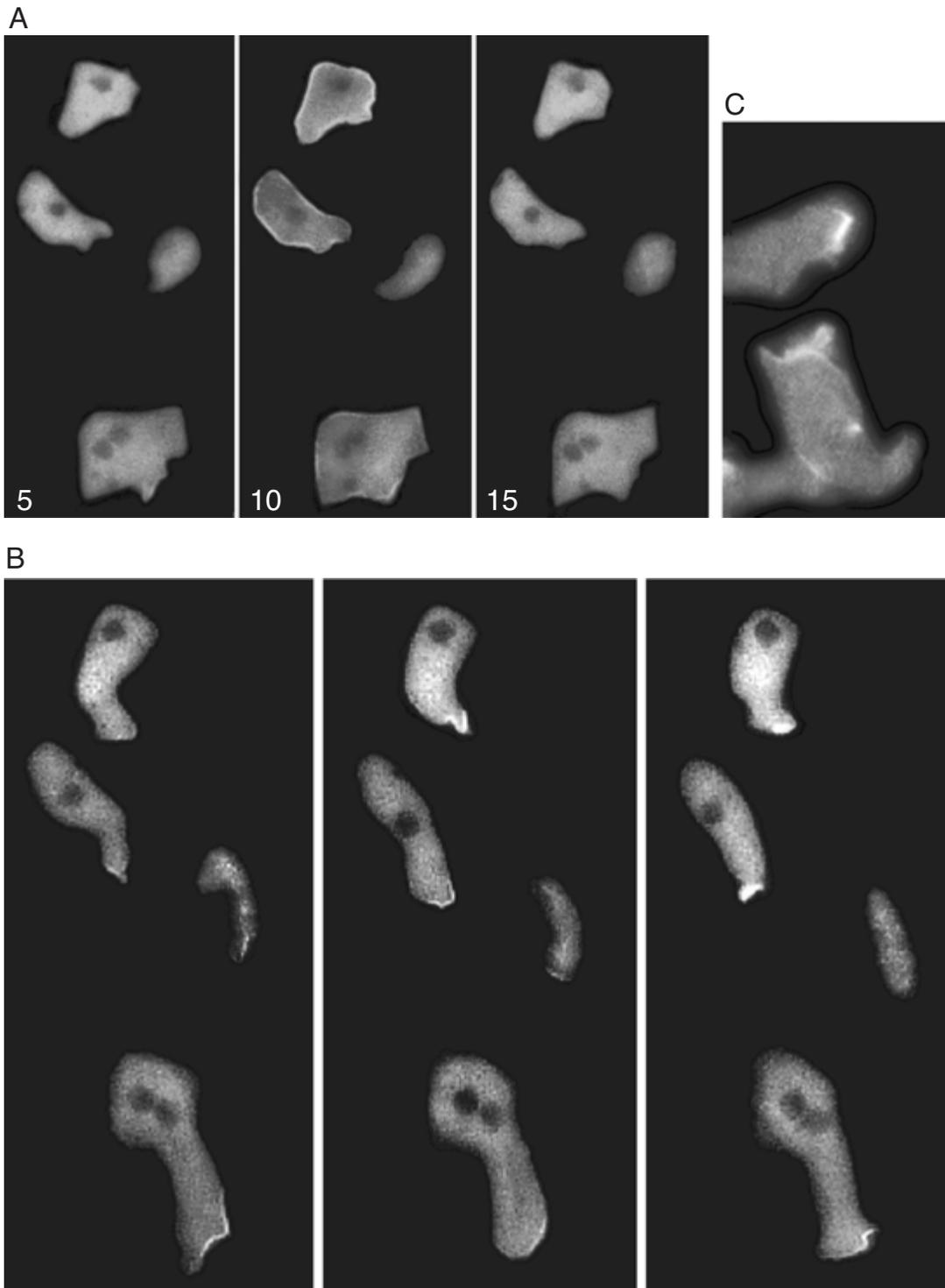


Figure 12.5.1 Translocation of pleckstrin homology (PH) domain-containing proteins to the plasma membrane at the leading edge of chemotaxing *D. discoideum* cells. Endogenous cytosolic regulator of adenylyl cyclase (CRAC), a PH domain-containing protein of *D. discoideum* amoebae, was replaced with a CRAC-GFP fusion protein. Transformants were grown, starved in the presence of pulses of cAMP, and processed for microscopy analysis. **(A)** Chemoattractant was applied uniformly by increasing the pressure on the microinjector (this can also be done by adding a bolus of attractant to the chamber). The cells respond with uniform translocation of CRAC to the plasma membrane. The colors were obtained by merging the 5-sec frame (in blue) with the 10- and 15-sec frames (in yellow). The association of CRAC-GFP appears in yellow. (*Legend continues on next page.*)

12.5.4

7. Shake 1 hr at 100 rpm.
8. To optimize development, while shaking at 100 rpm for 4 to 5 hr, add cAMP to a final concentration of 75 nM to the cells every 6 min using a peristaltic pump connected to a timer (see Commentary).
9. Remove 1 ml cells, centrifuge 4 min at $1500 \times g$, 22°C , and resuspend in 1 ml PB.
10. Vortex to break up cell aggregates.
11. Dilute 20 fold in PB and deposit 4 μl into each chamber of the eight-chambered coverglass.
12. Let cells adhere 5 min and gently add 300 μl PB to each well.

Acquire GFP images

13. Mount coverglass on the inverted microscope and set the acquisition software to time-lapse mode.

D. discoideum amoebae have a diameter of ~10 to 20 μm . Using 63 \times or 100 \times oil-immersion objectives will give the best results, increasing both spatial resolution and brightness. The exact time frame to be chosen will depend on the protein of interest.

14. Start the acquisition process and record baseline images from a chamber for about 4 to 5 frames.
15. Between two frames, delicately add 30 μl of cAMP stock solution to the chamber.

This will give a 10 \times dilution factor. Prepare stock solutions of cAMP to give final concentrations ranging from 10^{-9} to 10^{-5} M.

16. Finish data acquisition.

The length of the acquisition process will depend on the protein studied.

17. Move to a different chamber and repeat steps 14 to 16, using a different concentration of cAMP.

A dose-response curve with cAMP concentrations ranging from 10^{-9} to 10^{-5} M should be performed.

IMAGING GFP-LABELED PROTEINS IN A CHEMOATTRACTANT GRADIENT

**ALTERNATE
PROTOCOL 2**

The spatial localization of the GFP fusion protein in chemotaxing cells can be assessed by observing cells exposed to a chemoattractant gradient (Parent and Devreotes, 1999). This is achieved using a micropipet to deliver a constant amount of attractant on the coverglass (Figure 12.5.1B,C).

Figure 12.5.1 (continued from previous page) **(B)** These three images are from the same series as in (A). The gradient of cAMP was restored, and a polarized CRAC-GFP signal was observed as the cells moved up the gradient (from low concentrations at the top of the frame to high concentrations at the bottom). The micropipet was located just outside the bottom of the frames. In both (A) and (B), the numbers in the bottom left corner are seconds after the start of the experiment. **(C)** Wild-type amoebae expressing the PH domain of PKB (protein kinase B or Akt) linked to GFP were placed in a gradient of chemoattractant. As the cells were moving up the gradient, the fluorescent signal was observed at the front of the cells. The micropipet was located at the top right corner of the frame. Reprinted from Parent and Devreotes (1999) with permission from the American Association for the Advancement of Science. **See color plate.**

Cell Motility

12.5.5

Additional Materials (see *Basic Protocol 1* and *Alternate Protocol 1*)

- 10⁻⁵ to 10⁻⁶ M adenosine 3',5'-cyclic monophosphate (cAMP, sodium salt)
- One-chambered coverglass (Lab-Tek II, Nalge Nunc International) or Attofluor cell chamber (Molecular Probes) fitted with a 35-mm coverslip
- Micropipet consisting of pulled glass capillary with an opening of ~0.5 μm (e.g., Femtotips, Eppendorf Scientific)
- Microinjector (e.g., Eppendorf Scientific)
- Additional reagents and equipment for imaging GFP-labeled proteins following a uniform increase in chemoattractant (see *Alternate Protocol 1*)

NOTE: A Microloader (Eppendorf Scientific) can be used to load the micropipet.

1. Set up an inverted microscope and prepare developed transformed *D. discoideum* cells as described (see *Alternate Protocol 1*, steps 1 to 10).
2. Dilute cells 20 fold in PB and deposit 4 μl onto a one-chambered coverglass or on an Attofluor cell chamber fitted with a 35-mm coverslip.
3. Let cells adhere 5 min and gently add ~2 ml PB.
4. Mount coverglass or cell chamber on the inverted microscope.
5. Load a micropipet with 10⁻⁵ to 10⁻⁶ M cAMP and secure it in a microinjector.

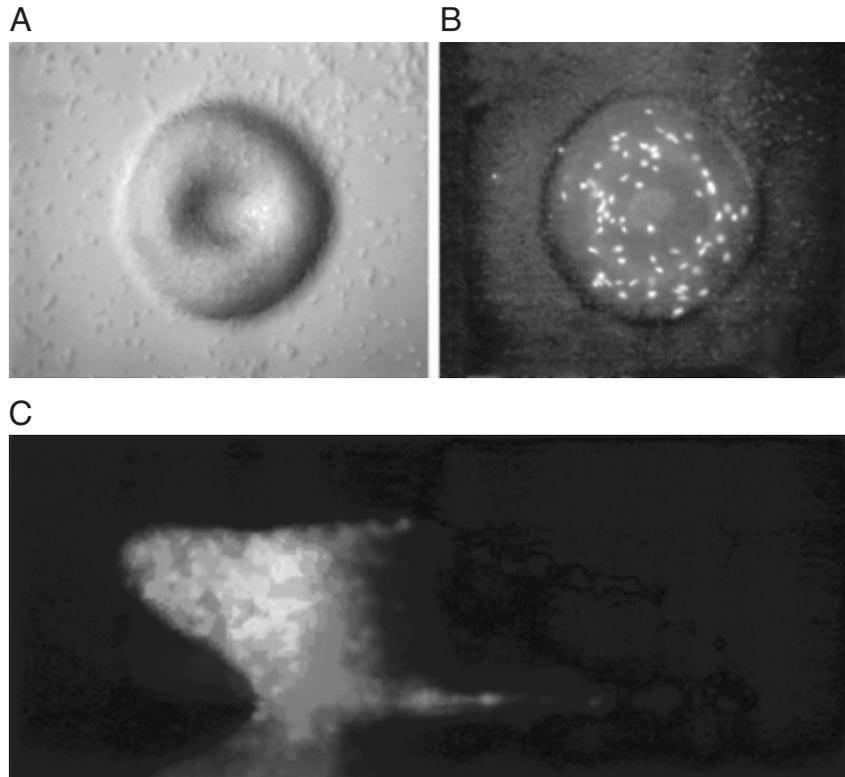


Figure 12.5.2 Visualizing GFP-labeled cells in *D. discoideum* mounds and slugs. **(A)** Bright field and **(B)** fluorescence images of a mound containing 0.1% cells expressing GFP under the actin 15 promoter, viewed at 10× magnification. **(C)** Merged image of a slug co-expressing wild-type GFP under the *ecmA* promoter and the red-shifted S65T GFP mutant under the *ecmO* promoter. The slug is viewed from the side at 10× magnification as described (see *Alternate Protocol 2*). Separate images were taken <0.5 sec apart using excitation at 400 and 485 nm, respectively, to show the wild-type (in green) and red-shifted (in red) GFP. These images were then merged to give the image shown. Regions that express both forms of GFP appear in yellow. Figure and methods generously provided by D. Dormann and C.J. Weijer (Dormann et al., 1996). **See color plate.**

- Using phase optics and a 40× oil-immersion objective, carefully position the micropipet in the vicinity of a group of cells.

A 40× objective is used to view larger field of cells.

Because the cells move rapidly towards cAMP (~15 μm/min), the micropipet should be positioned far enough from them so the cells do not aggregate on the micropipet and clog it.

- Adjust the microinjector to manual mode with a compensation pressure of ~20 hPa.

The gradient generated can be assessed by placing a diluted solution of rhodamine (which has a molecular weight similar to cAMP) in the micropipet and observing the fluorescence signal after the gradient reaches steady state (Parent et al., 1998).

- Set the acquisition software to time-lapse mode at one frame every 15 sec and begin data acquisition.

Acquisition can be performed for as long as desired.

The distribution of the fusion protein can be observed while the gradient is re-established if the micropipet is repositioned while the cells are chemotaxing.

IMAGING GFP-LABELED PROTEINS IN AGGREGATION STREAM AND MOUND PREPARATIONS

BASIC PROTOCOL 2

GFP-labeled cells can be used to study the patterns of cell movement during multicellular development in *D. discoideum*. This is achieved by mixing nonexpressing cells with cells expressing GFP under a constitutive promoter. Alternatively, GFP (and/or a color variant of GFP) can be fused to cell-specific promoters, thereby allowing the visualization of different cell types in a live organism. This technique not only allows the observation of wild-type organisms, but it can also be used to precisely study the developmental defects of mutants.

This protocol describes the procedures used to visualize aggregation streams and mounds. The aggregation streams are formed in early development as the cells are chemotaxing toward centers. Visualization of these streams is best observed at higher magnification. For the visualization of mounds and later structures, lower magnification is preferred (Fig. 12.5.2, panels A and B; also see Rietdorf et al., 1996).

Materials

Transformed *D. discoideum* cells (see Support Protocol 3) grown on non-nutrient agar (see Support Protocol 5)

Silicone oil (e.g., Dow Corning 200/20cs, BDH Chemicals)

Incubator, 22°C

Attofluor cell chamber (Molecular Probes) fitted with a 35-mm coverslip (optional)

Additional reagents and equipment for imaging GFP-labeled proteins (see Basic Protocol 1)

- Set up an inverted microscope and its associated hardware as described (see Basic Protocol 1, steps 1 to 2).
- Place transformed *D. discoideum* cells grown on non-nutrient agar in an incubator at 22°C until aggregation streams or mounds have formed (~6 to 9 hr).
- Directly mount the 35-mm petri dish containing the *D. discoideum* cells on the inverted microscope and observe with a 10× objective.

The agar layer is thin enough to allow imaging through the agar with a 10× objective.

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4. *For observation at higher magnifications (20×, 40×, and 63×):* Excise a small piece of agar containing aggregation streams or mounds with a scalpel and deposit the agar piece on its side onto a one-chambered coverglass or an Attofluor cell chamber fitted with a 35-mm coverslip.
5. Carefully pick up the agar piece, invert it, and lower it with the cells facing down onto the surface of the coverglass such that the mounds will be squashed against the glass.
6. Fill the chamber with silicone oil to prevent the agar from drying.
7. Mount the chamber on the microscope and start data acquisition.

GFP-LABELED PROTEINS IN SLUG PREPARATIONS

This protocol includes methods used to visualize structures appearing in later development such as slugs. Here again, optimal visualization will be obtained under lower magnification conditions.

Materials

- Transformed *D. discoideum* cells (see Support Protocol 3) grown to log phase in liquid medium (see Support Protocol 1)
- 35-mm petri dish containing 0.5 ml of 1% (w/v) aqueous Bacto Agar (Difco)
- Silicone oil (e.g., Dow Corning 200/20cs, BDH Chemicals)
- Incubator, 22°C
- Small rubber ring (1 mm high and 5 mm in diameter)
- Additional reagents and equipment for imaging GFP-labeled proteins (see Basic Protocol 1)

Prepare *D. discoideum* slugs

1. Set up an inverted microscope and its associated hardware as described (see Basic Protocol 1, steps 1 to 2).
2. Dispense transformed *D. discoideum* cells, grown to log phase in liquid medium, in 50-ml aliquots into sterile conical tubes.
3. Centrifuge 4 min at $1500 \times g$, 22°C, and wash cells in DB.
4. Centrifuge as in step 3 and wash cells in water.
5. Centrifuge again, count cells (*UNIT 1.1*), and resuspend at 10^8 cells/ml in water.
6. Spot 10 μ l cells on a 35-mm petri dish containing 0.5 ml of 1% aqueous Bacto Agar.
7. After 30 min, tilt the plate and blot excess fluid.

The 30-min period will allow cells to adhere.

8. Place plate in the dark in an incubator at 22°C until slugs are formed (~16 to 36 hr).
9. Place a small rubber ring around a slug and carefully push the ring against the agar surface.

This eliminates light scattering at the slug's surface.

10. Fill ring with a few drops of silicone oil to cover the slug.

View *D. discoideum* slugs

11. Directly mount the 35-mm petri dish on the inverted microscope and observe with a 10× objective.

The slug can be viewed through the agar.

- 12a. *To view the slug from its side:* Carefully cut an agar piece with a slug on it, turn agar 90° around its long axis (relative to the long axis of the slug), and place it on a one-chambered coverglass. Immediately fill chamber with silicone oil and mount on the microscope.
- 12b. *To view at higher magnification:* Carefully cut an agar piece with a slug on it, invert agar 180°, and deposit it on a one-chambered coverglass, slug-side down. Fill chamber partly with silicone oil and mount on the microscope.

The agar should be cut right next to the slug tip. If the tip protrudes between the edge of the agar and coverglass, the slug usually continues to migrate.

13. Start data acquisition.

GROWING *D. DISCOIDEUM* AXENICALLY

The availability of axenic strains (i.e., strains that can grow in liquid cultures) renders growth of *D. discoideum* amoebae in the laboratory very simple (Sussman, 1987). The most common wild-type strains used are Ax2 and Ax3. The standard growth medium, HL5, is easily made in the laboratory. Over 10¹¹ clonal amoebae can be grown and harvested in a few days without sophisticated equipment. The optimal growth temperature for the amoebae is 22°C; the cells will die at temperatures above 29°C. Amoebae are handled aseptically and grown in temperature-controlled incubators. Cells can be harvested at growth stage or at any developmental stage. In the early stages of development, the genetically identical cells differentiate synchronously and the population remains homogenous (Sussman, 1987). This allows biochemical analyses to be performed using a variety of physiologically relevant conditions. Amoebae can also easily and rapidly be grown on a lawn of bacteria (see Support Protocol 2). *D. discoideum* amoebae can readily be stored under liquid nitrogen as amoebae in the presence of glycerol or dimethyl sulfoxide. Alternatively, spores can be stored virtually indefinitely with silica gel at 4°C (Sussman, 1987).

Materials

D. discoideum cells (ATCC)
HL5 medium (see recipe)
Phosphate buffer (PB, see recipe)

100-mm tissue culture plate
250-ml tissue culture flask
Incubator, 22°C, with shaker

1. Aseptically grow *D. discoideum* cells to confluence in 12 ml HL5 medium in a 100-mm tissue culture plate in an incubator at 22°C.

The procedure can be scaled up as needed by increasing the number of plates used.

2. Using a 10-ml sterile pipet, transfer cells from the plate to a 250 ml tissue culture flask containing 100 ml HL5 medium.

This procedure can be scaled up or down depending on the need. A flask/medium volume ratio of ~2.5 is preferred for good aeration.

3. Shake at 200 rpm in the incubator at 22°C until log phase is reached.

Under these conditions, cells double every 10 to 12 hr and reach log-phase densities of 5 × 10⁶ cells/ml in ~2 days.

4. Collect log-phase cells as needed by centrifuging 4 min at 1500 × g, 22°C.

SUPPORT PROTOCOL 1

Cell Motility

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**SUPPORT
PROTOCOL 2**

5. Passage the mass culture plates every 3 to 5 days (split ratio of ~1:5) in HL5 medium (UNIT 1.1).

These cultures can usually be kept for ~2 months.

GROWING *D. DISCOIDEUM* ON BACTERIAL LAWNS

Alternatively, *D. discoideum* can be grown on bacterial lawns (Sussman, 1987). This type of growth is used when studying phagocytosis.

Materials

Klebsiella aerogenes (ATCC)
HL5 medium (see recipe) without dihydrostreptomycin
D. discoideum amoebae (ATCC)
100-mm SM plates (see recipe)
Phosphate buffer (PB; see recipe)
Incubator, 22°C

1. Grow a dense culture of *Klebsiella aerogenes* in HL5 medium without dihydrostreptomycin overnight at room temperature with shaking at 250 rpm.

Start cells from a single colony.

2. Mix $\sim 10^6$ *D. discoideum* amoebae with 200 μ l grown bacterial culture and plate on a 100-mm SM plate.

Use log-phase-grown amoebae.

The procedure can be scaled up as needed by increasing the number of plates used.

3. Store plate in a humidified environment in an incubator at 22°C.

Placing the plates in a closed plastic box in the incubator will provide an appropriately humid environment. Within 36 to 48 hr, the bacterial lawn will be cleared by the growing cells and $\sim 10^9$ amoebae can be recovered per 100-mm plate. It is important to recover the amoebae before they eat up the bacteria and enter the development program.

4. Add ~ 6 ml PB to the plate, scrape the layer of cells from the plate, and transfer to a 50-ml conical tube.

5. Repeat step 4 three times.

6. Centrifuge 1 min at $1000 \times g$, 22°C. Discard supernatant.

This supernatant should remain turbid from the remaining bacteria.

7. Resuspend pellet in 50 ml PB.

8. Repeat steps 6 to 7 until supernatant is clear (usually three times).

9. Resuspend pellet as required for subsequent analysis.

**SUPPORT
PROTOCOL 3**

PLASMID CONSTRUCTION AND TRANSFORMATION

Transformed lines of *D. discoideum* amoebae can be established using either integrating or episomal vectors (Nellen and Firtel, 1985; Manstein et al., 1995). Several selectable markers and promoters active during the developmental program have been well characterized (Egelhoff et al., 1991; Kay and Williams, 1999; Pang et al., 1999). Episomal vectors carrying Ddp1 sequence, which can amplify up to 100 copies per cell, and the actin 15 promoter/2H3 terminator expression cassette are used to establish cell lines expressing GFP fusion proteins (Hughes et al., 1994; Parent and Devreotes, 1996a,b).

This cassette gives rise to high constitutive levels of protein expression, and the extrachromosomal vector leads to little or no variation in protein expression between clones. Since the *D. discoideum* genome is ~70% A+T, a *recA* bacterial strain should be used to propagate the plasmid. In order for the gene to be translated, a ribosome binding site (RBS) must precede the ATG of the gene to be expressed. The 9-nucleotide RBS sequence, TTA-TAAAAA, has been routinely used for the constitutive expression of a variety of genes. The position of the GFP label within the protein will vary depending on the protein of interest. For information on plasmid construction, see *APPENDIX 3A*.

The enhanced version of GFP (eGFP; Clontech Laboratories), which harbors the F64L and S65T mutations and fluoresces about 35 times more intensely than wild-type GFP, has been used successfully in *D. discoideum*. This is the so-called red-shifted GFP because its maximal excitation peak is 490 nm compared to 395 nm for the wild-type GFP. Moreover, with the availability of eGFP variants in different colors (blue, cyan, green, and yellow; Clontech Laboratories), it is easy to perform double- and triple-labeling experiments.

When studying the distribution of a *D. discoideum* protein whose deletion by homologous recombination gives rise to a phenotype, gene replacement becomes a powerful tool to assess the functionality of the GFP fusion protein (see Commentary). Transformation of *D. discoideum* amoebae is accomplished by electroporation using supercoiled DNA preparations (Howard et al., 1988). The plasmid is introduced in both the wild-type and the appropriate null cells. Typically, transformants will appear 2 weeks after addition of G418. The cells should be kept under selection at all times during growth when episomal vectors are being used.

Materials

D. discoideum cells grown to log phase in liquid medium (see Support Protocol 1)

Electroporation buffer (EB; see recipe), ice cold

Supercoiled DNA vector encoding a GFP fusion protein

100-mm SM plate (see recipe)

Healing solution (see recipe)

HL5 medium (see recipe)

20 mg/ml (w/v) G418 solution (see recipe)

Klebsiella aerogenes prepared for bacterial lawns (see Support Protocol 2)

Electroporation cuvettes (0.2-cm electrode gap; e.g., Bio-Rad), ice cold

Electroporator (e.g., Gene Pulser II, Bio-Rad)

Incubator, 22°C, with shaker

24-well plates

Additional reagents and equipment for counting cells (*UNIT 1.1*), immunoblotting (see Support Protocol 4 and *UNIT 6.2*), and microscopic visualization (see Basic Protocols 1, 2, and 3; see Alternate Protocols 1 and 2)

NOTE: Unless otherwise mentioned, all manipulations are performed aseptically on ice.

Prepare cells

1. Dispense *D. discoideum* cells, grown to log phase in liquid medium, in 50-ml aliquots into sterile 50-ml conical tubes.
2. Centrifuge 4 min at $1500 \times g$, 22°C, count cells (*UNIT 1.1*), and resuspend at 4×10^7 cells/ml in ice-cold EB.
3. Remove 400 μ l cells and mix in a sterile microcentrifuge tube with 5 to 10 μ g supercoiled DNA vector encoding a GFP fusion protein.

A vector containing GFP alone should be used as a control.

4. Incubate tube 4 min on ice.

Transform cells

5. Transfer cells to ice-cold electroporation cuvettes.
6. Place cuvettes in an electroporator and electroporate at 1.1 to 1.2 kV and 3 μ F using a 5- Ω resistor in series with the chamber.
A τ value of 0.5 to 0.7 msec should be obtained.
7. Move cuvette to ice and incubate 10 min.
8. Transfer electroporated cells to a 100-mm tissue culture plate using a sterile Pasteur pipet.
9. Add 2 μ l healing solution and gently mix.
10. Incubate 15 min at room temperature.
11. Add 12 ml HL5 medium and incubate overnight in an incubator at 22°C.
12. Add G418 to 20 μ g/ml.

Clone cells

13. When the plate is confluent, isolate clones by clonally plating cells on *Klebsiella aerogenes* bacterial lawns at ~100 amoebae per plate (see Support Protocol 2, steps 1 to 3).
14. Grow cells 4 to 5 days at 22°C (Fig. 12.5.3).
15. Select individual clones by scraping the feeding edge of the plaques and transferring into 24-well plates containing 20 μ g/ml G418 in HL5 medium.
16. Move plate to 22°C incubator and allow growth.

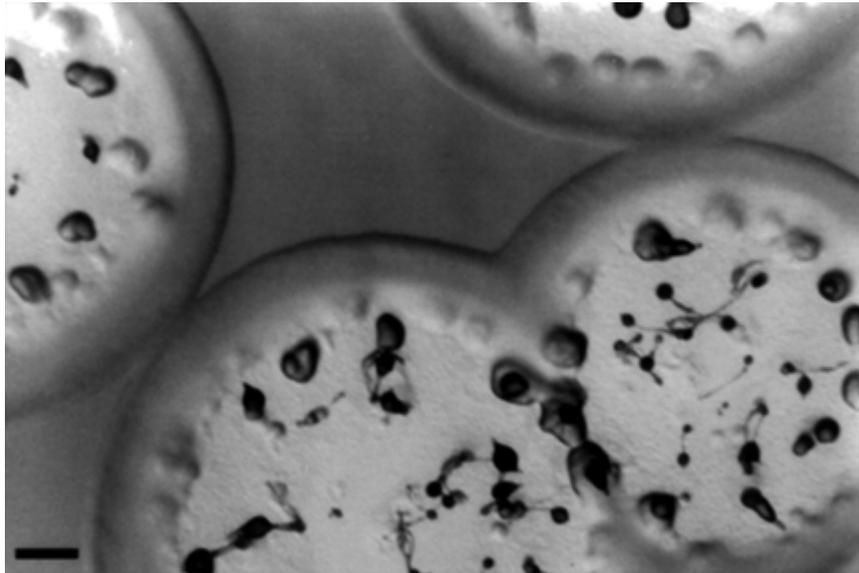


Figure 12.5.3 Cloning *D. discoideum* transformants on bacterial lawns. *D. discoideum* cells were mixed with *Klebsiella aerogenes* bacteria, plated on SM plates, and incubated at 22°C. This picture was taken 5 days after plating. Four cleared plaque regions are partially visible. Each plaque arose from a unique clone. Bar is 1.5 mm.

17. Analyze individual clones for GFP expression by immunoblotting (see Support Protocol 4 and *UNIT 6.2*) or microscopic visualization (see Basic Protocols 1, 2 and 3 and Alternate Protocols 1 and 2).

PHENOTYPIC SCREENING BY IMMUNOBLOTTING

The level of expression of the fusion protein is assessed by performing immunoblot analysis using commercially available antibodies directed against GFP (also see *UNIT 6.2*). This is especially important because it will confirm that the full-length GFP fusion is expressed and will provide information on the level of expression. In addition, probing with an antibody directed against the protein of interest allows one to compare the level of expression of the endogenous protein to that of the fusion protein. This is sometimes difficult as the epitope recognized by the antibody can be masked by the presence of the GFP.

Materials

Transformed *D. discoideum* cells (see Support Protocol 3) grown to log phase in liquid medium (see Support Protocol 1)
Developmental buffer (DB; see recipe)
4× SDS sample buffer (*APPENDIX 2A*)
Anti-GFP antibody (e.g., Clontech Laboratories)
Antibody directed against protein of interest (optional)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and immunoblotting (*UNIT 6.2*)

1. Dispense transformed *D. discoideum* cells, grown to log phase in liquid medium, into 50-ml tubes.

A cell line that does not express GFP should be used as a control.

2. Centrifuge 4 min at $1500 \times g$, 22°C, and wash cells in DB.
3. Centrifuge as in step 2 and resuspend at 2×10^7 cells/ml in DB.
4. Transfer cells to a flask and shake 2 hr at 100 rpm.

This starvation period will downregulate the expression of proteases and therefore limit protein degradation.

5. Mix 200 μ l cells with 67 μ l of 4× SDS sample buffer in a microcentrifuge tube.
6. Boil 2 min.
7. Carry out SDS-PAGE (*UNIT 6.1*), loading ~53 μ l sample (8×10^5 cells) per lane.
8. Transfer gel to nitrocellulose membrane.
9. Perform immunoblot analysis (*UNIT 6.2*) using an anti-GFP antibody and an antibody directed against the protein of interest (optional).

PHENOTYPIC SCREENING BY DEVELOPMENT ON NON-NUTRIENT AGAR

The functionality of the GFP fusion protein is assessed by determining if it behaves as its wild-type counterpart when expressed in the null background. In order to assess the effect (or noneffect) of the GFP fusion protein on normal development, this characterization is also performed when the fusion protein is expressed in wild-type cells. The procedure is simple and fast—wild-type cells will differentiate and form fruiting bodies 24 hr after the

SUPPORT PROTOCOL 4

SUPPORT PROTOCOL 5

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initiation of the starvation period (Fig. 12.5.4). Moreover, the whole developmental program can be readily recorded using time-lapse video. Alternatively, development can be assessed by clonally plating amoebae on bacterial lawns (see Support Protocol 2).

Materials

Transformed *D. discoideum* cells (see Support Protocol 3) grown to log phase in liquid medium (see Support Protocol 1)
Developmental buffer (DB; see recipe)
Developmental buffer agar (DB agar; see recipe)
35-mm petri dish
Incubator, 22°C

1. Dispense transformed *D. discoideum* cells, grown to log phase in liquid medium, in 50-ml aliquots into sterile conical tubes.

Wild-type cells should be used as controls.

2. Centrifuge 4 min at $1500 \times g$, 2°C, and wash cells in DB.
3. Centrifuge again, count cells (UNIT 1.1), and resuspend at 1×10^7 cells/ml in DB.
4. Transfer 1 ml cells to a 35-mm petri dish containing 0.5 ml DB agar.
5. Let cells adhere 5 min.
6. Tilt plate, wait 5 min, and aspirate excess buffer.
7. Let air dry 2 min.
8. Replace lid and store plate in a humidified environment in an incubator at 22°C.

Placing the plates in a closed plastic box in the incubator will provide an appropriately humid environment.

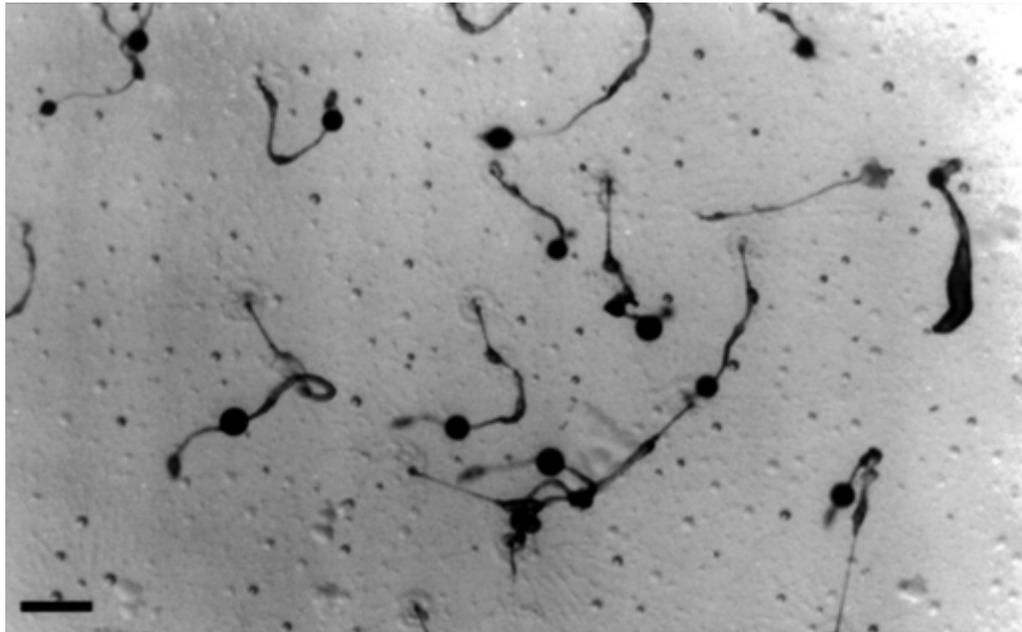


Figure 12.5.4 Development of *D. discoideum* on non-nutrient agar. Wild-type *D. discoideum* cells were grown axenically (see Support Protocol 1), plated on non-nutrient agar (see Support Protocol 5), and incubated at 22°C. This picture was taken 24 hr after plating. Bar is 1.5 mm. Note the characteristic spore heads on top of the stalks that form the fruiting bodies. Mutants can give rise to a wide variety of developmental phenotypes.

9. Assess development as appropriate.

The developmental program of the cells can be recorded so that the 24-hr period is compressed into a few minutes. This is easily accomplished by connecting a video camera to the microscope and using a time-lapse video recorder to capture images. For best results, a low-magnification objective should be used (2.5× to 5×). Since the cells are sensitive to high temperature, care should be taken to use a very dim light source. The functionality of the GFP fusion protein is then easily assessed by comparing the phenotype of the transformed cell line with that of wild-type cells. A comprehensive analysis of the developmental program of wild-type cells can be found in Kessin (2000) and references within.

REAGENTS AND SOLUTIONS

Use deionized distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Developmental buffer (DB)

5 mM Na₂HPO₄·7H₂O
5 mM KH₂PO₄
2 mM MgSO₄
200 μM CaCl₂
Final pH 6.2
Store up to 6 months at 4°C

Developmental buffer agar (DB agar)

Add 1% (w/v) Difco Bacto Agar to 50 ml DB (see recipe) in 100-ml bottles and autoclave on liquid cycle for 20 min. Store up to 6 months at room temperature. When ready for use, unscrew the cap and microwave until the agar is melted.

Electroporation buffer (EB)

10 mM sodium phosphate buffer, pH 6.1 (APPENDIX 2A)
50 mM sucrose
Final pH 6.1
Filter sterilize using 0.22-μm filters
Store up to 6 months at -20°C

G418 solution, 20 mg/ml

Prepare a 20 mg/ml solution in 10 mM HEPES, pH 7.2. Filter sterilize using 0.22-μm filters, dispense into 0.5-ml aliquots, and store at -20°C.

Healing solution

100 mM CaCl₂
100 mM MgCl₂
Filter sterilize using 0.22-μm filters
Store up to 6 month at 4°C

HL5 medium

10 g glucose (55 mM final)
10 g Difco Peptone (1% w/v final)
5 g yeast extract (0.5% w/v final)
0.95 g Na₂HPO₄·7H₂O (3.5 mM final)
0.5 g KH₂PO₄ (3.7 mM final)
0.03 g dihydrostreptomycin (41 μM final)
H₂O to 1 liter
Final pH 6.5

continued

Mix all components and divide into aliquots in bottles and flasks. Cover flasks with a stopper made of cotton cheesecloth. Autoclave 20 min on liquid cycle. Store up to 1 month at 22°C.

Autoclaving for longer periods will cause the glucose to caramelize (making the medium appear darker) and serious growth problems to occur.

Phosphate buffer (PB)

5 mM Na₂HPO₄·7H₂O

5 mM KH₂PO₄

Final pH 6.2

Store up to 6 months at 4°C

SM plates

10 g dextrose (55 mM final)

10 g Difco Bacto Peptone (1% w/v final)

1 g yeast extract (0.1% w/v final)

1.9 g KH₂PO₄ (13.9 mM final)

0.9 g K₂HPO₄ (5.2 mM final)

20 g Difco Bacto Agar (2% w/v final)

H₂O to 1 liter

Final pH 6.4

Autoclave 20 min on liquid cycle

Pour 25 ml per 100-mm petri dish

Let solidify at room temperature

Store up to 6 months at 4°C

COMMENTARY

Background Information

D. discoideum amoebae live in two distinct phases (Bonner, 1982). Vegetative or growth-stage cells function independently and use phagocytosis or pinocytosis to ingest bacteria or liquid medium. Similar to mammalian cells, amoebae grown in liquid medium divide using binary fission. Upon starvation, the amoebae acquire the capacity to communicate with one another and enter a developmental program that leads to the formation of a multicellular organism. In the early stages of development, ~10⁵ amoebae use chemotaxis to come together and form a tight aggregate. This aggregate then differentiates into stalk and spore cells and, through a series of morphological changes, forms a spore head atop a stalk of vacuolated cells—the so-called fruiting body. Once environmental conditions are favorable again, the spores germinate and repeat the life cycle. The unique feature of *D. discoideum*'s life cycle allows one to study in great detail the dynamic behavior of individual cells. Moreover, the advent of the GFP technology now allows one to readily assess the dynamic behavior of individual proteins within living, migrating cells.

The growth and developmental programs of *D. discoideum* are mediated via the evolution-

arily conserved G protein-coupled signaling cascade (Parent and Devreotes, 1996a; Aubry and Firtel, 1999). Four hours after the initiation of starvation, cells maximally synthesize and secrete cAMP. Through the action of an extracellular phosphodiesterase, cAMP is produced at 6-min intervals. These oscillations in the levels of cAMP produce propagating waves that generate gradients directing the cells toward the aggregating centers. At the same time, G protein-coupled receptors that specifically bind cAMP are expressed on the cell surface, and binding of the nucleotide to its receptor leads to a variety of biochemical responses. As is observed when leukocytes are stimulated with chemoattractants, rapid and transient increases in polymerized actin, Ca²⁺ influx, intracellular messengers such as IP₃, cAMP, and guanosine 3',5'-cyclic monophosphate (cGMP), and in the phosphorylation of myosin I and II have all been measured following cAMP addition to amoebae.

In the early aggregation period, these biochemical changes give rise to chemotactic movement and changes in gene expression, both of which are essential components of normal development. Genetic and biochemical analyses have established that, throughout

growth and development, four cAMP receptors (cAR1 to 4) coupled to eleven G proteins (composed of eleven distinct α subunits associated with a unique $\beta\gamma$ -complex) are expressed. Similarly, various conserved signaling effectors are expressed at specific times in the course of the *D. discoideum* life cycle. A number of these effectors have been identified by cloning and through information emerging from the genome and cDNA sequencing projects. These effectors include: adenylyl and guanylyl cyclases, phosphodiesterases, phospholipase C, protein kinase A, glycogen synthase 3, small guanosine triphosphatases and their regulators, actin and a plethora of actin binding proteins, conventional and unconventional myosins, MAP kinases, two-component histidine kinases, and STATs, which are not present in yeast (Harwood et al., 1995; Parent and Devreotes, 1996a; Kawata et al., 1997; Maeda et al., 1997; Brown and Firtel, 1998; Loomis, 1998; Loomis et al., 1998; Aubry and Firtel, 1999; Eichinger et al., 1999; Thomason et al., 1999; Williams, 1999).

D. discoideum's highly accessible genetics renders it a unique model system to study fundamental cellular processes including cell motility and directed migration (Eichinger et al., 1999). Most of the molecular genetic techniques typically associated with *S. cerevisiae* are available in *D. discoideum*. Random mutagenesis can be performed using high-efficiency extrachromosomal vectors (Parent and Devreotes, 1996a). Because the genome is haploid, novel genes can be identified using insertional mutagenesis or restriction-enzyme-mediated integration (REMI; Kuspa and Loomis, 1992). Nonessential genes can be easily disrupted by homologous recombination (De Lozanne and Spudich, 1987). So far, over 400 genes involved in cell motility, signal transduction, and cell differentiation have been targeted, and important generalizations for eukaryotic cells have been derived using these cell lines. Strains with multiple gene deletions can be constructed by consecutive transformations using different selectable markers. Since the cells are free living, gene deletions that might be lethal in other organisms can often be productively studied in *D. discoideum* (Kay and Williams, 1999).

Using phenotypic complementation of null mutants with GFP fusion proteins is extremely useful for cell biology. Because heterologous expression of yeast and mammalian genes is possible in *D. discoideum*, this technique can be used to assess the functionality of endo-

genous as well as heterologous genes (Manstein et al., 1995; Slade et al., 1997; Pang et al., 1999; Parent and Devreotes, unpub. observ.). Moreover, studying the phenotypic behavior of wild-type cells overexpressing wild-type or mutated genes is very useful in assessing the function of a given protein. Consequently, the high degree of conservation between amoebae and higher eukaryotes at the cellular and molecular levels coupled to the accessible genetics of this model system renders the study of fundamental biological processes observed in *D. discoideum* indispensable for deciphering complex biological responses.

Critical Parameters and Troubleshooting

Using *D. discoideum* to study cell motility has many advantages. Compared to mammalian cells, amoebae are easy to grow and manipulate. The medium is readily made in the laboratory and is inexpensive. As amoebae eat bacteria, the risk of bacterial contamination is small, and the optimal growth temperature of 22°C is very convenient. The cells adhere well to plastic and glass but do not require trypsinization procedures. Stable transformants are easily obtained and maintained, and phenotypic characterization is rapid. Nonetheless, there are some aspects of *D. discoideum* research that require special attention.

1. The quality of the peptone used to make the HL5 medium is critical for proper growth. It has been observed that the Difco brand gives by far the best results. Suboptimal medium will result in slow or no growth.

2. When growing cells on bacterial lawns, the author has found that using overgrown cultures of *Klebsiella aerogenes* results in serious growth problems. Growing the bacterial cultures overnight at room temperature gives rise to the best results.

3. A transformation efficiency of $\sim 1/1000$ is expected when using extrachromosomal vectors. Good quality DNA preparations free of contaminants and salt will give the best results. Also, because the heat generated by the electrical shock can kill the cells, prechilling the cuvette and using ice-cold buffer is critical. Looking at the cells 24 hr after electroporation, before the selection antibiotic is added, is a good way to see if the cells were harmed by the electrical burst. At that time, most of the cells should be adhering to the bottom of the plate.

4. Performing a dose-response curve analysis for the effectiveness of G418 on the survival of wild-type cells is preferable. This is

achieved by simply adding various concentrations of G418 to wild-type cells and monitoring cell survival. The optimal concentration should be close to 20 $\mu\text{g/ml}$.

5. Development on non-nutrient agar is influenced by environmental conditions, and most of the abnormal developmental problems are caused by an inadequate level of humidity on the plate. This can be fixed by not air drying the plate after aspirating the excess buffer and simply leaving it slightly tilted with the lid on.

6. When assessing the effect of receptor stimulation on the cellular distribution of the GFP fusion protein, it is essential to make sure that the cells are expressing cAMP receptors (a key point when studying cells that display a developmental phenotype). This is accomplished by performing immunoblot analysis using a specific polyclonal antibody directed against cAR1 (a receptor expressed in early development) on samples taken from developed cells.

Time Considerations

The limiting step in these procedures is obtaining the transformants, which under normal conditions will require ~2 to 3 weeks. Once the phenotypic characterization is completed (~1 week), the live-cell imaging is fast and rewarding.

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Key References

Kessin, 2000. See above.

This is the most recent book on Dictyostelium cell biology to date. It covers a wide variety of valuable information on the development of Dictyostelium.

Ludin, B. and Matus, A. in association with *Trends Cell Biol.* 1999. "GFP in motion".

This CD contains beautiful movies highlighting the use of GFP in live cells. Several sections describe results obtained using D. discoideum. Contact Trends in Cell Biology (www.elsevier.com) for more details.

Spudich, J.A. (ed.) 1987. *Dictyostelium discoideum: Molecular Approaches to Cell Biology. Methods Cell Biol.* Vol. 28.

This volume contains details of a variety of molecular and cell biological tools used to study D. discoideum.

Experientia Vol. 51. 1995.

This volume contains a series of reviews pertaining to genomics, cell biology, and biochemistry of D. discoideum.

Internet Resources

<http://dicty.cmb.nwu.edu/dicty/dicty.html>

This site serves as a single entry point for the online resources for the D. discoideum community. It provides access to an online database of investigators, current and past editions of the newsletter "Dicty News", links to cDNA and genome sequencing projects, and a wide variety of useful resources.

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Optical Microscopy–Based Migration Assay for Human Neutrophils

UNIT 12.6

This unit describes an *in vitro* microscopy assay for examining the migration of human neutrophils in two dimensions. The method is particularly useful for identifying the underlying cause of a migration defect, and it can be used to evaluate a variety of migration parameters (e.g., speed and directional persistence) that cannot be easily discerned from assays based on migration through a porous barrier (e.g., Boyden chamber assays; UNIT 12.4). For this assay, freshly isolated human neutrophils are added to microscopy chambers and then stimulated to migrate with a chemotactic agent. Images of fields of cells are acquired at multiple time points after stimulus addition. The images are used to create time-lapse movies that can be analyzed to determine the effects of specific pharmacological treatments on migration of individual cells.

The Basic Protocol describes the method for acquiring time-lapse images of migrating neutrophils, while support protocols describe isolation of human neutrophils from blood (see Support Protocol 1) and preparation of microscopy chambers (see Support Protocol 2).

NOTE: There are special ethical and safety considerations for working with human cells.

NOTE: All solutions and materials used with living neutrophils should be sterile and free of lipopolysaccharide (LPS, endotoxin) contamination, which will activate the neutrophils (see Critical Parameters).

ACQUIRING TIME-LAPSE IMAGES OF MIGRATING NEUTROPHILS

**BASIC
PROTOCOL**

A crucial initial step in an inflammatory response is the migration of neutrophils and other immune cells toward chemotactic stimuli. Cell migration requires a highly orchestrated series of attachment and detachment events, which occur in concert with protrusive and contractile forces. Importantly, each step in the process must be regulated in both space and time, and so investigations of migration mechanisms often warrant assays that provide both types of information. The assay described here measures the extent to which neutrophils translocate (i.e., migrate in two dimensions) in response to an isotropic bath of chemoattractant. This assay is based on the observation of cells by optical microscopy, which provides intracellular and extracellular spatial information. Temporal information is available because the cells are imaged at various times after stimulation, rather than at a single fixed timepoint.

Materials

- HBS, pH 7.4 (see recipe)
- 50× (0.5 M) glucose stock solution (filter sterilize and store at 4°C)
- Human neutrophils, freshly isolated (see Support Protocol 1)
- Formyl-Met-Leu-Phe (fMLF)
- 1.5-ml polypropylene microcentrifuge tubes
- End-over-end rotator or platform rocker
- Air-curtain heater (Arenberg Sage)
- 35-mm fibronectin-coated coverslip dishes (see Support Protocol 2)
- Humidified slide warmer (LabLine Instruments)
- Inverted microscope equipped for digital imaging with 20×, 40×, or 63× objective

Cell Motility

12.6.1

Contributed by Lynda M. Pierini and Frederick R. Maxfield

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Supplement 17

NOTE: Neutrophils are readily activated by shear forces or by contact with certain surfaces. Cells should be handled gently, transferred using wide-bore pipet tips, and kept in polypropylene (as opposed to glass or polystyrene) vessels.

Prepare cells, coverslip dishes, and microscope

1. On the day of the experiment, prepare ~25 ml of HBS, pH 7.4, with 10 mM glucose (added from 50× glucose stock) for every ten coverslip dishes to be used. Prewarm the HBS/glucose to 37°C and maintain at that temperature.
2. For every coverslip dish to be used, prepare 50 µl of neutrophil suspension at 1–2 × 10⁶ cells/ml in HBS/glucose. Prepare in bulk (up to 1.5 ml per 1.5-ml conical tube) and place on a slow-moving rotator or rocker at room temperature.

If testing the effects of a particular pretreatment (e.g., pharmacological agent) on the migration of neutrophils, split the cells into the appropriate number of aliquots and treat the test samples at this time.

3. Prewarm the microscope stage to 32° to 35°C with an air-curtain heater for 30 to 60 min before the start of the experiment.

Neutrophil migration is completely abrogated at temperatures above ~39°C, so it is essential that the temperature of the microscope stage and objective be carefully monitored throughout the experiment.

Typically, the air-curtain heater should be placed 4 to 6 in. (10 to 15 cm) away from the microscope stage and aimed so that warm air is blown across both the stage and the objective. As an alternative, a microscope objective heater may be used (e.g., Bioprotechs; <http://www.bioprotechs.com>).

If using an oil-immersion objective, prewarm the immersion oil to the same temperature as the stage and objective. This will minimize focus drift caused by temperature-dependent changes in oil viscosity.

4. When ready to begin, remove excess PBS from a 35-mm fibronectin-coated coverslip dish and add 50 µl of cell suspension to the well in the center of the dish. Place the coverslip dish onto a humidified slide warmer maintained at 37°C, and let cells settle onto the coverslip for 5 min.
5. Gently add 1 ml of warm HBS/glucose to the coverslip dish, adding the buffer off to the side of the dish so that cells do not become dislodged.

Neutrophils will not become tightly adherent until after they are stimulated. For this reason, it is easy to accidentally wash them away.

6. Transfer the coverslip dish to the microscope stage and look at the cells using transmitted light illumination and any contrast method available (e.g., phase contrast or differential interference contrast) to confirm by morphology that the cells are healthy and resting.

Neutrophils are relatively thick cells that are best imaged with differential interference contrast optics, which allows detailed observation of morphological changes. Phase-contrast imaging is usually less informative because the cells become extremely refractile at some points during migration.

This is a good time to evaluate the activation state of the neutrophils. As noted above, resting neutrophils should not be tightly adherent and they should not spread on the coverslip. Usually <10% of the cells will attach, spread, and become spontaneously polarized; the remainder of the cells will be rounded with smooth plasma membranes (see Fig. 12.6.1). If much greater than 10% of the neutrophils appear to be activated in the absence of exogenously added chemoattractant, subtle effects of experimental treatments may be difficult to detect. In this case, the investigator may wish to discard the samples and

begin again with a fresh blood draw from a different donor. If >40% of the neutrophils appear to be activated, neutrophils should be re-isolated from a new donor. If the basal level of activation of the neutrophils from various donors is consistently high, all solutions used in the isolation procedure should be discarded and prepared anew. LPS contamination should be considered (see Critical Parameters). Mechanical stimulation (e.g., from an unbalanced centrifuge) can also cause activation.

Add chemoattractant and begin image acquisition

7. Prepare a 20 nM solution of fMLF in HBS/10 mM glucose (see step 1) and maintain at 37°C in a water bath. Gently add 1 ml of the warm 20 nM fMLF solution to the periphery of the coverslip dish on the microscope stage and let stand for 2 min.

Because the cells are in an isotropic bath of chemoattractant (i.e., there is no gradient), they will become oriented in random directions.

8. Begin image acquisition. Acquire a transmitted-light image of the same field of cells every 10 to 20 sec for 4 min. Save this series of images, then acquire a second and third series of images of different fields from the same dish.

Alternatively, a single, longer time series can be acquired if detailed analyses of cell paths and behaviors are to be performed.

After data acquisition is complete, the sample can be discarded or used for immunofluorescence staining (see UNIT 4.3).

9. Repeat steps 4 to 8 with fresh cells and a new coverslip dish.

Analyze cell behavior

- 10a. *To determine the migration capacity of neutrophils:* Draw a circle of a fixed diameter around each cell within the first image of the time series. For each cell in the field, step through the images in the sequence and note whether the cell has moved out of its circle (i.e., for each cell, determine if the cell's centroid has translocated beyond the perimeter of the circle).

It is not sufficient just to compare the first and last images in a time series because cells may have moved out and then back into their circles and would thus be scored as nonmigratory. Alternatively, cells may become detached during the course of the assay and so may no longer reside within their starting circles; these cells would mistakenly be scored as migratory.

Some cells become very elongated. Depending on the intent of the experiment, a cell whose front, but not rear, moves outside the circle can be counted or not, as long as the criterion is specified and consistent throughout the analysis.

There are sophisticated image-analysis programs that can perform this sort of analysis in an automated fashion. A description of these programs and their use in analyzing time-lapse movies is beyond the scope of this unit. It should be noted that automated analysis of migration is most easily accomplished using fluorescence images of labeled cells rather than transmitted-light images in which object identification is more difficult.

Choosing the diameter of the circle to draw around the cells in the initial images is somewhat arbitrary. For historical reasons, the authors ordinarily use a 14- μ m-diameter circle, so a migrating neutrophil is defined as one that has translocated >7 μ m in 4 min. With this definition, 60% to 90% of control cells are migratory. All samples on a given day should be evaluated using circles with the same diameter.

The neutrophil preparation used for these studies will contain a small percentage (1% to 5%, depending on the donor) of eosinophils. Eosinophils are relatively easy to identify because their cytoplasm is full of distinct spherical granules that are generally larger and more refractile than the granules in neutrophils (see Fig. 12.6.3). Cells that are clearly identifiable as eosinophils should not be considered in the data analysis.

To compare data from experiments performed on different days, results should be normalized relative to the migratory level of the control cells.

- 10b. *To determine displacement, persistence, and turning behavior of individual cells:* Track and analyze each cell manually or by computer. To track cell paths manually, mark and record the positions of every cell's center in each field, then enter these positions into a graphing program for further analysis (Mandeville et al., 1995).

ISOLATING HUMAN NEUTROPHILS

Primary human neutrophils cannot be maintained in culture, making it necessary to isolate neutrophils from volunteers each day of an experiment. The first step of the isolation procedure is based on a centrifugal technique that separates neutrophils from red blood cells (RBCs) and most other leukocytes. The neutrophil-containing layer of cells isolated in this way will contain a small percentage of contaminating RBCs and eosinophils. RBCs are nearly completely removed from the preparation by using hypoosmotic shock to preferentially lyse the RBCs, leaving the neutrophils and eosinophils unharmed. Contaminating eosinophils typically comprise only 1% to 5% of the remaining cells and can be easily identified and disregarded during image analysis.

This separation technique is based on the density of human neutrophils and the osmolality of the separation medium. It is likely that the density of neutrophils from other animals will differ slightly from that of human neutrophils, so the density of the separation medium (i.e., Polymorphprep) will have to be adjusted if the protocol is to be used for isolation of neutrophils from animals other than humans. Suggestions for adjustments can be obtained from the manufacturer.

CAUTION: Only qualified and experienced personnel, following governmental and institutional guidelines for working with human subjects, should draw blood from donors.

NOTE: It is important to verify that the donor is healthy and not taking any medications because neutrophil function may be altered under these circumstances.

Materials

- Sodium heparin
- Healthy volunteer blood donor
- Polymorphprep density gradient medium (Accurate Chemical & Scientific Corp.)
- 5× PBS (see recipe)
- HBS/glucose: HBS, pH 7.4 (see recipe), containing 10 mM glucose added from 0.5 M stock (see Basic Protocol)
- 15- and 50-ml conical polypropylene centrifuge tubes
- Tourniquet
- Alcohol wipes
- 21-G needles
- 10-ml disposable syringes
- Tabletop centrifuge with swinging-bucket rotor
- Additional reagents and equipment for counting cells with a hemacytometer (*UNIT 1.1*)

NOTE: Use PBS without calcium or magnesium throughout the isolation procedure until the final resuspension step. Divalent cations in HBS will cause cells to aggregate.

Obtain donor blood sample

1. Place 200 U sodium heparin in a 15-ml conical tube.

The authors have avoided using Vacutainers because they tend to stimulate the neutrophils. Newer plastic Vacutainers have not been evaluated for this assay.

2. Place tourniquet on a healthy donor's arm, swab arm with alcohol wipe, and then draw 10 ml blood using a 21-G needle and a 10-ml syringe.

The health of the blood donor is essential to the success of the assay. For further details, see Critical Parameters.

3. Immediately transfer blood into the heparin-containing conical tube. Mix the heparin and blood by gently inverting the capped tube several times, and then let stand 10 min to allow blood to cool to room temperature.

The blood should be used within 2 hr of being drawn from the donor.

Isolate neutrophils

4. Place 5 ml Polymorphprep into each of two 15-ml conical tubes. Carefully layer 5 ml of the heparinized blood onto the top of the Polymorphprep in each tube using a sterile polypropylene transfer pipet. Cap the tubes and centrifuge 30 min at $450 \times g$, room temperature (18° to 22°C), in a swinging-bucket rotor.

At the end of the centrifugation step, several layers can be discerned within each tube, including (from bottom to top) a dark red layer consisting largely of RBCs, a clear layer containing Polymorphprep with some RBCs and some neutrophils, a fuzzy white band containing neutrophils and eosinophils, a tight white band of mononuclear cells (T cells, B cells, and monocytes) at the sample/medium interface, and a yellow layer of plasma. The two white bands of leukocytes are typically separated by 0.5 to 1.0 cm. If RBCs are clumped at the top of the clear Polymorphprep layer such that the band of neutrophils is not clearly defined, centrifuge the samples for an additional 5 to 10 min.

5. From each tube, carefully aspirate the yellow plasma layer and top tight white band (mononuclear cells), leaving the fuzzy white band (containing neutrophils and eosinophils). Remove the neutrophil-containing band from each tube with a sterile polypropylene transfer pipet and combine in a single 50-ml conical tube. Collect the clear Polymorphprep layer from each tube and add to the neutrophils.

When removing the tight white band of mononuclear cells (i.e., the upper band), it is best to err on the side of losing some neutrophils so that purity is optimized over yield. The number of neutrophils obtained from this procedure is far greater than needed for the migration assay.

6. Fill the 50-ml conical tube with $1 \times$ PBS and sediment cells by centrifuging 10 min at $400 \times g$, room temperature (18° to 22°C).
7. Aspirate the PBS. Resuspend neutrophil pellet in 1 ml of $1 \times$ PBS and transfer to a 1.5-ml microcentrifuge tube.

The cell suspension appears reddish because it contains contaminating RBCs in addition to neutrophils and eosinophils.

Remove contaminating RBCs

8. Microcentrifuge 6 to 8 sec at 10,000 rpm ($7,500 \times g$). Aspirate supernatant, leaving reddish pellet.
9. Using a wide-bore pipet tip, carefully resuspend pellet in 800 μl water.
10. Allow RBCs to lyse for 30 sec and then add 200 μl of $5 \times$ PBS. Rapidly mix the contents of the tube by capping the tube and inverting several times. Immediately sediment cells in microcentrifuge as in step 8, then aspirate the supernatant.

Keeping the cells in water for <30 sec will result in incomplete lysis of RBCs, while waiting significantly longer than 30 sec may damage the neutrophils.

11. Resuspend the pellet from step 10 in 1 ml of $1 \times$ PBS. Microcentrifuge as in step 8 and aspirate the supernatant. Repeat wash and resuspend final pellet in 1 ml HBS/glucose.

12. Determine concentration of cells using a hemocytometer (see *UNIT 1.1*).

Neutrophils can be kept on ice or at room temperature for several hours before use. In general, neutrophils should be used within 4 to 6 hr of isolation.

PREPARING COVERSLEIPS AND MICROSCOPY CHAMBERS

Long-term observation of live cells often requires specialized chambers to maintain cell viability. Neutrophil migration assays are typically completed within 30 min, so environmentally controlled perfusion chambers are unnecessary. Instead, the open-air chambers described below are adequate, and they are less expensive and easier to use than most perfusion setups. Prefabricated chambers with poly-D-lysine-coated coverslips can be purchased from commercial sources (e.g., MatTek) for approximately twice the cost of making them.

NOTE: These chambers are open to the air, so bicarbonate-buffered media cannot be used.

NOTE: Chemotaxis chambers can be constructed from these sample chambers by covalently attaching opsonized erythrocytes to the coverslip surface (Pytowski et al., 1990).

Materials

Nochromix glass cleaning reagent (Godax Laboratories) mixed with H₂SO₄ per manufacturer's instructions

Paraffin

Petroleum jelly

1× PBS (see recipe)

0.1 mg/ml fibronectin (see recipe)

Glass coverslips (thickness should be matched with recommendation of microscope manufacturer)

35-mm tissue culture dishes

Sheet metal hole punch

Cotton-tipped applicator or fine-tipped paint brush

Prepare coverslips

1. Clean glass coverslips by immersing in Nochromix/H₂SO₄ solution for 1 hr. Make sure that coverslips are well separated so that the surfaces of all of the coverslips are exposed to the cleaning solution.

CAUTION: Nochromix cleaning solution is extremely caustic. Use appropriate safety gear and procedures.

2. Remove the Nochromix/H₂SO₄ solution, rinse the coverslips with water three times, then soak for 1 hr in water. Repeat this water rinse and soaking process two more times.
3. Remove clean coverslips from the last wash one at a time using a pair of tweezers. Place coverslips in a single layer onto filter paper and let dry. Store clean, dry coverslips in a covered receptacle.

Prepare coverslip dishes

4. Prepare a mixture of paraffin/petroleum jelly at a ratio of 3:1 (v/v) by first melting enough paraffin over low-medium heat to yield 75 ml. Bring the volume of the liquefied paraffin up to 100 ml by adding petroleum jelly. Stir the mixture well.

This mixture can be stored for up to 6 months and remelted over low-medium heat when needed. The mixture should be stirred before each use, and the last ~25 ml of the mixture should be discarded because the composition changes with repeated heatings.

5. Punch a 1.2-cm-diameter hole in the bottom center of a 35-mm tissue culture dish using a sheet metal punch.
6. Keeping the dish in an inverted orientation, use a cotton-tipped applicator or a fine-tipped paint brush to spread a thin layer of melted paraffin/petroleum jelly around the perimeter of the hole on the outside surface of the dish. Allow paraffin/petroleum jelly to resolidify on the dish.
7. Place a single clean, dry coverslip atop the solid ring of paraffin/petroleum jelly on the underside of the dish.
8. To affix the coverslip to the dish, warm the inverted dish (with coverslip on top) briefly to liquify the paraffin/petroleum jelly, and then let cool to room temperature.

To warm the inverted dish, an inverted hot plate or an infrared lamp can be positioned such that heat from the hot plate or infrared lamp will warm items underneath. The heating rate should be set so that the paraffin/petroleum jelly mixture melts in ~5 sec when the inverted dish (with its coverslip on top) is warmed. Immediately after the paraffin/petroleum jelly melts, remove the dish from the heat and let it cool to room temperature.

The result will be a well in the center of the right-side-up dish. The coverslip forms the bottom of the well, and the sides of the hole in the dish form the sides of the well. The thin solidified ring of paraffin/petroleum jelly, which seals the coverslip to the dish, does not contribute significantly to the depth of the well.

9. Store coverslip dishes for up to 6 months at room temperature. Dishes should be stored in an inverted position to prevent scratching of the coverslips and to keep debris from collecting in the wells.

Coat coverslip dishes with fibronectin (perform on day of assay)

10. Rinse the well of each coverslip dish three times with 1× PBS. Apply 50 µl of 0.1 mg/ml fibronectin to the well of each coverslip dish and allow to stand at room temperature for 30 to 60 min. Carefully wash the coverslip dishes three times with 1× PBS, and then leave covered with PBS until use.

REAGENTS AND SOLUTIONS

Use endotoxin-free deionized water in all recipes and protocol steps. For common solutions see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Fibronectin, 0.1 mg/ml

Purchase lyophilized human fibronectin (1.0 mg per vial) from BD Biosciences. To rehydrate lyophilized fibronectin, add 1 ml of distilled water and let stand at room temperature for 1 hr, gently swirling the solution periodically to completely dissolve the fibronectin (do not vortex). Store fibronectin at 4°C and use within one month of rehydration. Dilute fibronectin 1:10 (v/v) with 1× PBS (see recipe) on day of use.

HEPES-buffered saline (HBS), pH 7.4

200 µl 5 M MgCl₂ (1 mM final)

0.147 g CaCl₂ (1 mM final)

0.37 g KCl (5 mM final)

8.8 g NaCl (150 mM final)

4.8 g HEPES (20 mM final)

H₂O to 1 liter

Adjust to pH 7.4 with 1 M NaOH or 1 M HCl

Filter sterilize and store up to 4 months at 4°C

The authors prefer to use a commercial MgCl₂ stock solution (e.g., 49 M MgCl₂ from Sigma) because MgCl₂ is very hygroscopic and difficult to weigh accurately.

Phosphate-buffered saline (PBS), 5×

1.0 g KH₂PO₄ (7.5 mM final)

40.0 g NaCl (685 mM)

1.0 g KCl (13.5 mM)

5.8 g Na₂HPO₄ (40.5 mM)

H₂O to 1 liter

Adjust to pH 7.4 with 1 M NaOH or 1 M HCl

Filter sterilize and store up to 4 months at 4°C

COMMENTARY

Background Information

Neutrophils, with their capacity to migrate in response to a variety of stimuli, are essential for maintaining human health. In the absence of functional neutrophils people would rapidly succumb to infection. It has been over a century since Metchnikoff suggested that neutrophils serve as active defenders against invaders (Metchnikoff, 1893), yet there are still many questions about neutrophil locomotion that remain to be answered. How do neutrophils polarize in an isotropic bath of stimuli? How can they migrate in persistent paths when there is no gradient of stimulus? What are the molecular components of the migration machinery? How are these components regulated? To address these sorts of questions, visual assays have been used for over fifty years and have provided detailed descriptions and quantification of cell locomotion (Dixon and McCutcheon, 1936; McCutcheon, 1946). Microscopy-based assays are still one of the most powerful means of gaining insight into the mechanics of cell migration.

Precise terminology has been developed to describe migration phenomena (Keller et al., 1977a,b,c), and brief definitions of the relevant terms (shown in italics) are given here. Cells may possess or lack an *intrinsic capacity* to migrate. For example, promyelocytic cells are nonmotile, and it is only after they differentiate into monocytes or neutrophils that they acquire the capacity for migration. Once migration has been induced, the cells' behavior can be described as *random* or *directed*. Cells that are migrating randomly move along a path with no particular orientation relative to the environment. Neutrophils and other cells have been shown to exhibit a "persistent random walk" as opposed to "Brownian" random movement (Dunn, 1981). The direction of persistence is completely random with no correlation between individual cells or between cells and environmental factors. In contrast, cells that migrate directionally move with a preference for (or avoidance of) a particular direction,

usually dictated by an environmental cue such as a *chemoattractant*, a soluble factor that activates cells to migrate (e.g., bacterially generated formyl-peptides and inflammatory chemokines). Cells within a gradient of a chemotactic chemoattractant will be oriented nonrandomly with their front-to-rear axis aligned parallel to the gradient (Zigmond, 1977). Chemoattractants can affect the speed or frequency of migration (*chemokinesis*) and/or the direction of migration (*chemotaxis*). Chemokinesis and chemotaxis are *not* synonymous with random and directed migration, respectively. Directed migration can result from either chemotaxis or chemokinesis coupled with *contact guidance*, where the physical properties of the environment dictate the direction of migration—e.g., aligned fibrils within tissue matrices (Mandeville et al., 1997; Wilkinson and Lackie, 1983). Refer to Keller et al. (1977a,b,c) for more in-depth definitions of the abovementioned italicized terms.

The assay described in this unit can only determine if an agent is chemokinetic; it cannot establish the chemotactic potential of a chemoattractant. To directly show that a chemoattractant is chemotactic, an orientation chamber, such as the one introduced by Zigmond (1977) and modified by others (Zicha et al., 1991, 1997) should be used. Alternatively, the assay chamber described here can be converted into a chemotaxis chamber by attaching bacterial spores (Allan and Wilkinson, 1978) or opsonized erythrocytes (Pytowski et al., 1990) to the surface of the coverslip. Addition of fresh serum to the chambers activates complement and generates radial gradients around the spores or erythrocytes when these particles are widely separated on the coverslip (~20 to 100 μm apart). Time-lapse imaging of neutrophils migrating towards these point sources provides a direct measure of chemotaxis. To address chemotactic potential, researchers have also used assays based on migration through micropore filters—e.g., Boyden chamber as-

says (Boyden, 1962). These are indirect assays that determine the end-point distribution of a cell population after exposure to chemoattractant. Although much larger numbers of cells are analyzed, filter assays can only provide information on cell populations as opposed to individual cells and/or subsets of cells. With a visual assay, the direction and speed of migration can be measured to directly demonstrate chemotaxis or chemokinesis, and displacement, persistence, and turning behavior of individual cells can be extracted from detailed analyses of cell paths (Haston and Wilkinson, 1987, 1988; Mandeville et al., 1995). Excellent discussions on the benefits and limitations of various filter assays can be found elsewhere (Wilkinson, 1996, 1998).

It should also be noted that the assay described here is a two-dimensional one. Thus, the analogous *in vivo* situation is likely to be cells migrating along the luminal surface of vascular endothelium rather than through the three-dimensional environment of connective tissues. Cells use somewhat different mechanisms for migration through three-dimensional matrices as compared to migration across two-dimensional surfaces, but there are enough similarities that a simple two-dimensional mi-

gration assay is an excellent starting point for dissecting the complexities of locomotion.

Clearly, no single type of migration assay is suitable for answering every question, so a variety of complementary assays should be used. See *UNITS 12.1, 12.2 & 12.4* for descriptions of other types of migration assays, and reviews by Wilkinson for an overview and critique of popular methods for studying cell migration (Wilkinson, 1996, 1998).

Critical Parameters

The success of neutrophil migration assays hinges on just two main parameters, the neutrophils and their environment. *In vivo*, the role of the neutrophil is to sense and respond to environmental signals that may indicate the presence of pathogens. The exquisite sensitivity of neutrophils to the presence of microbes is one of the main obstacles to performing an *in vitro* migration assay. Cells that are slightly prestimulated (or “primed”) often respond differently to chemoattractants than naïve cells, so it is important to start with truly unstimulated neutrophils. The neutrophils from a donor who is coming down with or just getting over a cold may be slightly activated and should not be used. Similarly, many medications are known to affect cell migration, and for that reason

Table 12.6.1 Troubleshooting Guide for Optical Microscopy–Based Neutrophil Migration Assays

Problem	Possible cause	Solution
Cells appear jagged prior to stimulation and migrate poorly or not at all in response to chemoattractant	Endotoxin contamination	Test H ₂ O supply for endotoxin contamination; decontaminate H ₂ O and remake all solutions
	Cells were activated during isolation	Isolate neutrophils from a different donor taking extra care not to activate cells by rough handling
Cells appear round and nonadherent	Chemoattractant not added	Remake solution of chemoattractant
	No divalent cations in assay buffer	Remake assay buffers; verify that HBS (not PBS) has been used as the assay buffer
Cells appear very spread and tightly adherent after stimulation, and they do not migrate	Temperature too high	Adjust temperature
	Substrate too adhesive	Prepare coverslip dishes with varying amounts of substrate to optimize conditions
	Chemoattractant concentration too high	Perform dose-response analysis with chemoattractant to optimize concentration
Cells are slow to polarize and migrate only a short distance after stimulation	Temperature too low	Adjust temperature

neutrophils should not be isolated from donors taking medication. Donor health needs to be carefully assessed prior to drawing blood, and all results ought to be confirmed with neutrophils from various donors.

Neutrophils are fragile cells that are readily activated by rough handling. Extreme care is required during the isolation procedure to minimize shear stress and to avoid cell contact with activating surfaces, such as glass. An unbalanced centrifuge, overzealous resuspension technique, or excessive handling of cells can all contribute to cell activation. Isolated neutrophils are still adept at sensing microorganisms in the environment, and they will become activated in the presence of even very low levels of bacterial lipopolysaccharides (LPS, endotoxin). It is crucial that the water supply be tested for the presence of LPSs prior to making isolation and assay buffers, and that all stock buffers be prepared and maintained under sterile conditions. Recirculating reverse osmosis (RO) water systems can become contaminated

with algae or other microorganisms. It is important to check for LPSs because contamination anywhere in a closed-loop system can release LPSs, which then spread throughout the loop. Filtration or UV light at the tap will not remove LPSs. The water supply can be tested for LPS contamination using a commercially available kit (e.g., Chromogenic Limulus Amebocyte Lysate Assay Kit from BioWhittaker).

After cells with a low basal level of activation have been isolated, they will be added to the assay chamber and stimulated to migrate. At this point, the ability of the cells to migrate and the extent of migration will depend upon the agent used to stimulate the cells, the matrix on which they will migrate, and the temperature during the assay. The absolute concentration of chemoattractant, even in the absence of a gradient, is an important determinant of migration. Neutrophils exhibit dose-dependent migration that varies between chemoattractants, making it necessary to generate dose-response curves to determine the optimal concentration of a

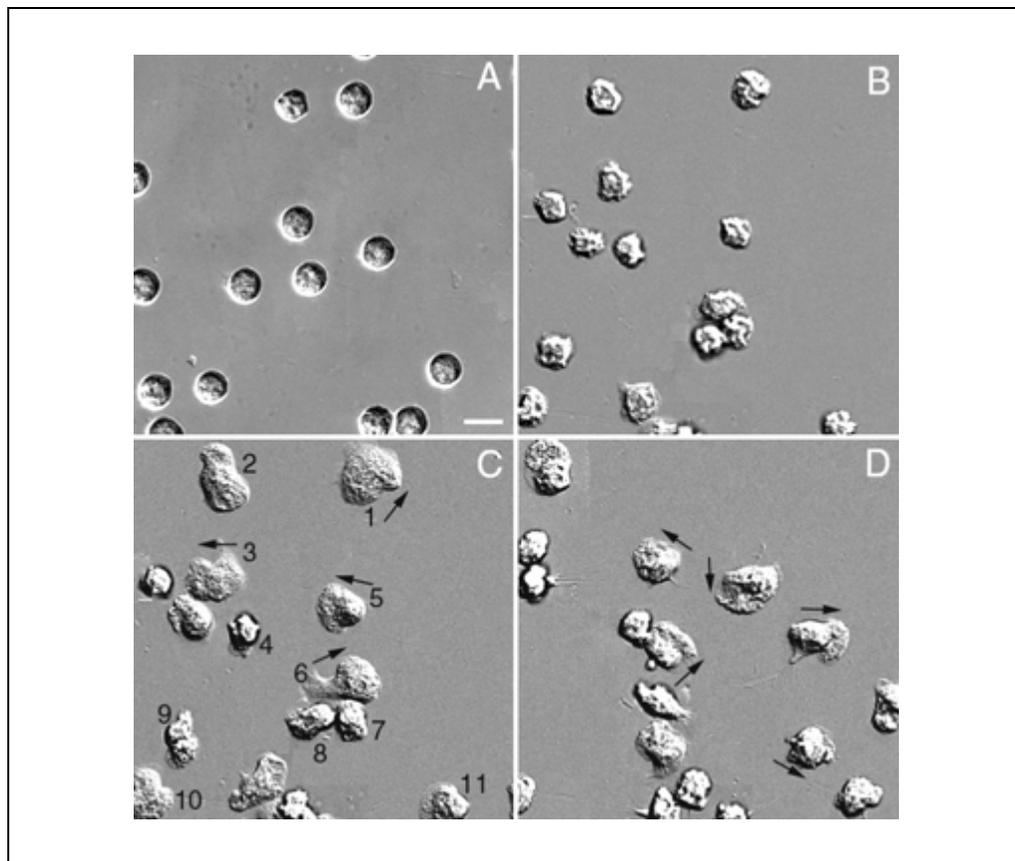


Figure 12.6.1 Morphological changes induced in human neutrophils by stimulation with the chemoattractant fMLF. Neutrophils were plated onto a fibronectin-coated coverslip dish for 5 min at 37°C and then left unstimulated (A) or stimulated with fMLF for 15 sec (B), 1 min (C), or 6 min (D). The images shown in panels A, B, and C are each from different fields of cells, whereas the image shown in panel D is the same field of cells shown in C, but imaged 5 min later. Numbers in C correspond to cell paths traced in Figure 12.6.2. Arrows indicate direction of neutrophil migration. Bar is equal to

particular chemoattractant. Because migration is dependent upon the precise regulation of cell attachments in space and time, the adhesive nature of the substratum is another critical parameter that can be varied. Substrata of differing adhesiveness can yield very different migration data in response to a given chemoattractant at a fixed concentration. If the substratum is too adhesive, cells will be unable to detach from the surface and therefore they will be unable to translocate. On the other hand, if the substratum is not adhesive enough, cells will not be able to form tight enough attachments to generate the tractional forces necessary for locomotion. Thus, the ability of cells to migrate, and their speed of migration, depend upon the tightness of the attachments that the cell forms with the substratum. This in turn depends upon the concentration of the ligand (e.g., the density of the matrix element deposited on the coverslip), the concentration of the ligand receptor (e.g., integrin expression levels), and the ligand receptor binding affinity (Palecek et al., 1997). Optimal migration conditions for substrates can be determined empirically by measuring migration of cells plated onto coverslips coated with varying concentra-

tions of substrate. When using a range of substrate concentrations, one should add an appropriate amount of albumin to keep the concentration of total protein constant, otherwise there may be areas of exposed glass at the low end of the substrate concentration range. Because contact guidance or exposed glass surfaces can bias cell migration, it is wise to verify that substrates have been deposited evenly. This can be accomplished by fluorescently labeling the substrate directly or indirectly via immunofluorescence (Marks et al., 1991) and evaluating the fluorescence intensity profile across the coverslips.

Finally, neutrophil migration is sensitive to temperature, with the complete abrogation of migration occurring at temperatures not much above 37°C. Neutrophils migrate well, though more slowly, even at room temperature, so it is sensible to perform migration assays slightly below 37°C (~35°C) to avoid deleterious effects of higher temperatures.

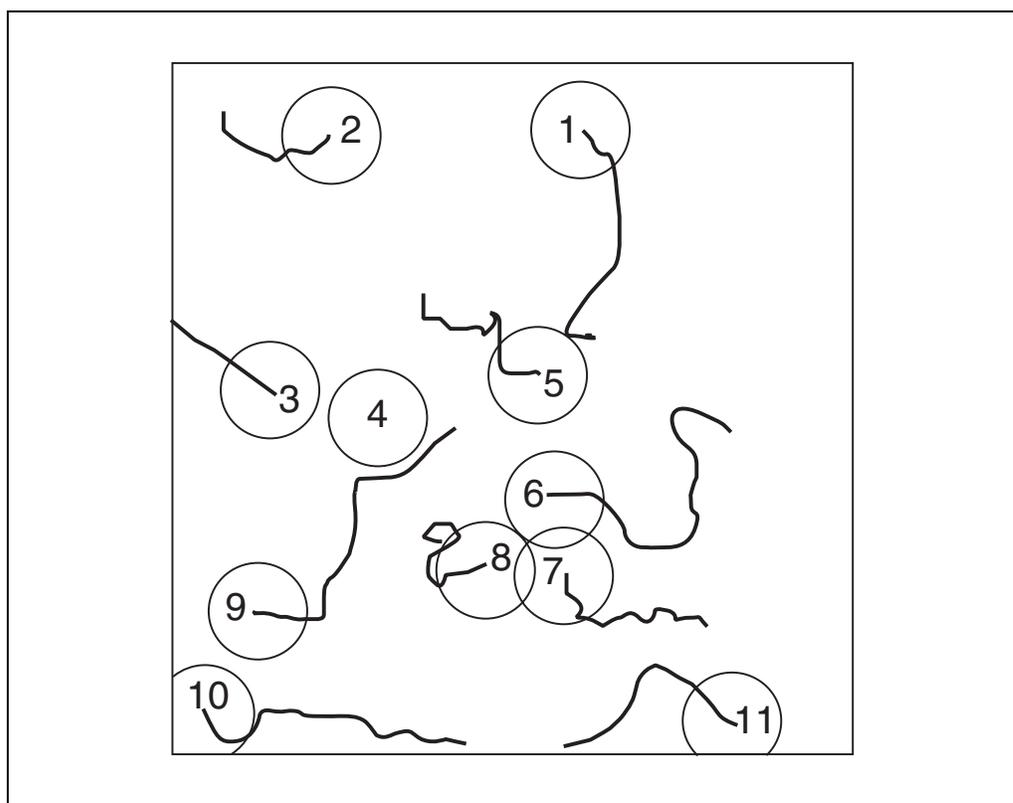


Figure 12.6.2 Tracings of cell paths. An image of a field of fMLF-stimulated neutrophils was acquired every 12 sec for 5 min (see Fig. 12.6.1C and D for initial and final images, respectively). 14- μm -diameter circles were drawn around the cells in the first image (Fig. 12.6.1C), and the positions of cell centroids were marked for each image in the sequence. Centroid positions were then connected to indicate the paths traveled by each cell.

Table 12.6.2 Possible Phenotypes of Neutrophils Observed in Optical Microscopy–Based Migration Assays

Phenotype	Potential cause	Reference
Prominent, elongated uropod (Fig. 12.6.4B)	Inability to detach from substratum. Cells with this defect will make attempts to move forward, but will usually “snap back” to their starting positions because their rears are tightly attached to the substratum. Inability to retract tail. Cells with this defect will move forward slowly, dragging along their elongated tails.	Hendey and Maxfield (1993); Marks et al. (1991); Pierini et al. (2000)
Few or no cells on dish, but remaining cells are still polarized	Adhesion inhibited. Neutrophils do not require adhesive interactions to polarize. Separate polarization and adhesion assays can be performed to verify conclusions.	Graham and Brown (1991); Wilkinson (1998)
Cells remain symmetrical in shape, without a discernible front or rear (Fig. 12.6.4D,F)	Inhibition of cell contraction. Cells with this defect can send out membrane protrusions, but cannot undergo the contractions required to form a polarized morphology. Inhibition of membrane extension. Cells with this defect may look as if they are unstimulated.	Eddy et al. (2000)

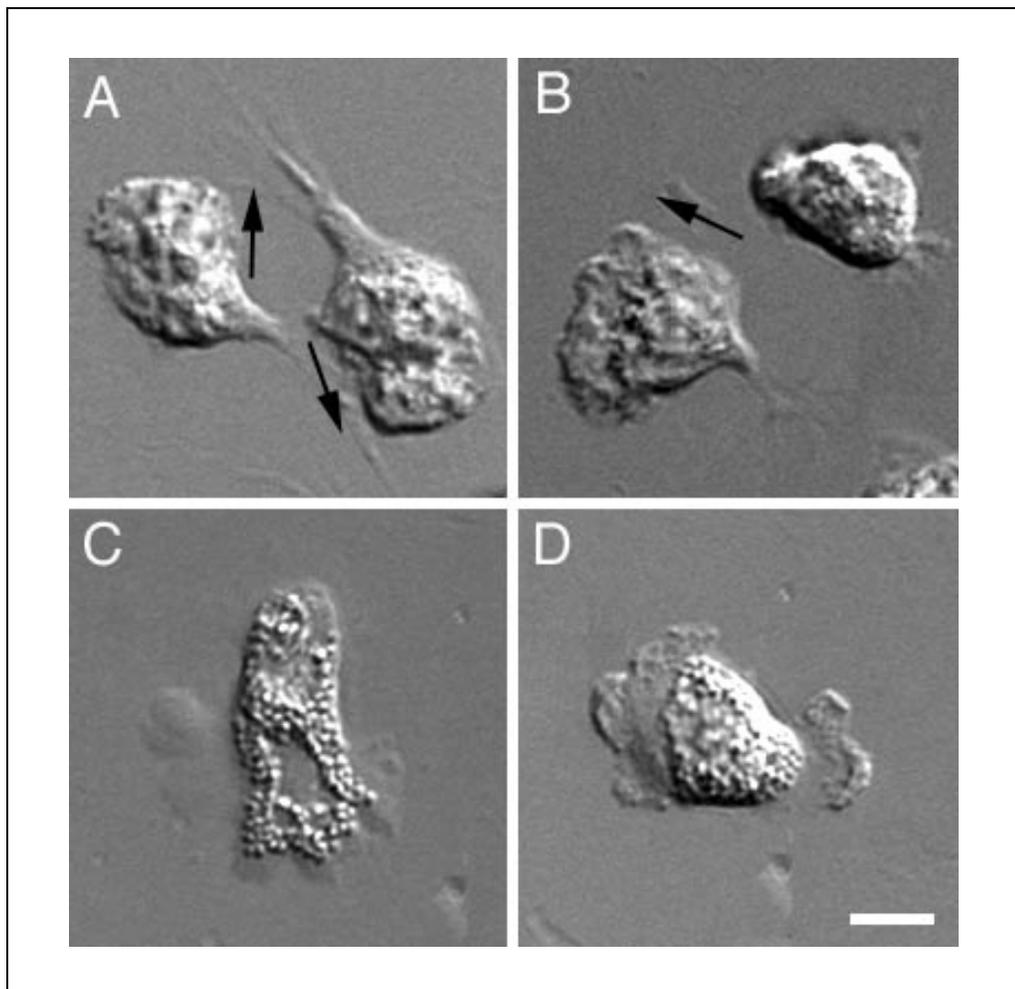


Figure 12.6.3 Comparison of eosinophil and neutrophil morphology. Differential interference contrast images of neutrophils (A, B) and eosinophils (C, D) are shown. Note the large refractile granules in the eosinophils. Arrows indicate direction of neutrophil migration. Bar is equal to 10 μm .

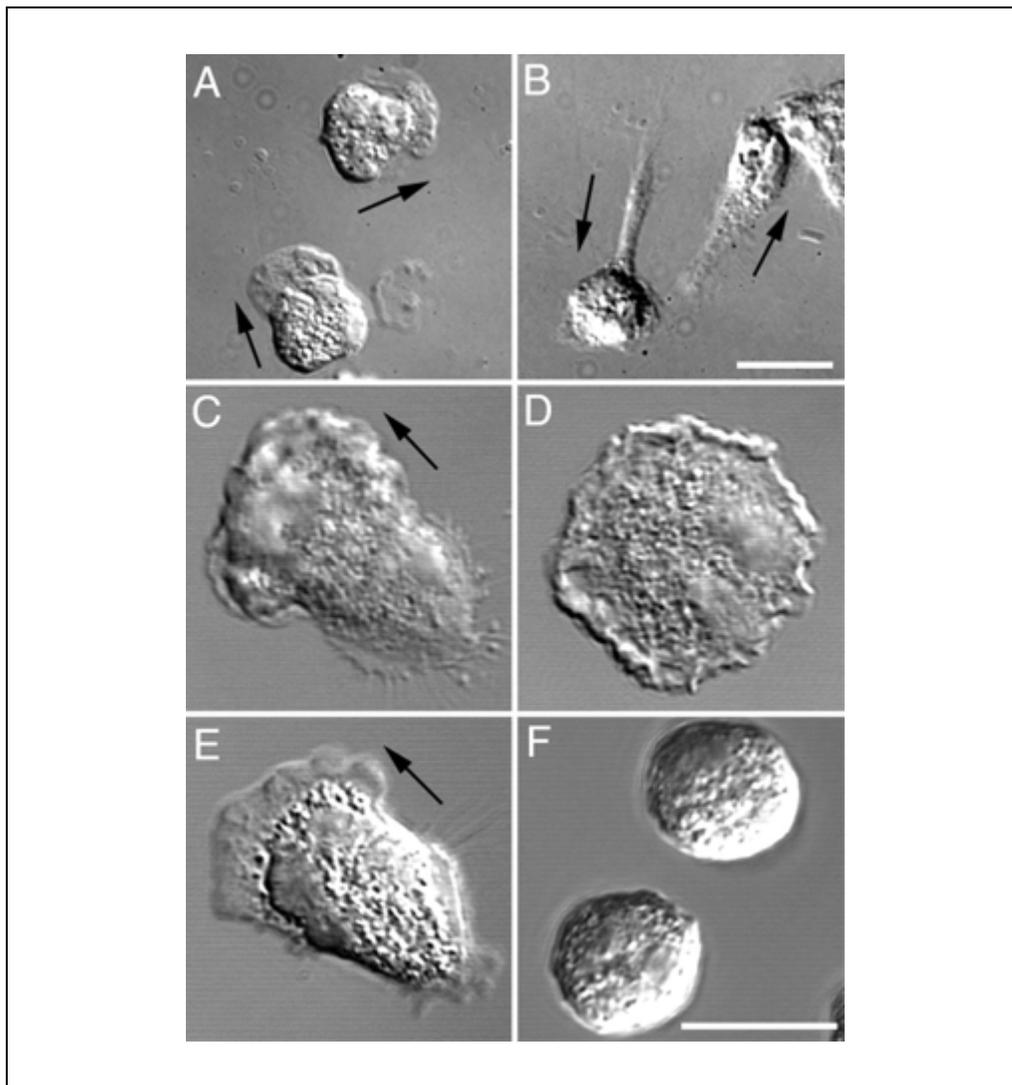


Figure 12.6.4 Examples of phenotypes resulting from various treatments of neutrophils. Images of control (**A, C, E**) and treated (**B, D, F**) neutrophils are shown after 2 min stimulation with fMLF. When intracellular calcium is buffered, neutrophils can polarize, but they cannot migrate because they are unable to detach from the substratum (Hendey and Maxfield, 1993; Marks et al., 1991; Pierini et al., 2000). Note the prominent elongated tails in panel B. In contrast, when either myosin light chain kinase is inhibited (Eddy et al., 2000; panel D) or cholesterol is depleted (Pierini and Maxfield, unpub. observ.; panel F), neutrophils can neither polarize nor migrate. These images make it evident that the underlying cause for each of these migration defects is different (see Table 12.6.2). Arrows indicate direction of neutrophil migration. Bars are equal to 10 μm . Images in panels C and D courtesy of Dr. Robert J. Eddy.

Troubleshooting

Table 12.6.1 provides a list of problems that can arise in the neutrophil migration assay, along with their possible causes and solutions.

Anticipated Results

Neutrophils go through a sequence of morphological changes following activation (Seveau et al., 2001; Zigmond and Sullivan, 1979; Fig. 12.6.1). When neutrophils are unstimulated, they are very nearly spherical in shape (Fig. 12.6.1A) and do not adhere tightly to the coverslip. Within 15 to 30 sec after the

addition of chemoattractant, the cell periphery becomes irregular, with small membrane protrusions and retractions occurring in every direction (Fig. 12.6.1B). By 2 min, the cells take on a polarized morphology with a distinct front (lamellipod) and rear (uropod) that will orient randomly in the field when chemoattractant is present isotropically (Fig. 12.6.1C).

Less than 10% of the neutrophils should polarize and migrate spontaneously (that is, in the absence of chemoattractant), compared to 60% to 90% of the cells polarizing and migrating after addition of chemoattractant (compare

panels A and C in Fig. 12.6.1). The numbers for spontaneous and stimulated migration will vary somewhat from day to day and will depend on the donor, the basal level of activation of the isolated neutrophils, the temperature of the microscope and buffers during the assay, and other parameters such as the migration matrix and chemoattractant used.

Representative of a typical data set, panels C and D in Figure 12.6.1 show images of a field of neutrophils at 1 min and 6 min after stimulation with fMLF, respectively. Figure 12.6.2 shows the paths traveled by the cells as they moved from their starting positions in panel C to their final positions in panel D. Note that most of the cells have moved several cell lengths during the 5-min course of the assay. Though not readily apparent from this relatively short migration assay, neutrophils normally exhibit a “persistent random walk,” with long stretches of approximately straight-line runs between infrequent turns (Fig. 12.6.2; see cells 1, 3, 9, and 10).

Up to 5% of the cells in the field may be eosinophils, as opposed to neutrophils, and it is important to be able to distinguish these cells from neutrophils so that they can be removed from the analysis. Eosinophils (Fig. 12.6.3C,D) tend to have more prominent granules than neutrophils (Fig. 12.6.3A,B).

Often it is informative to evaluate the effects of a pharmacological agent or other treatment on cell migration, using visual analysis to discern the likely basis for any effects on migration. Some phenotypes that can result, along with their possible causes, are listed in Table 12.6.2 and illustrated in Figure 12.6.4. This table is not meant to be comprehensive, only illustrative of how observation of cell morphology and/or behavior can lead to a mechanistic understanding. A particular treatment may have significantly subtler effects on cell migration that may only become apparent after careful analysis of cell paths and behavior.

Time Considerations

The entire procedure, from isolation of cells (~1.25 hr) and preparation of coverslip dishes (~1 hr) to acquisition of three 4-min image series from each of ten dishes can be accomplished in ~4 hr. Coverslips and coverslip dishes can be prepared and assembled ahead of time, with the exception of coating the coverslips with extracellular matrix component, which should be done on the day of the experiment. The time required for image analy-

sis will depend on the exact nature of the analysis and whether automation is employed.

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Actin-Based Motility Assay

UNIT 12.7

This unit describes an *in vitro* assay to study actin-based motility in which the propulsion of the bacteria *Listeria* and *Shigella* or functionalized microspheres is reconstituted from five pure proteins (see Basic Protocol). The pathogens *Listeria* and *Shigella* are acknowledged models of the leading edge of motile cells. By quantitating the dependence of motility parameters on the concentrations of components of the assay, the molecular processes that support the motile behavior of living cells can be understood, predictions of different theoretical models can be evaluated, and insight into the role of newly discovered proteins in motility can be obtained. Data analysis for the assay is described in Support Protocol 1.

This unit includes support protocols for biochemical purification of profilin (see Support Protocol 2), actin-depolymerizing factor (ADF; see Support Protocol 3), gelsolin (see Support Protocol 4), neural Wiskott-Aldrich syndrome protein (N-WASP; see Support Protocol 5), and Arp2/3 complex (actin-related protein; see Support Protocol 6), and for the preparation of N-WASP-coated microspheres (see Support Protocol 7).

STRATEGIC PLANNING

Performing a successful motility assay requires 1 month of preparatory biochemistry. Preparation of the main biochemical components for the motility assay is detailed in Support Protocols 2 to 6. These components must be prepared in advance, dispensed into aliquots of appropriate size, and stored at -80°C . Two different types of animal tissue must be obtained for the various preparations: bovine brain and bovine spleen. The tissues must be obtained from a freshly slaughtered cow in a slaughterhouse and used immediately or flash-frozen. Actin purification from rabbit muscle is also required for the motility assay (see UNIT 13.2).

The biochemical purifications represent a challenging task. The reader should also note that several proteins used in the assay (actin, rhodamine-labeled actin, and Arp2/3) are now commercially available (Cytoskeleton, Inc).

Motility assays are performed under optimal conditions using functionalized microspheres. Such particles can indeed be coated with N-WASP (see Support Protocol 7) or ActA in a chemically controlled and reproducible fashion. Use of bacteria (*Listeria* or *Shigella*) is recommended in restricted cases where *Listeria* strains mutated in ActA or *Shigella* strains mutated in IcsA (the bacterial activator of N-WASP) are to be examined.

ACTIN-BASED MOTILITY ASSAY

This protocol describes the execution of an assay that reconstitutes the propulsion of *Listeria*, *E. coli* expressing the *Shigella* protein IcsA, or N-WASP-functionalized microspheres in a minimum motility medium containing Arp2/3 complex, ADF, gelsolin, profilin, and actin (Loisel et al., 1999).

Materials

- Profilin (see Support Protocol 2)
- ADF (see Support Protocol 3)
- Gelsolin (see Support Protocol 4) or other capping protein
- WASP (for use with *Listeria* or ActA-functionalized microspheres only; prepare as for N-WASP, see Support Protocol 5)

**BASIC
PROTOCOL**

Cell Motility

12.7.1

Contributed by Christophe Le Clainche and Marie-France Carlier

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Supplement 24

- Arp2/3 complex *or* Alexa488-Arp2/3 complex (see Support Protocol 6)
 Assay buffer (see recipe) containing 1% (w/v) bovine serum albumin (BSA)
 10% (w/v) bovine serum albumin (BSA) in distilled water
 5 μM α -actinin (Sigma) in distilled H₂O
 48 μM Mg-F-actin (see recipe)
 1% (w/v) methylcellulose (see recipe)
 ATP/DABCO/DTT mix (see recipe)
 40 μM rhodamine-G-actin (Cytoskeleton, Inc.) in assay buffer/1% BSA (store up to 1 month at -80°C)
 4×10^9 microspheres/ml suspension of N-WASP-functionalized microspheres (see Support Protocol 7) *or* 4×10^9 bacteria/ml suspension of *Listeria* or *E. coli* IcsA in assay buffer/1% BSA
 Valap (UNIT 13.1)
 Glass slides (Superfrost Plus, Fisher, or Menzel-Gläser; $25 \times 75 \times$ /mm)
 Glass coverslips (22×22 mm)
 Microscope, phase-contrast or fluorescence (with appropriate filters; UNIT 4.2)
1. Rapidly thaw ADF, profilin, Arp2/3 complex, gelsolin, and VASP, and dilute to the appropriate stock concentrations (see Table 12.7.1) with assay buffer/1% BSA.
 2. Combine the components of the actin-based motility assay in a 0.5 ml tube in the (top-to-bottom) order indicated in Table 12.7.1.

Table 12.7.1 Components of Actin-Based Motility Assay

Ingredient ^a	Stock concentration	Volume added	Final concentration
BSA	10% (w/v)	1.2 μl	0.5%
ADF	55 μM	1.6 μl	3.7 μM
Profilin	50 μM	1.2 μl	2.5 μM
Gelsolin ^b	0.5 μM	2.4 μl	50 nM
α -Actinin	5 μM	1.2 μl	0.25 μM
Arp2/3 ^c	1 μM	1.8 μl	75 nM
VASP ^d	8 μM	1.4 μl	0.47 μM
Mg-F-actin	48 μM	3.8 μl	7.6 μM
Methylcellulose	1% (w/v)	5.4 μl	0.23%
ATP/DABCO/DTT mix	see Reagents and Solutions	3.0 μl	—
Rhodamin-G-actin	40 μM	0.6 μl	1 μM
N-WASP functionalized microspheres or <i>Listeria</i> or <i>E. coli</i> IcsA	4×10^9 microspheres or bacteria per ml	0.6 μl	2×10^8 microspheres or bacteria/ per ml
Final volume		24 μl	

^aAbbreviations: Arp2/3, actin-related protein; ADF, actin-depolymerizing factor; ATP, adenosine triphosphate; BSA, bovine serum albumin; DABCO, 1,4.-diazobicyclo[2,2,2]octane; DTT, dithiothreitol; N-WASP, neural Wiskott-Aldrich syndrome protein; VASP, vasodilator-stimulated phosphoprotein.

^bOther capping proteins may be used.

^cAlexa488-Arp2/3 (also prepared as in Support Protocol 6) may be used.

^dAdd only for assays using *Listeria* or ActA-functionalized microspheres in place of the N-WASP functionalized microspheres (see Strategic Planning).

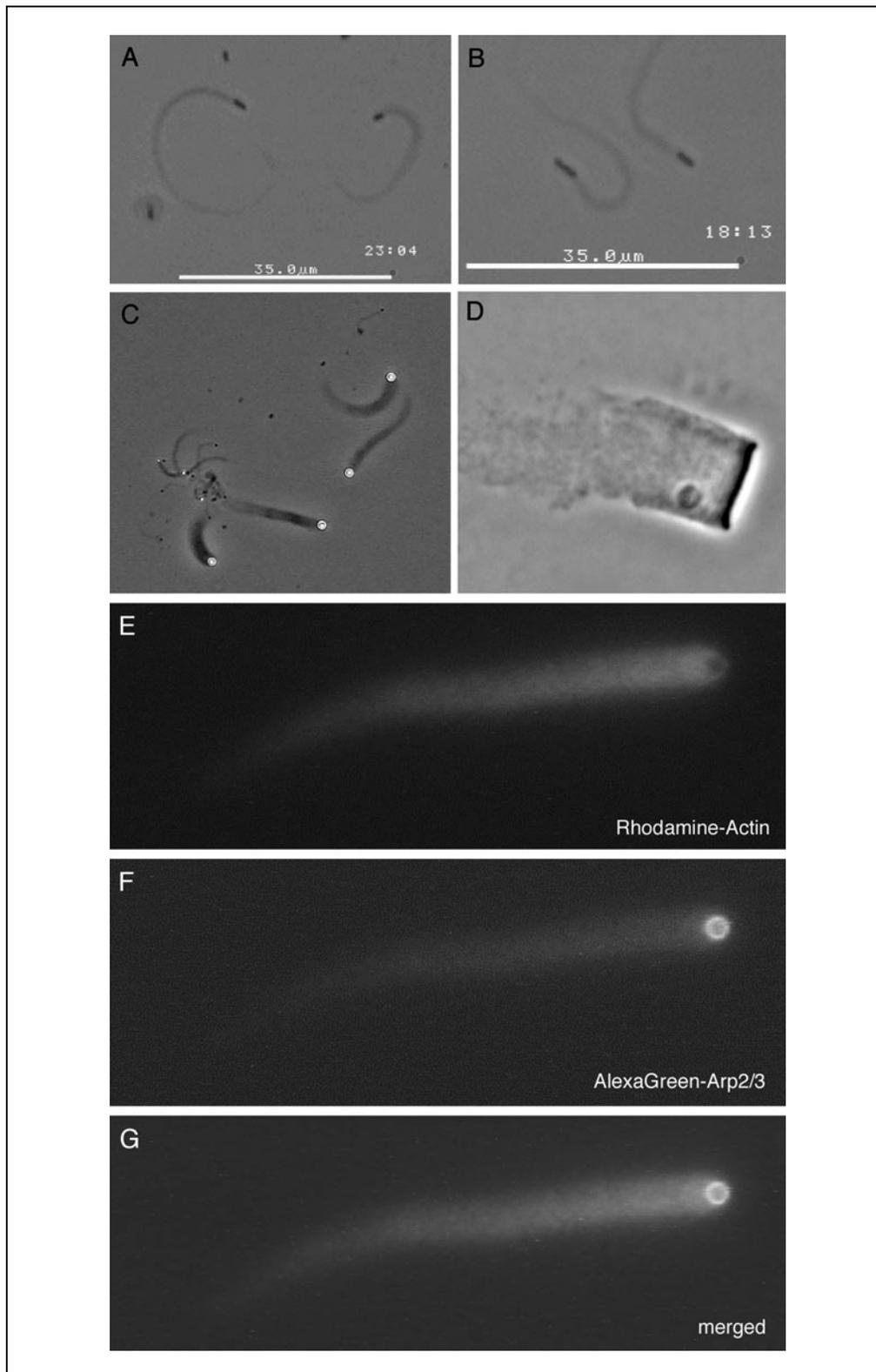


Figure 12.7.1 Examples of actin-based motility of bacteria or functionalized particles in the reconstituted motility assay. Synchronous films of up to four selected fields per chamber were recorded with a CCD camera on an Olympus AX70 microscope equipped with a motorized stage (Märzhäuser) using MetaMorph 4.6 for microscope control and image acquisition. **(A)** *Listeria monocytogenes*; **(B)** *E. coli lcsA* (substitute for *Shigella flexneri*); **(C)** N-WASP-coated polystyrene beads of different diameters; **(D)** N-WASP-coated glass rod (1 μm in diameter); **(E, F, G)** double-label experiment using N-WASP-coated beads in motility assay containing rhodamine-labeled actin and Alexa488-labeled Arp2/3 (from Wiesner et al., 2003). Both the surface of the bead and the tail are labeled by Arp2/3.

Because high concentrations of methylcellulose can cause excessive filament bundling, all components excluding methyl cellulose and F-actin must be mixed first.

Although α -actinin cross-links actin filaments and increases the density of the actin tail, this protein is not essential and can be omitted.

If the pipetting scheme given in Table 12.7.1 is modified (e.g., to vary the concentration of some component), the resulting change in volume of the assay should be such that all concentrations of other protein, BSA, and nonprotein components remain unchanged. To make up the final volume use assay buffer/1% BSA.

3. Pipet 3 μ l of the mixture on a glass slide and cover with a coverslip. Seal the preparation with Valap.

Be careful to avoid introducing bubbles between the slide and the coverslip.

For microspheres of diameter $>5 \mu\text{m}$, do not compress the coverslip onto the slide; rather, use a larger sample volume and chamber slide to avoid sticking of the bead to the glass surfaces of the slide and coverslip.

4. Observe under a phase-contrast or fluorescence microscope at room temperature (see Anticipated Results and Figure 12.7.1). Record up to four selected fields per chamber with a CCD camera, using MetaMorph 4.6 for microscope control and image acquisition. Analyze data (Support Protocol 1).

When optimal conditions are used, the actin tails can be observed in phase contrast using a $20\times$ phase-contrast objective (NA 0.5). Fluorescence is useful to colocalize a fluorescent protein like Alexa488-labeled Arp2/3 with the fluorescent rhodamine-actin tail.

If the rhodamine-labeled actin and/or Alexa488-labeled Arp2/3 photobleach rapidly, the concentrations of DABCO, an oxygen-scavenging component, and DTT, a reducing agent, should be checked (see Reagents and Solutions, recipe for ATP/DABCO/DTT mix).

SUPPORT PROTOCOL 1

DATA ANALYSIS

The reconstituted motility assay can be used as an analytical tool. Parameters that have been exploited are speed, percentage of motile particles, length of the actin tail, and density of actin and Arp2/3 in the actin tail.

Average rates of movement are determined by selecting freely moving microspheres from several different fields in the motility assay recordings. For microspheres of 0.5- μm diameter and larger, the template recognition-based tracking tool of MetaMorph (Universal Imaging) is used to measure mean velocities. In this assay, 100% of the beads move. Generally, when bacteria are used (see Strategic Planning), less than 100% of the bacteria move. The fraction of motile bacteria, in this case, is an additional parameter that should be considered in characterizing the motile behavior.

To determine F-actin and Arp2/3 densities in the actin tails, first, rhodamine-labeled actin and Alexa488-labeled Arp2/3 are used in the motility assays, and their respective fluorescence intensities are recorded with a LHESA 72LL CCD camera using appropriate filters and a 100 objective (NA 1.35). The extent of Arp2/3 labeling (100%) and of actin labeling (10%) is chosen to yield intensities in the linear response range of the camera for both fluorophores. Control experiments are performed to make sure that no emission overlap exists between the two fluorophores. Images of comets are recorded at steady state, and average emission intensities of rhodamine (IR) and Alexa488 (IA) are measured as mean gray values in a square section of the actin tail at least one bead diameter behind the bead. An average IR/IA ratio is determined from sets of at least five microspheres. To estimate the absolute stoichiometry of actin and Arp2/3 from these values, a calibration

is performed using solutions of the two fluorescent proteins at different concentrations (Wiesner et al., 2003).

The percentage of motile microspheres is determined manually by counting the ratio of motile particles to total particles. Only central regions are used because the edge of the preparation displays significant differences in the percentage of motile microspheres.

PROFILIN PURIFICATION

Recombinant profilin from different sources or profilin purified from bovine spleen can be used. Here, the purification of profilin from bovine spleen (Perelroizen et al., 1996) is described.

Materials

Bovine spleen (see Strategic Planning)

Profilin buffer A (see recipe) containing 0.5% (v/v) Triton X-100

200 mM PMSF stock in absolute ethanol

4 M and 7 M urea in profilin buffer A (see recipe for buffer)

Profilin buffer B (see recipe)

Liquid nitrogen

Centrifuge with Sorvall SLA 1500 rotor (or equivalent)

Cheesecloth

Ultracentrifuge with Sorvall A641 rotor (or equivalent)

15-cm length \times 2.5-cm inner diameter poly-L-proline-Sepharose column
(see recipe)

10,000-kDa MWCO dialysis membrane

Centriprep 10 centrifugal concentrator (Millipore; also see *APPENDIX 3C*)

Ultracentrifuge with Beckman TLA 100.3 rotor (or equivalent)

Additional reagents and equipment for dialysis and concentration of protein solutions (*APPENDIX 3C*) and determination of protein concentration (*APPENDIX 3B*)

Prepare tissue homogenate

1. Homogenize 240 g of frozen spleen in 350 ml of profilin buffer A/0.5 % Triton X-100. Add PMSF (from 200 mM stock) to 1 mM final and homogenize 45 sec.
2. Centrifuge 45 min at $22,000 \times g$ (12,000 rpm in a Sorvall SLA 1500 rotor), 4°C. Filter the supernatant through eight layers of cheesecloth.
3. Centrifuge the filtered supernatant for 1 hr and 45 min at $200,000 \times g$ (39,000 rpm in a Sorvall A641 rotor), 4°C. Filter again through eight layers of cheesecloth.

Purify by affinity chromatography

4. Add the sample to a 15-cm length \times 2.5-cm inner diameter poly-L-proline-Sepharose column equilibrated with profilin buffer A. Wash the column with 400 ml profilin buffer A.
5. Elute actin with 250 ml 4 M urea in profilin buffer A, monitoring absorbance at 280 nm.
6. Elute profilin with 250 ml 7 M urea in profilin buffer A, monitoring absorbance at 280 nm.
7. Wash the column with 500 ml water.

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Purify by dialysis

8. Combine fractions containing protein eluted by 7 M urea and dialyze 18 hr against two changes of 1000 ml profilin buffer B, using a 10,000-kDa MWCO dialysis membrane (*APPENDIX 3C*).
9. Concentrate dialyzed protein on Centriprep 10 centrifugal concentrator according to the manufacturer's instructions.
10. Dialyze again once for 18 hr against 1000 ml profilin buffer B, using a 10,000-kDa MWCO dialysis membrane (*APPENDIX 3C*).
11. Ultracentrifuge 15 min at $334,000 \times g$ (90,000 rpm in a Beckman TLA 100.3 rotor), 4°C. Retain supernatant.
12. Measure the protein concentration of the supernatant using UV absorbance (*APPENDIX 3B*). Freeze in liquid nitrogen in 0.1-ml aliquots and store at -80°C .

Profilin can be stored for months at -80°C . Thawed aliquots can be stored on ice for a few weeks without loss of activity.

ADF PURIFICATION

Bacterial recombinants of ADFs from different sources (plant, amoeba, and vertebrate) have been used successfully in the motility medium. Here the purification of recombinant human ADF expressed in *E. coli* (Carlier et al., 1997) is described.

Materials

- E. coli* BL21 DE3 strain transformed with pET16-ADF (see *APPENDIX 3A* for transformation of *E. coli* with plasmid)
- LB medium (*APPENDIX 2A*) containing 0.2 mg/ml ampicillin
- ADF buffers A, B, C, D, and E (see recipes)
- 10 mg/ml lysozyme
- 250 mM EDTA
- 200 mM PMSF in absolute ethanol
- 1 M MgCl_2
- 2 mg/ml DNase I
- Liquid nitrogen
- Centrifuge with Sorvall GSA rotor (or equivalent)
- Probe sonicator (Fisher)
- Centrifuge with Sorvall SS-34 rotor (or equivalent)
- 10,000-kDa MWCO dialysis membrane
- Ultracentrifuge with Beckman TL-100 rotor (or equivalent)
- DEAE (DE-52; Whatman) column (20×2.5 cm; *UNIT 10.11*)
- Stirred cell with Diaflo PM 10 ultrafiltration membrane (Amicon)
- SP trisacryl column (15×1.5 cm; BioSeptra)
- Additional reagents and equipment for transforming and growing bacteria and induction of protein expression with IPTG (*APPENDIX 3A*), dialysis (*APPENDIX 3C*), and determination of protein concentration (*APPENDIX 3B*)

Express the protein and lyse the cells

1. Grow *E. coli* BL21 DE3 strain transformed with pET16-ADF in LB medium containing 0.2 mg/ml ampicillin at 37°C . At $\text{OD} = 1$, add 0.5 mM IPTG to induce ADF expression. Incubate 2 hr and 30 min at 37°C .

APPENDIX 3A provides cross-references for the molecular biology techniques used in this step.

2. Centrifuge 10 min at $4000 \times g$ (5000 rpm in a Sorvall GSA rotor), 4°C. Resuspend pellet in 100 ml ADF buffer A. Rapidly freeze-thaw the homogenate.
3. Add 1 ml of 10 mg/ml lysozyme. Incubate 5 min, then add 1 ml of 250 mM EDTA and incubate an additional 5 min.
4. Add 250 μ l of 200 mM PMSF. Sonicate three times, each time for 1 min at 150 W power in a probe sonicator on ice.
5. Add 500 μ l of 1 M $MgCl_2$ and 1 ml of 2 mg/ml DNase I. Incubate 5 min at room temperature.

Purify the protein

6. Centrifuge 30 min at $17,000 \times g$ (12,000 rpm in a Sorvall SS-34 rotor), 4°C.
7. Dialyze supernatant overnight at 4°C against 1000 ml of ADF buffer B using a 10,000-kDa MWCO dialysis membrane (*APPENDIX 3C*).
8. Ultracentrifuge 45 min at $100,000 \times g$, 4°C.
9. Load the sample onto a 20×2.5 -cm size DEAE column. Elute with ADF buffer B. Collect fractions at 2 ml/min and 2 min/fraction.
10. Concentrate the flowthrough to 10 ml by ultrafiltration in a stirred cell with a Diaflo PM 10 membrane.
11. Dialyze overnight at 4°C against 1000 ml ADF buffer C using a 10,000-kDa MWCO dialysis membrane (*APPENDIX 3C*).
12. Load the sample onto a 1.5×15 size SP trisacryl column. Elute with 100 ml ADF buffer D. Collect fractions at 1 ml/min and 2 min/fraction.
13. Concentrate to 3 ml and dialyze overnight at 4°C against 1000 ml ADF buffer E using a 10,000-kDa MWCO dialysis membrane (*APPENDIX 3C*).
14. Ultracentrifuge the dialysate 15 min at $313,000 \times g$ (90,000 rpm in a Beckman TL-100 rotor), 4°C.
15. Measure the concentration using UV absorbance (*APPENDIX 3B*). Freeze 0.1-ml aliquots in liquid nitrogen and store at $-80^\circ C$.

ADF can be stored for months at $-80^\circ C$. Thawed aliquots can be stored on ice for a few weeks without loss of activity.

GELSOLIN PURIFICATION

Although in the original protocol published by Loisel et al. (1999) Capping Protein was used, further assays showed that any other capping protein can work as well as Capping Protein, i.e., the general barbed-end capping function is required for motility. In particular, gelsolin, which can easily be obtained as a bacterially expressed recombinant protein, supports motility.

Materials

E. coli BL21 DE3 strain expressing gelsolin (see *APPENDIX 3A* for transformation of *E. coli*)

Gelsolin buffers A, B, C, and D (see recipes)

1 M $MgCl_2$

2 mg/ml DNase I

500 mM NaCl in gelsolin buffer B (see recipe for buffer)

Anti-gelsolin antibody (Sigma)

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500 mM NaCl in gelsolin buffer C (see recipe for buffer)

Liquid nitrogen

Probe sonicator (Fisher)

Refrigerated centrifuge

Centriprep 30 centrifugal concentrator

30,000-kDa MWCO dialysis membrane

2.5 × 20-cm DEAE-cellulose (DE-52; Whatman) column (UNIT 10.11)

Gradient maker

11 × 1.25-cm SP trisacryl column (BioSeptra)

Additional reagents and equipment for transforming and growing bacteria and induction of protein expression with IPTG (APPENDIX 3A), dialysis (APPENDIX 3C), and determination of protein concentration (APPENDIX 3B), and immunodetection (UNIT 6.2)

1. Grow *E. coli* BL21 DE3 expressing gelsolin (see APPENDIX 3A for molecular biology techniques).
2. Add 100 ml gelsolin buffer A per liter of bacteria grown. Incubate 10 min at 4°C, then sonicate five times, each time for 1 min at 150 W power.
3. Add MgCl₂ from 1 M stock to a final concentration of 5 mM and 2 mg/ml DNase I to a final concentration of 0.02 mg/ml. Incubate 10 min at 4°C.
4. Centrifuge 30 min at 18,000 × g, 4°C.
5. Dialyze overnight at 4°C against 1000 ml gelsolin buffer B using a 30,000-kDa MWCO dialysis membrane (APPENDIX 3C).
6. Add the sample to a DEAE column equilibrated in gelsolin buffer B. Prepare a 1-liter, 0 to 500 mM linear NaCl gradient by placing 500 ml gelsolin buffer B in one compartment of a gradient maker and 500 ml of 500 mM NaCl (in gelsolin buffer B) in the other compartment. Apply the gradient to elute.
7. Identify the gelsolin-containing fractions by immunodetection (UNIT 6.2) using anti-gelsolin antibody. Combine desired fractions, then concentrate using a Centriprep 30 centrifugal concentrator and dialyze overnight at 4°C against 1000 ml gelsolin buffer C using a 30,000-kDa MWCO dialysis membrane (APPENDIX 3C).
8. Add the sample to an 11 × 1.25-cm SP trisacryl column equilibrated in gelsolin buffer C. Prepare a 300-ml, 0 to 500 mM linear NaCl gradient by placing 150 ml gelsolin buffer C in one compartment of the gradient maker and 150 ml of 500 mM NaCl (in gelsolin buffer C) in the other compartment. Apply the gradient to elute.
9. Identify the gelsolin-containing fractions by immunodetection (UNIT 6.2) using anti-gelsolin antibody. Combine desired fractions and dialyze overnight at 4°C against 1000 ml gelsolin buffer D. Freeze in liquid nitrogen and store at -80°C.

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N-WASP PURIFICATION

His-tagged human N-WASP is expressed in *Sf9* cells using the baculovirus system (Egile et al., 1999). His-tagged human VASP (see Basic Protocol and Table 12.7.1 for applications) is purified using the same protocol (Laurent et al., 1999).

Materials

Insect (*Sf9*) cells expressing N-WASP (see APPENDIX 3A for transformation of insect cells)

Phosphate-buffered saline (PBS; APPENDIX 2A)

PBS (*APPENDIX 2A*) containing 0.5 mM PMSF (add from 200 mM PMSF stock in absolute ethanol)
N-WASP buffers A, B, C, D, E, and F (see recipes)
NaCl
Imidazole
50% (w/v) suspension of Ni-NTA resin (Qiagen)
Liquid nitrogen
Cell scraper
Clinical (tabletop) centrifuge
Dounce homogenizer
Probe sonicator (Fisher)
Ultracentrifuge with Beckman TL-100 rotor (or equivalent)
2.5 × 20-cm DEAE-cellulose (DE-52; Whatman) column (*UNIT 10.11*)
1 × 5 cm, 10-ml chromatography column
30,000-kDa MWCO SpectraPor 2 dialysis membrane (Spectrum)
Additional reagents and equipment for protein expression in insect cells (*APPENDIX 3A*) and dialysis (*APPENDIX 3C*)

1. Collect 5×10^8 insect cells with a cell scraper in PBS. Centrifuge 5 min at $500 \times g$ (2000 rpm in a clinical centrifuge), room temperature.
2. Wash by adding 100 ml PBS containing 0.5 mM PMSF and centrifuging again as described above. Remove supernatant.
3. Resuspend cells in 10 ml N-WASP buffer A. Homogenize in a Dounce homogenizer, then sonicate three times, each time for 1 min at lowest power.
4. Ultracentrifuge 40 min at $334,000 \times g$ (90,000 rpm in a Beckman TL-100 rotor), 4°C.
5. Dilute the supernatant to obtain 0.1 M NaCl. Add the sample to a DEAE column equilibrated in N-WASP buffer B.
6. Save the flowthrough, adjust the NaCl concentration up to 0.3 M, and add imidazole to a final concentration of 10 mM.
7. Equilibrate 4 ml of a 50% suspension of agarose Ni-NTA resin with 30 ml N-WASP buffer C.
8. Mix the DEAE flowthrough and the Ni-NTA resin for 1 hr at 4°C on an end-over-end rotator. Load the sample/resin onto a 10-ml column and wash twice, each time with 4 ml N-WASP buffer D. Elute by adding ten 0.4-ml aliquots of N-WASP buffer E.
9. Dialyze eluate twice, each time for 30 min at 4°C in 500 ml N-WASP buffer F using a 30,000-kDa MWCO SpectraPor2 dialysis membrane (*APPENDIX 3C*). Freeze in liquid nitrogen and store at -80°C .

N-WASP can be stored for months at -80°C . Thawed aliquots kept on ice can be used for motility for a week.

ARP2/3 PURIFICATION

Arp2/3 can be purified from a variety of sources, including amoeba (Mullins et al., 1998) and vertebrate tissues like thymus (Higgs et al., 1999) or brain (Egile et al., 1999). It is also possible to purify recombinant Arp2/3 expressed in insect cells. Here, the purification of Arp2/3 from bovine brain (Egile et al., 1999) is described.

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Materials

Bovine brain (see Strategic Planning)
Arp2/3 buffers A, B, C, D, E, F, G, and H (see recipes)
2 M sucrose
100 μ M Alexa488-C5-maleimide (Molecular Probes) in Arp2/3 buffer H (see recipe for buffer)
DTT
Hammer
Waring blender
Refrigerated centrifuge
30,000-kDa MWCO dialysis membrane
SP trisacryl column (4 \times 30 cm; BioSeptra)
Sephacrose GSH-GST-VCA as described by Egile et al. (1999) 2-ml column containing
Disposable NAP-10 column (Amersham Biosciences)
Additional reagents and equipment for dialysis (APPENDIX 3C) and determination of protein concentration (APPENDIX 3B)

Purify Arp2/3 complex

1. Break 300 g frozen bovine brain in small pieces using a hammer and mix with 600 ml Arp2/3 buffer A at 4°C. Homogenize in a Waring blender three times at high speed, each time for 20 sec.
2. Centrifuge the homogenate 1 hr at 100,000 \times g, 4°C.
3. Dialyze the supernatant overnight at 4°C in 4 liters Arp2/3 buffer B using a 30,000-kDa MWCO dialysis membrane.
4. Centrifuge the supernatant 30 min at 100,000 \times g, 4°C.
5. Load the supernatant onto a 4 \times 30-cm SP trisacryl column equilibrated with Arp2/3 buffer B. Wash the resin with 5 volumes of Arp2/3 buffer B, wash again with 150 ml Arp2/3 buffer C, then elute with 150 ml Arp2/3 buffer D.
6. Dialyze the peak fractions 18 hr against 500 ml of Arp2/3 buffer E using a 30,000-kDa MWCO dialysis membrane (APPENDIX 3C).
7. Load the dialysate on a 2-ml column containing 1 ml of Sepharose-GSH-GST-VCA equilibrated in Arp2/3 buffer E.
8. Wash with 10 ml Arp2/3 buffer F, then elute with 10 ml Arp2/3 buffer G.
9. Combine the fractions showing absorbance at 290 nm and dialyze 18 hr against 1000 ml of Arp2/3 buffer F using a 30,000-kDa dialysis membrane.
10. Adjust the concentration of the solution to 200 mM sucrose using a 2 M sucrose stock solution. Measure protein concentration. Freeze in liquid nitrogen and store at -80°C .

Arp2/3 can be stored for months at -80°C . Thawed aliquots kept on ice can be used for 2 days.

The activity of Arp2/3 can be monitored during the purification by measuring the stimulation of actin assembly using pyrene fluorescence as a probe. In such an assay, the cuvette contains 2.5 μ M G-actin (10% pyrenyl-labeled), 0.5 μ M VCA, and an aliquot of the fraction to be tested, in polymerization buffer (see recipe).

Fluorescently label Arp2/3 complex (optional)

11. Filter Arp2/3 complex through a NAP-10 column equilibrated with Arp2/3 buffer H, to remove DTT.
12. Incubate Arp2/3 complex with 100 μ M Alexa488-C5-maleimide in Arp2/3 buffer H for 1 hr on ice, then add DTT to 1 mM to stop the labeling.
13. Add the sample to a NAP-10 column to separate the labeled protein from the free unreacted fluorophore. Elute with Arp2/3 buffer F. Keep Alexa488-Arp2/3 on ice and use within 3 days.

Determine the labeling stoichiometry from the UV-visible spectrum of the labeled protein.

PREPARATION OF N-WASP-FUNCTIONALIZED MICROSPHERES

The successful reconstitution of actin-based motility of *Listeria* and *Shigella* opened the way for the design of a biomimetic system using carboxylated polystyrene microspheres functionalized with an activator of the Arp2/3 complex (N-WASP or ActA) and placed in the minimum motility medium. This protocol describes the procedure to functionalize the beads with N-WASP (Wiesner et al., 2003). The same procedure can be used to make ActA-coated beads (Samarin et al., 2003).

Materials

Polystyrene carboxylated microspheres of diameters from 0.2 to 3 μ m
(Polysciences)
N-WASP buffers A and C (see recipes)
4 μ M N-WASP (see Support Protocol 5)
10 mg/ml BSA
Refrigerated centrifuge

1. Incubate polystyrene carboxylated microspheres of diameters from 0.2 to 3 μ m in N-WASP buffer A containing 400 nM N-WASP (added from 4 μ M stock) for 1 hr on ice.

To prepare ActA-functionalized microspheres, use the same protocol but incubate the microspheres with 0.7 μ M full-length ActA.

The quantity of microspheres in a 50- μ l suspension is adjusted so that the total surface of solid per unit volume of solution was 7.5×10^9 μ m²/ml, independent of the diameter of the microspheres.

2. Add 5 μ l of 10 mg/ml BSA to block free adsorption sites and incubate 15 min on ice.
3. Centrifuge the microspheres 5 min at $10,000 \times g$, 4°C, then wash twice, each time by adding 250 μ l N-WASP buffer A and centrifuging 5 min at $10,000 \times g$, 4°C. Resuspend final pellet in 50 μ l N-WASP buffer C containing 1 mg/ml BSA.

Functionalized microspheres have been stored on ice and used for up to 1 week without any change in their motile properties.

REAGENTS AND SOLUTIONS

Use deionized distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

ADF buffer A

10 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
1 mM EDTA
Store up to 1 week at 4°C

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ADF buffer B

10 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
50 mM NaCl
5 mM DTT
0.2 mM EGTA
0.01% (w/v) NaN₃
Store up to 1 week at 4°C

ADF buffer C

10 mM PIPES, pH 6.5
25 mM NaCl
5 mM DTT
0.2 mM EGTA
0.01% (w/v) NaN₃
Store up to 1 week at 4°C

ADF buffer D

10 mM PIPES, pH 6.5
50 mM NaCl
5 mM DTT
0.2 mM EGTA
0.01% (w/v) NaN₃
Store up to 1 week at 4°C

ADF buffer E

5 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 mM DTT
0.01% (w/v) NaN₃
Store up to 1 week at 4°C

Arp2/3 buffer A

10 mM PIPES, pH 6.8
5 mM EGTA
1 mM DTT
0.1 mM PMSF added just before use
Store up to 1 week at 4°C

Arp2/3 buffer B

20 mM PIPES pH 6.8
1 mM DTT
Store up to 1 week at 4°C

Arp2/3 buffer C

20 mM PIPES, pH 6.8
30 mM KCl
1 mM DTT
Store up to 1 week at 4°C

Arp2/3 buffer D

20 mM PIPES, pH 6.8
100 mM KCl
1 mM DTT
Store up to 1 week at 4°C

Arp2/3 buffer E

20 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
25 mM KCl
0.25 mM DTT
0.1 mM ATP
1 mM MgCl₂
0.5 mM EDTA
Store up to 1 week at 4°C

Arp2/3 buffer F

20 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
25 mM KCl
0.25 mM DTT
10 μM ATP
1 mM MgCl₂
0.5 mM EDTA
Store up to 1 week at 4°C

Arp2/3 buffer G

20 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
25 mM KCl
0.25 mM DTT
10 μM ATP
200 mM MgCl₂
0.5 mM EDTA
Store up to 1 week at 4°C

Arp2/3 buffer H

20 mM HEPES, pH 7.2
0.2 mM MgCl₂
0.2 mM ATP
Store up to 1 week at 4°C

Assay buffer

10 mM HEPES pH 7.5
0.1 M KCl
1 mM MgCl₂
0.1 mM CaCl₂
1 mM ATP
Store up to 1 week at 4°C

ATP/DABCO/DTT mix

Add 15 μl of 0.2 M ATP, pH 7.0, and 6 μl 1 M MgCl₂ to 79 μl of distilled water to prepare a 30 mM ATP/60 mM MgCl₂ stock solution. Next, prepare the

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ATP/DABCO/DTT mix by mixing 40 μ l of the 30 mM ATP/60 mM $MgCl_2$ stock solution with 20 μ l of 2.2 mM 1,4-diazabicyclo[2.2.2]octane (DABCO; Molecular Probes) and 10 μ l 0.2 M DTT. Store on ice and use within 3 days.

Gelsolin buffer A

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1 mM EDTA
100 mM NaCl
Store up to 1 week at 4°C
Just before use, add:
0.1 mg/ml lysozyme
0.5 mM PMSF (added just before use from 200 mM stock in absolute ethanol)
10 μ g/ml benzamidine

Gelsolin buffer B

20 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
1 mM EGTA
0.01% (w/v) NaN_3
Store up to 1 week at 4°C

Gelsolin buffer C

10 mM MES pH 6.5
1 mM EGTA
Store up to 1 week at 4°C

Gelsolin buffer D

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 mM EGTA
0.15 M NaCl
0.01% (w/v) NaN_3
Store up to 1 week at 4°C

Methylcellulose, 1%

Prepare 1% (w/v) methylcellulose in distilled water, heat to 60°C, and stir until the solution clears. Store up to 1 week at 4°C.

Mg-F-actin

Prepare KME:

2 M KCl
20 mM $MgCl_2$
4 mM EGTA

Prepare low-ionic-strength buffer:

5 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
0.1 mM $CaCl_2$
0.2 mM ATP
1 mM DTT
0.01% (w/v) NaN_3

Prepare 100 μ l of Mg-F-actin by adding 5 μ l of KME (see above) to 95 μ l Ca-G-actin (prepare as in *UNIT 13.2*) in low-ionic-strength buffer (see above). Store up to 1 week at 4°C.

N-WASP buffer A

20 mM sodium phosphate buffer, pH 7.8 (*APPENDIX 2A*)
5% (v/v) glycerol
0.3 M NaCl
0.1% (v/v) Tween 20
Store up to 1 week at 4°C
Just before use, add:
1 mM PMSF
5 µg/ml leupeptin
5 µg/ml chemostatin
5 µg/ml pepstatin
10 µg/ml benzamidine

N-WASP buffer B

20 mM sodium phosphate buffer, pH 7.8 (*APPENDIX 2A*)
5% (v/v) glycerol
0.1 M NaCl
Store up to 1 week at 4°C

N-WASP buffer C

20 mM sodium phosphate buffer, pH 7.8 (*APPENDIX 2A*)
5% (v/v) glycerol
0.3 M NaCl
10 mM imidazole
Store up to 1 week at 4°C

N-WASP buffer D

20 mM sodium phosphate buffer, pH 7.8 (*APPENDIX 2A*)
5% (v/v) glycerol
0.3 M NaCl
30 mM imidazole
Store up to 1 week at 4°C

N-WASP buffer E

20 mM sodium phosphate buffer, pH 7.8 (*APPENDIX 2A*)
5% (v/v) glycerol
0.3 M NaCl
100 mM imidazole
Store up to 1 week at 4°C

N-WASP buffer F

10 mM sodium phosphate buffer, pH 7.8 (*APPENDIX 2A*)
5% (v/v) glycerol
0.1 M NaCl
1 mM DTT
Store up to 1 week at 4°C

Poly-L-proline-Sepharose column

Conjugate poly-L-proline (Sigma) to Sepharose-CNBr (Amersham Biosciences) according to the coupling instructions supplied by the manufacturer of the Sepharose-CNBr. Pack the poly-L-proline-Sepharose in a 15-cm length × 2.5-cm inner diameter chromatography column.

Polymerization buffer

5 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
0.2 mM ATP
1 mM DTT
0.1 mM CaCl₂
1 mM MgCl₂
0.2 mM EGTA
0.1 M KCl
Store up to 1 week at 4°C

Profilin buffer A

10 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
0.1 M KCl
0.1 M glycine
1 mM DTT
Store up to 1 week at 4°C

Profilin buffer B

5 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 mM DTT
Store up to 1 week at 4°C

COMMENTARY

Background Information

Early studies of the movement of motile cells and associated dynamics of actin filaments revealed that the polarized array of actin filaments turns over rapidly in the lamellipodium. Filament barbed ends (dynamic ends) facing the plasma membrane grow, while filament pointed ends depolymerize at the rear of the lamellipodium in a treadmilling process. In the lamellipodium, actin filaments turn over 100 times faster than pure actin. To account for the rapid movement of eukaryotic cells, actin treadmilling is enhanced by several factors. ADF enhances the rate of pointed-end depolymerization and increases the concentration of actin monomers at steady state. Profilin specifically binds monomeric actin in a complex that has the property of associating exclusively at the barbed ends. The flux of actin monomer provided by ADF-mediated depolymerization is converted into the profilin-actin complex, which polymerizes at the barbed ends only. Hence, the processivity of treadmilling is enhanced by profilin in the presence of ADF. In addition to ADF and profilin, a capping protein is required for efficient motility in the reconstituted medium. By blocking a large fraction of the barbed ends, capping proteins funnel the flux of pointed-end depolymerization to feed the growth of a few noncapped filaments, which individually grow faster than if

the other filaments were not capped. In conclusion, ADF, profilin and capping proteins act synergistically in enhancing pointed-end depolymerization and barbed-end polymerization, resulting in an increase in treadmilling (Pantaloni et al., 2001).

In the treadmilling process, capping proteins are recycled after depolymerization of capped filaments and eventually cap the growing barbed ends. Maintenance of a steady number of transiently growing barbed ends requires the constant generation of barbed ends. The cellular factor that generates new filaments in a site-directed, signaling-controlled fashion is the Arp2/3 complex. The Arp2/3 complex, a conserved ubiquitous complex of seven subunits including the two actin-related proteins Arp2 and Arp3, stimulates the formation of actin filaments by branching pre-existing filaments. In higher eukaryotic cells, Arp2/3 alone is inactive and needs to be activated by a variety of activators like WASP family proteins (Rohatgi et al., 1999; Egile et al., 1999) and cortactin. (Weaver et al., 2001; Uruno et al., 2001). The multimodular proteins WASP interact with the Arp2/3 complex and G-actin via its C-terminal WA domain to stimulate branching of actin filaments. The activity of N-WASP is enhanced by the binding of effectors like Cdc42 (Egile et al., 1999; Rohatgi et al.,

1999), phosphatidylinositol bisphosphate (Rohatgi et al., 1999), Grb2 (Carlier et al., 2000), and Nck (Rohatgi et al., 2001).

The propulsive movement of the pathogens *Listeria* and *Shigella* involves the same molecular mechanism as the protrusive behavior of the leading edge of motile cells. The *Listeria* protein ActA directly activates Arp2/3 complex (Welch et al., 1998). The protein VASP interacts with the *Listeria* protein ActA and increases actin-based movement of *Listeria* 10-fold, but has no effect on *Shigella* (Laurent et al., 1999). The *Shigella* protein IcsA does not activate Arp2/3 complex but recruits and activates the host protein N-WASP, which in turn activates Arp2/3 complex (Egile et al., 1999).

In view of the abovementioned information, a number of biochemical assays have been developed to study actin-based propulsion of the bacteria *Listeria* and *Shigella*. First, the reconstitution of *Listeria* movement in cell extracts has converted the complex cell biology problem of motility into a simpler biochemistry problem (Theriot et al., 1994). This assay has been instrumental in identifying the Arp2/3 complex as the cellular factor responsible for stimulating actin assembly at a bacterial surface (Welch et al., 1997). This assay was also useful in understanding the function of the key players of actin-based motility, like VASP (Laurent et al., 1999) and ADF (Carlier et al., 1997). The design of a minimal motility medium using pure proteins (Loisel et al., 1999), presented in this unit, comes as a logical conclusion from discoveries of the factors involved in the reorganization of actin cytoskeleton at the surface of *Listeria* and *Shigella* and at the leading edge of motile cells.

The assay is based on two essential biochemical functions: (1) local nucleation of actin filaments by the Arp2/3 complex at the surface of a solid particle (bacteria or microsphere) coated with an activator of Arp2/3 and (2) constant renewal of the Arp2/3-induced actin array by treadmill regulating proteins (ADF, gelsolin, profilin; Loisel et al., 1999).

This chemically controlled medium offers advantages in analyzing the mechanism at work, because the dependence of velocity on the concentration of different factors and their interplay with the surface density of Arp2/3 activator can be addressed.

The minimum motility medium has the potential to test the function of proteins involved in signaling pathways or actin dynamics. For instance, the function of Grb2, a protein that

links N-WASP to receptor tyrosine kinase, has been tested in the motility medium. Grb2 shortens the delay preceding actin comet, supporting the biochemical demonstration that Grb2 is an activator of N-WASP (Carlier et al., 2000). Similarly, potential inhibitors or drugs can be tested as well. Another application is the study of the effect on motility of actin-binding proteins like thymosin β 4, actobindin, and ciboulot. Ciboulot and actobindin enhance motility and substitute for profilin in the minimum motility medium. In contrast, thymosin β 4 has no effect on motility and cannot replace profilin. These results and in-depth biochemical studies support the view that ciboulot-actin and actobindin-actin complexes assemble exclusively at the barbed end. Table 12.7.2 recapitulates examples of applications of the motility assay.

The physical mechanism of force protrusion can be addressed using the minimum motility medium. This biomimetic motility assay has been used to address the force-velocity relationship, by varying the size of the bead and the viscous drag (see Fig. 12.7.1C). Movement was not slowed down by increasing the diameter of the microspheres (0.2 to 3 μ m) or by increasing the viscosity of the medium by several thousand-fold. This result shows that forces due to actin polymerization are balanced by internal forces resulting from transient attachment of filament ends at the surface. These forces are greater than the viscous drag (Wiesner et al., 2003).

Critical Parameters and Troubleshooting

The actin-based motility assays described in this unit involve the purification of seven different proteins. The major source of problem is the loss of activity of one or more components of the motility medium. Several experiments that are commonly used to verify the activity of the proteins are listed below, but a detailed description of the protocols is outside the scope of this unit.

To verify actin assembly, measure the pyrenyl-labeled actin polymerization by the increase of pyrenyl fluorescence.

To verify the activity of Arp2/3 complex and N-WASP, measure the increase of pyrenyl-actin assembly in the presence of Arp2/3 activated by N-WASP.

The activity of gelsolin can be tested by its ability to bind NBD-labeled actin in a 1:2 complex. Add increasing concentrations of NBD-labeled actin in the presence of a fixed

Table 12.7.2 Applications of Actin-Based Motility Assay

Protein	Experiment	Result	Interpretation
Ciboulot	Substitution of profilin by ciboulot	Ciboulot functionally replaces profilin	The complex ciboulot-G-actin participates in barbed-end assembly in a profilin-like fashion (Boquet et al., 2000).
Actobindin	Substitution of profilin by actobindin	Actobindin functionally replaces profilin	The complex actobindin-G-actin participates in barbed-end assembly in a profilin-like fashion (Boquet et al., 2000).
Thymosin β 4	Substitution of profilin by thymosin β 4	Thymosin β 4 does not replace profilin	The complex actin-thymosin β 4 does not participate in barbed end (Boquet et al., 2000).
Grb2	Addition of Grb2	Grb2 shortens the delay preceding actin comet tail formation	The protein Grb2 is an activator of N-WASP (Carlier et al., 2000).
VASP	Addition of VASP in the motility medium containing <i>Listeria</i> or ActA- or WASP-coated microspheres	VASP enhances actin-based propulsion of <i>Listeria</i> (Laurent et al., 1998), of ActA-coated beads (Boujemaa-Paterski et al., 2001), and WASP-coated beads (Castellano et al., 2001)	VASP enhances the dissociation of the newly formed branch junction from the immobilized branching enzyme ActA (Samarin et al., 2003).

concentration of gelsolin. Monitor the formation of this complex by the increase of NBD-labeled actin fluorescence. At saturation, the ratio of gelsolin to actin is 1:2. Capping activity of gelsolin is highly dependent on the presence of Ca^{2+} at concentrations of the order of 10^{-4} M, at least. If one protein is added to the medium, be sure that the buffer in which it is equilibrated does not contain EDTA in amounts sufficient to chelate all Mg^{2+} and/or Ca^{2+} ions in the medium. ATP-Mg is the ligand of actin; the affinity of ATP decreases by six orders of magnitude upon removal of Mg^{2+} ions. Subsequent dissociation of ATP leads to irreversible denaturation of actin. EDTA can be removed by dialysis or gel filtration (on a G-25 column) in the appropriate buffer.

ADF binds to filaments and destabilizes them, causing partial depolymerization. The effect of ADF on the treadmilling is a suitable probe of its active state. In the presence of etheno-ATP-F-actin and a large excess of ATP, the decrease of etheno-ATP fluorescence reflects the rate of pointed-end depolymerization. ADF increases the rate of depolymerization. ADF activity is highly dependent on the pH, which should be >7.8 .

To verify the labeling of Alexa488-labeled Arp2/3, first resolve the different subunits

of Arp2/3 complex on SDS-PAGE and identify subunits by UV illumination of the gel. Typically 80% of the label is bound to the ARCP41 subunit, which contains five cysteines and 20% is bound to the Arp3 subunit (Wiesner et al., 2003).

To verify the branching activity of Alexa488-labeled Arp2/3 complex, polymerize actin (2.5 μM , 10% pyrenyl-labeled) in the presence of 0.5 μM VCA, in the absence or presence of 30 nM Arp2/3 before addition and after a 1-hr incubation with Alexa488-C5-maleimide. The authors find that the activity of Arp2/3 complex is not affected by Alexa488 labeling (Wiesner et al., 2003).

The reader will also find helpful information regarding the dependence of bacterial velocity on the concentration of essential components (Arp2/3, ADF, and gelsolin) and nonessential components (α -actinin, profilin, and VASP) in Figure 12.7.2.

Anticipated Results

A successfully completed assay is defined by the following criteria. Typically, the actin tails appear 5 to 15 min following addition to the motility medium. Typical examples of actin tails are shown in Figure 12.7.1. The percentage of motile bacteria/microspheres

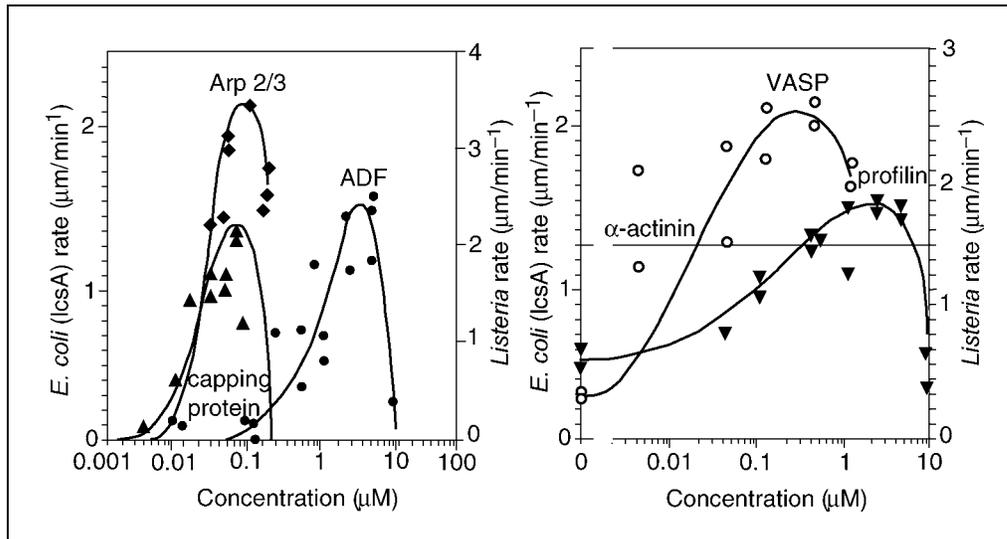


Figure 12.7.2 Dependence of bacterial rate on the concentration of essential components (left) and nonessential components (right). Filled symbols, *E. coli* (IcsA); open symbols, *Listeria*. Note the logarithmic abscissa scale. Duplicate data points represent independent measurements made by two experimentalists. Each data point is the average of 10 measurements. The standard deviation was generally 20%. From Loisel et al. (1999).

under optimal conditions falls between 70% and 80% for *Listeria* or *E. coli* IcsA and near 100% for N-WASP-coated polystyrene microspheres. At all surface densities of N-WASP, the steady-state velocity of microspheres was reached after 10 to 15 min incubation in the medium and remained constant for 1 hr. Average rates of movement at steady state are 1 to 2 $\mu\text{m}/\text{min}$ for N-WASP or ActA-coated microspheres, 2 $\mu\text{m}/\text{min}$ for *E. coli* IcsA and *Listeria*.

Time Considerations

Actin preparation requires 1 day to prepare an acetone powder from rabbit muscle and 6 days to purify actin extracted from acetone powder. Purifications of the proteins takes 3 days for Arp2/3 complex, 2 days for N-WASP, 2 days for gelsolin, 2 days for ADF, and 2 days for profilin. The motility assay only takes 1 hr.

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CHAPTER 13

Organelle Motility

INTRODUCTION

The cytoplasm of eukaryotic cells is comprised of a complex network of filaments, including microtubules and actin, that extend throughout the cytoplasm. These filaments are highly dynamic and continuously remodel as the cell changes shape, divides, and interacts with the environment. They play a key role in organizing the cytoplasm by connecting protein compartments and organelles in different regions of the cell. Microtubules, for example, are known to provide communication paths between organelles, controlling the spatial location of these structures. Actin filaments, on the other hand, form contractile bundles with myosin and mediate muscle contraction and contractile-ring formation during cytokinesis. In this chapter, assays are described for examining the characteristics and functions of the microtubule and actinomyosin filament systems. These assays are useful for identifying accessory proteins that attach microtubules to organelles and/or modulate sliding of myosin molecules across actin fibers.

UNIT 13.1 describes an in vitro assay for examining interactions between microtubules and isolated organelles. Microtubules are polar structures with their plus (i.e., rapidly growing) ends extending out to the cell periphery, and their minus ends associated with the centrosome adjacent to the nucleus. They emanate as a star-like network out from the centrosome, with new microtubules constantly extending out to replace old ones that have depolymerized. The endoplasmic reticulum (ER) uses microtubules to move out to the cell periphery, while membranes of the Golgi complex cluster inwards toward the centrosome along microtubules. These organelle movements are mediated by microtubule-associated motor proteins, including kinesin and cytoplasmic dynein. The in vitro motility assay described in this unit provides a useful system for studying organelle transport along microtubules. It can be used for dissecting the molecular machinery involved in such movement, including proteins that regulate microtubule motors or that cross-link motors to membranes. Effects of pharmacological or biochemical perturbations on microtubule structure and dynamics can also be investigated. In the assay described in *UNIT 13.1*, microtubules are nucleated from a stationary point (i.e., the flagellar axoneme), allowing their plus ends to grow and shorten in a way analogous to what happens in normal cells. Both motor-driven motility of organelles and microtubule polymerization-driven movement of organelles can be studied. The unit also provides detailed methods for preparation of purified tubulin, cytosol enriched in motor proteins, and axonemes from sea urchin sperm, as well as for isolation of membrane-bound organelles. The assay is performed in a simple perfusion chamber and the results are visualized using video-enhanced DIC microscopy.

UNIT 13.2 provides a motility assay for transport of actin by myosin. F-actin filaments labeled with rhodamine-phalloidin are imaged in an in vitro system that contains purified myosin, an ATP-activated motor protein that hydrolyzes ATP to ADP and P_i when stimulated by binding to actin filaments. This assay uses two purified proteins, actin and myosin, to study the contractile forces produced by the sliding of actin filaments along the myosin filaments. During muscle contraction, the head regions of myosin molecules engage in an ATP-driven cycle in which they attach to adjacent actin filaments, undergo

a conformational change that pulls the myosin filament, and then detach. This results in the sliding of actin filaments against the myosin, which can be directly visualized and quantified in this assay.

The use of GFP fusion proteins has revolutionized the study of organelle motility by providing a method to visualize diverse organelles directly under the microscope. Virtually any protein can be tagged with GFP, expressed in cells, then visualized by applying blue light. *UNIT 13.3* describes how to express and image GFP fusion proteins in plants in order to study organelle dynamics in these cell types. Both transient and viral-mediated expression methods are described for expressing GFP fusion proteins. Tips are given for imaging GFP proteins targeted to the endoplasmic reticulum and Golgi complex, two organelles that function in secretory transport. Solutions to common problems with expression and imaging GFP fusion proteins--e.g., misfolding and autofluorescence--are also discussed.

Nuclear migration (*UNIT 13.4*) occurs in a wide variety of cell types, including newly fertilized eggs, muscle, nerves, and dividing cells. It is vital for the cell to properly locate its nucleus and surrounding endomembrane system. This unit describes an in vitro assay for monitoring nuclear motility of the pronucleus from frog eggs. This motility is, as are other examples of nuclear motility, driven by microtubule-dependent motility. Purified nuclei are added to a motility extract that includes *Xenopus* cytosol, centrosomes, purified microtubules, dynein, and regulatory factors. The system is then visualized using video enhanced-differential interference contrast microscopy. Once they associate with microtubules, the nuclei normally move toward the centrosomes in microtubule minus end-directed (due the dynein in the extract) motility. There they accumulate over time. Regulators or inhibitors of this movement can be assayed, and the effects on motility can be quantified using this system.

Jennifer Lippincott-Schwartz

Microtubule/Organelle Motility Assays

UNIT 13.1

This unit describes an *in vitro* assay that uses video-enhanced differential interference contrast (VE-DIC) microscopy to examine the motile interactions between isolated organelle fractions and microtubules (MTs; see Basic Protocol). The method can be used to dissect the molecular requirements for organelle movement and membrane trafficking. A field of axoneme-nucleated MTs, growing and shortening as they would in a living cell (dynamic MTs), is generated in a simple microscope perfusion chamber. Various combinations of isolated endoplasmic reticulum (ER) and Golgi apparatus organelles, cytosol containing motor proteins and other soluble factors, nucleotides, and specific pharmacological reagents are then added to the dynamic MT, and the motile interactions between the organelles and MTs are observed by VE-DIC microscopy.

In addition, this unit includes protocols for biochemical preparation of phosphocellulose-purified tubulin from porcine brain (see Support Protocol 3), axonemes from sea urchin sperm (see Support Protocol 2), rat liver cytosol (see Support Protocol 4), and rat liver organelle fractions (see Support Protocol 5). To ensure more reproducible results, a protocol for preparing thoroughly cleaned (“squeaky clean”) coverslips and simple microscope perfusion chambers is also included (see Support Protocol 1).

STRATEGIC PLANNING

Performing a successful motility assay requires ~2 weeks of preparatory biochemistry and considerable skill in obtaining VE-DIC images. Detailed description of how to set up the sophisticated optical system required for imaging single MTs by VE-DIC is outside the scope of this unit and is not included here. Instead, the reader should consult the unit on microscopy by E.D. Salmon (UNIT 4.1) and other more comprehensive descriptions of the techniques required for achieving such images (Walker et al., 1988; Salmon and Tran, 1998).

Preparation of the principal biochemical components for the motility assay is detailed in Support Protocols 2 to 5. These components must be prepared in bulk in advance, dispensed into appropriately sized aliquots, and stored at -70°C . Unopened samples can be stored for >2 years. Three different types of animal tissue must be obtained for the various preparations. The animals that are most difficult to acquire are the sea urchins, *Strongylocentrotus purpuratus*, used for the preparation of axonemes. Sea urchins can be obtained from early winter through mid spring from Marinus, Inc., but their availability depends on the seasonal catch. Porcine brains for the tubulin preparation must be obtained from freshly slaughtered pigs, and the tubulin preparation should begin within 3 to 4 hr after the tissue is harvested. A local butcher can supply information regarding the location of the closest slaughterhouse. Fresh rat livers are fairly easy to obtain; alternatively, flash-frozen livers can be purchased from Pel-Freez.

In contrast to the biochemistry and microscopy, setting up the motility assay in the Basic Protocol is relatively simple. Note, however, that specific brands of microscope coverslips and slides are required for the preparation of the microscope perfusion chambers (see Support Protocol 1), and the coverslips should be cleaned according to the steps outlined. Rigorous attention to the detailed instructions presented in Support Protocol 1 is crucial to the success of the assay. Inexpensive microscopy supplies are often coated with oils and dirt that can lead to spurious and inconsistent results.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for care and use of laboratory animals.

Organelle Motility

13.1.1

Contributed by Clare M. Waterman-Storer

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MT/ORGANELLE MOTILITY ASSAYS

This protocol describes the set-up and execution of an assay that combines dynamic MTs with cellular organelle fractions and cytosolic proteins to reconstitute organelle motility in vitro (see Fig. 13.1.1).

Materials

- Axoneme fragments (see Support Protocol 2)
- Golgi or ER membranes (see Support Protocol 5)
- 45 μM purified brain tubulin (see Support Protocol 3)
- Rat liver cytosol (see Support Protocol 4)
- PM buffer (see recipe)
- PM buffer containing 1 mM GTP
- 20 \times energy regeneration system (see recipe)
- 15 mM MgGTP, prepared by diluting 100 mM MgGTP stock (see recipe) in PM buffer
- Valap (see recipe)
- Simple perfusion chambers (see Support Protocol 1)
- Filter paper cut into 2-cm squares

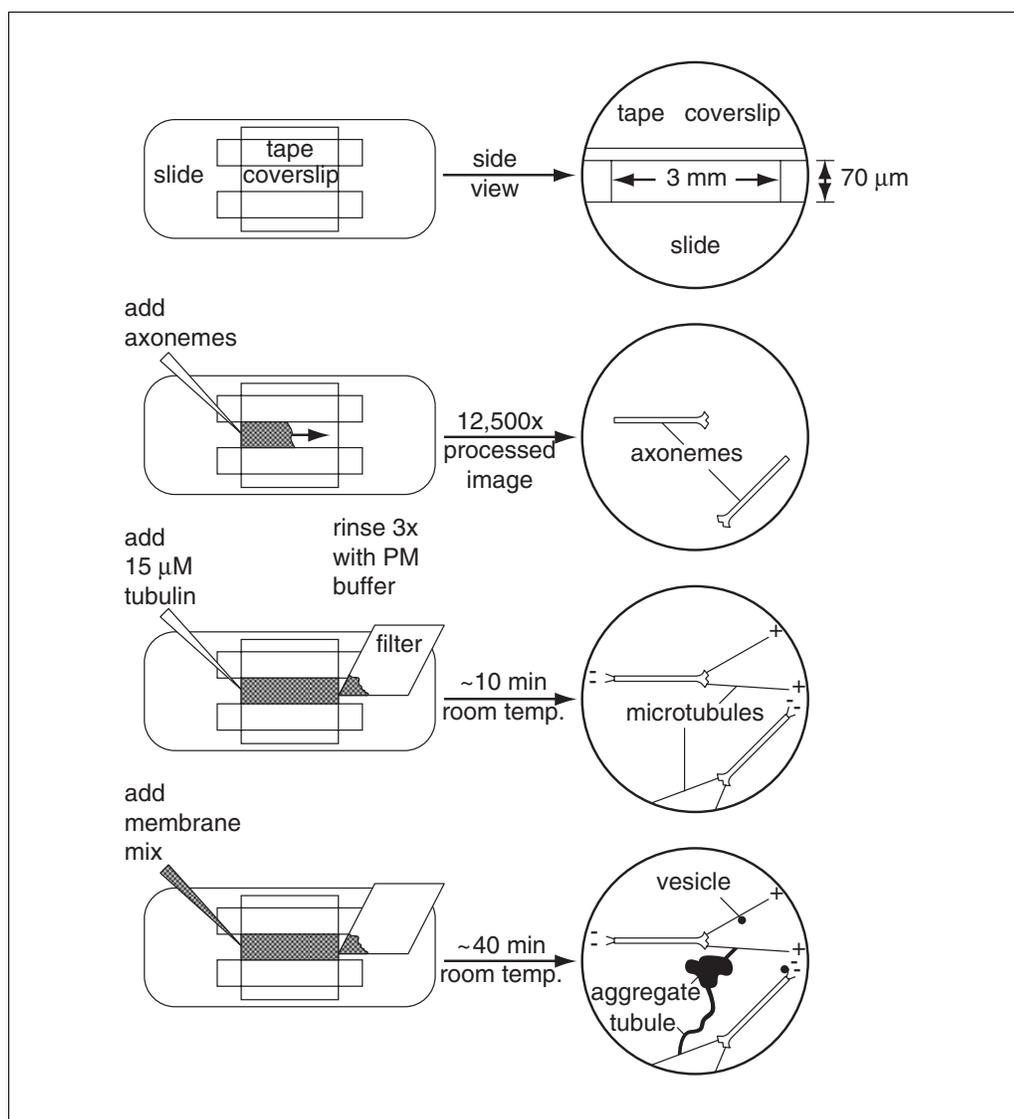


Figure 13.1.1 Flow chart for performing MT/organelle motility assay.

Humid chamber made of a 90-mm glass petri dish containing moist paper towels
High-resolution VE-DIC microscope system (as described in Salmon and Tran, 1998 or equivalent)

1. Rapidly thaw and immediately place on ice one aliquot each of axonemes, Golgi or ER membranes, 45 μM purified brain tubulin, rat liver cytosol, and 20 \times energy regeneration system.
2. Dilute axonemes with PM buffer to the proper concentration as determined in Support Protocol 2. Prepare 6 \times Golgi or ER membranes by diluting organelles with PM buffer/1 mM GTP (see Support Protocol 5, step 10).
3. Prepare and place on ice a 30- μl membrane mix:

5 μl 6 \times Golgi or ER membranes
1.5 μl 20 \times energy regeneration system
10 μl 45 μM tubulin
1 μl 15 mM MgGTP
12.5 μl cytosol.

4. Add axonemes to a simple perfusion chamber by slowly pipetting ~ 10 μl of diluted axonemes against one open end of the chamber and allowing the chamber to fill.

Be careful to avoid introducing large bubbles into the chamber.

5. Place the perfusion chamber into the humid chamber and incubate 10 min at room temperature to allow the axonemes to adhere to the glass.
6. Wash out unadhered axonemes by slowly pipetting 10 μl PM buffer against one end of the perfusion chamber while simultaneously wicking excess buffer from the opposite side of the chamber with the tip of a square of filter paper. Repeat wash two more times.
7. Dilute 5 μl of 45 μM tubulin with 10 μl PM buffer/1 mM MgGTP. Perfuse the diluted tubulin into the chamber containing the washed axonemes. Place a drop of immersion oil on the top and bottom of the slide, and transfer it to the VE-DIC microscope stage.

Briefly, the microscope system consists of illumination from an HBO100-W mercury arc lamp introduced into an upright microscope stand (equipped with optical components for DIC image formation) via a fiber-optic scrambler. Illumination is passed through IR reflecting and 546-nm narrow band-pass filters before being focused for Köhler illumination onto the specimen via a 1.4-NA oil-immersion condenser. The light is collected by a 100 \times 1.3- or 1.4-NA objective and magnified 12.5 \times before being collected by a scientific-grade Newvicon tube type video camera (equivalent of Hamamatsu C2400). The video signal is processed by frame averaging, background subtraction, and contrast enhancement by a real-time image processor (equivalent to the Hamamatsu Argus 10), and then recorded in real time onto high-resolution S-VHS video tape.

8. Focus on the axonemes with the 100 \times objective lens. Align the slide on the stage so that one edge of the double-stick tape that forms the perfusion chamber perfectly bisects the area illuminated by the microscope condenser lens. Immerse the 100 \times objective lens in oil and focus on the edge of the tape. With the edge of the tape in view, back off fine focus until the very edge of the tape begins to go out of focus. Move the slide so the lens is within the area coated with axonemes, which should now be quite close to focus.

It can be difficult to focus on axonemes on the surface of the coverslip because of their very small size and the very bright illumination needed for VE-DIC. This procedure should make focusing on the axonemes easier.

Table 13.1.1 Pharmacologic Agents for Addition to Membrane Mix^a

Pharmacologic agent	Final concentration	Function	Stock solution	Amount added to 30- μ l membrane mix
Brefeldin A	60 μ M	Removes Golgi coat proteins	Dilute 1.5 μ l of a 3.6 mM Brefeldin A stock in ethanol ^b 1:1 into PM buffer ^b prior to use	3 μ l
Aluminum fluoride		Activates heterotrimeric G proteins		Add 1 μ l of 30 \times NaF ^b and 1 μ l of 30 \times AlCl ₃ ^b
MgGTP- γ -S	1 mM	Activates heterotrimeric G proteins	30 mM MgGTP- γ -S ^b	1 μ l
MgAMP-PNP	1 mM	Inhibits kinesin-like proteins ^c	150 mM MgAMP-PNP ^b	1 μ l
Sodium orthovanadate	25 μ M	Inhibits cytoplasmic dynein ^d	Dilute 1 μ l of 100 mM stock into PM buffer ^b	1 μ l

^aAlso see APPENDIX 1B.

^bSee recipes for instructions on solution preparation. Abbreviations: MgAMP-PNP, 5' adenylylimidodiphosphate magnesium salt.

^cVale et al., 1985.

^dShpetner et al., 1988.

- Optimize the image for visualization of individual MTs by aligning the microscope for Köhler illumination. Use the real-time image processor to perform background subtraction, contrast enhancement, and frame averaging. Observe and record onto S-VHS video tape images of polymerization dynamics of individual MTs as they are nucleated off the axonemes.

Note the difference between the plus (longer, faster-growing MTs) and minus (shorter, slower-growing MTs) ends of the axonemes. For details on microscopy techniques, refer to UNIT 4.1 or Salmon and Tran (1998).

- During the observation of MT dynamics, allow the membrane mix to warm to room temperature.
- Perfuse 12 μ l of membrane mix into the simple perfusion chamber on the microscope stage. Seal the chamber edges on both sides with a drop of melted valap. Observe and record the dynamic interactions between the organelles and MTs.

Note that often it takes up to 45 min for motility to develop. This time period is proportional to room temperature.

Pharmacological agents (see Table 13.1.1) may be added to the membrane mix prepared in step 3 to test the involvement of Golgi coat proteins and MT motor proteins in organelle movement in vitro. For review of the effects of these pharmacological agents on membrane trafficking, see Klausner et al. (1992). These agents should be added to the mix, correcting all components for concentration, and incubated 15 min at 37°C prior to being introduced into the flow chamber.

Organelles may also be pretreated to strip them of specific subsets of peripheral proteins prior to addition to the mix. This will allow examination of the involvement of these proteins in organelle movement (see Support Protocol 5, steps 12 to 14).

PREPARATION OF SIMPLE PERFUSION CHAMBERS AND COVERSGLIPS

For the MT/organelle motility assay to be reproducible, the perfusion chambers and coverslips must be “squeaky clean.”

Materials

Versa Clean dish detergent (Fisher)
1 mM EDTA
70% and 100% ethanol
22 × 22–mm no. 1.5 coverslips (Corning)
Water-bath sonicator
Double-stick tape
Clay-Adams precleaned Goldseal 3 × 1–in. microscope slides (Becton Dickinson)
Dumont no. 5 forceps

Prepare “squeaky clean” coverslips

1. Place coverslips, one at a time, into a 500-ml glass beaker filled halfway with hot tap water containing ~5 ml Versa Clean detergent. Take care to separate coverslips that are stuck together. Sonicate 45 min in a water-bath sonicator.

Prepare three packages of coverslips at one time.

2. Rinse coverslips ten times by swirling with hot tap water. Sonicate 30 min in hot tap water.
3. Rinse coverslips ten times by swirling with double distilled water. Sonicate 30 min in double distilled water.
4. Rinse coverslips three times by swirling with 1 mM EDTA. Sonicate 30 min in 1 mM EDTA.
5. Rinse coverslips three times by swirling with 70% ethanol. Sonicate 30 min in 70% ethanol.
6. Rinse coverslips three times by swirling with 100% ethanol. Sonicate 30 min in 100% ethanol.
7. Rinse coverslips once with 100% ethanol. Transfer clean coverslips to a 500-ml screw cap jar, cover with 100% ethanol, and store at room temperature until use.

Prepare simple perfusion chambers

8. Cut pieces of double-stick tape into 25 × 5–mm strips. Apply two strips of tape to the center of a slide, side by side, 3 mm apart, and parallel to the long axis of the slide.
9. Using Dumont no. 5 forceps, retrieve a single squeaky clean coverslip from the storage jar. Dab the edge of the coverslip with a Kimwipe to remove any large drops of ethanol and quickly pass it through the flame of a Bunsen burner. Allow ethanol to burn off.
10. Center the coverslip over the tape strips on the slide and apply the coverslip to the strips of tape, pressing firmly with the back of the forceps to make a good seal.
11. Repeat until ~20 perfusion chambers have been prepared and store them in a slide storage box until use.

PREPARATION OF SEA URCHIN SPERM AXONEMES

This protocol (as adapted from Bell et al., 1982) should yield enough axoneme fragments to perform thousands of motility assays.

Materials

- 4 male *S. purpuratus* sea urchins (Marinus)
- 0.55 M KCl
- Artificial sea water (mixes available from aquarium supply stores; prepare per manufacturer's instructions)
- 20% (w/v) sucrose in distilled water
- Isolation buffer (see recipe)
- High-salt buffer (see recipe)
- Isolation buffer (see recipe) containing 50% glycerol
- 60-ml syringe with 18-G needle
- Tabletop clinical centrifuge
- Refrigerated superspeed centrifuge (Sorvall RC-5B) with Sorvall SS-34 rotor (or equivalent)
- 50-ml polycarbonate centrifuge tubes (e.g., Sorvall)
- 50-ml Dounce glass homogenizer with type A and B pestles

NOTE: Because it is impossible to tell what sex a sea urchin is until it sheds gametes from the pores located on its dorsal surface, order at least twice as many animals as needed.

Collect sperm

1. Sacrifice the sea urchins. Fill a 60-ml syringe with 0.55 M KCl and attach an 18-G needle. Pierce the urchin from the bottom center of the animal, insert the needle ~1 in., and inject body cavity with fluid until resistance is felt. Place the animal upright on paper toweling. Allow several minutes for gametes to begin to exude from the five pores located on top of the urchin.

The urchins must be injected and sacrificed to induce their gametes to shed. Upon arrival from shipment, some urchins may have shed a small amount of gamete. In this case, identifying the sex of the animal should be simple: sperm is white and eggs are peach or orange colored.

2. Using a glass Pasteur pipet with a rubber bulb, collect white sperm dropwise as it is exuded. Pool sperm from all four animals into a single test tube on ice.

Expect to get 3 to 4 ml of sperm per animal over a ~20-min collection period. Do not collect orange- or peach-colored eggs.

3. Dilute the sperm with 3 vol artificial sea water. Let stand 20 min on ice.
4. Centrifuge 5 min at 500 rpm in a tabletop clinical centrifuge to pellet debris.
5. Transfer supernatant to 50-ml polycarbonate centrifuge tubes. Pellet sperm by centrifuging 5 min at $3000 \times g$ (5000 rpm in SS-34 rotor), 4°C.

Isolate sperm tails

6. Remove and discard supernatant. Resuspend pellet by trituration in 5 vol of 20% sucrose to osmotically remove the plasma membranes.
7. Transfer the demembrated sperm to a Dounce glass homogenizer partially immersed in slushy ice. Homogenize with fifteen rapid passes of a type B pestle to break the sperm heads from the tails.

The pestle should be moved from the top of the liquid to the very bottom of the homogenizer, and upon return to the top, the pestle should not break the surface of the liquid.

8. Transfer the homogenate to 50-ml centrifuge tubes, pellet sperm heads by centrifuging 10 min at $12,000 \times g$ (10,000 rpm in SS-34 rotor), 4°C .
9. Collect the supernatant containing the demembrated tails into new 50-ml tubes and pellet the tails by centrifuging 15 min at $20,000 \times g$ (13,000 rpm in SS-34 rotor), 4°C .
10. Discard the supernatant. Using a metal weighing spatula and a Pasteur pipet, collect only the top white layer that contains the demembrated tails. Resuspend pelleted tails by trituration in 4 vol isolation buffer.

The bottom yellow layer of the pellet contains sperm heads and debris.

Disrupt tails and extract axonemes

11. Transfer resuspended tails to Dounce glass homogenizer on ice. Homogenize with five rapid passes of a type A pestle to break the tails into fragments. Transfer fragment suspension into 50-ml centrifuge tubes.
12. Pellet the tail fragments in 50-ml tubes by centrifuging 10 min at $12,000 \times g$, 4°C .

This step will also result in a two-layer pellet.

13. Discard the supernatant and collect only the top white layer. Resuspend this white pellet by trituration in 4 vol isolation buffer. Transfer to new tubes and centrifuge as in step 12. Repeat this cycle of resuspension and centrifugation one or two more times to completely separate the tail fragments from heads and debris. Continue until the pellet is a single layer of pure white.
14. Discard the supernatant and resuspend the white pellet in 4 vol high-salt buffer.
15. Transfer to Dounce glass homogenizer on ice. Homogenize with five passes of a type A pestle. Incubate 45 min on ice to extract dyneins and central pair MTs from the tail fragments.

Isolate axonemes

16. Transfer extraction suspension to 50-ml centrifuge tubes and separate the extracted axonemes from soluble proteins by centrifuging 15 min at $20,000 \times g$, 4°C .
17. Discard the supernatant and resuspend the axoneme pellet by trituration in 4 vol high-salt buffer. Transfer to a new 50-ml centrifuge tube and reextract the axoneme fragments by incubating 15 min on ice.
18. Pellet the extracted axonemes by centrifuging 15 min at $20,000 \times g$, 4°C .
19. Discard supernatant and resuspend extracted axoneme pellet by trituration in $\frac{1}{3}$ the original volume of sperm (in step 2) in isolation buffer/50% glycerol.
20. Distribute the axonemes into 20- μl aliquots in 0.5-ml microcentrifuge tubes and freeze by immersing tubes in liquid nitrogen.
21. Store frozen axonemes at -70°C until use.
22. Determine the proper axoneme dilution for use in the MT/organelle motility assay. Thaw an aliquot of axonemes and observe various dilutions by VE-DIC (at the same magnification as will be used in the assay) in a simple perfusion chamber (see Support Protocol 1). Note the dilution required such that two to three axonemes are visible per $\sim 30\text{-}\mu\text{m}^2$ microscopic field.

PREPARATION OF PORCINE BRAIN TUBULIN

Although many protocols for tubulin preparation are available, this procedure (as adapted from Walker et al., 1988) is the simplest and, in the author's experience, provides the highest yields of tubulin. Three pig brains should yield ~60 mg of purified tubulin.

Materials

- 100-g P-11 cellulose phosphate fibrous cation exchanger (Whatman)
- 0.1 M HCl
- 0.1 M and 10 M NaOH
- 0.1 M MgSO₄
- 10× and 1× column buffer (see recipe)
- 3 fresh pig brains (use <3 hr after slaughter)
- Homogenization buffer (see recipe; freshly prepared)
- PM buffer (see recipe)
- 100 mM MgATP (see recipe)
- PMG buffer (see recipe)
- 100 mM MgGTP (see recipe)
- 1 M dithiothreitol (DTT; *APPENDIX 2A*)
- Glutamic acid, sodium salt

- 2-liter sintered-glass filter funnel and 2-liter sidearm Erlenmeyer flask
- Waring blender
- Temperature-controlled ultracentrifuge (Beckman L7-55) with Beckman 50.2Ti rotor (or equivalent)
- 31.5-ml thick-walled polycarbonate ultracentrifuge tubes (e.g., Beckman) with screw caps
- 30-ml Dounce type A glass homogenizer
- 44 × 250-mm adjustable volume column for low-pressure liquid chromatography (e.g., Amicon model #95240 or equivalent)

- Additional reagents and equipment for determining protein concentration (*APPENDIX 3*)

DAY 1: Prepare phosphocellulose column

1. Add 90 g of Whatman P-11 phosphocellulose to 2 liters of 0.1 M NaOH in a 4-liter beaker while mixing very gently with a glass rod. Mix suspension gently 5 min, then allow the solids to settle 20 min. Aspirate and discard excess solution. Transfer remaining phosphocellulose slurry to a 2-liter sintered-glass funnel on a 2-liter sidearm Erlenmeyer flask connected to a vacuum line. Carefully vacuum filter the remaining 0.1 M NaOH from the phosphocellulose, but do not allow the resin to run dry.
2. Gently scrape the phosphocellulose from the funnel and return it to the 4-liter beaker. Add 2 liters of 0.1 M NaOH, mix gently 5 min, and check the pH with pH paper. If the pH of the slurry is not >12, repeat the settling, aspiration, and filtering treatment and resuspend phosphocellulose in 2 liters of 0.1 M NaOH. Repeat until pH >12.
3. Rinse phosphocellulose with 4 liters of distilled water by vacuum filtration, again being careful never to let the resin run dry.
4. Transfer phosphocellulose from the funnel to the 4-liter beaker and add 2 liters 0.1 M HCl. Mix gently, allow the resin to settle, aspirate excess solution, and vacuum filter the resin (as in step 1). Repeat the 0.1 M HCl treatment cycle until the pH of the phosphocellulose slurry is <3.
5. Rinse the phosphocellulose with 4 liters of distilled water by vacuum filtration.

6. Transfer the phosphocellulose resin to the 4-liter beaker and add 2 liters of 0.1 M MgSO₄. Mix, settle, aspirate, and vacuum filter.
7. Transfer the phosphocellulose resin to the 4-liter beaker and add 2 liters of 10× column buffer. Mix gently 10 to 15 min, allow the resin to settle, aspirate excess column buffer, and vacuum filter.
8. Transfer resin to the 4-liter beaker, add 2 liters of 1× column buffer, and mix gently for 5 min. Check the pH of the slurry with a pH meter and adjust to 6.6 with 10 M NaOH. Allow resin to settle, aspirate excess buffer, and vacuum filter. Repeat the 1× column buffer treatment cycle, using 10 M NaOH to adjust the pH of the slurry to 6.6, until the pH of the slurry is 6.6 without adjustment after resuspension and mixing. Allow resin to settle 20 min and aspirate buffer until the settled resin:buffer ratio is 3:1 (v/v).

Pour column

9. Mix the resin gently until evenly suspended in the buffer, then rapidly pour the slurry into an empty 44 × 250-mm liquid chromatography column (clamped to a support in the cold room). Fill column to the top. Cover with Parafilm and allow the resin to settle for several hours.
10. Fill the column adjuster plunger with column buffer, and very slowly insert it into the column, being very careful not to disturb the phosphocellulose resin. Insert the adjuster plunger until all air bubbles are expelled from column through the column inlet tubing which is immersed in a 4-liter reservoir of column buffer. Tighten and seal the adjuster plunger fittings, leaving the inlet tube in the buffer reservoir.
11. Attach the outlet tubing to a peristaltic pump and set the pump to run at 0.25 ml/min to allow the column to pack for ~48 hr.

A well-packed phosphocellulose column should be perfectly even in color with no evidence of cracks in the resin. The better the phosphocellulose column is packed, the more concentrated the peak of elution of tubulin protein.

DAY 2: Prepare MT protein

12. Transport brains from slaughterhouse to laboratory in an evacuated plastic ziplock bag buried in ice.

Brains must be kept on ice from the time the animals are slaughtered.

13. Working in a 4°C cold room, carefully and thoroughly remove the meninges and any blood-red tissue from the surface, stem, and within the folds of each brain. Pick tissue away by hand and use Kimwipes to peel membranes from surface of brain.

The dry wipe will stick to the meninges; as it is drawn gently across the surface of the brain, it will peel away the deep red membrane from the pinkish-gray nervous tissue underneath.

14. Cut cleaned brains into 2- to 3-cm² cubes and weigh the tissue. Transfer the tissue to a Waring blender and add 0.5 ml/g freshly prepared homogenization buffer containing 1 mM MgATP.

Homogenize brain tissue

15. Homogenize the tissue by blending 5 sec on high speed and then 45 sec on low speed.

This should result in a suspension with the color and consistency of a strawberry milkshake.

16. Using a 50-ml serological pipet with one-third of the tip cut off and a pipet bulb with strong suction, transfer the brain homogenate to several 31.5-ml polycarbonate

ultracentrifuge tubes. Note the homogenate volume and pair the tubes by weight (to 0.01 g). Discard any extra homogenate that does not fit into a full rotor-full of centrifuge tubes.

Tubulin is sensitive to proteases and easily denatured; keeping extra homogenate that must be left for 1 hr unprocessed while the first set of tubes is centrifuged does not significantly increase the final yield.

17. To remove undisrupted tissue from cell cytosol, centrifuge homogenate 60 min at $100,000 \times g$ (29,000 rpm in 50.2Ti rotor), 4°C.
18. Working at room temperature, carefully collect the cytosolic supernatants from the tubes with a pipet. Pool supernatants in a graduated cylinder and add an equal volume of PMG. To promote MT polymerization, add MgGTP to 0.2 mM. Distribute into 31.5-ml ultracentrifuge tubes and pair tubes by weight.

Isolate MT protein

19. Immerse the portion of the tubes containing the cytosol in a 37°C water bath and incubate 45 min to allow MT proteins to polymerize into MTs. During this incubation, warm the ultracentrifuge and 50.2Ti rotor to 25°C.
20. Pellet MTs from the cytosol by centrifuging 45 min at $100,000 \times g$, 25°C.
21. In the cold room, discard the supernatant and resuspend the MT pellet in a volume of PM buffer containing 0.2 mM GTP equal to one-fifth the volume of homogenate.

To resuspend the pellet, add a few milliliters of resuspension buffer to each centrifuge tube and to a small glass Dounce homogenizer (on ice).
22. Using a round-ended weighing spatula, scrape out the sticky pellets and transfer them to the Dounce homogenizer. Resuspend any pellet remaining in the ultracentrifuge tubes by trituration and transfer to the homogenizer.
23. Homogenize the pellets with five to ten passes of a type A pestle. Dispense the resuspended MTs into 31.5 ml-ultracentrifuge tubes and pair tubes by weight.
24. Incubate the resuspended MTs on ice for 30 min with gentle mixing every 5 min to allow for MT depolymerization. During this incubation, chill the ultracentrifuge and rotor to 4°C.
25. Clarify the MT protein by centrifuging 45 min at $100,000 \times g$, 4°C.
26. At room temperature, collect the supernatant containing the MT protein, add an equal volume of PMG, and add GTP to a concentration of 0.2 mM. Dispense into 31.5-ml centrifuge tubes and pair tubes by weight.
27. Immerse the portion of the tubes containing the MT protein solution in a 37°C water bath and incubate 45 min to allow MTs to polymerize. During the incubation, warm the ultracentrifuge and rotor to 25°C.
28. Pellet the MTs from polymerization-incompetent tubulin by centrifuging 45 min at $100,000 \times g$, 25°C.
29. In the cold room, discard the supernatant. Add 1 ml of column buffer containing 0.5 mM GTP to each tube. Making sure that the buffer is covering the pellet, immerse tube in liquid nitrogen to freeze pellet.

Store the tubes at -70°C until the next day or whenever phosphocellulose column purification of tubulin is to be carried out.

DAY 3: Purify tubulin from MT protein on phosphocellulose column

30. Chill ultracentrifuge and 50.2Ti rotor to 4°C.
31. Prepare and equilibrate phosphocellulose column with column buffer containing 0.5 mM GTP and 1 mM DTT. Turn off peristaltic pump, and if resin bed has settled, carefully loosen the seals on adjuster plunger. Slowly insert adjuster plunger further into column until it barely touches the top of the resin and retighten the seals. Switch the inlet tube from the reservoir of 1× column buffer to a 1-liter reservoir of 1× column buffer containing 0.5 mM MgGTP and 1 mM DTT. Be careful not to introduce bubbles into the inlet tube. Turn the peristaltic pump on and adjust speed to 1.8 ml/min. Allow ≥300 ml of buffer to be drawn through the column before loading the MT protein.
32. While column equilibrates, thaw MT pellets (from step 29) by immersing ultracentrifuge tubes in a 37°C water bath until pellets turn from chalky white to completely translucent white. When pellets are thawed, place tubes immediately on ice and put them in the cold room.
33. Add a volume of 1× column buffer containing 0.2 mM GTP equal to ~3× vol of pellets. Resuspend pellets on ice with a Dounce glass homogenizer and type A pestle (as in step 21). Transfer resuspended MTs to a 31.5-ml ultracentrifuge tube. Allow MTs to depolymerize by incubating on ice for 30 min with gentle mixing every 5 min.
34. Clarify the MT protein by centrifuging 45 min at 100,000 × g, 4°C.
35. In the cold room, collect the clarified MT protein supernatant and add MgGTP to a final concentration of 0.5 mM (an additional 0.3 mM) and DTT to 1 mM.

Load MT protein

36. When column has equilibrated, switch the inlet tube from the buffer reservoir to the clarified MT protein. Load the MT protein onto the column at 1.8 ml/min. When all of the protein is loaded, switch the inlet back to the buffer reservoir and begin to collect 10-ml fractions. As each aliquot comes off, add MgGTP to a final concentration of 1 mM (an additional 0.5 mM).

Tubulin will pass through the column and come off after ~100 ml. MT binding proteins will remain bound to the phosphocellulose resin.

37. Monitor the elution of tubulin by using a UV monitor to measure absorption at 280 nm. Pool fractions containing tubulin (usually ~100 ml).

Alternatively, the protein concentration of a 100-μl aliquot of each fraction can be determined by colorimetric assay (APPENDIX 3).

Separate MTs from denatured tubulin

38. Working at room temperature, add 0.186 g/ml glutamic acid (sodium salt) to the tubulin solution and stir slowly until dissolved. Dispense the solution into 31.5-ml ultracentrifuge tubes and pair tubes by weight.
39. Immerse the centrifuge tubes to the level of the liquid in a 37°C water bath and incubate 30 min to allow MTs to polymerize. During this time, warm the ultracentrifuge and rotor to 25°C.
40. Separate MTs from polymerization-incompetent tubulin by centrifuging 30 min at 100,000 × g, 25°C.
41. In the cold room, resuspend MT pellets on ice in 3 vol of PM buffer containing 0.5 mM MgGTP using a glass Dounce homogenizer. Incubate resuspended MTs on ice 30 min to allow MT depolymerization.

42. During this time, determine the tubulin concentration by measuring the absorbance at 280 nm (using PM buffer containing 0.5 mM MgGTP as a blank). Calculate the tubulin concentration using the formula: $[\text{tubulin}] = (A_{280} \times \text{dilution factor}) / \text{extinction coefficient}$, where the extinction coefficient of tubulin = 115,000 mol/cm. Add PM buffer containing 0.5 mM MgGTP to adjust the final protein concentration to 45 μM .
43. Dispense into several 1-ml aliquots (stock aliquots) and several 50- μl aliquots (to be used directly in the MT/organelle motility assays). Freeze by immersing in liquid nitrogen and store at -70°C until needed.

PREPARATION OF RAT LIVER CELL CYTOSOL

This protocol (modified from Donaldson et al., 1991) should yield enough cytosol for up to 200 MT/organelle motility assays.

Materials

Fresh or flash-frozen (Pel-Freez) rat livers
PBS (*APPENDIX 2A*)
Homogenization buffer (see recipe)
Homogenization buffer containing 0.5 mM MgGTP (from 100 mM MgGTP stock; see recipe)
PM buffer (see recipe) containing 0.25 M sucrose
Superspeed centrifuge (Sorvall RC-5B) with Sorvall SS-34 rotor (or equivalent)
Ultracentrifuge (Beckman L7-55) with Beckman 50.2Ti and SW-28 rotors (or equivalents)
20-ml glass homogenizer with Teflon pestle
Homogenizer or drill press
50-ml polycarbonate centrifuge tubes (e.g., Sorvall)
31.5-ml thick-walled polycarbonate ultracentrifuge tubes (e.g., Beckman) with screw caps
Additional reagents and equipment for determination of protein concentration (*APPENDIX 3*)

1. Chill the superspeed centrifuge, ultracentrifuge, and rotors to 4°C .
2. In the cold room, rinse liver well in PBS, then rinse in freshly prepared homogenization buffer.
3. Weigh liver, return to the cold room, and mince tissue finely with scissors. Transfer minced tissue to 20-ml glass homogenizer and add 1 vol homogenization buffer containing 0.5 mM MgGTP. Keeping the homogenizer immersed in slushy ice, homogenize tissue with a Teflon pestle attached to a homogenizer or drill press using six slow passes at 3000 rpm.
4. Transfer homogenate to 50-ml centrifuge tubes, pair tubes by weight, and remove cellular debris by centrifuging 10 min at $10,000 \times g$ (9000 rpm in SS-34 rotor), 4°C .
5. In the cold room, collect supernatant, transfer to 31.5-ml ultracentrifuge tubes, and pair tubes by weight. Clarify cytosol by centrifuging 60 min at $100,000 \times g$ (29,000 rpm in 50.2Ti rotor), 4°C .
6. Collect clarified cytosolic supernatant, dispense into 50- μl aliquots, and freeze by immersion in liquid nitrogen. Store at -70°C until use.

PREPARATION OF RAT LIVER ORGANELLE FRACTIONS

This protocol should yield enough organelles for up to 200 MT/organelle motility assays.

**SUPPORT
PROTOCOL 5**

Additional Materials (also see Support Protocol 4)

Homogenization buffer (see recipe) containing 0.5 mM MgGTP (from 100 mM stock; see recipe) and 0.25 M sucrose

Homogenization buffer containing 0.5 mM MgGTP

2.3 M sucrose

PM buffer (see recipe) containing 0.25 M sucrose and 0.5 mM GTP

3 M KI stock (optional)

0.5 M EDTA stock (optional; *APPENDIX 2A*)

0.5 M Na₂CO₃ stock, pH 11.5 (optional)

PM buffer containing 0.25 M sucrose (optional)

25-ml Ultraclear ultracentrifuge tubes (e.g., Beckman)

Beckman SW-28 and 50.2Ti rotors or equivalents

31.5-ml thick-walled polycarbonate ultracentrifuge tubes (e.g., Beckman) with screw caps

TLA tabletop ultracentrifuge (Beckman) with TLS-55 swinging bucket rotor or equivalent and mini-ultracentrifuge tubes (e.g., Beckman)

Additional reagents and equipment for determining protein concentration (*APPENDIX 3*)

Clarify rat liver homogenate

1. Rinse liver, mince, and perform initial centrifugation as in the preparation of rat liver cytosol (see Support Protocol 4, steps 1 to 4), except in step 2, homogenize minced liver in homogenization buffer containing 0.5 mM MgGTP and 0.25 M sucrose.
2. In the cold room, collect the supernatant from the clarified homogenate and add 2.3 M sucrose to a final concentration of 1.25 M sucrose.

Isolate Golgi and ER

3. Set up a sucrose density step gradient in a 25-ml Ultraclear ultracentrifuge tube as follows, using 2.0, 1.25, 1.1, and 0.25 M sucrose solutions prepared by diluting the 2.3 M sucrose stock solution with homogenization buffer containing 0.5 mM MgGTP, chilled to 4°C. Add a 2-ml 2.0 M sucrose cushion to the bottom of the tube. Being careful to avoid disturbing the sucrose cushion, layer 12 ml of clarified homogenate containing 1.25 M sucrose on the 2.0 M sucrose by using a pipet and allowing the solution to slowly run in a steady stream down the side of the centrifuge tube. Add a 12-ml layer of 1.1 M sucrose and then an 8-ml layer of 0.25 M sucrose. Discard any excess homogenate. Pair the gradient with a balance tube of equal weight.
4. Separate the organelles on the basis of density by centrifuging the gradient 3 hr at 100,000 × *g* (28,000 rpm in SW-28 rotor), 4°C.
5. Working in the cold room, use a Pasteur pipet to carefully harvest the off-white band of Golgi membrane at the interface between the 0.25 M and 1.1 M sucrose layers and the off-white band of ER membrane at the interface between the 1.1 M and 1.25 M sucrose layers. Place each in separate 31.5-ml ultracentrifuge tubes on ice.
6. Determine protein concentration of the Golgi and ER membrane fractions (*APPENDIX 3*).

Organelle Motility

13.1.13

7. Dilute both membrane fractions with 3 vol of 0.25 M sucrose in homogenization buffer containing 0.5 mM GTP. Pellet membranes by centrifuging 60 min at 100,000 × g (29,000 rpm in 50.2Ti rotor), 4°C.
8. In the cold room, discard the supernatants and resuspend the dense, sticky ER and Golgi membrane pellets separately by extensive trituration in PM buffer containing 0.25 M sucrose and 0.5 mM GTP to achieve a protein concentration of 5 mg/ml.
9. Dispense into 20-μl aliquots and freeze by immersion in liquid nitrogen. Store at -70°C until use.
10. To determine the proper dilution of ER of Golgi membranes for use in the motility assay, view various dilutions of membranes in PM buffer in simple perfusion chambers by VE-DIC microscopy. Note the dilution required such that ~20% of the area of the microscopic field is covered with organelles.
11. When preparing membranes prior to the MT motility assay, thaw one of the aliquots frozen in step 9 and make a stock membrane preparation 6× the dilution determined in step 10.

Prepare stripped membranes (optional)

12. To strip the membranes, incubate a small aliquot (~50 to 100 μl) of isolated organelles with one of the following solutions for 30 min on ice:

Salt-washed organelles: 0.6 M KI (from 3 M KI stock);

EDTA-stripped organelles: 10 mM EDTA (from 0.5 M EDTA stock);

Carbonate-washed organelles: 150 mM Na₂CO₃ (from 0.5 M Na₂CO₃ stock, pH 11.5).

13. Transfer to a mini-ultracentrifuge tube and pellet membranes by centrifuging 1 hr at 110,000 × g (50,000 rpm in TLS-55 rotor) at 4°C in a Beckman TLA tabletop ultracentrifuge.
14. Carefully remove supernatant and resuspend membrane pellet by gentle trituration in the original volume of PM buffer containing 0.25 M sucrose.

Treat membranes with pharmacological agents

15. Incubate membranes, cytosol, and energy mix with brefeldin A, aluminum fluoride, or GTP-γ-S (30 min at 37°C) prior to adding them to the mix (see Table 13.1.1). Isolate treated membranes by centrifugation as in step 13. Resuspend and use as described for stripped membranes.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

AlCl₃, 30× (1.5 mM)

20 mg AlCl₃

Distilled water to 100 ml

Store <1 year at room temperature

Brefeldin A, 3.6 mM

10 mg brefeldin A (Epicenter Technologies)

100% ethanol to 12 μl

Store <1 year at -20°C

Column buffer, 10×

500 ml 1 M PIPES (see recipe; 250 mM final)
40 ml 0.5 M EGTA (see recipe; 10 mM final)
40 ml 1 M MgSO₄ (5 mM final)
Distilled H₂O to 1800 ml
Adjust pH to 6.7
Add distilled H₂O to 2 liters
Store <1 month at 4°C

EGTA, 0.5 M

19.02 g EGTA sodium salt
Distilled H₂O to 90 ml
Adjust pH to 7.0
Distilled H₂O to 100 ml
Store <1 year at room temperature

Energy regeneration system, 20× (see Murray, 1991)

150 mM creatine phosphate (Boehringer Mannheim)
2 ml 100 mM MgATP (see recipe; 20 mM ATP/20 mM MgSO₄ final)
40 ml 0.5 M EGTA (see recipe; 2 mM final)
Distilled H₂O to 10 ml
Dispense into 100- μ l aliquots and store indefinitely at -20°C

HEPES, 1 M, pH 7.0

119.15 g *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (free acid)
Distilled H₂O to 400 ml
Add solid NaOH, a few pellets at a time while mixing, until the pH is ~6.8
Add concentrated NaOH dropwise to pH 7.0
Add distilled H₂O to 500 ml
Filter sterilize and store <6 months at 4°C

High-salt buffer

120 ml 5 M NaCl (0.6 M final)
4 ml 1 M MgSO₄ (4 mM final)
2 ml 0.5 M EDTA (*APPENDIX 2A*; 1 mM final)
10 ml 1 M HEPES (see recipe; 10 mM final)
Distilled water to 900 ml
Adjust pH to 7.0
Add H₂O to 1 liter
Store <1 year at 4°C
Add 2-mercaptoethanol (2-ME) to 7 mM final and DTT (*APPENDIX 2A*) to 1 mM final just before use

Homogenization buffer

300 ml PM buffer (see recipe)
52.3 mg PMSF (1 mM final)
3 g leupeptin (10 mg/ml final)
300 μ g pepstatin A (1 μ g/ml final)
3 mg *N*- α -*p*-tosyl-L-arginine methyl ester (TAME, 10 μ g/ml final)
Mix well to dissolve; use immediately

Isolation buffer

20 ml 5 M NaCl (0.1 M final)
4 ml 1 M MgSO₄ (4 mM final)
2 ml 0.5 M EDTA (*APPENDIX 2A*; 1 mM final)
10 ml 1 M HEPES (see recipe; 10 mM final)
Distilled H₂O to 900 ml
Adjust pH to 7.0
Add distilled H₂O to 1 liter
Store <1 year at 4°C
Add 2-mercaptoethanol (2-ME) to 7 mM final just before use

MgAMP-PNP, 150 mM

25 mg AMP-PNP (Boehringer Mannheim)
90 μl 1 M MgSO₄ (150 mM final)
Add 225 μl distilled H₂O (to 315 μl)
Store <2 years at -20°C

MgATP, 100 mM

Check formula weight of the lot of ATP and determine the amount required for 10 ml of 100 mM solution. Add 8.5 ml distilled H₂O to the determined amount of ATP. Add 1 ml 1 M MgSO₄ stock and adjust pH to 7.0 using NaOH. Add H₂O to 10 ml. Dispense into 200-μl aliquots and store <2 years at -20°C.

MgGTP, 100 mM

Check formula weight of the lot of GTP and determine the amount required for 10 ml of 100 mM solution. Add 8.5 ml distilled H₂O to the determined amount of GTP. Add 1 ml 1 M MgSO₄ stock and adjust pH to 7.0. Add H₂O to 10 ml. Dispense into 200-μl aliquots and store <2 years at -20°C.

MgGTP-γ-S, 30 mM

10 mg GTP-γ-S tetralithium salt (Boehringer Mannheim)
18 μl 1 M MgSO₄ stock (30 mM final)
Add 572 μl distilled H₂O
Store <2 years at -20°C

NaF, 30×

378 mg NaF (0.9 M final)
Distilled H₂O to 10 ml
Store indefinitely at -20°C

Na₂CO₃, 0.5 M

531 mg Na₂CO₃
Distilled H₂O to 10 ml
Store <1 year at room temperature

PIPES, 1 M, pH 6.9

151.2 g piperazine-*N,N'*-bis(2-hydroxypropanesulfonic acid), free acid
Distilled H₂O to 400 ml
Add solid NaOH a few pellets at a time while mixing until the pH is ~6.7
Add concentrated NaOH dropwise to pH 6.9
Add distilled H₂O to 500 ml
Filter sterilize and store <1 year at 4°C

PM buffer, pH 6.9

100 ml 1 M PIPES (see recipe; 100 mM final)
2 ml 1 M MgSO₄, (2 mM final)
2 ml 0.5 M EGTA (see recipe; 1 mM final)
Add distilled H₂O to 900 ml
Adjust pH to 6.9
Add distilled H₂O to 1 liter
Store <2 months at 4°C

PMG buffer

80 ml 1 M PIPES (see recipe; 80 mM final)
2 ml 1 M MgSO₄, (2 mM final)
2 ml 0.5 M EGTA (see recipe; 1 mM final)
600 ml glycerol
Distilled H₂O to 900 ml
Mix well
Adjust pH to 6.9
Add distilled H₂O to 1 liter
Store <1 month at 4°C

Sodium orthovanadate, 100 mM

1.839 g sodium orthovanadate
Distilled H₂O to 8 ml in a screw-cap tube
Adjust pH to 10 with NaOH or HCl
If solution is yellow, place in boiling water until clear, then recheck pH (repeat as necessary)
Adjust to final concentration by checking A_{265} and adding distilled water as needed
Store indefinitely at -20°C

The extinction coefficient for sodium orthovanadate is 2925 M⁻¹cm⁻¹.

Valap

50 g Vaseline (Fisher)
50 g lanolin (Fisher)
50 g paraffin (Fisher)

Place vaseline, lanolin, and paraffin in a 1-liter Pyrex beaker. Heat on low setting on a hot plate, stirring occasionally, until all components are melted and well mixed. Pour into several small screw-cap jars (~50 ml capacity). Store indefinitely at room temperature.

COMMENTARY**Background Information**

As proteins mature and are processed and secreted, they move continuously between the membranous network compartments of the endoplasmic reticulum (ER) and Golgi apparatus, and from the Golgi apparatus to the cell surface. In living cells, this dynamic membranous organelle traffic occurs through a process in which membrane tubules or vesicles bud from one membrane compartment and fuse with another to deliver their contents. Membrane tubulation, budding, and fusion is believed to be controlled by G proteins and specific “coat”

proteins that reversibly associate with the organelle surface (Klausner et al., 1992; Pfeffer, 1994; Bennett, 1995; Schmid and Damke, 1995). In addition, in living cells, ER and Golgi membrane tubules extend and branch in a microtubule (MT)-dependent fashion (Terasaki et al., 1986; Dailey and Bridgman, 1989; Lee et al., 1989; Cooper et al., 1990; Terasaki and Reese, 1994). MT-dependent membrane trafficking is believed to involve the MT-based motor proteins kinesin and cytoplasmic dynein (Lippincott-Schwartz et al., 1995; Presley et al., 1997; reviewed in Cole and Lippincott-

Schwartz, 1995; Sheetz, 1996). The MT/organelle motility assay provides an *in vitro* system in which MT-based membrane tubule movement and fusion can be observed and the effects of pharmacological, immunochemical, or biochemical treatments can be tested. As mentioned in the Basic Protocol, manipulations of the system may include: the addition of known effectors of coat proteins, G proteins, or MT-based motors to the membrane mix; the removal of subsets of peripheral proteins from the isolated organelles; and fractionation or immunoprecipitation of specific proteins from the cytosol prior to addition to the membrane mix.

ER-like membrane tubules have been shown to form *in vitro* in a MT-dependent fashion by two mechanisms. The first involves motor proteins and occurs when crude organelle fractions are mixed with cytosolic extracts and taxol-stabilized MTs in the presence of ATP. Here, membranes make gliding attachments to MTs or stable attachments to gliding MTs (Dabora and Sheetz, 1988; Vale and Hotani, 1988; Allan and Vale, 1991, 1994; Gill et al., 1991; Schroer and Sheetz, 1991; Allan, 1995). The second mechanism is independent of motor ATPase activity and occurs in extracts of *Xenopus laevis* eggs. In this case, membranes attach to the growing ends of MTs, and membrane tubules extend by a MT polymerization-dependent mechanism (Waterman-Storer et al., 1995). Similar MT polymerization-dependent movements of chromosomes have also been demonstrated *in vitro* (Coue et al., 1991; Lombillo et al., 1995a,b). The assay described in the Basic Protocol improves on previously published membrane motility assays in that the MTs are nucleated from a stationary point (a fragment of a flagellar axoneme), allowing their free ends to grow and shorten similar to the way they would behave in a living cell. This assay permits observation of both motor-driven motility of organelles and MT polymerization-driven mechanisms of organelle movement. Nucleation of MTs from stationary axonemes also provides a good marker for determining the polarity of organelle movement, as MTs grow more robustly from the plus ends of axonemes than from their minus ends. Thus axoneme-nucleated MTs with free plus ends are much longer than those with free minus ends (Walker et al., 1988).

Critical Parameters and Troubleshooting

The success of the membrane MT motility assay depends on two basic parameters: the

cleanliness of the slides, coverslips, and perfusion chamber, and the quality of the biochemical components used in the assay. Adherence to the recommended choice of brand names and proper cleaning of coverslips is tantamount to making a good perfusion chamber for the motility assay.

Motile interactions between membranes and MTs take 30 to 45 min to develop. If no motility occurs after this period of time, try raising the room temperature. Be aware that many of the manipulations of membrane mixes suggested in the Basic Protocol have not yet been described in published work. Thus they may not yield any results, much less interesting results.

For all biochemical preparations, pay attention to the cleanliness of the glassware. Use double glass-distilled water for the preparation of solutions. Plan manipulations carefully in advance and follow the protocols with expedience.

The most important step for getting pure axonemes (Support Protocol 2) with very little debris is the careful collection of only the top, white layer of the pellet (containing sperm tails) without collecting any of the yellowish bottom layer of the pellet (containing heads and debris). The pellet must be washed until it is pure white and free of debris before completing the protocol. When determining the proper axoneme dilution, it is possible to check the purity of the preparation. If excess non-axoneme material appears to be present, dilute the axonemes 1:4 with isolation buffer (final glycerol concentration is 12.5%), centrifuge, and resuspend pellet in isolation buffer plus 50% glycerol. Check the purity of the axonemes by microscopy. If the preparation still appears “dirty,” repeat the wash step until the axonemes appear “clean.”

Several steps are critical for the preparation of tubulin (Support Protocol 3). First, proper packing of the phosphocellulose column is necessary to obtain a good concentrated peak of tubulin. If the resin has a cracked appearance after packing, it should be emptied, resuspended in column buffer, and repoured. If this procedure requires an extra day, keep the pellets of MT protein frozen until the column is packed properly. Never use phosphocellulose resin that is >1 week old. Tubulin is susceptible to proteolysis and easily denatured; therefore time is critical throughout the preparation of MT protein. Thorough removal of brain meninges, which contain high levels of blood proteases, and addition of protease inhibitors to the homogenization buffer just prior to use is very

important. Improper resuspension of microtubule pellets in a Dounce homogenizer can greatly reduce tubulin yields; be very careful not to cause bubbling in the solution and always keep the homogenizer on ice when moving the pestle. Be sure that rotor temperatures are equilibrated prior to use and that enough centrifuge tubes are clean. Fortunately, even if timing is drastically off or slightly careless technique is used, the preparation should yield some usable protein.

When preparing rat liver organelles (Support Protocol 4), pour the step gradients so that there is as little mixing between steps as possible. This is the most critical step to clean separation of the different fractions of organelles. To avoid diluting organelles at the interface between the gradient steps, avoid collecting solution above and below the interface; it is better to leave some membrane behind than to collect too much from adjacent fractions that may contain soluble protein complexes. Finally, some labs insist that interactions between motor proteins and organelles are better preserved when organelle fractions are isolated on Nycodenz gradients. If active motor proteins are present in the MT/organelle motility assay (i.e., microtubules or whole axonemes glide on the coverslip surface), but organelles do not form motile interactions with microtubules, consider isolating organelles on Nycodenz gradients (McIlvain et al., 1993; Kumar et al., 1995).

Anticipated Results

The membrane MT motility assay should generate the formation of moving membrane tubules and vesicles similar to those seen in

Figure 13.1.2. Key observations may include the following: (1) What form do the membranes take on: tubular, vesicular, or both? (2) Are both static and dynamic interactions between membranes and MTs observed in the same preparation? (3) How are the membranes attached to the MT: along the shaft or at the end? (4) What direction do the membranes move on the MT? (5) Do axonemes or free MTs glide on the coverslip surface, and in what direction do they glide? (6) Does the motility change direction? Comparison of different assays, using differently treated membrane mixes, may yield interesting differences in these behaviors.

Sperm axonemes prepared from four sea urchins (Support Protocol 2) should yield enough fragments for thousands of motility assays. Isolation of tubulin from three pig brains (Support Protocol 3) should yield ~60 mg of purified tubulin. One rat liver should yield enough cytosol (Support Protocol 4) or organelles (Support Protocol 5) for up to 200 assays.

Time Considerations

Once all biochemical components are prepared, their proper dilutions determined, and a supply of perfusion chambers is made, the MT/organelle motility assay should take 2 hr to perform, allowing 30 to 45 min to record the motility on video tape. Thus, several different membrane mixes or treatment conditions can be tested in a single day. New aliquots of membrane and cytosol should be thawed for each new assay done in a single day.

Axonemes, rat liver organelles, and rat liver cytosol are all relatively easy to prepare; each requires two 3- to 6-hr days. Reagents and

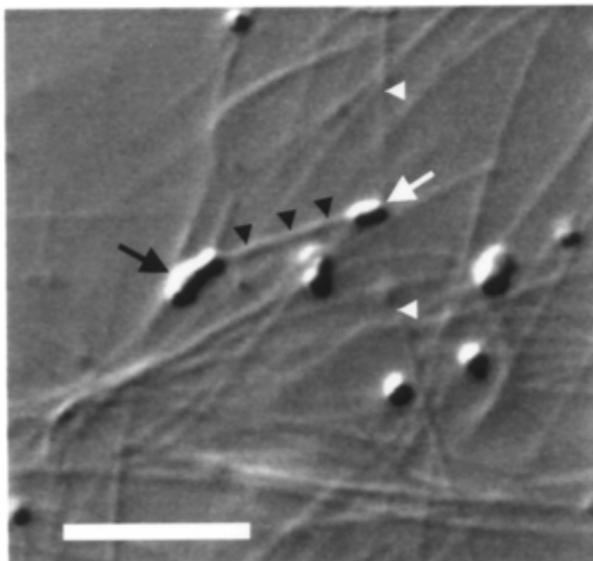


Figure 13.1.2 VE-DIC micrograph of a membrane/microtubule motility assay. Membrane associated with an axoneme fragment (black arrow) has extended a thin membrane tubule (black arrowheads) via a motile attachment (white arrow) to a single microtubule (white arrowheads). Many single microtubules and membrane vesicles can be seen in this field. Bar = 5 μ m.

buffers are prepared on the first day and the components are purified the second day. Tubulin purification is much more difficult, and requires 4 long (8- to 12-hr) days of work. Buffers and reagents are prepared on the first day. The phosphocellulose column is prepared during the second day. The pig brains should be acquired fresh on the third day for the preparation of MT protein, and on the fourth day tubulin is purified from the MT protein on the phosphocellulose column. The first tubulin preparation attempted will probably not give the best results. The organization, timing, and motor skills required for the preparation must be practiced and developed over time, and the preparation must often be adapted to the constraints of the particular laboratory in which it is performed (types of rotors available, cold room space available) before this purification results in high protein yields.

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Key References

Coue et al., 1991 and Waterman-Storer et al., 1995.
See above.

In these papers, in vitro assays show that organelles can be moved by microtubule polymerization and depolymerization in the absence of motor ATPase activity.

Dabora and Sheetz, 1988 and Vale and Hotani, 1988.
See above.

These papers were the first to use in vitro assays to reconstitute microtubule motor-based organelle motility.

Klausner et al., 1992. See above.

This review clearly describes the roles and regulation of coat proteins in the morphology and movement of organelles through the secretory pathway.

Salmon and Tran, 1998. See above.

This paper provides an in-depth description of the theory and practice of setting up the microscope system required to perform the MT/organelle motility assay.

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In Vitro Motility Assay to Study Translocation of Actin by Myosin

A most basic property of myosin is its ability to interact with and translocate actin. This unit describes an in vitro motility assay that can be used to study the translocation, or sliding, of actin filaments by myosin bound to a coverslip. The assay makes use of the ability to image single F-actin filaments labeled with rhodamine phalloidin, a high-affinity fluorescent ligand using fluorescence microscopy. The system is fast, easy to set up and maintain, uses only small amounts of protein, and yields quantitative results.

Several preliminary steps are required before the in vitro motility assay can be conducted. Special flow cells that are used in the assay must be prepared (see Support Protocol 1). The assay also requires purified myosin and actin. Other proteins, such as the regulatory proteins tropomyosin and troponin must be prepared if needed for assay variations. The method for preparing myosin varies greatly depending on the type of myosin being studied (see Table 13.2.1). Actin can be purified by the method in Support Protocol 2, and then it must be labeled with rhodamine phalloidin (see Support Protocol 3). When the in vitro motility assay is conducted (see Basic Protocol), the flow cell is placed under the objective of a fluorescent microscope, and the image is recorded on videotape. Afterward, the data from the videotape can be quantified.

ANALYZING ACTIN TRANSLOCATION BY MYOSIN

Translocation of actin by myosin is followed using rhodamine phalloidin–labeled actin exposed to immobilized myosin in a flow cell. The assay can be modified for specific purposes. For example, myosin can be applied to the coverslip surface either as filaments or as monomers. The enzymatically active fragments of myosin, heavy meromyosin (HMM) or subfragment-1 (S1), can also be used. The ionic strength can be varied over a range extending up to physiological levels and the temperature can be varied over a wide range. The movement of pure actin, or of actin complexed with regulatory proteins, can be measured.

The assay is best conducted with freshly prepared myosin, although it is possible to use myosin that has been flash frozen and stored in liquid nitrogen. The presence of damaged myosin heads (dead heads) that bind actin, but do not hydrolyze ATP, contributes to poor quality of movement. Two procedures can minimize this problem. The myosin-coated coverslip can be washed with a solution containing unlabeled actin in the presence of ATP before the rhodamine phalloidin–labeled actin is introduced, as is done in this protocol. This has the effect of complexing the dead heads with the unlabeled actin. If this simple treatment does not prove sufficient, a stoichiometric amount of F actin can be added to myosin in the presence of ATP at high ionic strength and sedimented at $480,000 \times g$ for 15 min in a Beckman TL100 ultracentrifuge. This removes dead heads before the myosin sample is applied to the coverslip.

Quantification of the images can be done in several ways. The most thorough method makes use of a dedicated automatic tracking system such as the Cell Trak system from Motion Analysis (Homsher et al., 1992). Another alternative involves the use of frame grabbers to digitize images coupled with the use of tracking software run on a desktop computer (Work and Warshaw, 1992; Marston et al., 1996).

BASIC PROTOCOL

Table 13.2.1 Purification Protocols for Myosins

Myosin type	Purification protocol
Rabbit skeletal muscle myosin	Margossian and Lowey, 1982
Invertebrate striated muscle myosin	Sellers, 1981
Chicken smooth muscle	Sellers et al., 1981
Nonmuscle myosin II	Daniel and Sellers, 1992
Native thick filaments	Sellers and Kachar, 1990
Myosin I	Collins et al., 1990
Myosin V	Nascimento et al., 1996
Baculovirus/ <i>Sf9</i> -expressed recombinant myosins	Trybus, 1994 ^a

^aIf the recombinant myosin is HIS or FLAG tagged, specific affinity columns can be used to aid in purification.

Materials

- 0.2 mg/ml myosin monomers (see Table 13.2.1)
- 1 mg/ml BSA in G-actin buffer (see recipe)
- Wash buffer (see recipe)
- Wash buffer containing 1 mM ATP and 5 μ M actin (see Support Protocol 2)
- Wash buffer containing 20 nM rhodamine phalloidin–labeled actin (see Support Protocol 3)
- Assay buffer (see recipe)
- Flow cell (see Support Protocol 1)
- Fluorescent microscope, with high-numerical-aperture objective (60 \times to 100 \times , 1.3 to 1.4 NA) and 100-W mercury lamp
- SIT camera or an intensified CCD
- VHS or sVHS video recorder
- Image processor
- Cell Trak image analysis system (Motion Analysis)

1. Fill the flow cell with myosin monomers (or the soluble fragments HMM and S1) at a concentration of 0.2 mg/ml. Allow the solution to remain in the cell for 1 to 2 min to allow myosin to bind.

Myosin monomers are applied in a high-ionic-strength buffer (e.g., 0.5 M NaCl/10 mM MOPS [pH 7.0]/0.1 mM EGTA/1 mM DTT), whereas HMM or S1 can be applied at a low ionic strength (e.g., 10 mM MOPS [pH 7.0]/0.1 mM EGTA/1 mM DTT). Myosin filaments must be applied at low ionic strength in a buffer where myosin filaments are stable such as 50 mM KCl/10 mM MOPS (pH 7.0)/1 mM MgCl₂/0.1 mM EGTA/1 mM DTT.

2. Wash the flow cell with 2 to 3 volumes of 1 mg/ml BSA in actin buffer. Allow the last volume of buffer to remain in the cell for 1 to 2 min to block non-specific binding.

This serves to wash unbound myosin from the flow cell and block the surface. Note that if myosin filaments are being applied to the surface, it is necessary to wash the flow cell with BSA in a low-ionic-strength buffer that will not depolymerize myosin filaments.

3. Wash the flow cell with 2 to 3 vol wash buffer containing 1 mM ATP and 5 μ M actin. Allow the last volume of solution to remain in the flow cell for 1 to 2 min.

Note that prior to use this actin/ATP–containing solution is vortexed intensely or passed through a narrow-gauge syringe in order to shear the unlabeled actin. The actin added in this step binds to damaged, noncycling myosins and dramatically improves the quality of measurements. The actin is sheared to increase the accessibility to the surface.

4. Wash the flow cell with 2 to 3 vol wash buffer to remove ATP, followed by 2 vol wash buffer containing 20 nM of rhodamine phalloidin–labeled actin. Allow to bind for 0.5 to 1 min.

5. Begin the motility assay by adding assay buffer.

Note that this buffer is viscous due to the methylcellulose and it may be necessary to use a piece of filter paper placed at the outflow of the flow cell as a wick to pull the solution into the cell.

6. Place the flow cell on the microscope stage and observe the motion of the rhodamine phalloidin–labeled actin using a filter cube for detecting rhodamine fluorescence and a high-NA 60× to 100× objective.

Movement of actin by myosin is dependent on temperature, and thus it is necessary to regulate the temperature of the slide. Most investigators use 25° or 30°C as standard. This can be accomplished in several ways. The least expensive way is to place a blow dryer at an appropriate distance to yield the desired temperature. It is also possible to regulate the temperature of the assay by jacketing the objective using either a water jacket or a Peltier-type device. Simply regulating the temperature of the stage is usually not sufficient to control the temperature because the objective constitutes a large heat source. The temperature of the sample in the flow cell can be estimated using a fine wire thermister mounted between a coverslip and a slide. This “dummy” flow cell is mounted on the stage, and the objective is brought to the same position as the one used when imaging a flow cell.

It is useful to have several neutral density filaments that can be placed in the light path to attenuate the light intensity to avoid bleaching of rhodamine fluorescence.

7. Process the results with either an intensified CCD (charge-coupled device), a neuvi-con camera, or a SIT (silicon-intensified target) camera to detect the image and an sVHS video recorder to record the image movements, which can be displayed on a standard black-and-white video monitor.

It is useful to have an image processor to conduct frame averaging and background subtraction. This is especially helpful if the myosin being studied moves actin filaments slowly. This can be accomplished using either a dedicated image processor such as the Argus-20 (Hamamatsu) or image-processing software running on a desktop computer. If an image processor is used, it is useful to have two monitors, one for the processed image and one for the raw image.

8. Quantify results by measuring the position of an actin filament over time, measuring five to ten time points for each actin filament. Calculate and record the mean velocity for the measurements.

Quantification of the rate of movement is most easily accomplished using an automated tracking system such as the Cell Track System by Motion Analysis. This system digitizes the image of all actin filaments in a video sequence, determines the centroid position, determines the path, and calculates the main velocity of each actin filament. In the absence of such an automated system, some investigators have written their own software using commercially available frame grabbers (Work and Warshaw, 1992; Winkleman et al., 1995).

**SUPPORT
PROTOCOL 1**

PREPARATION OF FLOW CELLS

The actin translocation assay requires nitrocellulose-coated coverslips applied to a simple flow cell.

Materials

1% (w/v) nitrocellulose (Ernest F. Fullham) in amyl acetate
Apiezon M grease (Thomas; optional)

Double-sided tape
18-mm² no. 1 coverslips
Glass microscope slides
24 × 60-mm no. 0 coverslips (optional)

1. Place a 2- μ l drop of 1% nitrocellulose in amyl acetate onto a no. 1 coverslip and spread the drop over the surface using the long axis of the pipet as a swab. Allow the surface to dry (5 min).

Alternatively, to create a thin film of nitrocellulose, apply 1 drop of 1% nitrocellulose from a Pasteur pipet to the surface of deionized water in a 10-cm round dish and allow the amyl acetate to evaporate. Place no. 1 coverslips onto the dried film. Tear excess film away from the area surrounding the coverslips using forceps. Remove the coated coverslips by submersing and inverting them while lifting them from the water. Air dry the coated coverslip with the film side up. Coverslips and microscope slides are typically used as they come from the box.

2. Apply two parallel strips of double-sided tape ~10 mm apart on a glass microscope slide. Place a nitrocellulose-coated coverslip with the coated side down on top of the tape strips to create a quick and simple flow cell.

Alternatively, cut spacers (24 × 3 to 4 mm) from a no. 0 coverslip using a diamond scribe. Place parallel tracks of Apiezon M grease ~10 mm apart and 25 mm long on the long axis of a glass microscope slide. Place the thin coverslip spacers on the outside of the grease tracks and cover them with a nitrocellulose-coated coverslip with the coated side down. It is also possible to glue the spacers and coverslip to the slide with fingernail polish.

**SUPPORT
PROTOCOL 2**

PURIFICATION OF ACTIN

Rabbit muscle actin used for the translocation assay is most often purified by this method (Spudich and Watt, 1971). After it is prepared, a portion is labeled with rhodamine phalloidin (see Support Protocol 3).

Materials

Back and leg muscles of freshly sacrificed 350-g rabbit
0.1 M KCl/0.15 M potassium phosphate, pH 6.5
0.05 M NaHCO₃
1 mM EDTA, pH 7.0
Acetone
G-actin buffer (see recipe)
2 M KCl (stock solution)
1 M MgCl₂ (stock solution)

Meat grinder, prechilled
Cheesecloth
Filter paper
Sorvall centrifuge and SS-34 rotor (or equivalent)
Beckman ultracentrifuge and 55 Ti rotor (or equivalent)
Potter-Elvehjem tissue grinder

Additional reagents and equipment for dialysis (*APPENDIX 3*)

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: All procedures are performed at 4°C unless otherwise specified and all buffers should be prechilled to this temperature.

Prepare actin powder

1. Excise the back and leg muscles of a freshly sacrificed rabbit, wash them free of blood with distilled water, and chill on ice.

Steps 2 to 6 should be carried out in a cold room at 4°C.

2. Grind the muscle in a meat grinder that has been prechilled.
3. Extract the mince with 1 liter of 0.1 M KCl/0.15 M potassium phosphate, pH 6.5, for 10 min with stirring. Filter the mince by squeezing it through several layers of cheesecloth.

Stirring in steps 3 to 7 is done by hand using a glass rod or spatula or by using an overhead motorized stirrer. The suspension is too viscous to use a magnetic stirrer.

Filtration is most easily accomplished by spreading several layers of cheesecloth over a large funnel which is supported by a ring stand. This method of filtering is also used in steps 4 to 7.

4. Extract the retained mince with 2 liters of 0.05 M NaHCO₃ for exactly 10 min with stirring, and filter as in step 3.
5. Extract the filtered mince with 1 liter of 1 mM EDTA, pH 7.0, for 10 min with stirring.
6. Extract the mince twice with 2 liters distilled water for 5 min each time.
7. Extract the mince five times at room temperature with chilled acetone for 10 min each time. Stir to break up residue clumps and filter the extract.
8. Spread out the acetone-washed, filtered residue on a large piece of filter paper and dry overnight in a hood.

The resulting acetone powder can be stored for months at -20°C.

Extract the acetone powder

9. Extract the acetone powder (typically 5 g) in 20 ml of G-actin buffer per gram of powder on ice, 30 min with stirring.
10. Filter the extract through cheesecloth (see step 3 annotation) and retain the filtrate. Reextract the residue using 20 ml of the same buffer per gram of original dried acetone powder, 10 min with stirring.
11. Filter through cheesecloth again, retaining the filtrate, and combine the filtrates.
12. Centrifuge 1 hr at 40,000 × g, 4°C. Carefully decant the supernatant and discard the pellet.

Polymerize the actin

13. Add KCl to 50 mM and MgCl₂ to 2 mM to polymerize actin. Allow polymerization to continue for 2 hr on ice.
14. Slowly add solid KCl to a final concentration of 0.8 M and stir gently 30 min.

This step helps to remove tropomyosin and α-actinin contaminants.

15. Centrifuge 1.5 hr at $150,000 \times g$, 4°C , to sediment the polymerized actin.
16. Resuspend the F-actin pellets by homogenization in a Teflon-lined homogenizer using 3 ml G-actin buffer per gram of acetone powder. Dialyze for 2 days in G-actin buffer with repeated changes to depolymerize the actin.
17. Centrifuge the depolymerized actin 1.5 hr at $150,000 \times g$, 4°C . Retain the supernatant.
18. Add KCl and MgCl_2 to concentrations of 50 mM and 2 mM, respectively, to polymerize the actin. Dialyze in the buffer of choice containing 1 to 2 mM MgCl_2 to maintain the polymerized state. Store on ice at a concentration of 100 to 200 μM for ~1 month.

If the actin is stored longer than 1 month, it should be put through a new depolymerization/polymerization cycle by repeating steps 16 to 18.

**SUPPORT
PROTOCOL 3**

PREPARATION OF RHODAMINE PHALLOIDIN-LABELED ACTIN

Actin is labeled with rhodamine phalloidin so that its translocation can be followed in the assay described in the Basic Protocol. Rhodamine phalloidin serves two purposes: the phalloidin moiety binds to actin and stabilizes the filaments against depolymerization; and the rhodamine moiety is a common fluorophore that is excitable by green light.

Materials

Rhodamine phalloidin (Molecular Probes)
Methanol
Labeling buffer (see recipe)
Actin (see Support Protocol 2, step 18)
Wash buffer (see recipe)
Speed-Vac evaporator (Savant) or equivalent

1. Place 60 μl of rhodamine phalloidin in a microcentrifuge tube and dry in an evaporator.
2. Redissolve the dried powder in 5 μl methanol.
3. Add 85 μl labeling buffer.
4. Dilute actin to 20 μM in labeling buffer. Add 10 μl of 20 μM actin and incubate overnight. Store on ice up to 1 month.
5. Dilute an aliquot to 20 nM actin with wash buffer the day of use.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Assay buffer

Wash buffer (see recipe) containing:
0.7% (w/v) methylcellulose (see recipe)
1 mM ATP
50 mM DTT
2.5 mg/ml glucose
0.1 mg/ml glucose oxidase
0.02 mg/ml catalase
Prepare fresh daily

continued

Assay buffer should be prepared fresh daily from stocks of ATP (0.1 M ATP, pH 7.0, stored at -20°C), DTT (1 M, prepared fresh daily), glucose (2.5 mg/ml, stored at -20°C), glucose oxidase (Sigma), and catalase (Sigma).

The ionic conditions of this buffer (KCl, MgCl_2 , CaCl_2 concentrations) and the pH and temperature can be varied according to the experimental protocol.

G-actin buffer

2 mM Tris base
0.2 mM Na_2ATP
0.5 mM DTT
0.2 mM CaCl_2
3 mM NaN_3
Adjust pH to 8.5 with HCl
Store at 4°C

Labeling buffer

10 mM MOPS [3-(*N*-morpholino) propane sulfonic acid], pH 7.0
0.1 mM EGTA
3 mM NaN_3
Store at 4°C

Methylcellulose, 14% (w/v)

Prepare a 1.4% (w/v) solution of methylcellulose (Sigma). Dialyze the solution against deionized water for 24 hr. Store at -20°C until used.

Methylcellulose should have a viscosity of 4000 centipoise for a 2% solution at 20°C .

Wash buffer

80 mM KCl
5 mM MgCl_2
20 mM MOPS, pH 7.4
0.1 mM EGTA
Store at 4°C

COMMENTARY

Background Information

Myosin is an ATP-dependent molecular motor that interacts with actin (Sellers and Goodson, 1995). In muscle fibers actin and myosin are arranged in interdigitating filaments that slide past each other during contraction. There is a superfamily of myosins that also perform mechanical functions such as cytokinesis and vesicle transport in nonmuscle cells. The in vitro motility assay to study actomyosin interactions fills a void between biochemical measurements of the actin-activated Mg-ATPase activity of myosin and mechanical measurements of skinned muscle fibers. In this assay, myosin molecules bound to a nitrocellulose-coated surface interact with actin filaments in solution and translocate them in an ATP-dependent process. This is a simple assay that reconstitutes the essence of a muscle fiber using only two purified proteins, actin and myosin. It requires only small quantities of these proteins (e.g., 1 to 2

μg myosin per assay) and is quick to perform. Thin filament regulatory proteins can be added to examine actin-linked regulatory systems (Shirinsky et al., 1992; Fraser and Marston, 1995; Lin et al., 1996). The ionic conditions can be easily varied as needed. The assay is much simpler than a previous in vitro motility assay that followed the movement of myosin-coated microbeads over actin cables exposed by dissection of the long cells of *Nitella axillaris*, a green alga (Sheetz and Spudich, 1983).

Many novel techniques for the study of actomyosin interaction have evolved from the in vitro motility assay, including assays that allow for the imaging of both the myosin and actin filaments (Sellers and Kachar, 1990), assays in which the force generation of myosin is measured, and assays in which single molecules of myosin can be imaged as they interact with actin. Force measurement is accomplished by two methods. In the first case, actin filaments

attached to flexible microneedles are lowered onto a myosin-coated surface (Kishino and Yanagida, 1988; VanBuren et al., 1995). Force per unit length of actin is monitored by imaging the bending of the needle. In the other mechanical assay, beads are attached to the opposite ends of an actin filament, and optical trapping techniques are used in which the position of each bead is controlled by dual optical traps. The filament is lowered to a surface coated with low-density myosin, and force-producing events are measured by monitoring the position of the beads at the end of the actin filament (Finer et al., 1994; Molloy et al., 1995). The use of single-molecule fluorescence methods allows one to image individual myosin molecules or observe their interactions with fluorescently labeled nucleotides (Funatsu et al., 1995; Vale et al., 1996).

Critical Parameters

Myosin can be attached to the coverslip surface by a variety of methods. It can be bound as filaments or as monomers (Toyoshima et al., 1987; Umemoto and Sellers, 1990). Fragments of myosin such as HMM or S1 can also be used (Toyoshima et al., 1987). A more specific attachment of myosin to the surface is accomplished using site-specific antibodies, usually directed against the carboxyl terminus of the myosin or subfragment (Winkelmann et al., 1995). Isoform-specific antibodies can also be used to select a specific myosin isoform from a mixture of myosins or from a crude cell or tissue extract (Cuda et al., 1993; Kelley et al., 1996). In these cases, the affinity-purified antibody (0.2 mg/ml) is first bound to the nitrocellulose surface; this is followed by washing and blocking with BSA. Myosin (or an extract) is then added and allowed to complex with the antibody for 10 to 30 min. Subsequent steps are performed as described in the Basic Protocol.

The rate of actin-filament sliding generated by myosin varies greatly among myosin isoforms (Sellers and Goodson, 1995). In addition, assay conditions such as ionic strength, temperature, and pH can modulate the rate of sliding. Various parameters of the assay must therefore be varied. With slowly moving myosins (<0.2 $\mu\text{m}/\text{sec}$), longer imaging intervals are required, necessitating lower illumination levels, and as a consequence, it is very useful to average 16 to 64 frames to improve the signal-to-noise ratio. With more rapidly moving myosins (4 to 10 $\mu\text{m}/\text{sec}$), short imaging intervals can be used along with higher illumination levels. Under these conditions, it is preferable

to have an intensified CCD camera as opposed to a SIT camera, which has a slower temporal response that may result in the appearance of comet-tail-like images of the actin filaments.

There are many high-quality manufacturers of the required equipment. It is advisable to have representatives demonstrate the various pieces of equipment before purchase. A detailed treatise on video microscopy is available (Inoue and Spring, 1997).

In general, the assay does not work well at pH 6.5 or below, and the rate of actin filament sliding by myosin slows dramatically as the temperature falls below 20°C.

Troubleshooting

There are several common problems in the assay that are usually easily remedied.

1. *The actin filaments are too dim.* Increase the light intensity or the number of frames that are averaged. Remove any unnecessary glass surfaces between the actin filaments and the camera. If necessary, prepare fresh actin. It is important that the dried rhodamine phalloidin powder be predissolved in methanol to ensure complete solubilization.

2. *Rhodamine label attached to actin filaments photobleaches rapidly.* The light intensity should be reduced by means of neutral density filters, and frame averaging should be used to increase signal-to-noise ratio. The oxygen-scavenging components of the in vitro motility assay (glucose oxidase and catalase) should be checked to ensure they are active. Use 50 mM DTT, if not already doing so.

3. *The actin filaments move intermittently or rapidly shear into small pieces.* This is typical in myosin preparations containing rigorlike, noncycling “dead heads” (see Basic Protocol). Reducing the surface density of myosin also leads to less shearing. If necessary, prepare fresh myosin.

4. *Actin filaments detach from the surface or exhibit wobbly motions.* Increase the density of myosin attached to the surface or decrease the ionic strength. Use of methylcellulose generally allows for movement over lower myosin surface densities by decreasing the lateral Brownian movement of actin filaments. In the presence of methylcellulose, Brownian movement occurs only in the long dimension of the actin filament.

Anticipated Results

A successfully completed assay should allow measurement of translocation of actin by myosin and dissection of the biochemistry of

the process. Typically, translocation rates range from $<0.2 \mu\text{m}/\text{sec}$ to $10 \mu\text{m}/\text{sec}$ depending upon the source of myosin and the assay conditions.

Time Considerations

Actin preparation requires ~1 day to prepare an acetone powder and 3 to 4 days to prepare polymerized filaments. Myosin preparation takes from 1 day to 5 days, depending on the myosin isoform. Performing the translocation assay requires 30 min to set up. Data acquisition is in real time. Data analysis can be very slow and laborious unless an automated tracking system is used. The time required for data analysis is a function of how many filaments are being followed and the system being used for analysis.

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Organelle Motility in Plant Cells: Imaging Golgi and ER Dynamics with GFP

UNIT 13.3

This unit describes the use of green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* as a vital marker for endoplasmic reticulum (ER) and Golgi apparatus (GA) in higher plant cells. GFP is expressed as a chimeric protein either with selected peptide targeting sequences or with complete proteins that are resident in the ER or GA. Expression of GFP constructs can be observed in living material using conventional, or preferably, confocal laser-scanning epifluorescence microscopes. GFP has a number of intrinsic properties that make it attractive as a marker for cell biological studies. For instance, it is highly fluorescent, yet requires no cofactors or substrates. In addition, it is relatively stable, is pH insensitive, shows no detectable level of cytotoxicity (when expressed in organelles), and in many instances, does not interfere with the functioning of native proteins when expressed as chimeric constructs. Moreover, there are genetically modified variants of GFP with altered spectral and other properties, such as folding rate (Cubitt et al., 1995).

This unit describes two protocols for rapid transient expression of GFP in the plant endomembrane system using *Agrobacterium* and virus vectors. These protocols can be used for the expression of GFP targeted to most organelles. Besides *Agrobacterium*-mediated transformation (see Basic Protocol), virus transformation (see Alternate Protocol), has been optimized for GFP expression in some *Nicotiana* species.

TRANSIENT EXPRESSION FOR VISUALIZATION OF ER AND GOLGI PROBES IN LEAVES

**BASIC
PROTOCOL**

This protocol describes the production of GFP-transcripts in *Nicotiana* using an *Agrobacterium tumefaciens*-mediated transient expression system. There are a number of techniques for transient expression of reporter constructs in plant cells, such as electroporation and PEG-mediated transformation of protoplasts, and microprojectile bombardment and virus-mediated expression in tissues; however, transient expression mediated by *Agrobacterium* containing a reporter construct in a suitable binary vector (e.g., pVKH18En6) is one of the easiest, quickest, and more reliable methods. It requires minimum laboratory equipment, and it allows the study of intact cells and tissues from leaves still attached to the plant with minimal tissue disruption, although it is not clear to what extent the bacterial infection alters cell physiology. Moreover, transformed leaf tissue may be used to generate stable transformants.

The basic principle of the transformation is infiltration of the intracellular spaces of *Nicotiana* leaves with a suspension of *A. tumefaciens*, which is injected into the leaf tissue by pressure through the stomata of the abaxial leaf epidermis. The infectious *Agrobacterium* cells then transfer the T-DNA carrying the genes to be transferred to the host cell resulting in a transient build-up of reporter transcripts in the cell. With GFP-based reporters, fluorescence can be detected in the infected area from abaxial and adaxial epidermis, guard cells, and palisade and spongy mesophylls, but rarely trichomes and vascular tissue. The technique gives high expression levels of GFP constructs, which may fade in a week to ten days after inoculation of the leaves due to progressive depletion of the foreign DNA.

This method offers a number of advantages including speed and relatively little variability between cells within the infected area in which most, if not all cells, appear to express the construct. Moreover, Rossi et al. (1993) have shown that expression from T-DNA

Organelle Motility

13.3.1

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Supplement 9

varies linearly with bacterial concentration over three orders of magnitude offering the opportunity to control GFP expression levels. Finally, the geometry of epidermal cells, which have large vacuoles and a thin layer of cytoplasm immediately below the cuticular wall, greatly facilitates observation by both conventional epifluorescence microscopy (UNIT 4.2) and confocal imaging (UNIT 4.5). For example, the authors have been able to distinguish the distribution of ER- and Golgi-targeted GFP in these cells using a conventional epifluorescence microscope.

Materials

Suitable *Agrobacterium* vector (e.g., pVKH18En6) with multiple cloning site and appropriate selectable marker

Agrobacterium tumefaciens (e.g., GV3101::pMP90)

YEB medium containing appropriate selective antibiotic (see recipe)

5% (v/v) sodium hypochlorite or 1% (w/v) Virkon (Amtec Int. Ltd.)

Infiltration medium (INM; see recipe)

Four-week-old greenhouse plants of *Nicotiana tabacum*, *N. clevelandii*, and *N. benthamiana*

Shaking incubator, 28°C

1.5-ml microcentrifuge tubes, sterile

Spectrophotometer

1-ml disposable plastic syringe, without needle

22° to 25°C greenhouse for growing plants

Permanent marker pen

Fine scissors

Slides and coverslips (use thickness 0 for confocal microscopes)

Electrical or waterproof tape

Conventional epifluorescence microscope, laser-scanning confocal microscope (preferred), or equivalent, with appropriate filters (e.g., standard FITC filter block)

Confocal-imaging time-lapse software (Zeiss, Leica, Biorad)

Additional reagents and equipment for vector construction, transformation, and cell culture (see APPENDIX 3) adapted for plants

CAUTION: All solutions used to culture and wash bacteria should be treated with a suitable disinfectant before discarding in an autoclavable waste container. Resulting plant material should be treated as biological hazard and handled accordingly.

NOTE: Sterile conditions are required for culture and handling of *Agrobacterium*, but not during tobacco leaf infiltration and subsequent incubation of the plant. All equipment coming into contact with bacteria should be autoclaved.

Clone GFP into bacteria and grow *Agrobacterium*

1. Using standard molecular biology techniques (see APPENDIX 3), clone GFP chimera into a suitable vector with an appropriate selectable marker (e.g., kan^r, amp^r) and transform into a suitable *Agrobacterium* strain.

A number of binary Agrobacterium vectors can be used for the cloning of GFP (Bevan, 1984). In the authors' laboratories a vector with an enhanced 35S promoter, pVKH18En6, is used to drive high levels of GFP expression (Hawes et al., 2000).

2. Pick a single colony of *Agrobacterium* from a selection plate and inoculate 2 to 5 ml YEB medium containing appropriate selectable antibiotic.

3. Culture *Agrobacterium* at 28°C in a shaking incubator to stationary phase (24 to 48 hr).
4. Transfer 1.0 ml of culture to a 1.5-ml sterile microcentrifuge tube and pellet bacteria by microcentrifuging for 5 min at 4000 rpm.
5. Discard the supernatant in a disinfecting solution of either 5% (v/v) sodium hypochlorite (NaOCl₃) or 1% (w/v) Virkon.
6. Wash the bacterial pellet twice with 1.0 ml INM each.
7. Microcentrifuge for 5 min at 4000 rpm.
8. Resuspend in INM to OD₆₀₀ 0.5 to 0.6 or higher to increase the level of expression.

Inoculate plants

9. Inject bacterial suspension into the abaxial epidermis of plant leaves from a 1-ml disposable plastic syringe by simply pressing the nozzle against the leaf surface (do not use a needle), holding the leaf on the other side with a gloved finger at the point of contact to support the pressure.

The infiltration is easier when the leaf stomata are open. Illuminating the plants from underneath with a bright light prior to infiltration is recommended.

The spread of liquid entering the leaf via stomata is visualized by a darkening of the leaf tissue. The boundaries of the infiltrated area should be outlined with a permanent marker pen.

Expression can also be achieved in whole Arabidopsis plants by immersing plantlets in bacterial suspension under vacuum (Rakousky et al., 1998).

10. Incubate plants for 2 to 3 days under normal growing conditions in a greenhouse, except at 20° to 22°C to optimize infection.
- 11a. *For observation with a UV lamp:* Check for GFP fluorescence in leaves with a hand-held long-wavelength UV lamp after 2 days (assuming a GFP variant with a UV-excitation peak).

*The time required for the fluorescence to appear may depend on the construct. It is wise to check a piece of leaf tissue 2 days after the inoculation of *Agrobacterium* cells into the leaf, and every 4 to 6 hr thereafter, to establish the optimal time for expression of each construct used.*

- 11b. *For observation using a microscope:* After 2 days, cut out a segment of leaf tissue with fine scissors, mount in a drop of water on a microscope slide, and cover with a long coverslip held in position with strips of electrical or waterproof tape at either end.

It is important to remember that GFP fluorochrome cyclization will not occur in an anoxic environment so it is preferable that microscope preparations are not sealed.

Visualize expression

12. Observe the specimen with a conventional epifluorescence microscope or laser-scanning confocal microscope (preferred) and appropriate filters.

Most forms of GFP can be observed with a standard FITC filter block; however, the exact filter configuration will depend upon the excitation and emission wavelengths of the GFP (or its spectral variant) used.

Depending on the levels of expression, for ER- or GA-targeted constructs, image capture by confocal microscopy may require high laser power. This is possible with GFP as it is relatively resistant to photobleaching. Moreover, a wide pinhole aperture may be required

to obtain a satisfactory signal-to-noise ratio. Although, this strategy reduces confocality, and the ultimate resolution of the image, it can help overcome problems of image blurring due to organelle movement during the image capture process.

13. (Optional) To make a confocal video of endomembrane dynamics, select a region of interest, increase the laser power to the maximum available, select a wide pinhole aperture, and collect images as fast as possible using confocal-imaging time-lapse software.

If cytoplasmic movement is not dramatic then line (not sequential) image averaging can be used to improve the signal-to-noise ratio.

ALTERNATE PROTOCOL

VIRUS MEDIATED EXPRESSION OF GFP CONSTRUCTS

Viruses are often used as vectors for transient expression of DNA in foreign organisms, due to the high level of replication of the virus and the high level of protein synthesis (results may be obtained 4 to 10 days after inoculation of leaves of the host plants). In general the level of protein expression is high because of the activity of the strong viral promoter. The limitation associated with the virus expression technique resides in the fact that not all cells in plants expressing the viral genome become infected. This may in turn limit the reproducibility of biochemical assays. Moreover, the high levels of expression of the desired protein may be a reason for their mistargeting in the cell. Some inserts may be unstable in the vector, and the likelihood of instability increases with the size of the insert, such that 3 kb may be close to the practical limit. Finally, the pathogenic effects of the viral infection must be taken into account.

For plants, potato virus X (PVX) has been used successfully to express the marker proteins GFP and β -glucuronidase (GUS; Chapman et al., 1992; Baulcombe et al., 1995), and numerous other foreign proteins, often as fusions with GFP (Blackman et al., 1998; Boevink et al., 1998, 1999). The foreign genes inserted into the multiple cloning site are under the transcriptional control of the subgenomic promoter. A site at the 3' end of the PVX sequence is used to linearize the plasmid before infectious run-off transcripts are made with T7 RNA polymerase (the T7 promoter is present immediately upstream of the PVX sequence).

Additional Materials (also see Basic Protocol)

- GFP chimera construct
- PVX vector (pTXS.P3C2) with multiple cloning site and T7 promoter (available from various laboratories)
- Midiprep kit, without RNase (Qiagen)
- T7 RNA polymerase transcription kit (e.g., Ambion T7 message in machine kit)
- Spe*I or *Sph*I restriction enzymes (see APPENDIX 3)
- Aluminum oxide
- Aluminum oxide dispenser: small glass flask containing abrasive, sealed with miracloth
- Additional equipment and reagents for restriction digestion, phenol/chloroform extraction, and ethanol precipitation (see APPENDIX 3), and quantification of DNA concentration by spectroscopy (see APPENDIX 3D)

IMPORTANT NOTE: In some countries in order to handle plant viruses as vectors, a license must be obtained from the appropriate authorities.

IMPORTANT NOTE: All solutions used for virus work must be RNase-free.

1. Insert the GFP chimera in the multiple cloning site of the PVX vector in the correct orientation with respect to the T7 promoter (see *APPENDIX 3*).
2. Prepare a high-quality, medium-scale, RNase-free DNA preparation using a midiprep kit. Avoid using RNase in preparation of the DNA (i.e., do not add RNases to solution P1 and increase the volume of the wash buffer as recommended by the manufacturer).
3. Linearize the vector with *SpeI* or *SphI* restriction enzymes to make infectious RNA.
4. (Optional) Clean the DNA by phenol/chloroform extraction and ethanol precipitation (see *APPENDIX 3*).
5. Synthesize capped transcripts using 0.2 to 1.0 μg linearized DNA for a single infection, as template for the T7 RNA polymerase transcription kit, following the manufacturer's instructions.

The transcripts are generally not phenol/chloroform extracted or precipitated before inoculating.
6. Dust host plants *lightly* with abrasive aluminum oxide using an aluminum oxide dispenser.
7. Drop 5 μl of transcripts (1 to 5 $\mu\text{g}/\mu\text{l}$) onto four leaves and spread lightly with a glove-protected fingertip.
8. After inoculation wash the abrasive off the leaves. Keep plants at $\sim 23^\circ\text{C}$ in a culture room or sealed greenhouse.
9. Four to seven days post inoculation with PVX-GFP virus, screen plants for small fluorescent lesions on inoculated leaves with a hand-held long-wavelength UV-light or an epifluorescence microscope.
10. Analyze as described elsewhere (see Basic Protocol, steps 11 to 13).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

Infiltration medium (INM)

In a 10-ml measuring cylinder, add 50 mg glucose, 1 ml 0.5 M MES, pH 5.6 (see recipe), 1 ml 20 mM Na_3PO_4 (see recipe), and 5 μl 200 mM acetosyringone (Aldrich) in dimethyl sulfoxide (DMSO; final concentration 100 μM). Bring volume to 10 ml with water, seal the cylinder with 1 cm^2 Parafilm, and mix thoroughly by inversion. Make fresh prior to use.

200 mM acetosyringone in DMSO and 20 mM Na_3PO_4 can be stored at 4°C up to two weeks, or aliquoted and kept at -20°C for longer (i.e., 6 months) storage.

NOTE: No antibiotic must be added to this medium.

MES, 0.5 M, pH 5.6

In a 20-ml beaker add 1.95 g MES and 18 ml water. Adjust the pH to 5.6 with KOH and bring the volume to 20 ml with water.

Store up to 2 weeks at 4°C , or for longer storage (i.e., 6 months), aliquot and store at -20°C .

Na_3PO_4 , 20 mM

Dissolve 0.152 g $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ in 20 ml water. Store up to 2 weeks at 4°C , or for longer storage (i.e., 6 months) aliquot and store at -20°C .

YEB medium

In a 1-liter beaker, dissolve 5 g beef extract (Difco), 1 g yeast extract (Merck), 5 g peptone (Difco), 5 g sucrose (BDH Laboratory), and 0.5 g MgSO₄·7H₂O, in 0.8 liters of water. Add water to 1 liter, stirring continuously. Autoclave and store up to 6 months at room temperature.

It may take some time to dissolve completely.

COMMENTARY

Background Information

While many experimental programs may ultimately require the production of stable transformants, much useful information can be obtained through the use of transient expression systems. The production of transgenic plants and culture cell lines expressing GFP can be achieved using standard transformation and gene expression technologies. One of the major advantages of GFP and its wavelength-shifted derivatives, when used to tag proteins of interest, is that cellular events can be studied *in vivo* in planta and monitored by conventional epifluorescence microscopy. However, with tissue samples it may be necessary to use either confocal microscopy or conventional fluorescence microscopy combined with low-light-level cameras and deconvolution software.

The introduction of jellyfish fluorescent protein has provided cell biologists with a powerful new tool with which to study cell structure and function *in vivo*.

GFP chimeras were first used in plant cell biology to investigate the movement of viruses by the construction of both fluorescent viruses and fluorescent viral proteins, such as the movement proteins (Baulcombe et al., 1995; Oparika et al., 1995; Itaya et al., 1997). Subsequently, most major organelles have been successfully labeled with GFP chimeras, including the nucleus (Grebenok et al., 1997), vacuole (Di Sansebastiano et al., 1998), mitochondria (Köhler et al., 1997), plastids (Köhler and Hanson, 2000), cell plate (Gu and Verma, 1997), GA and ER (Boevink et al., 1998, 1999), and cytoskeleton (Köst et al., 1998; Marc et al., 1998).

In plant cells, GFP can be expressed on its own, fused to targeting peptides, or as a chimera with a complete protein of interest. Targeting the protein into the ER can be achieved by the addition of an appropriate N-terminal signal peptide (such as sporamin, patatin, or chitinase signal; Boevink et al., 1999; Haseloff et al., 1997) to translocate the protein into the ER lumen, and a C-terminal His/Lys, Asp, Glu, Leu (H/KDEL) retrieval sequence to maintain the

protein in the ER (see *APPENDIX 1C*). For GA visualization, targeting (signal-anchor) sequences from plant or mammalian transferases can be spliced onto the C terminus of GFP to locate the Golgi (Boevink et al., 1998), as can complete Golgi enzyme-coding sequences (Nebenführ et al., 1999). For instance the signal-anchor sequence of a rat sialyl transferase, incorporating the transmembrane and cytoplasmic amino acid domains, targets GFP to the *trans*-Golgi, while a soybean mannosidase I-GFP fusion locates towards the *cis*-face (Nebenführ et al., 1999). Both ER and Golgi can be targeted with the *Arabidopsis* homolog of the yeast H/KDEL receptor, *aERD2* (Boevink et al., 1998).

The transient expression protocol described in this unit can be used for species other than *Nicotiana*. The authors have been able to transiently express ER-targeted GFP in *Arabidopsis*, *Petunia*, and cucumber.

Critical Parameters

There are now a number of variants of GFP, obtained by genetic modification of the wild-type gene, which have been optimized to change various characteristics of the protein. This reengineering has been aimed at changing the spectral properties of the protein and improving the speed of maturation of the chromophore, including photoisomerisation and reduction in photobleaching of the protein (Cubitt et al., 1995, 1999); therefore, it is advisable to make a careful choice of the GFP to be used before embarking on extensive transformation procedures. For instance, mutations with enhanced blue excitation peaks may have reduced UV excitation, making rapid screening of plants with UV lamps impossible.

In order to avoid possible artifacts, it is important to ensure that the detected GFP fluorescence in transiently expressing plant cells is not derived from *Agrobacteria* synthesizing the protein. With other reporter systems this problem has been overcome by insertion of a plant intron into the reporter gene. Unfortunately such intron-containing GFPs have yet to be

constructed. Furthermore, as high expression levels may result in mistargeting, it is advisable that serial dilutions of bacteria be used to optimize expression levels.

Due to the availability of the spectral variants of GFP, including yellow (YFP), cyan (CFP) and the newly released red fluorescent proteins (dsRed), co-expression of constructs is possible. Care must then be taken with the choice of filter sets to prevent bleed through of signal when excitation and emission spectra are close. This of course is not the case for specific techniques, such as fluorescence resonance energy transfer (FRET; *UNIT 17.1*), in which the overlapping spectra are essential parameters (Gadella et al., 1999).

GFP will remain fluorescent after both paraformaldehyde and light glutaraldehyde fixation, thus permitting the use of other fluorescent probes, such as rhodamine conjugated phalloidin (Boevink et al., 1998), or standard immunofluorescence protocols, with red or far-red emitting fluorochromes. GFP will also retain antigenicity after fixation and preparation for immunogold labeling using the progressive-lowering-of-temperature technique and embedding in acrylic resins (VandenBosch, 1991; Boevink et al., 1998; *UNIT 4.7*). Thus, it is possible to confirm the location of GFP fusion proteins at the ultrastructural level. This is important when proteins are targeted to some of the smaller organelles, such as mitochondria, plastids, and Golgi. One may encounter

difficulties in obtaining GFP antibodies that are suitable for immunogold labeling of plant tissue. Consequently, it may be advisable to incorporate one of the widely used epitope tags into the GFP fusion if at some stage in the future immunogold labeling is envisaged.

Finally, with the observation of living plant material, specific problems, such as autofluorescence of chlorophyll and rapid cytoplasmic streaming, or organelle movements, are often encountered; these problems can be overcome by selective use of filter sets and optimization of microscope settings.

Troubleshooting

Sometimes a GFP chimera does not fluoresce, which can be due to several reasons. For example, frame shifts when GFP-fusions are used. If the resulting cDNAs have been checked and confirmed by sequencing, it is advisable to check that the binary plasmid carrying the construct is stable in *Agrobacterium*. Another possible explanation for lack of fluorescence may be that the GFP chimera is misfolded. Levels of expression can be checked by conventional immunoblot assay of protein extract (*UNIT 6.2*). This is also useful when with a particular construct, one encounters a relatively low-fluorescence level, which may result from protein instability or reduced folding kinetics.

On occasion a mislocalization of the fusion protein can be observed. If the quality of the targeting signals is trusted, then the phenome-

Table 13.3.1 Optimization of Images of GFP Targeted to GA and ER at the Confocal Microscope

Problem	Solution	Result
Autofluorescence of chlorophylls	Use a narrow-band emission filter set (e.g., 515–525 nm) in combination with optimum brightness and contrast settings	Reduction of fluorescence from chloroplasts
Blurred image due to the dynamics of the ER and GA	Increase laser intensity and/or increase pinhole aperture in combination with single-line scan or collect data from a defined region of interest (ROI)	Reduction in resolution and confocality may be experienced. A collection of time-resolved data sets for presentation as movies can be obtained
Low signal from GFP that is not compensated by change of brightness/contrast settings	Increase the laser intensity and/or increase the pinhole aperture	Increase of signal from GFP and reduction of confocality and resolution
Photobleaching of GFP fluorescence	Reduce the laser intensity, the number of image scans for averaging, and the number of sections per Z-series	Reduction of resolution and photobleaching damage

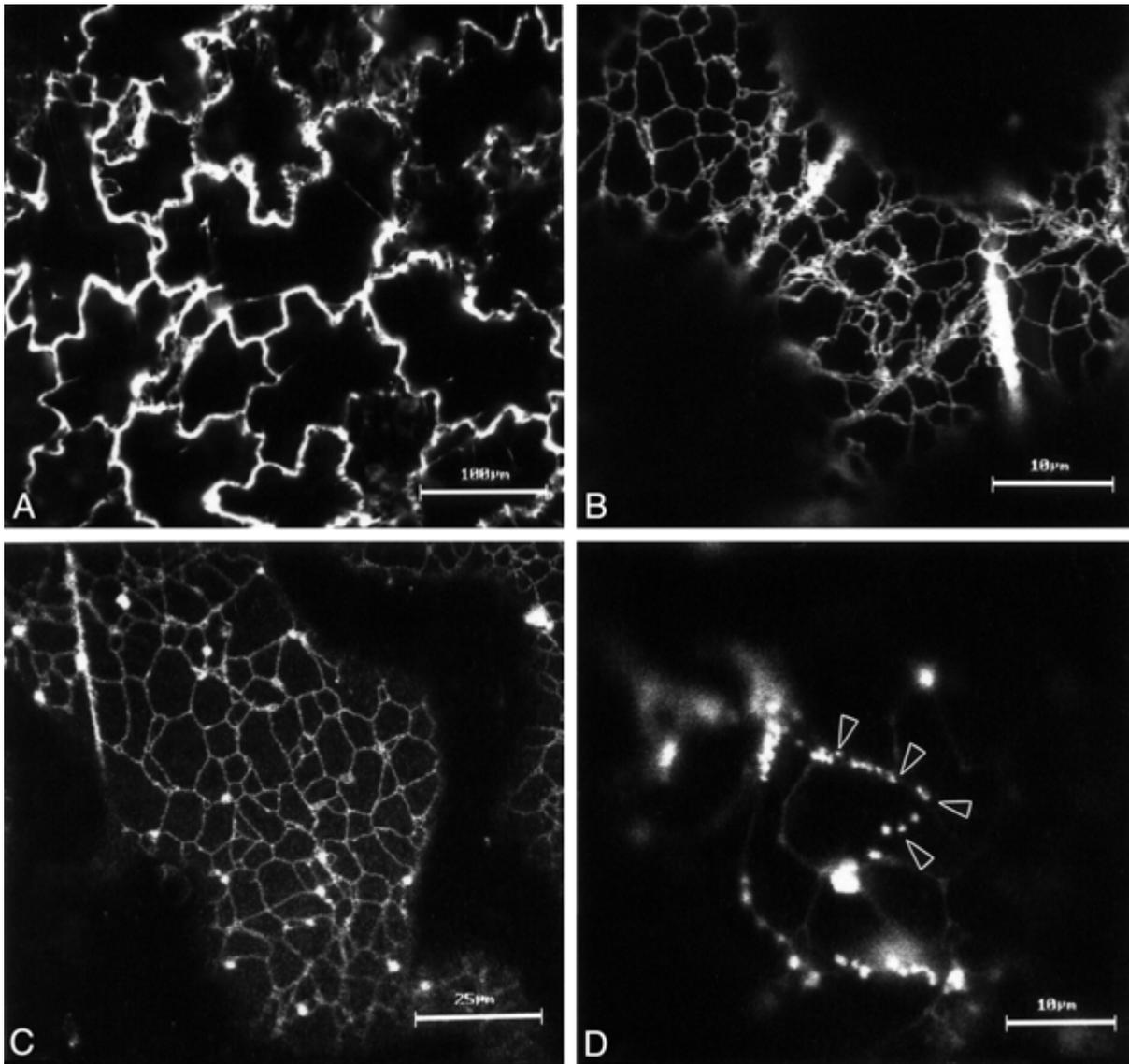


Figure 13.3.1 (A) *Agrobacterium*-mediated expression of a signal peptide-GFP-HDEL construct in leaf epidermal cells of *Nicotiana clevelandii*. The low magnification micrograph shows high levels of expression in the endoplasmic reticulum at the cortex of the cells. (B) Potato virus X-mediated expression of a signal peptide-GFP-calreticulin construct in a leaf epidermal cell of *N. clevelandii*. (C) Virus-mediated expression of an ER/Golgi-targeted construct (aERD2-GFP) in a leaf epidermal cell. Note the bright Golgi bodies are associated with the cortical ER tubules. (D) Golgi dynamics. Sequential frame capture of Golgi (arrows) moving over static cortical ER tubules. All micrographs taken with a Zeiss LSM 410 laser scanning confocal microscope.

non may be due to overexpression of the construct. Optimization of the bacterial concentration (as measured by optical density) is then suggested. Mislocalization can be also due to instability of the targeting signal within the fusion protein.

During observation of leaf tissue, the autofluorescence of the chlorophylls can mask low levels of GFP fluorescence. The use of a narrow-band emission filter set (e.g., 515 to 525 nm) in combination with optimum brightness and contrast settings will reduce the autofluo-

rescence from chloroplasts. Increasing the laser power and/or altering brightness and contrast settings can usually compensate for the overall reduction in signal that may result. Movement of labeled organelles in plant cells may result in a blurred image. Modifying laser and/or pinhole settings in combination with single-line scanning can help limit this problem. If necessary, the authors recommend collecting data from a defined region of interest (ROI). A reduction in resolution and confocality will occur, but quick collection of time-resolved

data sets for presentation as movies can be obtained. GFP is an ideal marker for time-lapse studies because of its relative resistance to photobleaching; however, if a reduction of signal from GFP and its variants due to photobleaching is observed, the laser intensity, the number of scans for image averaging, and the number of sections per Z-series should be reduced. Recommendations for the optimization of the ER and GA image at the confocal microscope are summarized in Table 13.3.1.

Anticipated Results

Typical results can be seen in Figure 13.3.1. The results illustrated in the figure are typical for those organelles to which GFP has been deliberately targeted. The percentage of transformed cells can vary with the time and with the concentration of bacteria. With the experimental conditions suggested in the Basic Protocol usually, 3 to 4 days after inoculation of plants, 20% to 40% of the cells are transformed within the area of initial bacterial penetration. Similarly, the virus transformation may produce approximately the same yield of transformed cells (after 4 to 5 days), and this number of cells may increase with time due to the virus spread which is not a feature of *Agrobacterium* transformation.

Time Consideration

As mentioned above, fluorescence may be detected 2 days after plant transformation. In the authors' experience, this depends on the particular construct, but in general, a detectable expression is obtained from 2 to 4 days and may last about 7 to 10 days.

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This paper illustrates the production of GFP targeted to the ER of living plant cells using a PVX-based expression system.

Boevink et al., 1998. See above.

The authors describe the fusion of the wild-type GFP to the transmembrane domain of a rat sialyl transferase in Nicotiana cells and its localization at the GA. Moreover, they describe the splicing of the wild-type GFP to the C-terminus of the Arabidopsis homologue of the yeast HDEL receptor, aERD2 and the localization of the protein chimera at the ER and GA, using the potato virus X expression system.

Haseloff et al., 1997. See above.

In this paper the authors describe how the wild-type GFP was engineered to be expressed in plants in a non-virus mediated system and to be targeted to the ER.

Nebenführ et al., 1999. See above.

The authors describe the fusion of a soybean Gm-Man1, encoding the resident Golgi protein α -1,2 mannosidase-1, to the green fluorescent protein, and its targeting to the Golgi of Bright Yellow 2 suspension-cultured cells.

Internet Resources

<http://www.brookes.uk/schools/bms/research/molcell/hawes/gfp/gfpold.html>

Web site for movies obtained at the confocal laser microscope in Nicotiana clevelandii epidermal cells expressing GFP fused to the C-terminus of the transmembrane domain of a rat sialyl transferase (localized at the GA) and the GFP spliced to the C-terminus of the Arabidopsis homologue of the yeast HDEL receptor, aERD2 (localized at the ER and GA).

<http://www.plantsci.cam.ac.uk/Haseloff/Home.html>

Web site for movies of GFP expressed in Arabidopsis root tips showing different patterns of GFP expression generated by enhancer detection.

http://www.mbg.cornell.edu/kohler/kohler_Trends.html

Web site with movies showing GFP targeting to plastids and mitochondria.

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Movement of Nuclei

UNIT 13.4

This unit presents an *in vitro* assay for nuclear motility along microtubules (see Basic Protocol). This assay system mimics the movement of the female pronucleus of the fertilized frog egg. Dynamic microtubule asters are assembled from purified centrosomes in *Xenopus* cytosol. Purified nuclei are then added and the motility is observed using video-enhanced differential interference contrast (VE-DIC) microscopy. This assay has been used to demonstrate that cytoplasmic dynein drives the motility of nuclei along microtubules in this system. Dynein activity causes nuclei to move to microtubule minus-ends. Therefore, the nuclei move to and accumulate at centrosomes. Other components of the nuclear motility apparatus can be defined by this assay including regulatory factors present in the cytosol, or binding partners for dynein on the nuclear membrane. Pharmacological and biological inhibitors of nuclear motility can be assayed and the effects on motility can be quantitated.

The assay system requires the following components: purified nuclei assembled in *Xenopus* egg extracts (see Support Protocol 5); purified centrosomes (see Support Protocols 1 and 2); and *Xenopus* cytosol that has been clarified by high-speed centrifugation (see Support Protocol 4) from fractionated interphase extracts (see Support Protocol 3). The clarified extract provides the subunits for microtubule assembly, the dynein motor complex that drives motility, and regulatory factors that have not yet been defined.

STRATEGIC PLANNING

These experiments require familiarity with handling *Xenopus laevis* for obtaining eggs used in this protocol. Protocols for handling *Xenopus* for egg production and preparation of active cytoplasmic extracts are described elsewhere and should be reviewed (Murray, 1991; UNIT 11.10). Included here are those procedures specifically required for nuclear motility experiments. These experiments require significant microscopy expertise, especially using VE-DIC. VE-DIC theory and techniques are not presented here. Refer to UNIT 4.1 for basic microscopy theory and techniques. Specific techniques for imaging microtubules and organelles using VE-DIC are presented elsewhere (Walker et al., 1988; Salmon and Tran, 1998; UNIT 13.1). The support protocols describe the procedures to prepare the different components used in the Basic Protocol.

NUCLEAR MOTILITY ASSAY

This protocol describes the set-up and execution of an assay that combines dynamic microtubules with synthetic nuclei assembled in *Xenopus* egg extracts (see Fig. 13.4.1 for a schematic of the assay). All assay components are stored separately in small aliquots at -70°C and recombined during the assay. (Assay components have been stored as long as 6 months without any loss of activity. Longer storage is conceivable, but has not been tested.) The use of aliquotted, frozen components allows reproducibility between assays as well as the possibility to inactivate or modify individual components to study the function of individual proteins in microtubule-mediated nuclear motility.

BASIC PROTOCOL

Contributed by Sigrid Reinsch

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Organelle Motility

13.4.1

Supplement 10

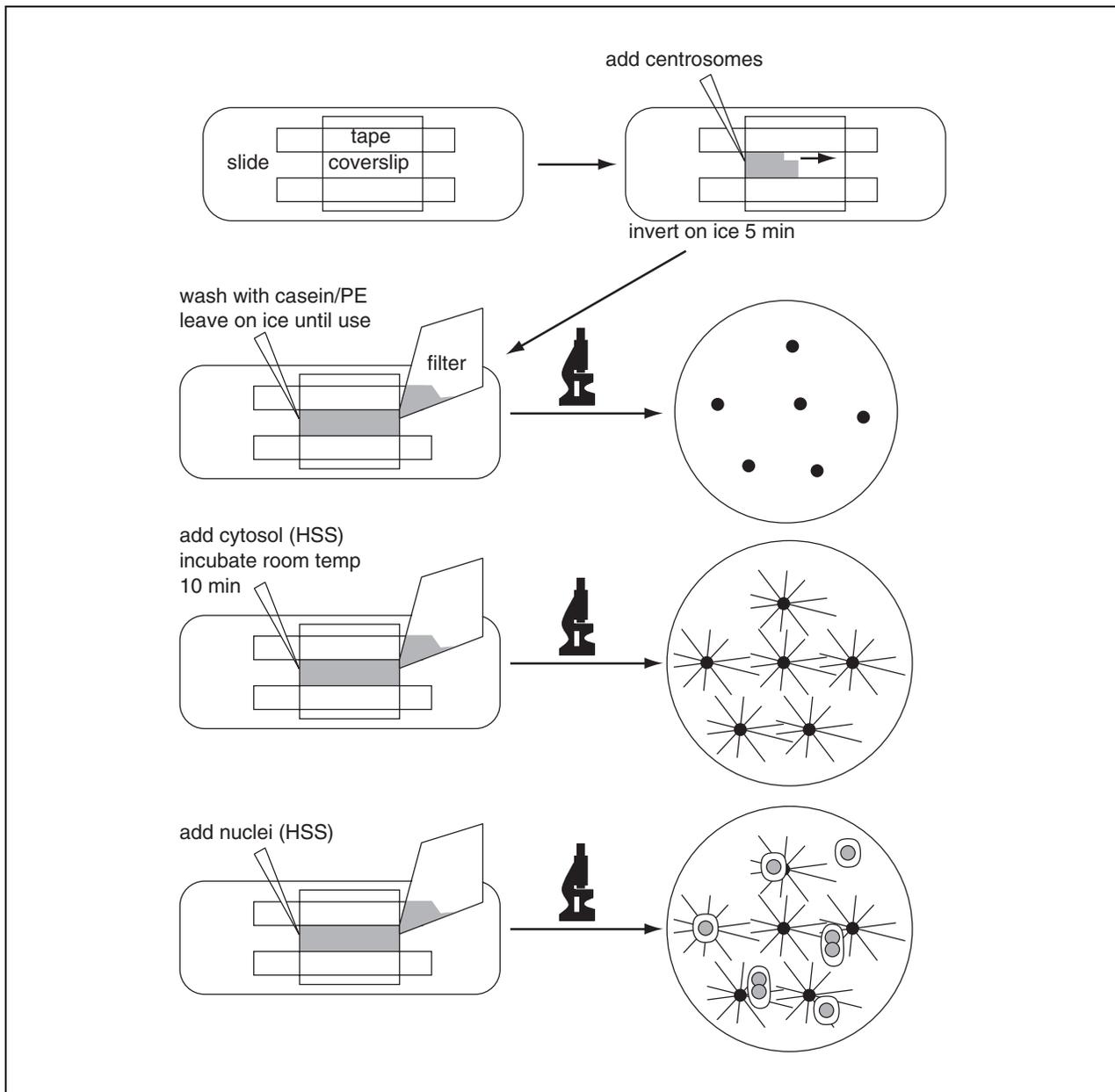


Figure 13.4.1 Flow chart of nuclear motility assay. Schematic showing the slide/cover slip chamber assembly used in this assay, and the flow-through technique. The individual steps are shown on the left panels and a representative microscope field is shown on the right panels. Centrosomes appear as round dots scattered on the field, microtubules are the lines emanating from the centrosomes in the second step, and nuclei moving on the asters are shown in the third step.

Materials

- Centrosomes (see Support Protocol 1)
- PE buffer (see recipe)
- 5 mg/ml casein in PE buffer (see recipe)
- ABC buffer (see recipe)
- HSS cytosol (see Support Protocol 4)
- DNA-bead nuclei (see Support Protocol 5)
- Valap (*UNIT 13.1*) at 37°C
- Immersion oil
- Assay reagent (e.g., drug, antibody, expressed protein)
- Antibodies bound to beads (for immunodepletion experiments)

Inverted microscope equipped for high-resolution DIC optics
Simple perfusion chambers made from: clean slide, double-stick tape, and clean
18 × 18–mm coverslips (for preparation see *UNIT 13.1*, Support Protocol 1)
Humid chamber: a covered 10- or 15-cm glass petri dish with strip of moist filter
paper around perimeter
Magnetic particle concentrator (Dynal)
Inverted microscope
Camera (e.g., Hamamatsu CCD C307, Hamamatsu)
Image processor (e.g., Argus 10, Hamamatsu or equivalent)

Assay nuclear motility

1. Prior to the experiment, optimize inverted microscope for DIC imaging of microtubules.
2. Assemble perfusion chambers according to the instructions in *UNIT 13.1*, Support Protocol 1. Assemble by placing two pieces of double-sided tape 2 mm apart on a microscope slide and attach an 18 × 18–mm coverslip.

The chambers should have an approximate volume of 7 μ l.

3. Thaw an aliquot of centrosomes. Dilute with PE buffer to the optimal concentration determined in Support Protocol 2. Flow 7 μ l of diluted centrosomes into each chamber to be used in the day's set of experiments. Be careful not to introduce bubbles into the chamber. Invert perfusion chambers onto ice and allow the centrosomes to settle onto the coverslip and attach for 5 min.

For all perfusion steps, samples are pipetted at one opening of the perfusion chamber and wicked on the opposite side with a small piece of filter paper.

4. Flow 30 μ l of 5 mg/ml casein in PE through perfusion chamber to block binding sites on the glass and rinse out unattached centrosomes. Incubate on ice until use (up to 4 hr maximum).

From this point, only one chamber will be used at a time because each must be viewed individually on the microscope.

5. Ten minutes before the start of the assay, rinse chamber with 30 μ l of ABC buffer. Flow 10 μ l of HSS cytosol into chamber and incubate for 10 min at room temperature in the humid chamber.

This allows recruitment of pericentriolar material from the cytosol, nucleation of microtubules, and aster formation.

6. During the 10-min incubation, quickly thaw an aliquot of DNA-bead nuclei (see Support Protocol 5) and immediately place on ice. Resuspend in 150 μ l ABC buffer. Retrieve on a magnetic particle concentrator, carefully remove the ABC buffer, and resuspend in 10 ml HSS cytosol.

7. Flow the nuclear suspension into the chamber to start the assay. Seal the chamber with Valap, oil top and bottom of chamber with immersion oil, and observe at 20° to 22°C. Use DIC optics, a 100× objective, and a high-resolution condenser.

If appropriate, use additional magnification to obtain an optimal on-screen field size ~50 μ m. Use image background subtraction to enhance the microtubules (Walker et al., 1988; Salmon and Tran, 1998).

Simultaneous imaging of nuclei and microtubules is a bit trickier than imaging only microtubules or other organelles due to the high refringence of the nuclei (especially magnetic-bead nuclei). Nuclei move rapidly on the microtubules. By 20 min, most nuclei have stopped moving and reached the centrosomes.



Figure 13.4.2 DIC images of a nucleus moving on a microtubule aster. A sequence of four stills are shown from a motility assay over a period of 10 min. The centrosome is in the lower left corner of each panel, and a robust microtubule aster emanates from this focus. A synthetic nucleus containing several paramagnetic beads starts in the upper right corner and moves to the centrosome during the course of the experiment. The nucleus appears refractory under AVEC-DIC imaging conditions. Image processing, including background subtraction and frame averaging, enhances the microtubules for clearer visualization. The time points are not indicated, but would occur during the first 20 min following assembly of the assay. By the 20-min time-point where quantitation is performed, 90% of nuclei will have reached the centrosome.

Collect images

8. To film nuclear movements, collect shuttered images with a camera. Perform rolling image averaging and background subtraction with an Argus 10 image processor, or equivalent image processing program.

An appropriate interval between shuttered images is 5 sec. Figure 13.4.2 shows several sequential frames of a movie of a nucleus migrating on a microtubule aster.

Quantitate nuclear motility

9. To perform a quantitative assay for accumulation at centrosomes, repeat steps 1 through 8. Starting at 20 min after adding DNA-bead nuclei, for an additional 10 min, count as many fields as possible to determine percentage of nuclei present at centrosomes versus those at a distance ($>5 \mu\text{m}$) from centrosomes.

One should be able to count 50 to 100 nuclei in the 10-min interval. Typical values are 90% to 95% of the nuclei present at centrosomes. Because many nuclei contain multiple DNA beads, each centrosome containing beads/nuclei should be counted as "1". Therefore numbers for accumulation are minimal values. Values $<90\%$ to 95% indicate difficulties with the assay that must be addressed before continuing (see Troubleshooting). If the assay looks optimized, then proceed to the next step.

Perform addition experiments

To assess pharmacological or biological reagents for effect

- 10a. Perform steps 1 through 4.
- 11a. Add $2 \mu\text{l}$ of drug, antibody, or expressed protein to $18 \mu\text{l}$ of HSS cytosol.
- 12a. Resuspend an aliquot of DNA-bead nuclei in $150 \mu\text{l}$ of ABC buffer. Retrieve on a magnet particle concentrator and discard supernatant.
- 13a. Resuspend DNA-bead nuclei in $10 \mu\text{l}$ of HSS/test compound mixture (step 11a). Use the remaining $10 \mu\text{l}$ to flow through perfusion chamber after the nucleation step (step 4) to replace the normal HSS cytosol with that containing the test compound.
- 14a. Flow in the nuclei/HSS/test compound mixture, incubate 20 min at 20° to 22°C and count as in step 9.

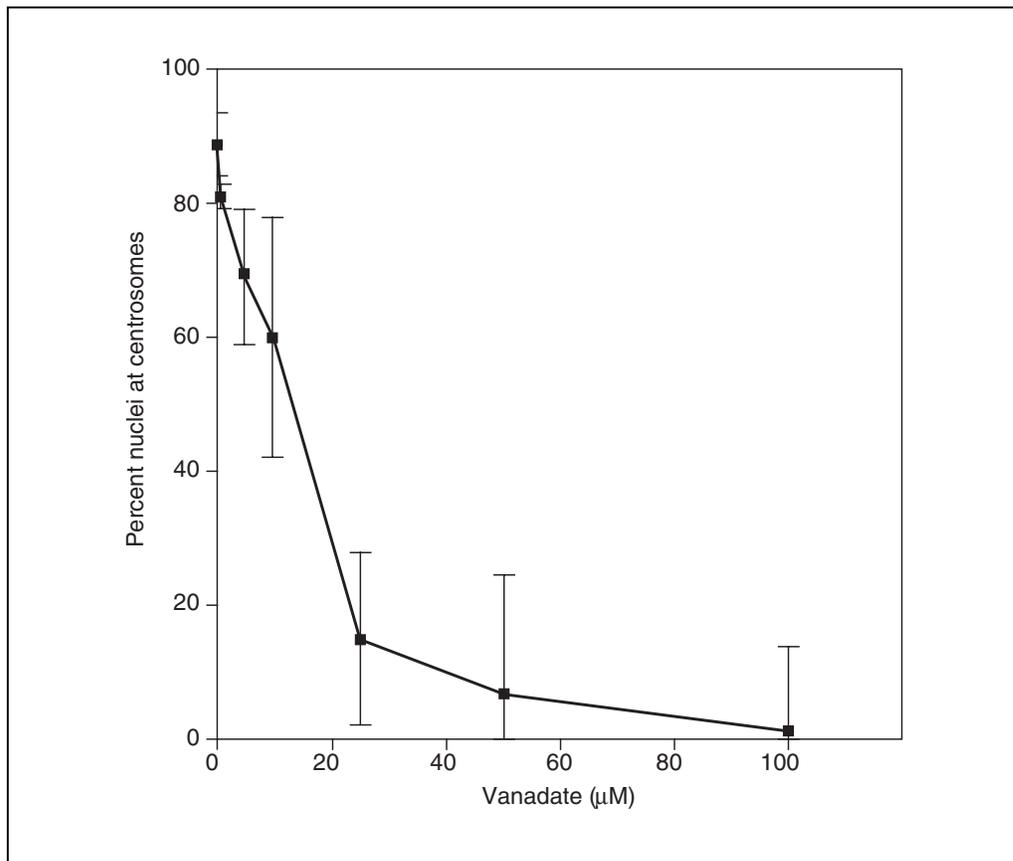


Figure 13.4.3 Effect of vanadate on accumulation of nuclei at centrosomes is an example of how this assay is used to derive quantitative data. Accumulation of nuclei at centrosomes was compared in HSS containing varying amounts of sodium orthovanadate, a general ATPase inhibitor. Assays were performed in triplicate for each vanadate concentration to generate a dose-response curve. The experiment indicates that vanadate significantly inhibits nuclear accumulation at centrosomes at a concentration of 20 mM. This concentration has been shown to be effective in inhibiting the motility of cytoplasmic dynein, while inhibition of kinesins requires concentrations of vanadate in the 100 mM range.

Perform each assay three to four times to obtain statistically significant data. The control mixture should contain 2 µl of appropriate buffer. Figure 13.4.3 shows a titration curve for the effects of different concentrations of vanadate on the accumulation of nuclei at centrosomes.

For some reagents, it is important to incubate the cytosol with the reagent for 30 to 60 min before the assay. This is especially true for proteins expressed in bacteria, which may need to undergo refolding, or other post-translational modification in the extract to be effective (S. Reinsch, unpub. observ.).

To perform depletion experiments

10b. Immunodeplete specific components from HSS cytosol using antibodies bound to beads. Use depleted HSS cytosol for motility cytosol in the assay.

For examples of successful depletion experiments using Xenopus extracts see Merdes et al. (1996); Walczak et al. (1996); or Tournebize et al. (2000).

These experiments are technically more challenging.

CENTROSOME ISOLATION FROM LYMPHOCYTES

Centrosomes in fibroblastic and lymphoid cells are anchored in the cytoplasm by microtubules and interact with the nucleus in an unknown way. The centrosome-nucleus association is dynamic *in vivo* but becomes irreversible *in vitro* if cells are lysed at physiological ionic strength. Most procedures for making nuclei lead to the preparation of nucleus-centrosome complexes that are almost impossible to dissociate. Therefore, in this protocol, the nucleus-centrosome interaction *in vivo* is first disrupted, then cells are lysed under conditions where centrosomes can be purified away from the nucleus.

Human lymphoid cells in culture are used here because they grow in suspension and have a low cytoplasm/nucleus ratio. Evidence indicates that both the microtubule cytoskeleton and the actin network are involved in centrosome anchoring in the cytoplasm (Euteneuer and Schliwa, 1985; Buendia et al., 1990; Mack and Rattner, 1993). To purify centrosomes, cells are first preincubated with cytochalasin and nocodazole to disrupt connections between centrosomes and nucleus (Bornens et al., 1987). Extremely fast lysis of cells at very low ionic strength in the presence of Triton X-100 leads to chromatin dispersion and release of centrosomes in suspension. The addition of a low concentration of magnesium ions to the lysis buffer leads to partial chromatin stabilization and still allows centrosome release. This reduces contamination of centrosomes by chromatin components. Centrosomes are rapidly sedimenting particles. Their purification is therefore achieved by separation on sucrose gradients.

It takes ~1 week to grow enough cells for the preparation. The preparation itself takes 1 day. One or two people can do the preparation, and a minimum of two people are needed to perform steps 6 to 7, which need to be done very rapidly. It is important to have highly concentrated centrosomes ($\sim 2 \times 10^8/\text{ml}$) for this assay. The yield is typically 30% to 50%, with several fractions containing centrosomes at the optimal concentration.

Materials

- KE37 human lymphoblastic cells (ACC46; DSMZ German Collection of Microorganisms and Cell Cultures)
- RPMI-10: RPMI 1640 medium with 10% (w/v) FBS
- 10 mM nocodazole stock solution in DMSO (see recipe)
- 10 mg/ml cytochalasin D stock solution in DMSO (see recipe)
- PBS (see recipe), ice cold
- PBS/10 with 8% sucrose (see recipe)
- Lysis buffer (see recipe)
- 4 ml 0.5 M K-PIPES (pH 7.2)/1 mM EDTA (see recipe)
- 1 mg/ml DNase I (see recipe)
- 40%, 50%, and 70% sucrose in gradient buffer (see recipe)
- Liquid nitrogen
- PE buffer (see recipe)
- Methanol, -20°C
- PBS/0.1% Triton X-100
- Monoclonal anti-tubulin antibody (Amersham)
- Secondary antibodies
- Hoechst
- Polyclonal anti-pericentriolar antibody (e.g., γ -tubulin, pericentrin; optional)
- 250-ml plastic flask
- 2-liter spinner culture flask
- 500-ml centrifuge bottles
- Clinical centrifuge or equivalent with swinging bucket rotor for 50-ml tubes

Super-speed centrifuge (e.g., Sorvall RC-26 or equivalent)
Large volume rotor (GSA or equivalent)
50-ml capped conical tubes
10-ml plastic pipet
125- μ m nylon mesh (Millipore)
Beckman SW28 and SW28.1 centrifuge tubes
Ultracentrifuge
Ultracentrifuge SW28 rotor with SW28 and SW28.1 buckets
18-G needles
Refractometer
Modified Corex tubes (Evans et al., 1985; *UNIT 11.13*)
11- to 12-mm diameter coverslips (acid-washed)
HB-4 or HB-6 rotor
Forceps
Fixing jar with coverslip holder

Additional reagents and equipment for immunofluorescence staining (*UNIT 4.3*) and analyzing centrosomes by spinning onto coverslips (*UNIT 11.13*)

Grow KE37 cells

1. Grow 1 liter of KE37 cells in four 250-ml plastic flasks in RPMI 1640-10.
2. One day before the centrosome preparation, transfer 1-liter cells to a 2-liter spinner culture bottle and add 1 liter RPMI 1640-10.
3. On the day of purification have 2 liters of cells at a density close to 1.5×10^6 cells/ml (3×10^9 cells total).

It is important that the cells are maintained in exponential phase at densities between 1 and 4×10^6 cells/ml.

4. Add 60 μ l of 10 mM nocodazole to a final concentration of 33 μ M and 200 μ l of 10 mg/ml cytochalasin D to 1 μ g/ml final. Incubate 1 hr at 37°C with continuous stirring.
5. Transfer to 500-ml centrifuge bottles and centrifuge for 15 min at $650 \times g$ (2000 rpm in GSA rotor), 4°C.

During all subsequent centrifugations, do not decant supernatant, but rather aspirate supernatant.

6. Resuspend cells in ice-cold PBS to a maximum total volume of 160 ml.
7. Transfer to four 50-ml capped conical tubes. Centrifuge for 5 min at $500 \times g$ (in a clinical centrifuge), 4°C. Repeat PBS wash and centrifugation once.
8. Resuspend cells in ≥ 25 ml/tube PBS/10 with 8% sucrose. Centrifuge for 5 min at $250 \times g$ (1000 rpm in a clinical centrifuge), 4°C.

Do this step and step 9 as quickly and gently as possible.

9. Lyse cells in each tube by adding 10 ml of lysis buffer to pellet. Disperse pellet by pipetting up and down with a 10-ml plastic pipet without generating bubbles. Bring volume in each tube up to 20 ml with lysis buffer. Invert 2 to 3 times slowly. Incubate 5 min on ice.

Cells should not form an aggregate. They should lyse immediately. The maximum total volume for the preparation should not exceed 90 ml at this point.

10. Centrifuge 10 min at $2000 \times g$ (3000 rpm in a clinical centrifuge), 4°C.

11. Filter supernatant through 125- μ m nylon mesh. Remove a 300- μ l sample for counting.
12. To the pooled 90 ml supernatants add 1.8 ml of 0.5 M K-PIPES (pH 7.2)/1 mM EDTA and 90 μ l DNase I stock solution.

Purify centrosomes on sucrose gradients

13. Load 30 ml of lysate into three Beckman SW28 ultracentrifuge tubes and using a 10-ml plastic pipet underlay with 5 ml of 50% sucrose in gradient buffer.
14. Ultracentrifuge 20 min at 20,000 \times g (11,100 rpm in SW28 rotor), 4°C.
15. Aspirate supernatant leaving ~2 ml of lysate above the cushion in each tube.

To estimate the 2-ml volume above the cushion, prepare a blank tube containing the cushion volume and 2 ml above this volume. Mark tube at the appropriate height to compare with the tubes containing the centrosome samples.

16. Take the 2 ml of remaining supernatant and 2 ml of 50% sucrose cushion. Pool 12-ml total volume from the three step gradients. Keep a 100- μ l aliquot for counting.
17. Mix well and layer over a discontinuous sucrose gradient in a SW28.1 ultracentrifuge tube.

2.0 ml 70% sucrose
1.5 ml 50% sucrose
1.5 ml 40% sucrose
12.0 ml of centriole suspension (~25% sucrose: pooled volume from the three-step gradients).

18. Ultracentrifuge 75 min at 110,000 \times g (25,000 rpm in SW28.1 rotor), 4°C.
19. Eliminate top of the gradient to the 40% solution. Pierce bottom of tube with an 18-G needle and collect 0.4-ml fractions manually.

Analyze gradient fractions and count centrosomes

20. Read the fractions with a refractometer. Take 5- μ l aliquots from fractions between 40% and 70% sucrose to count centrosomes by immunofluorescence. Snap freeze the remaining volume of these fractions in liquid nitrogen without further aliquotting. Store at -70°C. Discard fractions at <40% and >70% sucrose.
21. Prepare one modified Corex tube for each 5- μ l aliquot and one each for the samples from steps 11 and 16. Place the removable chuck on top of the bottom spacer. Place a clean 11-mm diameter coverslip on top of the chuck.
22. Mix the 5- μ l aliquots each with 5 ml of PE buffer by inverting several times in a 15-ml capped tube. Pipet into the prepared Corex tube. Centrifuge 10 min at 23,600 \times g (12,000 rpm in HB4 rotor), 4°C.
23. Carefully remove coverslips from the tube: use a thin spatula with a bent tip to lift the chuck out of the tube, then use forceps to transfer coverslip into cold methanol. Fix coverslips 5 min in methanol at -20°C.

It is important to remember which side of the coverslip the centrosomes are on; maintain all of the coverslips in the same orientation.

24. Remove coverslips from fix and place in PBS/ 0.1% Triton X-100.

For a more detailed description of the procedure in steps 21 through 25, see UNIT 11.13, Support Protocol 4.

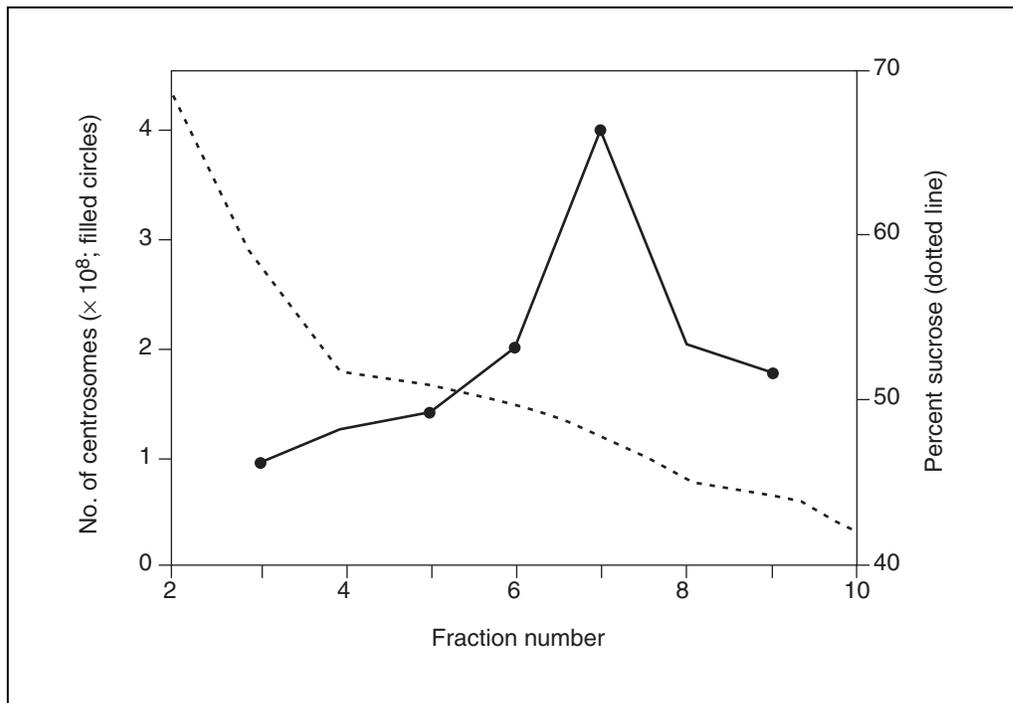


Figure 13.4.4 Fractionation of centrosomes in sucrose step gradient. A typical graph obtained after fractionation of centrosomes on a sucrose step gradient. The sucrose concentration (dotted line) of each fraction was determined using a refractometer, while the centrosome concentration (solid line) was determined as in Support Protocol 1. The peak fractions, containing centrosomes sufficiently concentrated for use in this assay, typically sediment between 45% and 50% sucrose.

25. Perform immunofluorescence using a monoclonal anti-tubulin antibody, followed by appropriate secondary antibodies (UNIT 4.3). Add Hoechst to secondary antibodies to check for DNA contamination.

If possible, also use a polyclonal antibody against pericentriolar material (anti- γ -tubulin). This helps to confirm that the dots are centrosomes. Santa Cruz Biotechnology offers polyclonal anti- γ -tubulin antibody, but it has not been tested in this protocol.

26. Observe samples at a magnification where centrosomes are easily visualized (usually as pairs of dots, with some single dots). Count the total number of centrosomes in several microscope fields to get an average number. Use a stage micrometer to measure the size of the field. Extrapolate to the total cross-sectional area of the Corex tube to get the total number of centrosomes in a particular 5- μ l aliquot or from intermediate steps 11 and 16 (see Fig. 13.4.4 for a typical determination of centrosome concentration from a sucrose gradient).
27. Maintain the sucrose gradient fractions of centrosomes frozen at -80°C .

Centrosomes can be stored this way for ≥ 1 year. See Support Protocol 2 for subaliquotting the centrosomes and for titration in the motility assay.

TITRATION OF CONCENTRATED CENTROSOMES

The assay presented in the Basic Protocol requires standardized components so that assays performed on different days can be compared. In particular, the same concentration of centrosomes must be used from one assay to the next as the plating density of centrosomes affects assay results.

SUPPORT PROTOCOL 2

Organelle Motility 13.4.9

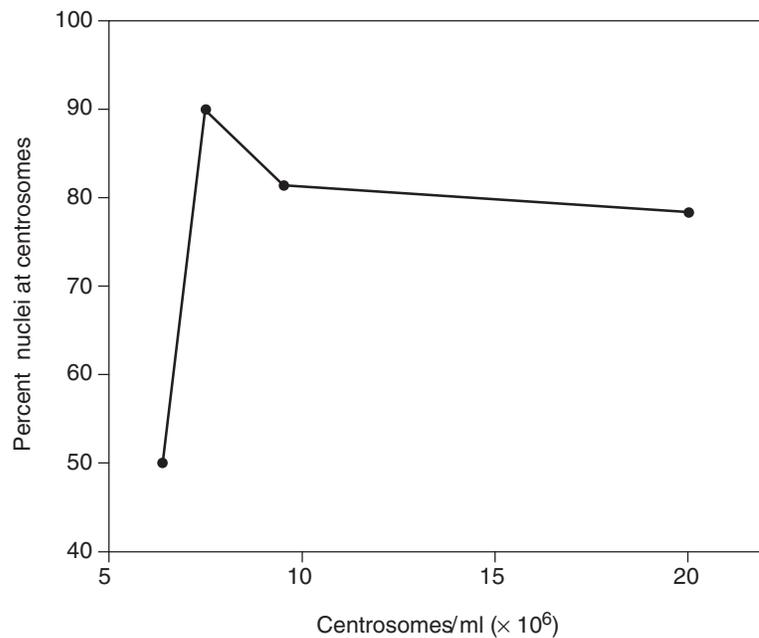


Figure 13.4.5 Titration of centrosomes in motility assay. An assay showing how centrosome concentration affects the accumulation of nuclei at centrosomes. If centrosomes are plated to sparsely, microtubules form spontaneously rather than at centrosomes preferentially, and nuclei will move in a random fashion on these microtubules rather than accumulating at centrosomes. At high centrosome concentrations, elevated concentrations beyond a certain value do not enhance accumulation of nuclei at centrosomes. Steps for titrating centrosomes for this assay are described for Support Protocol 2.

Additional Materials (also see Basic Protocol)

- Large aliquot of centrosomes (see Support Protocol 1)
- Liquid nitrogen
- 0.5-ml microcentrifuge tubes for aliquotting

Prepare centrosome aliquots

1. Prepare about one hundred 0.5-ml microcentrifuge tubes on ice.
2. Rapidly thaw one large 400- to 500- μ l aliquot of centrosomes. Mix the aliquot well by inverting tube several times or pipetting up and down without introducing bubbles. Make 5- μ l aliquots. Snap freeze in liquid nitrogen.

It is important that the large aliquot is well mixed before dividing into smaller aliquots so that the concentration of centrosomes is the same in each tube. Since centrosomes are large particles they will sediment in the tube over time.

Titrate centrosomes

3. Prepare 8 perfusion chambers according to UNIT 13.1, Support Protocol 1.
4. Thaw four 5- μ l aliquots of centrosomes. Resuspend each in a different volume to bracket the range of final concentrations of 5-20 $\times 10^6$ centrosomes/ml.
5. Flow each centrosome sample into two perfusion chambers to serve as duplicates.
6. Perform Basic Protocol, steps 2 through 7 on each sample to determine which centrosome concentration yields the highest value for number of nuclei that accumulate at the centrosomes.

A typical plot is shown in Figure 13.4.5.

Empirically, a final concentration in the range of 7.5×10^6 centrosomes/ml gives consistently good values. This corresponds to a plating density of 2 to 3 centrosomes/50 μm^2 microscope field. The optimum value is a result of the competition between free nucleation of microtubules and that nucleated by centrosomes. If there are too few centrosomes, there is a lot of spontaneous nucleation of microtubules that generates random arrays. With too many centrosomes, there is little or no spontaneous microtubule assembly, but microtubules will have a shorter overall length at the 20-min time point.

PREPARING FRACTIONATED INTERPHASE EXTRACTS FOR NUCLEAR ASSEMBLY

Functional nuclei can be assembled in vitro using *Xenopus* extracts (for review see Newmeyer and Wilson, 1991; Gant and Wilson, 1997; UNIT 11.10). Freshly made $16,000 \times g$ interphase extracts generally give the best results for nuclear assembly and can be used with the DNA-magnetic beads (stop at step 11). However, extracts that have been fractionated into membraneous and cytosolic components are useful for many types of functional studies and are therefore presented here. The cytosolic and membrane fractions are stored frozen and are recombined with template DNA to assemble chromatin and nuclei. Many published protocols for nuclear assembly use sperm nuclei. These are not appropriate for studies of nuclear motility that mimic movement of the female pronucleus along microtubules. Sperm nuclei have an associated centrosome, and microtubules nucleated from this centrosome will generate motility events in their own right (reviewed in Reinsch and Gonczy, 1998). For motility assays one can use nuclei assembled with magnetic beads coupled with plasmid DNA, or nuclei assembled around larger purified DNA such as lambda DNA. The use of magnetic beads as substrates for nuclear assembly allows rapid and gentle purification of the nuclei.

For handling of frogs and general methodologies for preparing *Xenopus* egg extracts see Murray (1991) and Newmeyer and Wilson (1991); also see UNIT 11.10. The following protocol uses eggs that have been collected after overnight stimulation of the frogs with human chorionic gonadotropin (HCG), and is based on a published protocol (Hartl et al., 1994; also see UNIT 11.10).

The general principles of this procedure are illustrated in Figure 13.4.6.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

- 5 to 7 frogs
- 100 U pregnant mare serum gonadotropin (PMSG; see recipe)
- 500 U HCG (see recipe)
- MMR (see recipe)
- Dejelling solution (see recipe)
- S-lysis buffer (see recipe)
- S-lysis-plus buffer (see recipe)
- 10 mg/ml cytochalasin D stock solution in DMSO (see recipe)
- 1 M DTT (see recipe)
- Protease inhibitors (LPC; see recipe)
- 2.5 M sucrose (see recipe)
- Glycerol
- Liquid nitrogen for freezing aliquots
- S-lysis-plus/500 mM sucrose solution (see recipe)

SUPPORT PROTOCOL 3

Organelle Motility 13.4.11

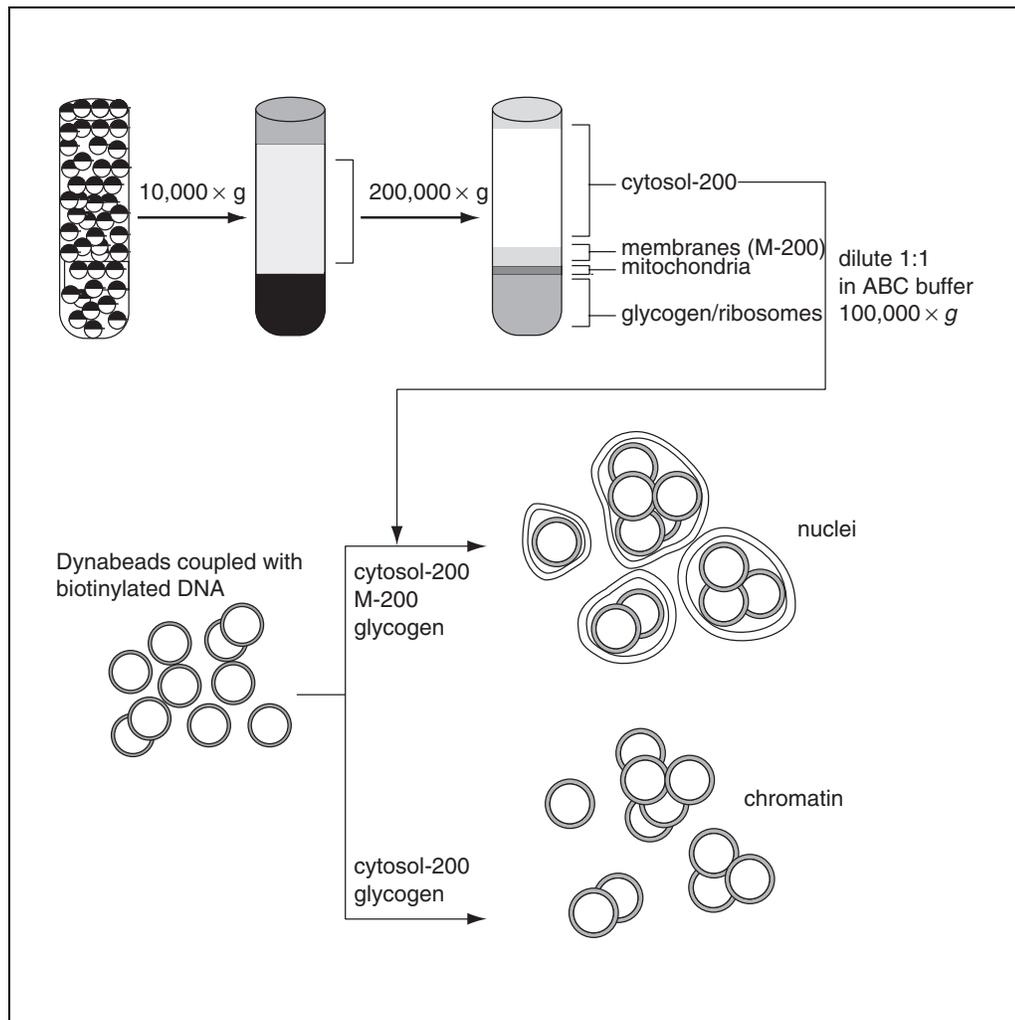


Figure 13.4.6 Nuclear assembly from fractionated extracts. Schematic showing the basic steps required to assemble synthetic nuclei using fractionated extracts. In the protocol presented here, generation of the cytosol-200 and M200 fractions are described in Support Protocol 3. These are frozen separately and then recombined with DNA-coupled Dynabeads as described in Support Protocol 5. Preparation of DNA-coupled Dynabeads is described in *UNIT 11.13* (Heald et al., 1998). If the M200 membranes are omitted during the assembly process, interphase chromatin assembles on the DNA, but full nuclear assembly requires the M200 fraction. Synthetic nuclei often contain multiple Dynabeads which behave very similarly in the assays presented here.

1-ml syringes and 27-G needles
 400-ml beakers
 SW50 ultraclear centrifuge tubes (Beckman)
 Pasteur pipet cut to a wide-mouth bore with a file, fire polished
 Sarstedt 13-ml adaptor tubes
 2-ml syringe and 18-G needle
 Clinical centrifuge
 Sorvall RC5 centrifuge
 HB-4 rotor with rubber adapters (Sorvall)
 5-ml polypropionate tubes with caps
 Ultracentrifuge
 SW55 rotor
 500-ml microcentrifuge tubes for aliquotting cytosol and membranes

NOTE: Make sure that all glassware is clean and rinsed with distilled water before use. Additionally, wet all glassware with buffer before contact with the eggs as they will stick to the glassware and activate or lyse.

Collect the eggs

1. Inject 5 to 7 frogs subcutaneously into the dorsal lymph sac with 0.5 ml of 100 U PMSG, using a 1-ml syringe and 27-G needle. Perform the injection ≥ 4 days (and ≤ 10 days) before the extract is to be prepared. Do not feed frogs after PMSG injection.
2. Twelve to 18 hr before use, inject each frog subcutaneously with 0.5 ml of 500 U HCG. Place frogs in individual containers with 500 ml MMR and maintain the frogs overnight at 16°C.
3. Collect eggs into 400-ml beakers using a separate beaker for each frog. Discard batches of eggs containing $>5\%$ of lysed, mottled, or stringy eggs.

Keep batches of eggs separate through dejelling and even through the $16,000 \times g$ centrifugation, if possible. Note that there is no need to activate the eggs to send them into interphase. They will spontaneously enter interphase due to lysis in the presence of Ca^{2+} ions. Cycloheximide added to the buffers will maintain the interphasic state of the extract. Any batches of eggs that lyse during dejelling, loading into tubes, or packing in the low-speed centrifuge should be discarded.

Dejelly the eggs

4. Pour off MMR and add 50 to 100 ml dejelling solution to each beaker. Swirl beaker gently and change the solution several times until the eggs start to pack (usually ~ 5 min).

The eggs should be left in dejelling solution for the minimal amount of time required to completely remove the jelly coat. Further incubation seriously compromises egg quality.

5. Rinse three times with 50 to 100 ml MMR. Work quickly through the next steps, until lysis/centrifugation, as the eggs are quite fragile once the jelly coat is removed. Ensure that the eggs are always covered with buffer. Remove bad eggs (white and puffy, or dark pigment retracted or mottled).

Lyse the eggs

6. Pour off as much MMR as possible while keeping eggs immersed in a minimal quantity. Rinse two times with S-lysis buffer in the same manner.
7. Rinse once with S-lysis-plus buffer. Use a wide-bore Pasteur pipet to load eggs into SW50 ultraclear centrifuge tubes containing 1 ml S-lysis-plus buffer and 10 μ l of 10 mg/ml cytochalasin D stock (to 100 μ g/ml final concentration). Fill tubes as full as possible and remove any buffer on top of eggs.

Always place the tip of the pipet containing the eggs into the buffer in the tube before expelling the eggs. This ensures that the eggs do not contact air which would cause lysis.

8. Place each filled SW50 tube into a Sarstedt 13-ml adapter tube containing ~ 0.5 ml water in the bottom. Pack eggs by centrifuging 30 sec at low speed, $150 \times g$, room temperature, in a clinical centrifuge, then 30 sec at $700 \times g$. Remove all the buffer above the eggs and discard any tubes with significant lysis.
9. Lyse eggs by centrifuging 15 min at $\sim 16,000 \times g$ (10,000 rpm in HB-4 rotor containing rubber adapters), 4°C. Remove tubes from adapters and place upright on ice.

Collect cytoplasm

10. Attach an 18-G needle to a 2-ml syringe. Move syringe plunger back and forth once to loosen before piercing tube. Wipe sides of tube with a tissue and then pierce the tube just above the bottom of the clear cytoplasmic layer. Make sure the opening of the needle faces upwards. Withdraw the clear straw-colored cytoplasm avoiding the yellow lipid layer above and the heavier layers containing yolk, mitochondria, and cortex fragments below. Remove the needle from the syringe and expel the cytosol from the syringe into an appropriate sized polypropionate tube with a cap.

Bracing the centrifuge tube against the inside of an ice bucket will help avoid accidents during piercing.

One 5-ml tube of eggs will yield 1 to 2 ml of cytosol.

11. Pool cytosols that look good. Add 1/1000 volume each of cytochalasin D (final 10 µg/ml), DTT (to 1 mM) and protease inhibitors.
12. If there is any contamination of the cytosol, then repeat the 16,000 × g centrifugation to clarify cytosol of any residual yolk or heavy cortex fragments. Remove clear cytosol as above and add 2.5 M sucrose to 125 mM to the cytosol.

This helps separate the membranes out of the cytosol in the following centrifugation.

Isolate membrane and cytosol fractions

13. Ultracentrifuge cytosol for 60 min at 200,000 × g (555,000 rpm in SW55 rotor), 4°C.

There will be the following fractions from top to bottom: lipid, clear cytosol, golden membranes, grayish mitochondrial membranes, then translucent pellet consisting of glycogen and ribosomes.

14. Carefully aspirate clear lipid layer and discard. Remove the clear cytosol to a separate tube using a 200-µl tip (do not use a 1000-µl tip).

From a 5-ml tube one should collect ~2.5 ml. It is difficult to remove the clear cytosol without pulling up some of the golden membranes. Go slowly when near the membrane layer.

15. Remove and save golden membrane fractions (they are the nuclear assembly membranes) and follow with them at step 17. Carefully avoid the gray mitochondrial layer.
16. Recentrifuge cytosol 30 min at 200,000 × g (55,000 rpm in TLS55 rotor), 4°C, to remove residual membranes and lipid. Remove cytosol as in step 14. To cytosol, add glycerol to a final concentration of 3%. Snap freeze in liquid nitrogen in 50-µl aliquots. Store aliquots at -70°C.

This cytosol fraction is called cytosol-200.

17. Resuspend the golden membranes in excess S-lysis-plus (≥10 vol) and centrifuge over a 0.5-ml cushion of S-lysis-plus/500 mM sucrose for 20 min at 26,000 × g (14,000 rpm in SW55 rotor), 4°C. Resuspend as a 10× stock in S-lysis-plus/500mM sucrose solution and freeze in 10-µl aliquots (10× stock means 1/10th the volume of cytosol).

This membrane stock is called M-200.

PREPARATION OF HIGH-SPEED SUPERNATANT (HSS)

The cytosol used for the motility assay is clarified of all membranes by dilution of concentrated $16,000 \times g$ cytosol followed by centrifugation. The concentrated cytosol is prepared, dispensed into aliquots, and stored frozen until the day of the experiment, when the dilution and centrifugation steps are performed.

Additional Materials (see Support Protocol 3)

ABC buffer (see recipe)

Table top ultracentrifuge and TLA-100 rotor or Airfuge (Beckman) and rotor

1. Perform Support Protocol 3, steps 1 through 11. However, in step 11 lower the sucrose concentration to 100 mM rather than 125 mM.
2. Pipet the cytosol into 50- μ l aliquots and snap freeze in liquid nitrogen. Store aliquots at -80°C until use.
3. On the day of the assay, thaw an aliquot of cytosol and dilute with 2 vol ABC buffer. Centrifuge for 20 min at $100,000 \times g$ (15 min, 30 psi in Beckman Airfuge or 550,000 rpm in TLA-100 rotor,), 4°C , to pellet membranes.
4. Pipet the cytosol to a fresh tube and maintain on ice until use (~ 4 hr max).

NUCLEAR ASSEMBLY USING DNA-COATED MAGNETIC BEADS AS TEMPLATE

This protocol describes the assembly of nuclei around DNA-coated magnetic beads. The protocol for binding of DNA to the beads is presented in *UNIT 11.13* (Heald et al., 1998). The nuclei can be assembled by simply adding DNA beads to fresh $16,000 \times g$ cytosol (see Support Protocol 2, step 11). However, to perform manipulations of the nuclei to test specific components of the nucleus for a role in nuclear motility, use this alternate approach. Each batch of fractionated cytosol has to be titrated for nuclear assembly using DNA-magnetic beads. Given below is a starting protocol. One may have to vary the glycogen, amount of membranes, and/or dilution of cytosol with ABC buffer to get better assembly. Assembly using beads is much trickier than assembly with either sperm nuclei or lambda DNA. The beads must have a high concentration of DNA. Only one end of the DNA should be attached to the beads (i.e., fill in only one end of the DNA with biotinylated nucleotides), and the plasmid cannot be too short. (The MCP plasmid is ~ 8 kb and assembles nuclei much better than a 6-kb plasmid. Much shorter plasmids do not assemble at all.) Once the parameters for assembly with the extract preparation has been optimized, then scale up to make a nuclear prep for dispensing aliquots and freezing.

Materials

Cytosol-200 (see Support Protocol 2)

ABC buffer (see recipe)

DNA-Dynabeads (for preparation see *UNIT 11.13*, Heald et al., 1998)

PBS/1% (w/v) BSA (see recipe)

150 mg/ml glycogen stock (see recipe)

M-200 (see Support Protocol 2)

50 mM Mg-ATP (see recipe)

0.5 M creatine phosphate (see recipe)

8 mg/ml creatine kinase (see recipe)

1.4 mg/ml TRITC-BSA-NLS (transport substrate; *UNIT 11.7*, Support Protocol 2)

Fix solution (see recipe)

Beckman table top ultracentrifuge and TL100 rotor (or Beckman airfuge and rotor)

Magnetic particle concentrator

**SUPPORT
PROTOCOL 4**

**SUPPORT
PROTOCOL 5**

Organelle Motility

13.4.15

Microscope slides and clean coverslips
Nail polish
Microscope equipped with epifluorescence optics

1. Thaw one 50- μ l aliquot of cytosol-200.
2. Add 50 μ l ABC buffer and centrifuge for 30 min at $100,000 \times g$ (55,000 rpm in TL100 rotor or 15 min, 30 psi in airfuge), 4°C.
3. During spin, prepare DNA beads. Use 10 μ l beads (70 μ g/ml) for a 100- μ l reaction. Resuspend the DNA-bead stock well before pipetting. Pipet 10 μ l beads to a clean tube. Add 200 μ l PBS/1% BSA and pipet beads up and down several times. Retrieve beads on the magnetic particle concentrator, carefully remove the buffer and repeat washing step.
4. Resuspend washed beads in the diluted and centrifuged cytosol (step 2, ~100 μ l). Pipet thoroughly but carefully to disrupt any aggregates. Do not introduce air bubbles into the cytosol.

Failure to disrupt aggregates results in large masses of beads that do not properly assemble into nuclei.

5. Add and mix by pipetting:
 - 10 μ l 150 mg/ml glycogen
 - 10 μ l membranes (M-200)
 - 2 μ l 50 mM Mg-ATP
 - 2 μ l 0.5 M creatine phosphate
 - 1 μ l 8 mg/ml creatine kinase.

Incubate 2 to 3 hr at 20°C.

Fully formed nuclei are first apparent at 40 min and increase progressively until 3 hr.

6. To assay for functional nuclei, remove a 10- μ l aliquot and add 0.5 μ l of 1.4 mg/ml TRITC-BSA-NLS. Incubate 30 min at 4°C. Pipet 2 μ l of this reaction onto a clean slide. Add 5 μ l of fix solution and apply a clean coverslip. Seal the edges with nail polish if retention of the sample is desired. Observe nuclei with a microscope equipped with epifluorescence optics to detect nuclei that have accumulated the transport substrate (see Fig. 13.4.7 for images of nuclear uptake).

Since Dynabeads are autofluorescent with emissions in the red wavelengths, a nuclear uptake substrate that will emit in the green wavelengths is often preferable, such as GFP or fluorescein-labeled. If desired, prepare FITC-BSA-NLS as in UNIT 11.7, Support Protocol 2, by substituting fluorescein-5-EX succinimidyl ester (Molecular Probes) for TRITC in steps 2 to 6 of the protocol. Follow manufacturer's recommendations for coupling buffer and pH conditions. The rest of the protocol remains the same. Alternatively, prepare GFP-GST-NLS (UNIT 11.7, Support Protocol 3). Use both as indicated here for TRITC-BSA-NLS.

7. When functional nuclei are observed, then scale up the reaction 10- to 20-fold and repeat all steps including the assay step. To freeze, add glycerol to 10% (v/v), dispense 10- μ l aliquots, and freeze in liquid nitrogen. Store at -70°C.

Nuclei can be stored for several years in this state. Frozen nuclei can be thawed on ice and washed in ABC buffer, retrieved on a magnet for several minutes (on ice) and resuspended in cytosol, or other buffers. Typically, they retain ~90% activity or higher after freezing. One 10- μ l aliquot of nuclei is used for each motility assay.

If the membranes are omitted from the reaction, interphase chromatin assembles onto the DNA, but no nuclear envelope forms. The motility of the chromatin can be compared with that of bona fide nuclei (Reinsch and Karsenti, 1997).

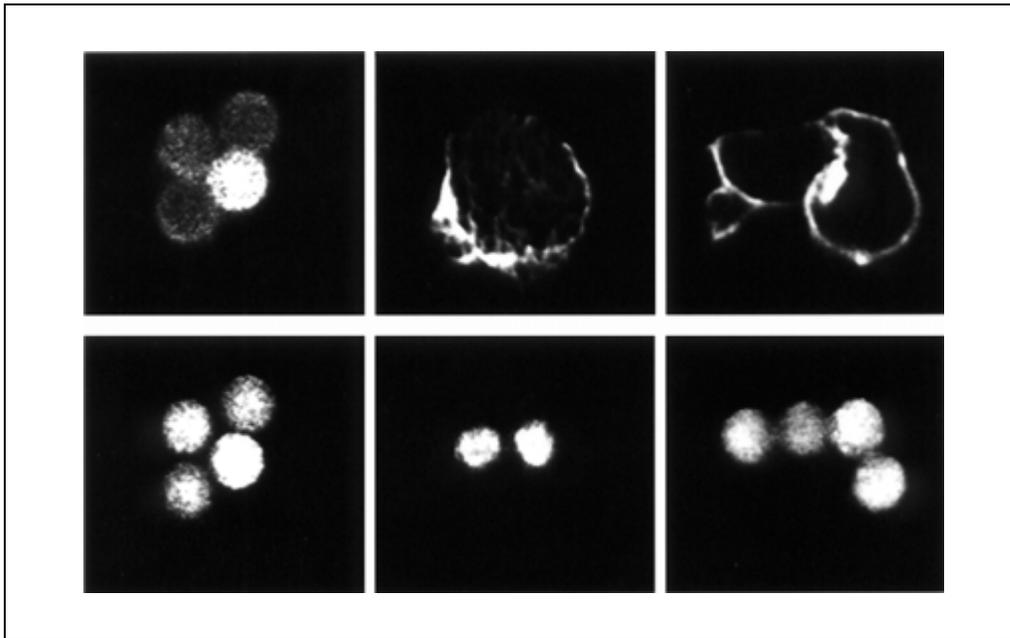


Figure 13.4.7 Nuclei assembled around magnetic DNA-beads are functional for nuclear transport and contain nuclear antigens. Confocal fluorescence images of synthetic nuclei assembled using the protocols described here. Autofluorescence of the beads is shown below (rhodamine filter), while the upper panels show the same beads in the fluorescein channel. FITC-BSA-NLS: typical fluorescence following uptake of the nuclear transport substrate FITC-BSA NLS in an unfixed sample. Staining for lamins and nuclear pores gives the typical peripheral staining pattern for these antigens.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

ABC (complete acetate buffer)

Acetate buffer (see recipe) supplemented with:
 7.5 mM creatine phosphate (see recipe for 0.5 M)
 1 mM MgATP
 80 µg/ml creatine kinase (see recipe for 8 mg/ml)
 10 mg/ml protease inhibitors
 Store as 1- to 2-ml aliquots 1 year at -20°C
 Thaw once and discard

Acetate buffer

To prepare 1 liter:
 9.8 g potassium-acetate (100 mM final)
 0.64 g magnesium-acetate (3 mM final)
 1.9 g EGTA (5 mM final)
 2.38 g HEPES (10 mM final, pH 7.4)
 51.34 g sucrose (150 mM final)
 H₂O to 950 ml
 Adjust the pH to 7.4
 H₂O to 1 liter
 Aliquot in 50-ml tubes
 Store 1 year at -20°C

Casein in PE

100 mg casein
Add 20 ml PE (see recipe)
Vortex vigorously
Incubate at 37°C with intermittent vortexing to aid solubilization of casein
Aliquot and store 1 year at -20°C

Creatine kinase, 8 mg/ml

8 mg creatine kinase
Add 475 µl distilled water
Add 20 µl 1 M HEPES-KOH, pH 7.5
Add 5 µl 1 M DTT
Add 500 µl glycerol
Snap freeze in 10-µl aliquots in liquid nitrogen
Store 1 year at -70°C

Creatine phosphate, 0.5 M

127.55 mg creatine phosphate
1 ml H₂O
Store 1 year at -20°C in 50-µl aliquots
This solution can be thawed and refrozen

Cytochalasin D, 10 mg/ml

10 mg of cytochalasin
1 ml DMSO
Store 1 year at -20°C in 50-µl aliquots

Dejelling solution (2% L-cysteine, pH 7.8)

20 g cysteine
H₂O to 970 ml
Adjust pH to 7.8 with 6 M NaOH
Prepare fresh just before use

DNase I, 1 mg/ml

10 mg DNase I
10 ml H₂O
Store 1 week at 4°C

DTT, 1 M

0.77 g of DTT
5 ml H₂O
Keep on ice. Dissolve the DTT precipitate by moderate heating just before use.
Prepare fresh.

Fix solution

600 µl 80% glycerol
300 µl 37% formaldehyde
100 µl 10× MMR (see recipe)
0.5 µl 10 mg/ml Hoechst dye
Always prepare fresh on day of use

Glycogen, 150 mg/ml

750 mg glycogen
Dissolve in 5 ml acetate buffer (see recipe)
Aliquot and store 1 year at -20°C

Aliquots can be repeatedly thawed and refrozen

Gradient buffer

3.02 g PIPES (10 mM final)
H₂O to 950 ml
Add:
2 ml 0.5 M EDTA stock solution (1 mM final)
1 ml pure 2-β-mercaptoethanol (1% final)
10 ml of 10% Triton X-100 stock solution (0.1% final)
Bring to pH 7.2 with KOH
H₂O to 1 liter
Use immediately to prepare sucrose gradient solutions
Store remainder 1 year at -20°C

HCG, 500 U/ml

10,000 U human chorionic gonadotropin (Sigma)
Add 10 ml sterile distilled water
Keep sterile
Store at 4°C
Use within 10 days

HEPES/KOH, pH 7.5 (1 M)

59.6 g HEPES
200 ml H₂O
Adjust pH to 7.5 with concentrated KOH
H₂O to 250 ml
Filter sterilize
Store 1 year at -20°C in 5-ml aliquots

K-PIPES, pH 7.2, 0.5 M, 1 mM EDTA

7.55 g PIPES
40-ml H₂O
Add 0.1 ml 0.5 M EDTA stock solution
Bring pH to 7.2 with KOH
H₂O to 50 ml
Store 1 year at -20°C in 2-ml aliquots

Lysis buffer

0.5 ml Tris-HCl, pH 8.0, 1 M stock (*APPENDIX 2A*; 1 mM final)
490 ml H₂O
0.5 ml pure β-mercaptoethanol (0.1% v/v final)
2.5 ml pure NP-40 (0.5% v/v final)
51 μl 4.9 M MgCl₂ (Sigma; 0.5 mM final)
87.1 g PMSF
0.5 ml 10 mg/ml stock aprotinin
50 μl 10 mg/ml stock leupeptin
50 μl 10 mg/ml stock pepstatin
H₂O to 500 ml

This buffer can be freshly prepared or stored in aliquots at -20°C.

NOTE: NP40 is chemically identical to IGEPAL CA-630 (Sigma).

Mg-ATP, 50 mM

0.275 mg ATP
9 ml H₂O
0.1 g MgCl₂
Adjust pH to 7.5 with NaOH
H₂O to 10 ml
Store 1 year at -20°C as 500-μl aliquots

MMR, 10×

58.4 g NaCl (100 mM final)
1.49 g KCl (20 mM final)
2.04 ml of 4.9 M MgCl₂ stock solution (Sigma) (10 mM final)
2.94 g CaCl₂ (20 mM final)
372 mg EDTA (0.1 mM final)
11.9 g HEPES (50 mM final, pH 7.8)
900 ml H₂O
Adjust pH to 7.8 with 6 M NaOH
H₂O to 1 liter
Autoclave and store 6 months at room temperature

For MMR, dilute 10× stock 1:10 with water.

Nocodazole, 10 mM

To prepare 1 ml:
3 mg nocodazole
1 ml DMSO
Store 1 year at -20°C in 50-μl aliquots

PBS, 10×

14.2 g Na₂HPO₄ dissolved in 500 ml H₂O
2.67 g NaH₂PO₄ dissolved in 100 ml H₂O
Mix the 2 solutions
Add 90 g NaCl
H₂O to 1 liter
Check that solution is at pH 7.4
Store 1 year at -20°C

For PBS: Dilute 10× PBS 1:10 with water.

PBS/10 with 8% sucrose

10 ml 10× PBS (see recipe) diluted to 1 liter with H₂O (PBS/10)
80 g sucrose
Bring weight to 1 kg with PBS/10
Store 1 year at -20°C

PBS/1% (v/v) BSA

Weigh 500 mg BSA
Dissolve in 50 ml PBS (see recipe)
Filter and store 1 week at 4°C or aliquot and store 1 year at -20°C

PE buffer

To prepare 500 ml:

1.51 g K-PIPES (10 mM final; see recipe)
480 ml H₂O
Add 1 ml 0.5 M EDTA stock solution (final 1 mM)
Bring to pH 7.2 with KOH
H₂O to 500 ml
Filter
Store 3 months at 4°C

PMSG, 200 U/ml

Add 5 ml sterile water to vial containing 1000 U pregnant mare serum gonadotropin
Store 2 weeks at 4°C

Protease inhibitor stock (LPC, 10 mg/ml)

10 mg each of leupeptin, pepstatin, and chymostatin
Dissolve in 1 ml DMSO
Store 1 year at -20°C in 50- μ l aliquots

S-lysis buffer

50 ml 2.5 M sucrose (250 mM final)
255 μ l 4.9 M MgCl₂ (Sigma; 2.5 mM final)
12.5 ml 2M KCl (50 mM final)
5 ml 1 M HEPES-KOH, pH 7.5 (10 mM final)
H₂O to 500 ml
Adjust pH to 7.5 with KOH
Prepare fresh

S-lysis-plus buffer

150 ml S-lysis buffer (see recipe)
150 μ l protease inhibitor stock (LPC; see recipe)
150 μ l 1 M DTT (see recipe)
15 mg cycloheximide (final 100 μ g/ml)
Stir until dissolved and store on ice until use

Wear gloves when handling cycloheximide.

S-lysis-plus/500 mM sucrose

3.4 g sucrose
S-lysis buffer (see recipe) to 20 ml
Incubate 37°C with intermittent vortexing until dissolved
20 μ l protease inhibitor stock (LPC; see recipe)
20 μ l 1 M DTT (see recipe)
20 mg cycloheximide
Store on ice until use

Wear gloves when handling cycloheximide.

Sucrose, 2.5 M (85% w/v)

85 g sucrose
Adjust to a total volume of 100 ml with H₂O
Allow overnight for dissolving (at 60°C)
Filter
Store 6 months at 4°C

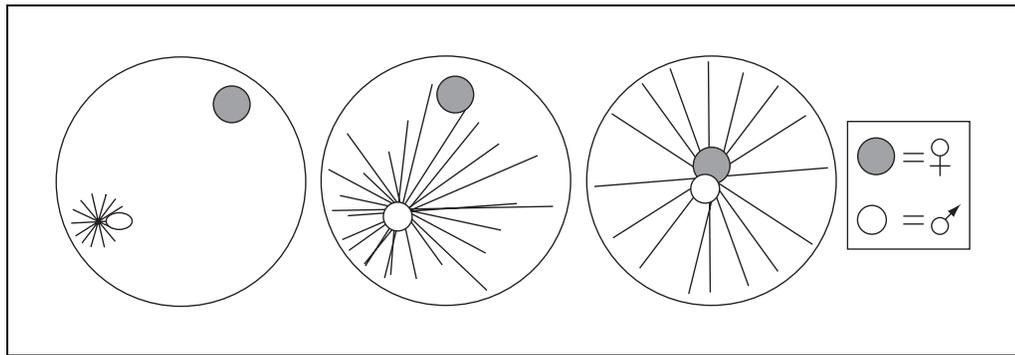


Figure 13.4.8 Movements of the male and female pronuclei in newly fertilized eggs. Schematic showing the microtubule-mediated positioning events of the male and female pronuclei in a typical metazoan early embryo. See text for details. Microtubules are represented by lines, and the male and female pronuclei by open and shaded circles respectively.

Sucrose in gradient buffer, 40% (w/w)

40 g sucrose
 Gradient buffer (see recipe) to 100 g
 Prepare fresh or store 6 months at -20°C

Sucrose in gradient buffer, 50% (w/w)

50 g sucrose
 Gradient buffer (see recipe) to 100 g
 Prepare fresh or store 6 months at -20°C

Sucrose in gradient buffer, 70% (w/w)

70 g sucrose
 Gradient buffer (see recipe) to 100 g
 Prepare fresh or store 6 months at -20°C

COMMENTARY

Background Information

The microtubule cytoskeleton plays a crucial role in positioning of the nucleus (reviewed in Reinsch and Gonczy, 1998). Nuclear migration processes occur in numerous cell types such as muscle, nerve, and somatic dividing cells. In newly fertilized eggs, long-range microtubule-dependent movements of the male and female pronuclei are essential for the two nuclei to meet and form the zygotic nucleus (Schatten, 1994).

During oogenesis in metazoan organisms, including *Xenopus*, the centrosome of the egg degenerates (Schatten, 1994). At fertilization, the basal body of the sperm flagellum converts to the centrosome by recruiting pericentriolar material from the egg cytosol, and nucleates a large microtubule aster. The male pronucleus (Fig. 13.4.8, open circle) remains at the center of the aster, which grows to fill the egg, thus transporting the male pronucleus to the center of the egg. The female pronucleus (Fig. 13.4.8, shaded circle) has no associated centrosome or

microtubule-nucleating activity. Instead, the female pronucleus translocates along the microtubules of the sperm aster from the cell cortex to the centrosome located in the center of the aster. Thus, the two nuclei meet at the center of the aster prior to the first cleavage. Pronuclei of some species traverse hundreds of microns in a fraction of the first cell cycle (Stewart-Savage and Grey, 1982). The motility of the two nuclei differs in that the female pronucleus translocates along microtubules towards the microtubule minus-end while the movement of the entire sperm aster drives the movement of the male pronucleus.

The assay presented here recapitulates the movement of the female pronucleus *in vivo* and is the first system to demonstrate the translocation of nuclei along microtubules *in vitro*. Using this extract-based assay, it has been determined that cytoplasmic dynein is required for nuclear movements along microtubules (Reinsch and Karsenti, 1997). This system can be further used to determine how cytoplasmic

dynein is targeted to the nuclear membrane to mediate the organelle-like movement along microtubules.

Until the development of this assay, microtubule-dependent nuclear motility had only been studied *in vivo* because of the complexity of the process. Perturbation studies *in vivo* have used pharmacological agents to demonstrate the role of the microtubule cytoskeleton in pronuclear movement (Aronson, 1971; Schatten, 1982; Rouviere et al., 1994). Genetic studies in *C. elegans* have only very recently begun to address this process (Gonczy et al., 1999b). Recent experiments using RNA-mediated inhibition of expression in *C. elegans* embryos have confirmed the role of dynein and associated proteins in pronuclear migration (Gonczy et al., 1999a). These genetic experiments, thus, validate the biochemical approach presented here to demonstrate that dynein is required for the motility of synthetic nuclei along microtubules (Reinsch and Karsenti, 1997), and indicate that this approach is a valid means to identify candidate molecules involved in complex processes in the early embryo.

There were two important constraints on the choice of nuclei for the motility assay. First, it is important to use nuclei lacking an associated centrosome to resemble the female pronucleus. Nuclei purified from many tissues and from cells in culture generally have an associated centrosome whose activity could potentially generate motility events resembling those of the male rather than the female pronucleus (Reinsch and Gonczy, 1998). Second, it is important to choose a source of nuclei that would be competent for motility. Nuclei from many tissue sources may not be functional for motility if nuclear migration events are not occurring in the selected tissue at the time of purification.

Interphase *Xenopus* egg extracts spontaneously assemble nuclei from soluble and membrane components stored in the egg (Forbes et al., 1983; Newport, 1987; Newmeyer and Wilson, 1991). Upon the addition of DNA or chromatin to a lysed extract, nuclei containing double nuclear membranes, a nuclear lamina, and nuclear pores are quickly assembled (UNIT 11.10). These nuclei are capable of nuclear import, DNA replication, and normal mitotic disassembly. The most commonly used and convenient substrate for nuclear assembly studies is demembrated sperm nuclei. However, the sperm has an associated basal body that converts into a centrosome in the egg (or extract). Sperm nuclei are therefore not appropriate substrates for this assay. Recently, magnetic beads

coupled to plasmid DNA have been demonstrated to function as artificial chromosomes capable of inducing nuclear assembly in interphase extracts and bipolar spindles in mitotic extracts (Heald et al., 1996; Reinsch and Karsenti, 1997; UNIT 11.13). Since DNA beads are paramagnetic, they can be simply retrieved from the extract. Centrosomes do not spontaneously assemble in these extracts and eggs contain no centrosomes. Therefore, the nuclei assembled around magnetic beads in this extract lack an associated centrosome. These synthetic nuclei provide a novel approach to studying nuclear motility since they allow rapid and gentle isolation of large numbers of nuclei in a motility competent state.

An alternative to magnetic bead nuclei, is to use DNA purified from phage lambda as a substrate for nuclear assembly. The DNA is simply added to a $16,000 \times g$ fresh extract and allowed to assemble (see Support Protocol 3, steps 1 through 11). However, the nuclei must then be purified over sucrose gradients (S. Reinsch, unpub. observ.). The advantage of the lambda DNA–nuclei is that they are much smaller than the DNA-bead nuclei used here and more suitable for certain analyses such as electron microscopy.

The benefits of using this extract-based assay system are numerous. This is an ideal system for the identification of the molecular components involved in nuclear motility as it is amenable to biochemical dissection of both nuclear and cytoplasmic components and allows high-resolution microscopic observation of nuclear motility.

This assay allows pronuclear migration to be studied in isolation of other simultaneous or upstream processes. Both microtubules and cytoplasmic dynein function in numerous processes that occur both prior to and after fertilization. By using this assay, the molecular components that are specific to this process as well as those which are shared with other processes can be identified. The ease of biochemical manipulation of these extracts allows experiments that are impossible in intact cells or organisms.

Nuclear motility can be observed using much cruder preparations from *Xenopus* eggs than those described here (S. Reinsch, unpub. observ.; Murray et al., 1996). However, this assay provides a means to define the minimal essential components required for nuclear motility along microtubules.

Critical Parameters and Troubleshooting

The system presented here uses largely homologous components. Only the centrosomes are purified from humans rather than frog. In theory, purified centrosomes from any source can be used. The use of homologous cytosol and nuclei is critical. Cytosol from other sources (e.g., bovine brain, HeLa cells, rat liver) does not support the movement of nuclei assembled *Xenopus* egg extracts (S. Reinsch, unpub. observ.). The use of heterologous nuclei has not been rigorously tested to determine whether heterologous nuclei will move on microtubules in *Xenopus* egg extracts. However, nuclei purified from other sources may undergo "repair" when incubated in *Xenopus* egg extracts and acquire components from the cytosol (Leno and Munshi, 1994) that may enable them to use the *Xenopus* cytosolic machinery for motility. It is conceivable then, that they will demonstrate motility events in this system. However, it is important to realize that nuclei purified from tissues or cultured cells may have associated centrosomes. Microtubules nucleated from these associated centrosomes may generate motility events in their own right that are independent of the translocation of nuclei along microtubules (reviewed in Reinsch and Gonczy, 1998).

As stated previously, nuclear motility per se can be visualized using less purified extract-based components. Freshly prepared low-speed ($10,000 \times g$) extracts support both nuclear assembly and motility. Visualization of nuclear motility in this simpler system is a bit more challenging and requires the use of fluorescent components to label microtubules and nuclei. The assay presented here has been developed to allow for biochemical manipulations of both the cytosol and the nuclei to dissect the motility apparatus while simultaneously allowing visualization of motility events. It has also been optimized so that only frozen aliquoted components are used. This allows reproducibility from one assay to the next so that the effects of drugs or other added components can be determined.

For the motility assay, each component can present specific difficulties as outlined below. Once the assay has been optimized and is working well, then it is quite reproducible. To minimize difficulties, prepare larger stocks with aliquots that are the appropriate size for individual experiments. Never refreeze aliquots. Prepare a new stock (centrosomes, nuclei, HSS) well before the last aliquots are used. Try

to switch out only one component at a time. Test the new batch of the component alongside the old batch to ensure that it functions properly.

Careful biochemical preparation of the individual components of the Basic Protocol is absolutely critical for the success of the quantitative motility assay. Several steps are critical in preparing extracts of *Xenopus* eggs. Please read the general references for handling *Xenopus* and extract preparation (Murray, 1991; Newmeyer and Wilson, 1991; UNIT 11.10). It is also worthwhile to understand the scientific literature on using *Xenopus* extracts for nuclear assembly (for review see Lohka, 1998 and UNIT 11.10). Eggs for preparation of nuclear assembly extracts and for HSS must be of top quality. Carefully read each support protocol and have all materials on hand before starting the preparations. Do not let the preparations sit on ice for long periods. Proceed immediately through the steps to the finished frozen product.

One of the most critical parameters in preparing fractionated extracts for nuclear assembly is to avoid contaminating the nuclear membranes with mitochondria. The mitochondria contribute to apoptotic events (Newmeyer et al., 1994; UNIT 11.12). The mitochondrial layer appears as grayish as opposed to the nuclear membranes, which have a golden tint. During the $200,000 \times g$ centrifugation, sometimes the membranes do not band optimally and multiple bands are visible. Recentrifuge for a longer time to allow better separation. Too low a sucrose concentration in the extract can also contribute to this. The prep can be done on a smaller scale using a TLS-55 rotor instead of an SW-55 rotor.

For nuclear assembly using magnetic beads, the quality of DNA-beads is very important. Be sure to precisely follow the instructions in UNIT 11.13 for bead preparation. By Hoechst staining, the DNA should make a uniform bright rim around the bead. There should not be a punctate pattern around the bead. Make sure to use long DNA fragments. An 8-kb fragment works very well. Shorter fragments do not assemble well into nuclei. The DNA must be filled in with biotin at only one end so that only one end attaches to the bead. This assures good chromatin assembly on the DNA bead. DNA-beads can be stored for months at 4°C . Do not freeze.

When assembling the bead-nuclei from fractionated extracts make sure that DNA-beads are well suspended in extract so that they don't clump too much. Otherwise all the nuclei will contain many beads. An average of 1 to 3 beads per nucleus is optimal for the motility assay. Glycogen is essential for assembly of

chromatin onto naked DNA (Hartl et al., 1994). The source of the glycogen is not important. Commercial oyster glycogen works quite well. The length of time for the nuclear assembly reaction is not critical and can be from 1 to 3 hr. The longer the nuclei are left in the assembly reaction, the larger they tend to get. They do not acquire more beads, but they will import proteins from the extract and become more swollen. This is not really a problem and often is helpful for visualizing uptake of the transport substrate TRITC-BSA-NLS. When performing the transport reaction, one should be able to clearly see uptake of nuclear transport substrate into 90% to 95% of nuclei. After assembly, nuclei can be stored overnight at 4°C without loss of function. Longer storage at this temperature is not advised. If one encounters difficulties in assembling nuclei from fractionated components, then try a different substrate, such as sperm nuclei (see *UNIT 11.13* for preparation) or lambda DNA to be convinced that the problem is not the DNA-bead substrate.

The centrosome preparation presented here has been optimized for high yield and high concentration of centrosomes. It is a variation of a published method (Moudjou and Bornens, 1998). Other preparations of centrosomes have not been tested for use in this assay, but would be interesting to compare (e.g., Blomberg-Wirschell and Doxsey, 1998). In theory, any centrosome preparation should be acceptable for use in this assay as long as robust microtubule asters are generated upon addition of HSS. For this particular preparation, it is essential that the lymphocytes be grown in exponential phase. The treatments with the cytoskeletal inhibitors, nocodazole and cytochalasin, separate the centrosome from the nuclear membrane so that centrosome and nucleus do not copurify. The incubation time for this treatment allows depolymerization of microtubules and actin filaments without causing cells to accumulate in mitosis. The washes with high osmotic strength buffers cause the cells to be very fragile. These steps should be done as rapidly as possible to minimize cell lysis. However, do not pipet so vigorously that bubbles are produced. The centrifugation steps are straightforward and can be adapted for other rotors and volumes. Do not leave the centrosomes unfrozen for long periods. Freeze the large aliquots as quickly as possible. Thaw only once to re-aliquot and freeze again. The size for the small aliquots should be dictated by experiment design. Do not refreeze the small aliquots.

Preparations of HSS can vary in activity largely due to egg quality. Cycloheximide is added to ensure that the extract stays in interphase. It is important to add sucrose as a cryoprotectant to the $16,000 \times g$ extract before freezing. The prep is frozen as a $16,000 \times g$ extract for several reasons. First, this takes up less freezer space, and the subsequent dilution and centrifugation steps to be done on the day of preparation are very reproducible. Second, preps that were carried through the subsequent dilution and centrifugation and then frozen as aliquots did not function as well in the motility assay. On the day of the motility assay, the dilution before high-speed centrifugation ensures that all of the membranes are sedimented. This allows optimal DIC imaging of nuclei without extraneous membranes. The dilution recommended here has been optimized for microtubule aster assembly. Too low a dilution causes a lot of spontaneous microtubule assembly. Sometimes asters generated in HSS become fragmented before the 20-min time point. Throw these batches of cytosol away since they will consistently give the same result, and prepare a new batch of $16,000 \times g$ extract. These poor-quality batches often also show high apoptotic activity. Try keeping egg batches from different frogs separate all the way through the preparation if poor quality cytosol is a problem.

The quantitative assay for nuclear motility requires nuclei to accumulate at centrosomes. Therefore, robust aster morphology with nice spacing between the asters is crucial. Human lymphocytes assemble asters with only a few microtubules in the presence of pure tubulin. Incubating centrosomes in HSS allows pericentriolar material to be recruited to the centrosomes. After incubation in *Xenopus* egg extract, their nucleation capacity increases significantly (Buendia et al., 1992) so that robust microtubule asters are generated. Plating density of the centrosomes can affect the outcome of the assay. Steps for optimizing this parameter are outlined in Support Protocol 3. The asters should be spaced so that there are not more than 1 to 3 centrosomes/50- μm field. This allows nuclei to reach centrosomes so that quantitation is possible. If asters are too widely spaced, then there will be a lot of free microtubule nucleation and random movement of the nuclei rather than nuclei moving to and accumulating at centrosomes. Make sure the incubation to allow centrosomes to attach to the glass coverslip is consistently 5 min. Several blocking agents have been tested; casein gives satisfactory re-

sults to prevent nuclei from sticking. The casein also inhibits dynein present in HSS from binding to the coverslip. This decreases the amount of microtubule gliding that occurs.

If robust, well-spaced asters are not obtained, the problems can either be in the density of the centrosomes or more likely in the preparation of HSS as described above. Adding agents such as drugs and antibodies can also seriously affect the ability of asters to be nucleated and should only be added after the nucleation step. For example, addition of agents that inhibit dynein activity cause random microtubules to assemble, rather than preferential assembly off centrosomes (S. Reinsch, unpub. observ.). If one does not plan to use the motility assay as a quantitative assay, but only for visualizing nuclear movements without perturbing agents, then the nucleation step can be completely omitted. Many agents also compromise microtubule assembly. For example, BAPTA, a calcium chelator, completely inhibits microtubule assembly in this assay (S. Reinsch, unpub. observ.). Since the microtubules are dynamic in this assay, any factor that affects microtubule dynamics may compromise the success of the assay.

Anticipated Results

The nuclear motility assay allows clear documentation of nuclear motility events. When bead-nuclei are purified on the magnet, they do come with a considerable amount of associated endoplasmic reticulum (ER). Therefore, both nuclear movements and ER tubule elongation will be visible. In HSS, the nuclei move exclusively towards the minus-ends of the microtubules, that is, towards the centrosome. The average rate of movement of nuclei assembled in *Xenopus* extracts is 1 $\mu\text{m}/\text{sec}$ with a range from 0.2 to 1.8 $\mu\text{m}/\text{sec}$. Neither DNA-beads (unassembled) nor chromatin-beads (assembled in cytosol without adding membranes) will move to the microtubule minus-ends. In the presence of inhibitors of dynein (e.g., >15 μM vanadate) minus-end directed movements are blocked. The nuclei then are moved to the aster periphery due to the action of growing microtubules. This is not plus-end directed movement. Instead nuclei appear to be pulled out by growing microtubules due to ER tubule attachment to the tips of growing microtubules (Waterman-Storer et al., 1995; Reinsch and Karsenti, 1997).

For the quantitative assay, 90% to 95% of the nuclei should accumulate at centrosomes by the 20-min time point under control condi-

tions. One should be able to easily count between 50 and 100 nuclei in the 10-min counting interval. Dynein inhibitors significantly inhibit accumulation at centrosomes (see Fig. 13.4.3; Reinsch and Karsenti, 1997). An antibody against the intermediate chain of cytoplasmic dynein (mAb 70.1, Sigma) also inhibits nuclear motility and accumulation of nuclei at centrosomes when directly added to HSS (Reinsch and Karsenti, 1997). Not all antibodies against cytoplasmic dynein successfully inhibit nuclear motility (S. Reinsch, unpub. observ.).

Each assay should be performed at least three times to generate statistically significant data. Once a control reaction has been performed on a given batch of centrosomes/nuclei/HSS, the control reaction should be consistent from day to day and should always give 90% to 95% accumulation. This includes adding buffer to account for added experimental agents. Therefore, on a given experimental day, the control reaction can be performed once or possibly twice, to confirm that the system looks the same as on the previous experimental day, rather than having to perform multiple control experiments on each day an experiment is performed. This gives more time for experimental manipulation (see below).

The HSS should contain no membraneous materials. When incubated at room temperature, microtubule assembly should be visible within several minutes using VE-DIC optics. In the absence of centrosomes, only random microtubule assembly occurs. In the presence of centrosomes, microtubules first assemble randomly, but over the 10-min nucleation period, the centrosomes acquire pericentriolar material and nucleate more microtubules. By 10 min almost all microtubules are nucleated by centrosomes. Microtubule dynamics do occur and by VE-DIC both growing and shrinking microtubules should be visible.

The typical yield for this centrosome preparation is 30% to 50%. Therefore, a 2-liter preparation of lymphocytes (3×10^9 cells) generally yields $\sim 10^9$ centrosomes with several fractions containing centrosomes at or above the optimal concentration ($\sim 2 \times 10^8/\text{ml}$). A single centrosome preparation generates enough centrosomes for at least 1000 motility assays.

Eggs from one frog (5 ml dejellied eggs) generally yields 1 ml of $16,000 \times g$ cytosol. Each milliliter of cytosol generates ~ 3 ml of HSS. Each assay uses 20 μl of HSS. Therefore, one frog yields enough HSS for ~ 150 assays.

Eggs from one frog (5 ml dejellied eggs) will generally yield $\sim 300 \mu\text{l}$ of cytosol-200 (and

membranes). This will make a ~700- μ l nuclear assembly reaction, which is aliquotted by 10 μ l at the end. Each 10- μ l aliquot contains nuclei for one motility assay. Therefore, eggs from one frog generate nuclei for ~70 motility assays.

Time Considerations

Once all of the support protocols have been followed to generate the components of the motility assay and the learning period has been overcome, then each motility assay takes ~40 min to perform. One to 2 hr of preparation time is required before each assay period. Therefore, only a few experimental samples can be compared on 1 day if assays are performed in triplicate with appropriate buffer controls.

The centrosome preparation requires minimal time for 10 to 14 days to grow the lymphocytes, 1 day to prepare the buffers, and 1 day to do the actual preparation. Titration of the centrosomes in the motility assay requires another full day, but should only be done once all the other components have been prepared.

Preparation of fractionated extracts for nuclear assembly requires 1 full day. Frogs must be primed 4 to 10 days in advance with PMSG and the night before the prep with HCG.

For time considerations in preparing magnetic DNA-beads, see *UNIT 11.13*. Working out the conditions for optimal nuclear assembly using DNA beads and fractionated extracts can optimistically be done in an afternoon, but often can take several tries. Actual preparative scale nuclear assembly takes half a day.

Preparation of 16,000 \times g extracts for generation of HSS takes half a day once frogs are primed as above.

Preparation of slide chambers for motility assays takes parts of a day, during which many other activities can be undertaken simultaneously.

Time considerations for familiarization with VE-DIC and optimization for visualization of microtubules are beyond the scope of this protocol. Please refer to *UNITS 4.1 & 13.1* and to Salmon and Tran (1998) and Walker et al. (1988).

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Key References

Reinsch and Gonczy, 1998. See above.

This review describes the different mechanisms that drive microtubule-mediated nuclear motility events in different cell types and different organisms. Nuclear motility following fertilization is particularly emphasized.

Reinsch and Karsenti, 1997. See above.

This work demonstrates that nuclei can translocate along microtubules in Xenopus egg extracts similar to other organelles, and that cytoplasmic dynein cytoplasmic dynein drives the translocation of nuclei along microtubules.

Internet Resources

<http://current-biology.com/supmat/cub/bb7325s1.mov>

<http://current-biology.com/supmat/cub/bb7325s2.mov>

<http://current-biology.com/supmat/cub/bb7325s3.mov>

These movies are supplemental material for Reinsch and Karsenti (1997). They are videos of nuclei moving along microtubule asters as described in the Basic Protocol. The nuclei used in these movies were assembled as described in Support Protocols 3 and 5.

<http://www.indiana.edu/~elegans/>

*This is the webpage for the laboratory of Susan Strome at Indiana University. Movies within this site show pronuclear migration in vivo in the nematode *C. elegans*.*

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Measuring Dynamics of Nuclear Proteins by Photobleaching

Photobleaching techniques offer the possibility of obtaining information on molecular motion and interactions in a specific part of a cell (McNally and Smith, 2002). The three main advantages of photobleaching techniques are fast experimental turn around, good spatial and temporal resolution, and the ability to measure kinetics inside of living cells. The main disadvantage of these techniques is the requirement for fluorescently tagged proteins (in this case by creating a gene fusion with GFP). Alternatively, other genetically engineered fluorescent tags may be used for a fusion with the protein of interest—e.g., cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP). Also, in some cases, addition of this moiety can functionally alter the protein. Thus, prior to proceeding with photobleaching studies, the fusion protein must be rigorously tested to ensure it has the same properties and function as its native counterpart.

Ideally, a fusion protein is tested in a functional *in vivo* assay. The best is a functional complementation assay, for example, in a knock-out cell line or yeast. However, for many mammalian proteins such assays are not readily available. A minimal requirement for testing GFP-fusion proteins is their stability and proper localization in cells. The stability of a GFP-fusion protein can be tested by immunoblot (UNIT 6.2), using either a specific antibody against the endogenous protein of interest or an antibody against GFP. An immunoblot ensures that the protein is of the expected molecular weight and that it is not degraded upon expression. Furthermore, the GFP-fusion protein must also colocalize with its endogenous counterpart. If the immunoblot shows degradation products of the protein chimera or the protein is mislocalized, the GFP-fusion protein is likely not fully functional. In this case, it is worth trying to introduce the GFP tag at the opposite end of the protein molecule. In some cases, the introduction of a longer linker between the protein and GFP is also helpful.

Three major methods of photobleaching microscopy are commonly used: fluorescence recovery after photobleaching (FRAP; see Basic Protocol), fluorescence loss in photobleaching (FLIP; see Alternate Protocol 1), and inverse fluorescence recovery after photobleaching (iFRAP; see Alternate Protocol 2). As summarized in Table 13.5.1, each of these techniques has specific characteristics permitting the determination of distinct particular parameters of protein behavior *in vivo*. In addition, transfection methods are given for introducing GFP-expression vectors into mammalian cells by electroporation (see Support Protocol 1) or by lipofection (see Support Protocol 2). Finally, since quantification of GFP-fusion proteins is critical, a method for the determination of the number of GFP molecules in a single, living cell is also described (see Support Protocol 3).

NOTE: The reader is referred to UNIT 4.5 for a general discussion of confocal microscopy.

Table 13.5.1 Comparison of Photobleaching Methods for Detecting Movement of Nuclear Proteins^a

	Mobility	Diffusion constant	Immobile fraction	Distinct pools	Compartment continuity
FRAP	+++	+++	+++	+	+
FLIP	+++	+	+	+++	+++
iFRAP	–	–	+	+++	–

^a+++ optimal, + suitable, –not suitable.

Contributed by Miroslav Dundr and Tom Misteli
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FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP)

In a FRAP experiment, an intense focused laser beam of the appropriate wavelength bleaches a relatively small region of interest. The laser light irreversibly bleaches the fluorescent signal of molecules in the region of interest and, immediately following bleaching, the recovery of the fluorescence signal in the bleached area is monitored using an attenuated laser beam. The signal in the region of interest is measured and a fluorescence recovery curve $F_{rec}(t)$ (fluorescence intensity as a function of time after photobleaching) is generated (Fig. 13.5.1). Acquisition of a FRAP data set involves three phases: prebleach images, bleach pulse, and postbleach monitoring of fluorescence recovery.

FRAP experiments are most easily performed on any modern confocal microscope (e.g., Zeiss 510 and newer, Leica SP and newer), as these microscopes have integrated FRAP routines. Alternatively, it is possible to custom write macros for many older microscopes that will automatically perform FRAP routines (Ellenberg et al., 1998). This protocol, as well as its alternatives (see Alternate Protocols 1 and 2), are designed for the Zeiss LSM

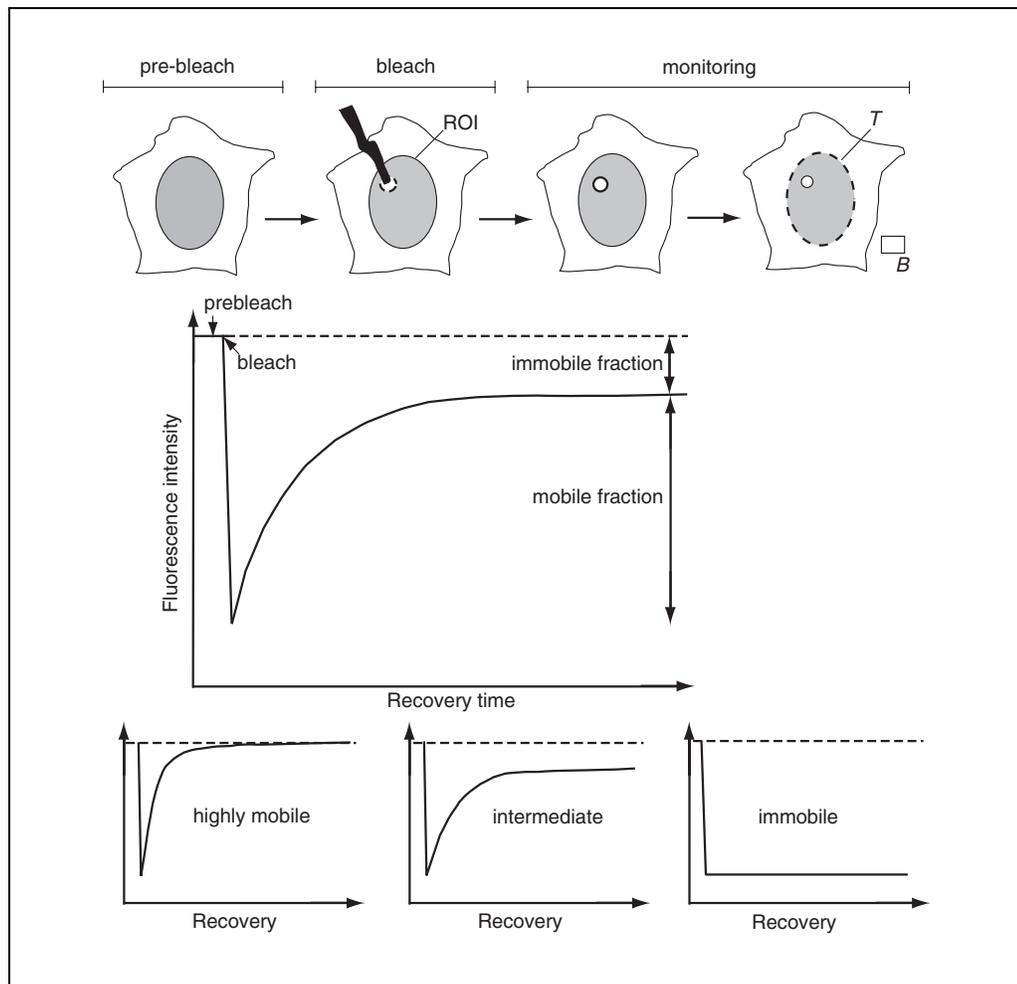


Figure 13.5.1 General scheme of a FRAP experiment. A cell is imaged before a short bleach pulse is applied to a defined region of interest (ROI). The recovery of the average fluorescence signal in the ROI is then monitored. To quantify and normalize FRAP data, the average fluorescence signal in the ROI, average total nuclear signal (T), and average signal in a random area outside of the cell for background correction (B) are measured during the entire experiment. The FRAP curve shows the prebleach intensity, depth of bleach, and recovery of the signal. The immobile fraction is determined as the difference between the prebleach signal and the signal after complete recovery. Based on different profiles of recovery curves, proteins can be considered highly mobile with virtually no immobile fraction, intermediately mobility with an immobile fraction, or immobile.

510 confocal microscope, but similar protocols can be applied to other confocal microscopes.

When FRAP experiments are carried out on confocal microscopes, the fluorescence recovery in the region of interest can be considered largely the result of two-dimensional exchange of fluorescent protein molecules within the same focal plane as the bleached area. This condition is approximately valid when objectives with high-numerical-aperture (N.A. of 1.2-1.45) lenses are used; a large cone of out-of-focus light bleaches the fluorescent molecules above and below the focal plane so that only nonbleached molecules from the focal plane participate in the recovery in the region of interest (Ellenberg et al., 1998).

There are several essential conditions that must be fulfilled for a successful FRAP experiment. (1) The fluorescent signal to be bleached must be clearly detectable over any background signal. (2) The photobleaching must be fast relative to the period of recovery to provide sufficient temporal resolution for analysis of the recovery curve and to allow measurement of the half-time of recovery. (3) The monitoring beam must be of low intensity to minimize photobleaching. To obtain a sufficiently high signal during the recovery period, the sensitivity of the photomultiplier tube may be increased.

Materials

2- or 4-chambered Lab-Tek II coverglass (Nalgene Nunc) containing cells, 50% to 70% confluent

Complete normal growth medium with 25 mM HEPES, but without phenol red
(UNIT 1.2)

Confocal microscope

Air stream incubator (Nevtek)

1. On the stage of a confocal microscope, place a 2- or 4-chambered Lab-Tek II coverglass containing cells in complete normal growth medium with 25 mM HEPES, but without phenol red. Maintain growth temperature with a Nevtek air incubator. Examine the cells with fluorescent light of the appropriate wavelength.
2. Choose the appropriate lens (at least 63 \times magnification) and zoom setting to clearly observe the region of interest.

If the same zoom is used for all observed cells, one can easily compare individual data sets without correction for bleach-area size.

3. Image a cell of interest using a low level of laser power to prevent photobleaching.

On the Zeiss LSM 510 microscope, 20% to 50% output from a 40-mW laser with the beam attenuated to 0.1% is routinely used for the image acquisition.

4. Choose a small pinhole diameter.

The authors recommend using a pinhole diameter in the range of 1 Airy unit (minimal resolvable distance which corresponds to the diameter of the peak of the bright spot of the point source of light; UNIT 4.1). A larger pinhole diameter improves the signal intensity, but increases the optical thickness of the collected section. This could affect monitoring of the signal in the region of interest by collecting data from a thicker optical section including unbleached fluorescent molecules. When a smaller pinhole diameter is used, the monitoring of signal recovery is restricted to the optical section most completely bleached. Therefore, it is advisable to use the smallest pinhole diameter which gives a sufficient signal-to-noise ratio.

Setup imaging sequence

5. Adjust the intensity of fluorescence signal to slightly below the saturation level by adjusting the gain on the photomultiplier tube.

Use at least 8- but preferably 12-bit imaging. It is important to ensure that no pixels are saturated since the change in fluorescence intensity in these pixels cannot be measured accurately.

6. Adjust laser power to 100% for bleaching.

The bleach time should be minimized. The efficiency of the bleach should be tested on a fixed sample. The average intensity in a bleached spot in a fixed sample should be reduced by at least 70% compared to its surroundings. If the bleach depth is insufficient, use several, rapid, consecutive bleach pulses. If the bleach spot in a living cell is distinct from the bleach spot in a fixed cell, do not increase the bleach time or intensity. The discrepancy is an important indication that the observed protein contains a very rapidly moving fraction. These highly mobile molecules enter the bleached spot between the time the bleach pulse is terminated and the acquisition of the first image. Recovery of freely mobile proteins in the nucleus can occur on the time scale of ≤ 50 msec.

7. Adjust the number of prebleach images for five to ten images and acquire five to ten prebleach images.

It is important to acquire more than one prebleach image, as many fluorescent molecules are disproportionately bleached when first excited.

8. Adjust the number of postbleach intervals and the time between images.

The number of intervals is determined by the resolution needed. The length of intervals should cover the curve until it reaches a plateau. Be careful not to collect an unnecessarily high number of images as this will bleach the sample.

9. Define the region to be bleached.

The size of the bleach region is critical for quantitative analysis of FRAP recovery. The authors recommend using a small circular spot as the bleach region over the desired region of interest in the nucleus. The size of the bleach region should be small compared to the size of the nucleus. The advantage of a circular bleach spot is its symmetry, which permits finding it even when the cell moves. To check the dimensions of the bleach region, it is necessary to fix the sample, bleach the region of interest, and collect a series of optical sections through the cell. When the bleached region is monitored for signal recovery, the same size area or slightly smaller can be used for measurement of signal recovery. Using a larger monitoring area allows one to obtain the recovery data even if the bleached area moved slightly during monitoring; however, the relatively unchanged fluorescence intensity in the neighborhood of the bleached region reduces the sensitivity of the measurement.

10. Initiate data collection (i.e., prebleach images, bleach, postbleach images) and monitor the recovery of the fluorescence signal. Check the position of the cell and the focal plane to ensure that the region of interest is not moving from its original position due to focal-plane movement.

It is essential to determine that recovery is complete—i.e., the curve should reach a clear plateau. The shape of a recovery curve should resemble the one in Figure 13.5.1. If the recovery is not complete, it is essential to increase the monitoring time of recovery by increasing the number of acquired images during monitoring or the time between images (step 8).

Perform quantitative analysis of FRAP

11. Measure the average intensity of the region of interest at each time point (I_t).

Most confocal microscope software packages allow the researcher to perform this measurement on a whole stack of time-lapse data.

12. To correct for signal bleaching during monitoring, measure the average intensity of the entire nucleus at each time point (T_t).
13. Measure the background intensity (B_t) in a randomly selected region outside of the cell at each time point.
14. Calculate the relative intensity for each time point:

$$I_{\text{rel}} = (T_0 - B_0) \times (I_t - B_t) / (T_t - B_t) \times (I_0 - B_0)$$

where T_0 is the average intensity of the entire nucleus during prebleach and I_0 is the average intensity of the region of interest during prebleach (step 6).

This formula is derived in three steps. First, background is subtracted from all measured values, then the fluorescence loss due to monitor bleaching is normalized, and finally fluorescence intensity is normalized to one.

15. Plot I_{rel} as function of time.

For convenient calculation, it is useful to import FRAP data sheets from the microscope software to a spread sheet program such as Microsoft Excel.

16. Evaluate the individual curves and determine the average and standard deviations for each time point.

It is advisable to plot all acquired curves before determining the average. Jumps in the recovery curves or declining curves are frequently caused by cell or focal plane movement. These curves can either be discarded from analysis, or if possible, they should be manually remeasured. For many applications, it is sufficient to collect data for 10 to 20 individual cells. Typical error bars are on the order of 5% to 10% of the measured value.

Alternative normalization procedures have been published. For example, some investigators assign the value 1 to the prebleach intensity and the value 0 to the postbleach image in the bleached spot. (Kruhlak et al., 2000). This method is only appropriate for relatively slow-moving molecules where the signal in the bleached region is reduced by $\geq 75\%$. For fast moving molecules where the reduction of signal might only be $\leq 50\%$, this normalization method overestimates the recovery kinetics.

In the case of FRAP analyses of proteins which are enriched in small nuclear compartments such as the nucleolus or dot-like nuclear bodies and present at only low concentrations in the surrounding nucleoplasm, the relative fluorescent intensity in the bleached nuclear compartment can be normalized against the nonbleached nuclear compartment in the same nucleus (Chen and Huang, 2001). For each time point, the relative intensity is determined by the formula $I_{\text{rel}} = (N_e N I_0) / (N_e N I_t)$, where N_e and $N I_t$ are the average intensities of the bleached compartment and control nonbleached compartment at each time point, respectively, and N_e and $N I_0$ are the average intensities of the bleached compartment and control nonbleached compartment in the same nucleus during prebleach, respectively.

Determine the immobile fraction and diffusion constant

17. Calculate the immobile fraction of fluorescent molecules as the difference between the relative fluorescence intensity in the region of interest after the recovery curve has reached plateau, and the prebleach fluorescence signal intensity (normalized as 100%; Fig. 13.5.1).

Failure to recover to 100% prebleach fluorescence signal intensity even for a protein without immobile fraction such as GFP alone may represent the loss of total nuclear fluorescence due to bleaching during monitoring.

18. Determine the diffusion constant.

The mobility of a protein is characterized by its diffusion constant (D). FRAP experiments allow the determination of D assuming lateral diffusion in the focal plane (Axelrod et al.,

1976); however, it is essential to understand that the measured diffusion constant is only an apparent diffusion constant. The overall mobility of a protein is not only determined by its diffusional properties, but more significantly, by its functional properties such as incorporation into multiprotein complexes and binding to relatively immobile structures such as chromatin or a karyoskeleton (Misteli, 2001). Therefore, although a diffusion constant is a convenient method to compare the recovery kinetics of various proteins, one must be cautious in interpreting diffusion constants as a true indicator of the translational mobility of a protein. For discussion of diffusion constants see Lippincott-Schwartz et al. (2001). For determination of diffusion constants using a bleached strip of defined size see Ellenberg et al. (1998) Alternatively, diffusion constants can be determined directly from fitting the experimental data to the theoretical curve by computer simulation (Phair and Misteli, 2000; Houtsmuller and Vermeulen, 2001; Reits and Neeffjes, 2001).

ALTERNATE PROTOCOL 1

FLUORESCENCE LOSS IN PHOTBLEACHING (FLIP)

In FLIP, a region in the nucleus is repeatedly bleached and the loss of fluorescence in a monitoring region of interest some distance away from bleached region is recorded (Fig. 13.5.2). The basis of FLIP is the bleaching of mobile molecules as they pass through a bleached region. If all or a fraction of the fluorescently-tagged molecules in the nucleus are immobile, they will never enter or exit the repeatedly bleached region; therefore, they will not be bleached and cannot distribute the bleached signal elsewhere. As a consequence, the fluorescence signal in the regions surrounding the bleached spot will remain constant. In contrast, if molecules are highly mobile, they will eventually pass through the bleached region. The fluorescence signal in the nucleus will decrease. If all fluorescently-tagged molecules inside of the monitoring region of interest move freely into the bleached region, repeated bleaching will reduce the fluorescent signal in the monitoring region of interest at a constant rate. On the other hand, if multiple, kinetically distinct fractions of molecules exist, the loss curve will contain multiple distinct slopes. FLIP is also a particularly effective technique for testing continuity between nuclear compartments such as nucleoli, splicing factor compartments, Cajal bodies, or PML bodies.

FLIP is complementary to FRAP. Since in FLIP the signal in an unbleached region is measured, the technique is often used to ensure that the mobility of a protein of interest observed in FRAP experiments is not due to photodamage of the protein at the bleached spot.

See Basic Protocol for materials.

1. Set up the cells on the confocal microscope as described (see Basic Protocol, steps 1 and 2).
2. Image two cells of interest close to each other using a low level of laser power to prevent photobleaching.

On the Zeiss LSM 510 microscope, 20% to 50% output from a 40-mW laser with the beam attenuated to 0.1% is routinely used for the image acquisition.

3. Adjust settings as described for FRAP (see Basic Protocol, steps 4 to 9).

A successful FLIP experiment depends on the successful loss of fluorescence in regions outside the bleached region. It is therefore more practical to use bleach areas that are larger in size than the ones used in the FRAP experiments.

4. Initiate data collection (i.e., prebleach images, bleach, postbleach images) and monitor fluorescence loss. Check the position of the cell and the focal plane to be sure that the monitoring spot is not moving from the original position.

The acquisition routine should run in a loop between bleach pulse and single image acquisition separated by the predetermined time interval.

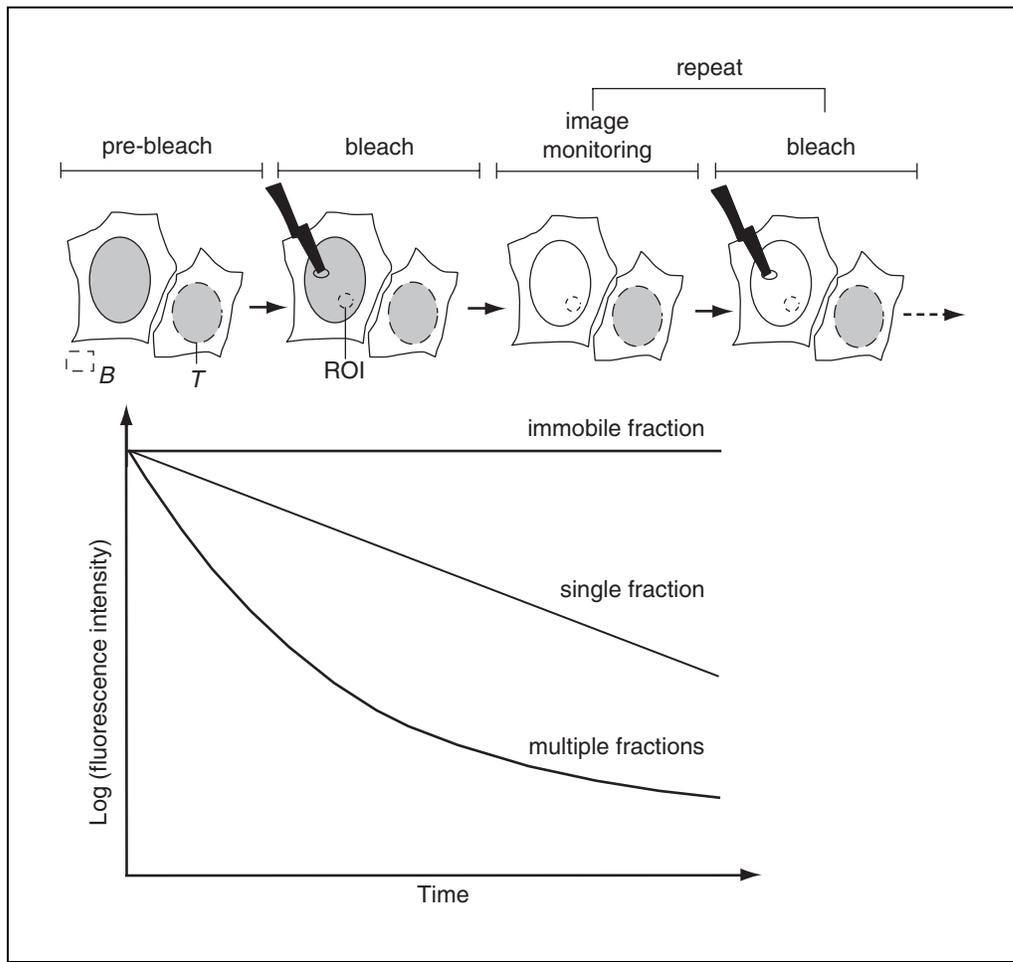


Figure 13.5.2 General scheme of a FLIP experiment. A cell is imaged before a short bleach pulse is applied to a defined bleach region. The image-bleach routine is repeated with the two steps separated by a predetermined time interval. To quantify and normalize FLIP data, measure the average fluorescence signal in a region of interest (ROI) distinct from the bleached region, measure the average total nuclear fluorescence signal (T) in a neighboring cell, and the average signal in a random region outside of the cell for background correction (B), for the duration of the experiment. When the FLIP data are plotted on a semilog scale, the shape of the curve indicates whether the GFP-fusion protein exists in the cell in a single fraction, in multiple fractions or whether it is immobile.

It is essential that the level of fluorescence signal at the end of the experiment be below 15% of the prebleach level. The fluorescence loss curve should resemble the FLIP curve in Figure 13.5.2. If the loss of fluorescence is not below 15%, it is essential to increase the monitoring time. Note that the slope of the loss curve depends on the size, intensity, and duration of the bleach pulse.

Perform quantitative analysis of FLIP

5. Measure the average intensity of the monitoring region of interest as a function of time I_t .
6. Measure the average intensity of the total nuclear area of a neighbor cell at each time point (T_t).
7. Measure the background intensity in a random region outside of the cells at each time point (B_t).
8. For each time point, calculate the relative intensity:

$$I_{\text{rel}} = (I_t - B_t) / [(I_0 - B_0) \times (T_t - B_t)]$$

where I_0 is the average intensity of the region of interest during prebleach.

This formula is derived in three steps. First, background is subtracted from all measured values, then fluorescence loss due to monitor bleaching is normalized, and finally fluorescence intensity is normalized to one.

9. Plot I_{rel} as a function of time and determine average and standard deviations as described for FRAP (see Basic Protocol, steps 16 and 17).

If all fluorescently tagged proteins in the compartment are kinetically identical, the region of interest will lose its fluorescence linearly. If the nuclear compartment contains kinetically distinct pools of proteins (e.g., due to binding of one fraction of the protein to chromatin or incorporation of a fraction into a large complex), the loss of fluorescence will be multiphasic. The number of distinct fractions can be determined by plotting the loss curve as a function of time in a semilog plot. Each kink in the semilog plot corresponds to a kinetically distinct fraction of molecules (Fig. 13.5.2).

As a negative control, fixed cells should be used. No significant loss of signal should be observed outside of the bleached region. The amount of loss that is observed corresponds to bleaching due to imaging.

ALTERNATE PROTOCOL 2

INVERSE FLUORESCENCE RECOVERY AFTER PHOTBLEACHING (iFRAP)

iFRAP is a hybrid method between FRAP and FLIP. In iFRAP, the entire nucleus except a small region of interest is bleached using a single bleach pulse. The loss of fluorescence signal from the unbleached region of interest is then monitored over time (Fig. 13.5.3). Because the number of unbleached molecules in the region of interest is small compared to the number of bleached molecules outside the region of interest, the unbleached molecules do not contribute to new binding events because their pool is proportionally too small in comparison to the large pool of bleached molecules. Thus, iFRAP is ideally suited to provide information about off-rates of proteins from binding sites (e.g., those on chromatin) or from nuclear compartments. iFRAP is particularly well suited for proteins which are enriched in relatively small structures (e.g., replication sites, intranuclear compartments) and for proteins which are bound to a substrate for relatively long periods of time.

See Basic Protocol for materials.

1. Set up cells on the confocal microscope and image as described for FLIP (see Alternate Protocol 1, steps 1 to 2). Adjust the microscope settings as described for FRAP (see Basic Protocol, steps 4 to 6).
2. Adjust laser power to 100% for bleaching.

The bleach time should be minimized and the efficiency of the bleach should be tested on a fixed sample. The average intensity in a bleached region in a fixed sample should be reduced by at least 70% compared to the same region of the neighboring cell. If the bleach depth is insufficient, use several, rapid, consecutive bleach pulses.

3. Adjust the number of prebleach images for five to ten images.
4. Adjust the number of postbleach intervals and the time intervals.

The number of intervals is determined by the resolution needed and the length of intervals should cover the curve until it reaches a plateau. Be careful not to collect an unnecessarily high number of images as this will bleach the sample.

5. Define the region to be bleached excluding the region of interest.

This must be performed quickly as the region of interest can move while the bleached region is being outlined. The bleach has to be as fast as possible to obtain information about rapidly moving molecules in the unbleached region of interest.

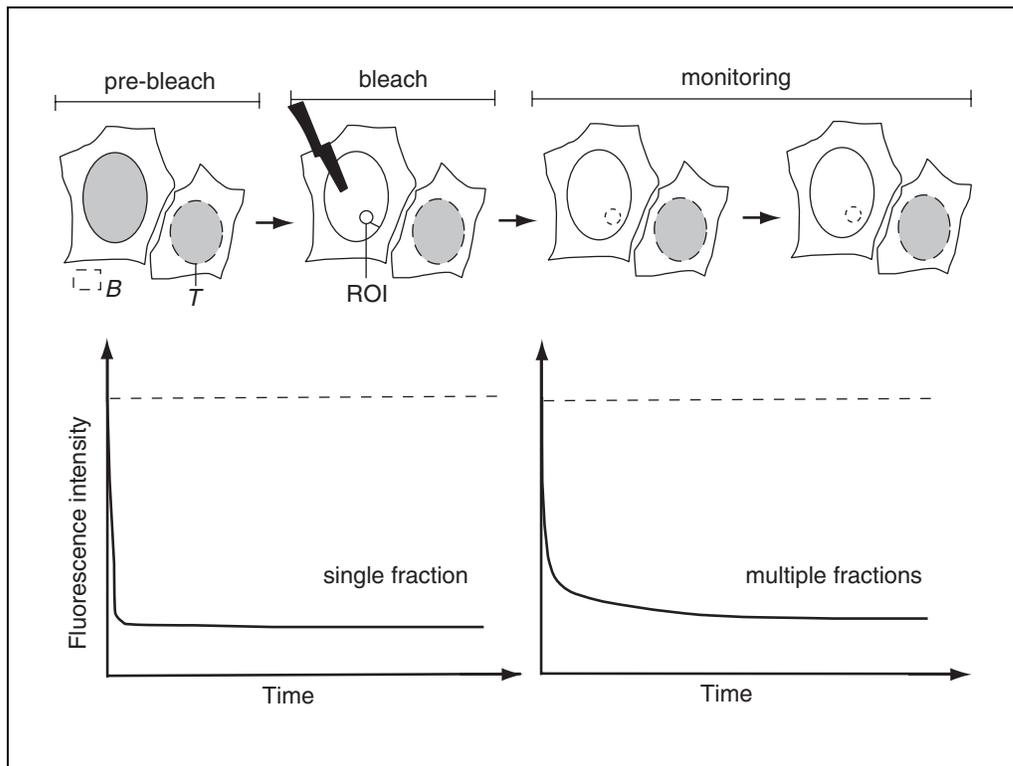


Figure 13.5.3 General scheme of an iFRAP experiment. A cell is imaged before a short bleach pulse is applied to the whole area of the nucleus except a small region of interest (ROI). The loss of fluorescence signal in the ROI is then monitored. To quantify and normalize iFRAP data, measure the average fluorescence signal in the ROI, the average total nuclear fluorescence signal (T) in a neighboring cell, and the average signal in random place outside of the cell for background correction (B), for the duration of the experiment. If the GFP-fusion protein in the ROI exists as a single population, the iFRAP curve should resemble the iFRAP curve on the left. If the GFP-fusion protein exists in the region of interest in multiple populations, the iFRAP curve should resemble the curve on right.

- Initiate data collection (i.e., prebleach images, bleach, postbleach images) and monitor fluorescence loss in the region of interest. Check the position of the cell and the focal plane to be sure that the region of interest is not moving from the original position.

The shape of the fluorescence loss curve should resemble the iFRAP curve in Figure 13.5.3. The region of interest has to be monitored until the loss of fluorescence signal reaches a plateau.

Perform quantitative analysis of iFRAP

- Measure the average intensity of the region of interest at each time point (I_t).
- To correct for the photobleaching effect due to monitoring, measure the average intensity of the entire nucleus of a neighboring cell at each time point (T_t).
- Measure the background intensity in a randomly selected region outside of the cell at each time point (B_t).
- Calculate the relative intensity (I_{rel}) each time point:

$$I_{rel} = \frac{(I_t - B_t) \times (T_0 - B_0)}{(I_0 - B_0) \times (T_t - B_t)}$$

where I_0 is the average intensity of the region of interest during prebleach (step 1) and T_0 is the average intensity of the total nuclear area of a neighbor cell during prebleach.

This formula is derived in three steps: first background is subtracted from all measured values, then fluorescence loss due to monitor bleaching is normalized, and finally fluorescence intensity is normalized to one.

11. Plot I_{rel} as a function of time and determine average and standard deviations as described for FRAP (see Basic Protocol).

It is advisable to plot all acquired curves before determining the average. Jumps in the recovery curves or declining curves are frequently caused by cell or focal plane movement. These curves can either be discarded for analysis or, if possible, they should be manually remeasured. For many applications it is sufficient to collect data for 10 to 20 individual cells. Typical error bars are on the order of 5% to 10% of the measured value.

If all GFP-fusion proteins in the compartment are fast moving components from the same pool, the fluorescent signal in the region of interest after the bleach will drop dramatically and then reach a plateau immediately (Fig. 13.5.3). If the nuclear compartment contains kinetically distinct pools of proteins (due to binding of one fraction of the protein to chromatin or substrate for longer time or incorporation of a fraction into a large complex), the curve of loss of fluorescence in the region of interest will drop dramatically and then show a linear decline until it finally reaches a plateau (Fig. 13.5.3).

As a control, fixed cells should be used. In this case the loss of fluorescence in the region of interest should show no significant loss of signal after the bleach over the monitoring.

SUPPORT PROTOCOL 1

TRANSIENT TRANSFECTION OF MAMMALIAN CELLS BY ELECTROPORATION

To study the dynamics of nuclear proteins in living cells, the specific protein-GFP chimera needs to be expressed. Ideally, a cell line stably expressing the GFP fusion protein from an integrated transgene is generated. The use of stable cell lines and inducible promoters such as the tetracycline- or ecdysone-inducible expression systems (No et al., 1996) are recommended when the overexpression of a fusion protein might be toxic or interfere with essential biological functions (Freundlieb et al., 1998). Detailed protocols for establishing stable cell lines are presented elsewhere (e.g., Stenmark and Zerial, 1998).

A quicker and often sufficient option for expression of GFP-fusion proteins in living cells is the introduction of a GFP-fusion protein by transient transfection encoded on a vector which does not integrate into the cell's genome. These vectors result in short-term, high-level expression of the fusion protein, but they will eventually be eliminated from the cell and expression will cease. Regardless of whether GFP fusion proteins are stably or transiently expressed, a vector containing the GFP-fusion protein cDNA must be introduced into cells by transfection. Two of the most effective and reliable methods of transfection are transfer of DNA via electrical current (presented here) or through one of the commercially available lipid-mediated delivery protocols (see Support Protocol 2). These protocols are for transfection of adherent cells, but both methods can also be used for transfection of suspension cells.

This protocol has been tested for the following adherent cell lines: HeLa, COS, CMT3, NIH 3T3, BHK, and CHO cells. The protocol must be optimized for other cell types. The important parameters for optimal results are the strength of applied electric field, duration of the electrical pulse, number of pulses applied, and concentrations of cells and plasmid DNA.

Materials

Cells

Complete growth medium (e.g., *UNIT 1.2*)

Plasmid DNA

Carrier DNA: sheared salmon sperm DNA

1× PBS (*APPENDIX 2A*)

0.25% (w/v) trypsin/1 mM EDTA, 37°C

Complete growth medium with 25 mM HEPES, but without phenol red

10-cm Petri dish

37°C warming tray

15-ml test tubes

Electroporation cuvettes with 2-mm gap

BTX ECM830 electroporator (BTX, a division of Genetronics, Inc.) or equivalent

Culture dish or incubation chamber (e.g., 2- or 4-chambered Lab-Tek II coverglass; Nalgene Nunc)

Additional reagents and equipment for mammalian cell culture (*UNIT 1.1*)

NOTE: All solutions and materials coming into contact with cells must be sterile, and proper aseptic technique must be used.

NOTE: All cell culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise indicated.

NOTE: Avoid repeated freezing and defrosting of plasmid DNA stock solution. It may cause nicks in the plasmid DNA which leads to linearization of supercoiled DNA. Prepare aliquots of plasmid DNA and store them at –20°C.

1. Prepare an ~70% confluent monolayer of cells in a 10-cm Petri-dish (*UNIT 1.1*) in complete growth medium.
2. Add 3 to 7 µg plasmid DNA to a 1.5-ml test tube. Add sufficient carrier DNA to bring the total amount of DNA to 20 µg. Adjust the volume to 30 µl with water.
3. Wash cells once with 1× PBS.
4. Add enough 0.25% (w/v) trypsin/1 mM EDTA, 37°C to the culture to cover the adhering cell layer. Place the plate on a 37°C warming tray 1 to 2 min.
5. When cells round up, but before they detach from the substratum, add 7 ml complete growth medium. Harvest by collecting the cells and transferring them to a 15-ml centrifuge tube. Pellet cells by centrifuging 2 min at 1000 rpm in a benchtop centrifuge, room temperature.
6. Decant the aqueous layer and resuspend cells in 200 µl fresh complete medium. Pipet the cell suspension into the 1.5-ml test tube containing the prepared DNA solution (step 2). Mix well by pipetting up and down three times, let stand 2 min, and transfer to an electroporation cuvette with 2-mm gap.
7. For HeLa, COS, CMT3, NIH 3T3, BHK, or CHO cells, electroporate on a BTX ECM830 electroporator using the following settings: 150 V, 1-msec pulse, 4 pulses, and 0.5-sec interval.

For BTX electroporator settings for other cell types refer to <http://www.btxonline.com/btx> and the manufacturer's lab manual at http://www.btxonline.com/products/pdfs/ECM_830/ECM_830_Manual.pdf. For electroporators from other manufacturers see the appropriate company's web pages.

**SUPPORT
PROTOCOL 2**

- Using a Pasteur pipet, transfer the cells from the cuvette to a well in a culture dish or to an incubation chamber.

For HeLa, COS, CMT3, NIH 3T3, BHK, or CHO cells, one drop of cell suspension in a 35-mm well gives a confluent layer after overnight incubation. For other cell types, the amount of cells must be optimized.

- Change the medium 8 to 10 hr after transfection.
- Before imaging living cells (see Basic Protocol and Alternate Protocols 1 and 2), replace the medium with complete medium containing 25 mM HEPES, but no phenol red.

Phenol red increases background fluorescence.

TRANSIENT TRANSFECTION OF MAMMALIAN CELLS USING FuGENE 6

FuGENE 6 is a transfection reagent that highly efficiently transfects a wide variety of cells including primary cultures and hard-to-transfect cell lines. The advantage of FuGENE 6 is that it demonstrates virtually no cytotoxicity even with primary cell cultures. The authors have successfully tested several adherent transformed cell lines—HeLa, COS, CMT3, NIH 3T3, CHO—and mouse primary fibroblasts. The FuGENE 6 reagent web page—<http://biochem.roche.com/techserv/fugene.htm>—has a current list of 250 successfully transfected cell lines.

Materials

- Cells
- Serum-containing and serum-free medium (UNIT 1.2)
- FuGENE 6 reagent (Roche)
- Plasmid DNA solution
- Serum-containing medium (UNIT 1.2)
- Serum (optional)
- Glass coverslips or Lab Tek II incubation chambers (Nalgene Nunc)
- Additional reagents and equipment for mammalian cell culture (UNIT 1.1)

NOTE: All solutions and materials coming into contact with cells must be sterile, and proper aseptic technique must be used.

NOTE: All cell culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise indicated.

NOTE: Avoid repeated freezing and defrosting of plasmid DNA stock solution. It may cause nicks in the plasmid DNA which leads to linearization of supercoiled DNA. Prepare aliquots of plasmid DNA and store them at –20°C.

- Prepare ~50% to 70% confluent monolayers on glass coverslips or in Nalgene Lab Tek II incubation chambers (UNIT 1.1) in serum-containing medium.
- In a 1.5-ml sterile test tube, add 97 µl serum-free medium, then add 3 µl FuGENE6 reagent directly into this medium.

The order of addition is critical. The serum-free medium must be aliquoted into the test tube first to avoid adversely affecting transfection efficiency by contact of the undiluted FuGENE with plastic surfaces.

- Add 1 to 2 µg plasmid DNA solution in a volume of 0.5 to 50 µl to the diluted FuGENE 6 reagent.

The total volume of DNA solution has to be in this range.

4. Gently tap the test tube to mix the contents, but do not vortex. Incubate a minimum of 15 min at room temperature.

Continued incubation for up to 45 min will not affect the transfection efficiency in most cell types.

5. Dissolve the mixture into 2 ml serum-containing medium. Add to the cells dropwise, distributing it around the well or incubation chamber. Swirl the wells or incubation chamber to ensure even dispersal.
6. Return the cells to the incubator. After 3 to 8 hr, replace the medium with serum-containing medium or add serum directly to wells. Continue incubating (12 to 20 hr) until imaging is performed (see Basic Protocol and Alternate Protocols 1 and 2).

QUANTIFICATION OF FLUORESCENTLY TAGGED PROTEIN MOLECULES IN NUCLEAR COMPARTMENTS OF SINGLE LIVING CELLS

The number of fluorescently tagged molecules in nuclear compartments in single living cells can be determined using individual fluorescently labeled virus-like particles (VLP) as a standard for the observed signals. VLPs contain a precise number of GFP molecules and the number of GFP molecules in a test volume can therefore be determined by comparing the total fluorescent intensity of VLP and the total fluorescent intensity of a nuclear volume. Since VLPs are small spherical particles, this technique is especially useful for the determination of the GFP content of small spherical cellular compartments and organelles such as Cajal bodies, PML bodies, fibrillar centers within the nucleoli, mitochondria, or vesicles.

VLPs are in vitro-assembled rotavirus particles (Charpilienne et al., 2001). Rotaviruses are large icosahedral particles, which contain three concentric capsid layers. While the outermost layer is composed of the VP4 and VP7 proteins, the intermediate capsid layer is composed of trimers of VP6. The innermost layer contains exactly 120 molecules of VP2. When VP2 fused to GFP is coexpressed with the intermediate capsid protein VP6 in a baculovirus-insect cell system, double-layered icosahedral VLP are completely assembled. Each such VLP measures ~100 nm in diameter and contains exactly 120 VP2-GFP molecules (Charpilienne et al., 2001).

Materials

Cells expressing GFP-tagged protein of interest (see Support Protocols 1 and 2)

Complete growth medium (*UNIT 1.2*)

1× PBS (*APPENDIX 2A*)

GFP-labeled VLP particles (Charpilienne et al., 2001; Dundr et al., 2002)

22 × 22-mm glass coverslips

Confocal microscope with 100× objective

Additional reagents and materials for mammalian cell culture (*UNIT 1.1*)

1. Culture cells expressing the GFP-tagged protein of interest on 22 × 22-mm glass coverslips for 16 to 20 hr (*UNIT 1.1*) in complete growth medium.
2. Wash cells twice with 1× PBS for 5 min each time.
3. Dilute purified GFP-labeled VLP particles to a concentration of 1 to 10 μg/ml in PBS.
4. Place ~30 to 40 μl diluted VLP onto the coverslip (step 1).

It is advisable to perform a pilot experiment in which VLP particles are imaged in solution without cells. This allows for their easy detection later when they are mixed with the cells.

SUPPORT PROTOCOL 3

Organelle Motility

13.5.13

5. Examine cells using a confocal microscope with 100× objective.

The powerful objective is needed because VLPs are difficult to see at low magnifications.

Alternatively, a conventional wide-field microscope fitted with a cooled CCD camera can be used.

6. Adjust the photomultiplier tube intensity such that the fluorescence signal of the GFP fusion protein is slightly below the saturation level.

Use at least 8- but preferably 12-bit imaging. It is important to ensure that no pixels are saturated since the fluorescence intensity in these pixels cannot be measured accurately.

7. Collect a series of 50 single images of the cells expressing the GFP-fusion protein.
8. Using identical settings, collect 50 single images of the VLP-GFP particles in a different field of view in the same specimen.
9. Measure the average area and the average intensity of at least fifty compartments of interest containing the GFP-fusion protein and at least fifty VLPs.
10. Measure the background intensity in a randomly selected area that does not contain cells or VLPs.
11. Subtract the background intensity from the average intensity value of compartments and VLPs.
12. Multiply the average area with the background-corrected average intensity to obtain average fluorescence intensities for the compartment and VLPs.
13. Calculate the number of GFP molecules per compartment:

$$\text{no. GFP molecules/compartment} = (TI_C \times 120)/(TI_{VLP})$$

where TI_C is the average intensity of the compartments and TI_{VLP} is the average intensity of the VLPs.

When using a confocal microscope, not all of the imaged VLP particles will fall within the focal plane and a range of signals will be observed. To determine the average intensity of a single VLP particle, it is helpful to perform three-dimensional reconstruction of VLPs with a distance of 100 nm between optical sections. Collect the maximum number of sections possible and observe them for quantification as a maximum projection. In this case, the total intensities of VLPs should fall within a 10% range of intensities.

COMMENTARY

Background Information

It has been established that the eukaryotic cell nucleus is not a homogeneous organelle but contains several specific membrane-less compartments. Recent observations using photobleaching methods have demonstrated that many nuclear proteins are highly mobile and do not permanently reside in any specific nuclear compartment but are continuously exchanged between the compartment and the surrounding nucleoplasm (Kruhlak et al., 2000; Phair and Misteli, 2000; Chen and Huang, 2001). In this view, nuclear compartments are the consequence of the steady-state dynamic behavior of their components. Nuclear proteins

are not primarily targeted to the nuclear compartments by specific targeting signals but rather retained there as a result of binding or collision with other nuclear components (i.e., protein-protein interactions; DNA-protein interactions: engagement in replication, DNA repair or RNA-protein interactions, and engagement in transcription etc.). The exchange rate of the nuclear proteins is strongly determined by the roles they play in nuclear function.

The localization of proteins within cells has traditionally been studied by indirect immunofluorescence microscopy using fluorescently labeled antibodies. This method is limited by the requirement for chemical fixation

of cells and yields only static snapshots of a protein's distribution. The development of the green fluorescent protein (GFP) as a genetically encoded fluorescent protein reporter has revolutionized the study of protein localization in living cells.

Apart from the use of GFP as a convenient and rapid method to determine where a known protein or one encoded by a newly cloned cDNA is localized, GFP has also been extensively used to visualize proteins in living cells by time-lapse microscopy. These latter approaches allow the study of dynamic aspects of protein function. GFP fusion proteins have been particularly insightful in the study of the cell nucleus. Using time-lapse-microscopy approaches, it has become clear that many nuclear compartments are highly dynamic both in interphase and during mitosis, and that many proteins continuously shuttle between the nucleus and the cytoplasm. (Ellenberg et al., 1997; Misteli et al., 1997; Boudonck et al., 1999; Dunder et al., 2000; Platani et al., 2000; Tsukamoto et al., 2000; Kamath et al., 2001). More recently, nuclear GFP fusion proteins have also been used in combination with photobleaching techniques to study the dynamic organization of nuclear compartments *in vivo*.

Fluorescence photobleaching techniques were originally developed in the 1970s to study the mobility of lipids and proteins in the lipid bilayer of the plasma membrane (Axelrod et al., 1976). They have now become a standard method for studying the dynamics of nuclear proteins in living cells (Ellenberg et al., 1997; Houtsmuller et al., 1999; Kruhlak et al., 2000; Phair and Misteli, 2000; Snaar et al., 2000; Misteli, 2001; Chen and Huang, 2001; Boisvert et al., 2001). The noninvasive nature of the photobleaching approach allows one to label nuclear compartments in living cells with high specificity, but often without functional interference. The GFP-protein marker is particularly useful for photobleaching experiments, because the signal is stable and does not bleach significantly at the low-intensity levels used to monitor bleach recovery. In addition, the bleaching of GFP in living cells can be considered irreversible and does not cause any detectable damage to the cell (but see Verkman, 2002).

What are the advantages of using photobleaching techniques for studying the dynamics of nuclear proteins in living cells? Recent observations using photobleaching techniques have demonstrated that many nuclear proteins are highly mobile and do not perma-

nently reside in any specific domain of the nucleus (Kruhlak et al., 2000; Phair and Misteli, 2000; Chen and Huang, 2001). The various available photobleaching techniques can provide information about the *in vivo* kinetics of the protein of interest and since the kinetic behavior of a protein is often directly related to its functional status, information about a protein's biological role may also be obtained (Phair and Misteli, 2001). Furthermore, photobleaching methods can be used to determine how many different pools of a protein are present in a cell and whether an immobile statically bound fraction exists.

Critical Parameters

Transfection efficiency

Transfection efficiency plays an important role in photobleaching experiments. Very low numbers of transfected cells can affect the evaluation of the pattern of GFP-fusion protein localization and selection of optimal cells for bleaching. In the case of FLIP or iFRAP experiments, high transfection efficiency is essential so that images of two neighboring cells expressing the GFP protein can be obtained, which is required for correction of the signal due to photobleaching. Therefore special attention should be paid to optimization of transfection efficiency.

Cell type

A cell type that tolerates introduction of plasmid DNA should be selected. If there is an option, select preferentially transformed cells over primary cells, which are usually more difficult to optimize for transfection. Cells should be in logarithmic growth when used for transfection.

Quality of plasmid DNA

One critical parameter of good transfection efficiency is the quality of plasmid DNA. It should be pure and the stock DNA solution should not be repeatedly frozen and defrosted. When transfection efficiency is low, a higher concentration of plasmid DNA can be used.

Electroporation

When electroporation is used, three parameters should be varied for optimization: electrical field, duration, and number of pulses. When the electrical field is too high, the survival rate of the cells is lowered. When it is too low, the efficiency of transfection is usually poor. When

a low electric field is used, longer pulses should be applied and vice versa.

Expression levels

For photobleaching experiments, it is advisable to select only cells expressing low or moderate levels of GFP fusion protein. Overexpression of a protein of interest can dramatically affect its localization and may change its behavior as well as influence the overall metabolism of the cell.

Critical elements

Special attention must be paid to the viability of the cells during photobleaching measurements. Cells in a chambered coverglass should not be on the microscope stage for more than 1 hr. Longer exposure of cells can affect cell metabolism and can cause changes in protein mobility. After 1 hr cells should be replaced with another sample or put back in the incubator for at least 30 min.

Controls

Fixed cells should be used as a control for all photobleaching experiments. The fixed sample permits determination of the x , y , and z dimensions of the bleach region and depth of bleach, if the series of optical sections through the sample is collected. The size of the bleach spot should be small relative to the size of the nucleus. Typically a bleach spot 1 μm in diameter works for most applications. Bleaching a region that is larger than the compartment of interest will affect the accuracy of measurement

by mixing the populations of GFP-molecules outside and inside of the compartment. Therefore, it is advisable to adjust the size of compartment relative to the size of the bleach spot by using the appropriate zoom.

Each individual recovery curve should be evaluated. Any dramatic change in the shape of the curve may indicate that the monitoring region of interest changed position or focal plane due to cell movement. These data should be discarded.

Anticipated Results

In a typical transient transfection, a fluorescent signal should be detected 12 to 20 hr after transfection, but weak signals can often be observed as early as 4 to 6 hr. The differences in expression level can vary depending on the purity of DNA, the vector and promoter used, and the cell type used in the study.

The recovery curve of the GFP fusion protein of interest contains information about the degree of mobility of a protein of interest within the nucleus. Generally, qualitative analysis of any photobleaching curves allows one to conclude whether a protein is completely mobile, mobile with a fraction of immobile molecules, or almost completely immobile. In many cases this basic information has significant ramifications for protein function. Note that coincidence of recovery curves for multiple proteins does not necessarily mean that these proteins are found in a complex and move together. Conversely, however, distinct curves of several proteins are generally a good indication that the

Table 13.5.2 Comparison of the Mobility of Nuclear Proteins

GFP-fusion protein	FRAP recovery (sec)	Diffusion coefficient ($\mu\text{m}^2/\text{s}$)	Reference
EGFP	~0.5	27	Swaminathan et al., 1997
580 kDa dextran	7	0.95	Calapez et al., 2002
GFP-ASF/SF2	20	0.24	Phair and Misteli, 2000
GFP-HMG17	30	0.45	Phair and Misteli, 2000
GFP-PABP2	7	0.6	Calapez et al., 2002
GFP-TAP	3	1.2	Calapez et al., 2002
Fibrillarin-GFP	30	0.53	Phair and Misteli, 2000
GFP-UBF1	60	0.14 (nucleolus) 0.57 (nucleoplasm)	Chen and Huang, 2001
GFP-Nucleolin	60	0.14 (nucleolus) 1.15 (nucleoplasm)	Chen and Huang, 2001
TFIIH-GFP	30	5.1	Hoogstraten et al., 2002
ERCC1-GFP/XPF	8	15 (absence of DNA damage) (immobile with DNA damage)	Houtsmuller et al., 1999
	—		

majority of the proteins of interest are not present in a complex *in vivo*.

Examples of recovery times and diffusion coefficients are given in Table 13.5.2.

As a positive control for the evaluation of the mobility of GFP protein of interest, GFP alone can be used as a standard for relatively freely mobile protein. The recovery of the fluorescent signal of the GFP alone is very fast (within 1 sec) and complete recovery with virtually no immobile fraction should be observed. Convenient negative controls are GFP fusions with the core histones (H2A-GFP, H3-GFP, H4-GFP; Kimura and Cook, 2001). In this case, there should be no recovery over the monitoring period. If recovery of fluorescence is observed using either fixed cells or core histone fusion proteins, it should be ensured that the correct excitation wavelength of the laser (488 nm for GFP) is used. Similarly, if no bleaching is observed, the laser settings should be checked.

In FLIP experiments, the kinetic status of specific nuclear compartments is evaluated. Specifically it is tested whether a GFP-fusion protein resides statically or moves rapidly in and out of a compartment. If two or more compartments are in physical continuity, bleaching one will result in loss of fluorescence in the other(s).

In iFRAP experiments, loss of fluorescence in region of interest reflects predominantly how the GFP-fusion protein is lost from its binding site.

Most photobleaching experiments should be completely reproducible with a typical error among cells in a population on the order of 5% to 10% of measured values. To achieve this goal, each acquired curve has to be evaluated independently, and every curve with jumps due to cell or focal plane movement should be discarded before final averaging. Since these are single-cell experiments, variations in cell populations might be observed as relatively large errors in a population measurement. For many proteins the position of the cell in the cell cycle can affect their behavior and mobility. If large fluctuation in measurements is observed, it is recommended to use cell-cycle-synchronized cells.

Special care must be applied to minimize photobleaching due to imaging during monitoring. The number of images collected during monitoring should be a compromise between the resolution of measurements needed and the length of the interval. The decline of the signal intensity due to photobleaching during monitoring should generally not exceed ~5% to 10% of the prebleach value. If bleaching is a prob-

lem, the laser power or the interval between images can be reduced.

GFP levels can be quantified with VLPs by comparing total fluorescent intensities of VLPs with a defined number of GFP molecules and fluorescently labeled specific small nuclear compartments of interest (see Support Protocol 3). This measurement provides the average number of GFP-fusion protein molecules in the compartments in single living cells. Since in 12-bit imaging, 4096 gray levels can be distinguished and the typical background intensity is ~100 to 200 units, the observable ratio of VLP to compartment signal is ~20-fold. Given that each virus particle contains 120 GFP molecules, only compartments which contain ~2500 molecules of interest can be measured using this method. The level of GFP molecules found in many nuclear compartments is well within this range.

Time Considerations

A transient transfection can be done in 30 to 45 min and the cells can be used for microscopy 12 to 20 hr after transfection. The time for a FRAP and iFRAP experiment depends on the mobility of the GFP fusion-protein, but is typically on the order of seconds to <10 min. A FLIP experiment takes on the order of minutes up to 1 hr. Each GFP fusion protein should be tested on 15 to 20 cells. The normalization of one sheet of FRAP data using Microsoft Excel takes several minutes. Electroporation takes ~20 min and the transfection procedure using FuGENE 6 takes 25 min to 1 hr. Acquiring a sufficient number of images of cells expressing protein-GFP and VLPs requires 0.5 to 1.0 hr. Measuring the average area and the average intensity of at least fifty compartments of interest and fifty VLPs using microscope or Metamorph software requires ~2 to 3 hr.

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CHAPTER 14

Signal Transduction: Protein Phosphorylation

INTRODUCTION

Cells use selective phosphorylation of proteins to regulate a vast number of intracellular processes. Enzymes termed protein kinases couple phosphate groups to tyrosine, serine, or threonine residues in specific amino acid sequence motifs of target proteins. The process can be reversed by protein phosphatases. Specificity is determined by the ability of each of the many different types of protein kinases and phosphatases to recognize specific motifs and proteins. By such site-specific regulation of phosphorylation, which affects 10% or more of all proteins, cells can switch on or modulate many major signaling and metabolic pathways, as well as regulate cell behavior in biological events such as migration and embryonic development. The importance of phosphorylation to cell biological regulation is underscored by the fact that cells have over a thousand different protein kinases.

One class of protein phosphorylations regulates enzymes, and the addition or removal of a key phosphate activates or suppresses activity of the enzyme. Other protein phosphorylations enable a protein to bind to another to form a complex—e.g., via the binding of an SH2 domain to a specific tyrosine-phosphorylated site in a target protein. Another general cellular strategy is to activate cascades of protein phosphorylation in signal-transduction pathways. For example, complex linear and interconnecting pathways of sequential phosphorylation of proteins leading to the various types of MAP kinases are important regulators of cell growth, differentiation, and gene expression. A current overview of this large field of protein phosphorylation is presented in *UNIT 14.1*, and a more specific review of MAP kinase pathways is presented in *UNIT 14.3*; both provide a number of relevant literature references.

Although phosphorylation has classically been characterized by the incorporation of ^{32}P using radioactive inorganic phosphate, a recent methodological breakthrough of particular value to cell biologists involves powerful nonradioactive approaches to the study of protein phosphorylation. Studies of complex signaling pathways in cells and tissues are now possible even for nonexperts by using immunoblotting and immunofluorescence or immunohistochemical methods. These new approaches are based on specific antibodies that recognize a phosphate group on one or more amino acids selectively—e.g., phosphotyrosine residues on any protein, or the presence of a certain type of phosphate linkage on a specific protein such as an activated MAP kinase. A wide selection of these immunological tools is becoming available commercially. *UNIT 14.2* provides methods for rapid direct characterization of phosphorylated proteins using a specific antibody. If antibodies of sufficiently high specificity with respect to a single protein are not available, this unit also provides a more indirect approach using immunoprecipitation by antibodies against the protein of interest, followed by anti-phosphotyrosine immunodetection. It also describes methods for antibody localization of key phosphorylated regulatory molecules. This approach permits an investigator to follow the expression patterns or intracellular movements of key phosphorylated proteins in cells, or even in various tissues of intact organisms.

MAP kinase signaling is central to many critical cell biological regulatory events, and *UNIT 14.3* provides methods for quantitative characterization of this important signaling

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Signal
Transduction:
Protein
Phosphorylation

14.0.1

Supplement 4

process. Because of the daunting complexity of signaling via MAP kinases, specific antibodies are needed to isolate each phosphoprotein, and phosphorylation is measured by determining amounts of incorporated ^{32}P . Because novel kinases may be involved in a specific cell biological regulatory event, *UNIT 14.3* also presents methods for detecting unknown kinases using an in-gel kinase assay. A test substrate is incorporated into an SDS-polyacrylamide gel, and electrophoretically separated crude protein extracts are evaluated for enzyme bands with ability to produce phosphorylation in vitro.

Although the recent proliferation of immunological methods for detecting specific types of protein phosphorylation has made the analysis of phosphorylation much easier, the "gold standard" for characterizing phosphorylation of individual proteins continues to be radioactive labeling with ^{32}P followed by biochemical analysis of the radiolabeled phosphoproteins. *UNIT 14.4* provides cell culture and biochemical protocols for incorporating ^{32}P -labeled inorganic phosphate and for characterizing radiolabeled phosphoproteins. *UNIT 14.5* presents current methods for unambiguously identifying the specific phosphorylated amino acids in individual phosphoproteins. Appropriate combinations of radioactive and immunological approaches should permit full characterization of the protein phosphorylation cascades and pathways that regulate many important cell biological functions.

Kenneth M. Yamada

HISTORY

Phosphorylation is the most common and important mechanism of acute and reversible regulation of protein function. Studies of mammalian cells metabolically labeled with [³²P]orthophosphate suggest that as many as one-third of all cellular proteins are covalently modified by protein phosphorylation. Protein phosphorylation has an important role in essentially all aspects of cell biology. Most polypeptide growth factors (platelet-derived growth factor and epidermal growth factor are among the best studied; Heldin, 1995) and cytokines (e.g., interleukin 2, colony stimulating factor 1, and γ -interferon; Ihle et al., 1994) stimulate phosphorylation upon binding to their receptors. Induced phosphorylation in turn activates cytoplasmic protein kinases, such as Raf, the activators of the mitogen-activated protein (MAP) kinases SEK and MEK, the MAP kinases ERK, JNK, and p38 (Marshall, 1995; see also UNIT 14.3), the Janus/JAK kinases (Ihle et al., 1994), the p21 activated kinases (PAKs) (Lim et al., 1996), and the phosphatidylinositol 3'-kinase-activated kinase, protein kinase B/Akt (Alessi and Cohen, 1998). Additionally, in all nucleated organisms, cell cycle progression is regulated at both the G1/S and the G2/M transitions by cyclin-dependent protein kinases (Doree and Galas, 1994; also see Chapter 8). These kinases regulate the G1/S transition by the phosphorylation of cell cycle regulators such as Rb protein (Reed, 1997) and the G2/M transition through the phosphorylation of nuclear lamins (Peter et al., 1990) and histones (Arion et al., 1988).

Differentiation and development are also controlled by phosphorylation. Development of the R7 cell in the *Drosophila* retina (Simon, 1994) and of the vulva in *Caenorhabditis elegans* (Eisenmann and Kim, 1994) are both dependent on the function of receptor and cytoplasmic protein kinases, such as the sevenless and LET-23 receptors and the RAK kinase (Perrimon et al., 1995). Additionally, adhesion is regulated by the FAK kinase (Hanks and Polte, 1997) and motility is regulated, in part, by Met, the hepatocyte growth factor/scatter factor receptor (Furlong, 1992). Finally, metabolism—in particular, the interconversion of glucose and glycogen and the transport of glucose—is regulated by phosphorylation (Cohen, 1985). Cell biologists of all stripes therefore

find, often unexpectedly and occasionally reluctantly, that they must study protein phosphorylation in order to understand the regulation and function of their favorite gene and its product.

LABELING STUDIES

Protein phosphorylation is usually studied by biosynthetic labeling with ³²P-labeled inorganic phosphate (³²P_i). This is intrinsically quite simple—the label is just added to growth medium. It is this step of an experiment, however, that makes many investigators the most nervous, given the perceived danger of radioactive exposure and the real danger of contamination of laboratory equipment with radioactivity. Neither problem is insurmountable. With proper shielding and technique, exposure of the investigator can be limited to the hands and contamination of the laboratory can be avoided. A general protocol for biosynthetic labeling with ³²P_i that maximizes incorporation and minimizes radioactive exposure of workers in the lab and contamination of lab equipment will be provided. (For a general discussion of radiation safety consult Safe Use of Radioisotopes, APPENDIX 1D.)

SITES OF PHOSPHORYLATION

Most proteins are found to be phosphorylated at serine or threonine residues, and many proteins involved in signal transduction are also phosphorylated at tyrosine residues. These three hydroxyphosphoamino acids exhibit sufficient chemical stability at acidic pH that they can be recovered after acid hydrolysis and identified in a straightforward manner. Proteins that contain covalently bound phosphate at histidine, cysteine, and aspartic acid residues, either as phosphoenzyme intermediates or as stable modifications, have also been described. Each of these phosphoamino acids is chemically labile and impossible to study with the standard techniques used for the acid-stable phosphoamino acids. Indeed, they are often identified by inference or elimination. A technique for identifying phosphoserine, phosphothreonine, and phosphotyrosine by acid hydrolysis and two-dimensional thin-layer electrophoresis will be presented. Techniques for analyzing acid-labile forms of protein phosphorylation are described in Ringer, 1991; Kamps, 1991; and Duclos et al., 1991.

Phosphotyrosine is not an abundant phosphoamino acid. Its detection in samples labeled with $^{32}\text{P}_i$ is often difficult, therefore, especially if the samples contain large quantities of proteins phosphorylated at serine residues or if they are contaminated with RNA. Detection of phosphotyrosine, as well as of phosphothreonine, can be enhanced considerably by incubation of gel-fractionated samples in alkali. This hydrolyzes RNA and dephosphorylates phosphoserine, allowing visualization of minor tyrosine- and threonine-phosphorylated proteins. A simple procedure for alkaline treatment will be described.

DETECTION OF UNLABELED PHOSPHOAMINO ACIDS

If a protein is modified by phosphorylation, identification of the phosphoamino acid can often be accomplished without resorting to biosynthetic labeling. For example, tyrosine phosphorylation can be studied because proteins containing this rare phosphoamino acid can be detected with great specificity and sensitivity by antibodies to phosphotyrosine (Kamps and Sefton, 1988). Attempts to generate antibodies that recognize phosphoserine or phosphothreonine have failed to produce reagents with the required specificity and/or sufficient sensitivity to be useful. Once the primary sequence around a phosphorylation site containing phosphoserine or phosphothreonine has been determined, however, it is possible to make antibodies against synthetic phosphopeptides modeled on these phosphorylation sites (Czernik et al., 1991). Such anti-phosphopeptide antibodies have been very useful tools for monitoring phosphorylation of the parent protein at specific sites (also see *UNIT 14.2*).

More generally, because phosphorylation often alters the mobility of a protein during SDS-polyacrylamide gel electrophoresis and almost always alters its isoelectric point, the presence of phosphorylated residues in an unlabeled protein can be deduced from altered gel mobility after incubation of the protein with a phosphatase.

PROTEIN KINASES

Most protein kinases exhibit a strict specificity for phosphorylation of either serine/threonine or tyrosine residues. Protein kinases comprising a third group more closely resemble the serine/threonine kinases in their primary sequence but phosphorylate both serine/threonine and tyrosine residues. MEK, a MAP kinase activator (Crews and Erickson,

1992), and *wee1*, an inhibitor of *cdc2* kinase (Parker et al., 1992), are examples of such dual-specificity protein kinases that phosphorylate threonine and tyrosine residues that are closely located in substrate proteins.

PROTEIN PHOSPHATASES

Many protein phosphatases also show a strict specificity for either phosphoserine/phosphothreonine or phosphotyrosine residues. Unlike kinases, however, the serine/threonine and tyrosine phosphatases are not evolutionarily related and exhibit no primary sequence homology (Shenolikar and Nairn, 1991; Charbonneau and Tonks, 1992). Acid and alkali phosphatases can also dephosphorylate phosphoproteins in vitro but share no structural homology with protein phosphatases. A new family of phosphatases, such as *cdc25*, *CL100*, and *VH-1*, are distantly related to the tyrosine phosphatases but dephosphorylate both phosphotyrosine and phosphothreonine in their target substrates (Fauman and Saper, 1996).

A number of potent phosphatase inhibitors have been identified in recent years. Okadaic acid and several other toxins inhibit protein (serine/threonine) phosphatase 1 and 2A, and vanadate and phenylarsenoxide inhibit tyrosine phosphatase. These compounds have implicated reversible phosphorylation as a regulatory mechanism in many physiological processes (Cohen, 1989; Hardie et al., 1991; Shenolikar and Nairn, 1991; Shenolikar, 1994); under certain conditions, they may be the dominant regulators of these cellular processes. These phosphatase inhibitors can also be used to distinguish protein dephosphorylation from proteolysis in crude tissue extracts (Cohen, 1991). By far the most important contribution of these reagents has been that they have allowed assessment of the role played by phosphorylation in cellular processes where neither the identity of the phosphoprotein involved nor that of the kinase(s) that regulates its function are known.

The units in this chapter will describe techniques that detect protein phosphorylation and identify amino acids that have been covalently modified.

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Incorporation of phosphates into serine, threonine, and tyrosine acceptors in proteins is a common mechanism for regulating protein function. This unit presents protocols that use specific anti-phosphoamino acid (PAA) and anti-phosphoprotein antibodies to detect protein phosphorylation and protein kinase activity. The first protocol describes the use of protein blots (immunoblotting; see Basic Protocol 1) to detect protein phosphorylation using either anti-PAA or anti-phosphoprotein antibodies. This is a convenient method that usually yields impressive results. Phosphorylation can also be detected by immunoprecipitation followed by immunoblot analysis (see Basic Protocol 2) or by immunofluorescent staining (see Basic Protocol 3); these methods are typically more complicated and time consuming. All three methods have been successfully used to detect protein phosphorylation with a wide variety of antibodies and most phosphorylated proteins.

IMMUNODETECTION OF PROTEIN PHOSPHORYLATION BY IMMUNOBLOTTING

**BASIC
PROTOCOL 1**

This method describes the immunodetection of phosphoproteins using detergent extraction of proteins followed by immunoblot analysis (also see *UNIT 6.2*). Phosphorylation of mitogen-activated protein kinase (MAP kinase, MAPK) is used here as an example; however, this immunodetection protocol can be used with most specific anti-PAA or anti-phosphoprotein antibodies. This protocol minimizes the time phosphorylated proteins are exposed to phosphatases, allowing reliable and quantitative detection of the phosphorylated proteins, and can be completed within 7 to 10 hr. This protocol describes EGF stimulation of Rat1 cells, but with minor changes can be used with almost all tissue culture cell lines, homogenized animal organs, and even whole lower organisms.

Materials

- Rat1 cells
- Dulbecco's modified Eagle medium containing 10% heat-inactivated fetal bovine serum (DMEM/10% FBS; *APPENDIX 2A*)
- Starvation medium: DMEM/0.1% FBS
- Epidermal growth factor (EGF) buffer: phosphate-buffered saline (PBS)/0.5 mg/ml bovine serum albumin (BSA, crystalline)
- 50 µg/ml EGF in EGF buffer
- PBS (*APPENDIX 2A*), ice cold
- Homogenization buffer (see recipe), ice cold
- Lysis buffer: homogenization buffer/1% Triton X-100, ice cold
- Kinase buffer (see recipe), ice cold
- Coomassie protein assay reagent (Pierce)
- Protein standards: 5, 10, 20, 50, 100, and 200 µg/ml BSA in homogenization buffer/0.03% Triton X-100
- 4× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (see recipe)
- Prestained protein markers (16 to 200 kDa)
- 12% SDS-polyacrylamide gel (12% polyacrylamide/0.32% bisacrylamide; *UNIT 6.1*)
- Transfer buffer: 50 mM Tris·Cl (pH ~8.8)/50 mM glycine
- Tris-buffered saline/Tween 20 (TBST; see recipe)
- Blocking solution: TBST/2% BSA
- Primary antibodies: monoclonal anti-active MAP kinase *and* polyclonal anti-general MAP kinase
- Secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-rabbit *and* alkaline phosphatase (AP)-conjugated goat anti-mouse

**Signal
Transduction:
Protein
Phosphorylation**

AP detection system (e.g., Promega)
Enhanced chemiluminescence (ECL) detection system (see recipe)
6-cm tissue culture plates
1.5-ml microcentrifuge tubes, prechilled to 4°C (four sets of six, each set labeled 1 to 6)
Stopwatch
1-ml pipet tips, prechilled to 4°C
Microcentrifuge, 4°C
96-well flat-bottomed microtiter plate
Microtiter plate reader, 595-nm wavelength
Nitrocellulose membrane cut to size of gel
Whatman 3MM filter paper, two pieces, cut to size of gel
Transfer apparatus for electroblots (e.g., Bio-Rad)
Flat container for washing nitrocellulose membrane
Additional reagents and equipment for tissue culture (UNIT 1.1), chromogenic and luminescent visualization (UNIT 6.2), and SDS-PAGE (UNIT 6.1)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All tissue culture incubations should be performed in a humidified 37°C, 5% to 10% CO₂ incubator unless otherwise specified.

Prepare cellular extracts

1. Grow six 6-cm tissue culture plates of Rat1 cells in DMEM/10% FBS to subconfluency ($\sim 0.5 \times 10^6$ cells/plate).
2. Remove culture medium, add 2 ml starvation medium to each plate, and culture a further 18 hr.

Make sure that the plates remain flat and that the medium covers the entire plate. Serum starvation makes the cells quiescent, which can be achieved under these conditions within 14 to 24 hr. Starvation for too long or any change in temperature or pH may be stressful to the cells and may induce activation of one or more signaling pathways.

3. Add 2.5 μ l of 50 μ g/ml EGF to three plates (stimulated) and 2.5 μ l EGF buffer to three plates (control). Return the plates to the incubator for 5, 15, and 45 min.

Add EGF or EGF buffer first to the plates with the longest incubation, then at appropriate intervals to the plates with the next longest incubation. It is useful to make and use a time chart to ensure that stimuli will be given at the appropriate times and the cells harvested within a short period of time (5 to 10 min).

If the influence of the stimulating agent on the particular cells being used is not yet known, a positive control should be included—for example, peroxovanadate (0.1 mM vanadate/0.2 mM hydrogen peroxide), which nonspecifically activates many signaling events in most tissue culture cells.

4. After 5, 15, or 45 min, remove medium from plates. Rinse plates twice with 5 ml ice-cold PBS and once with 5 ml ice-cold homogenization buffer.

Since slowing and arresting of biological processes is desired at this stage, the plates should be placed on ice. Washing and harvesting each plate takes 0.5 to 1.5 min; all six plates should be harvested within 5 to 10 min.

5. Add 350 μ l ice-cold lysis buffer to each plate, tilt the plate gently (on ice), and scrape the cells into the buffer using a plastic scraper or rubber policeman. Using prechilled

1-ml pipet tips, transfer the cells and buffer to prelabeled, prechilled 1.5-ml microcentrifuge tubes.

The sample being harvested may become slightly viscous; the 1-ml pipet tips can be cut to make the tip opening wider, allowing easy collection of these samples.

Detergents other than Triton X-100, such as 0.5% Nonidet P-40 (NP-40; Igepal), can also be used.

6. Continue the cell lysis for an additional 10 min on ice.

A nonionic detergent is used for membrane disruption because it extracts proteins from membranous, cytosolic, and some nuclear fractions of the cell, and because the determination of protein concentration in such extracts is relatively easy (steps 9 to 12). Alternatively, a more stringent mix of detergents (RadioImmunoPrecipitation Assay [RIPA] buffer; see Reagents and Solutions), which causes complete extraction of proteins from most cell compartments, can be used for membrane disruption, although it might interfere with the protein determination. Cells can also be disrupted by sonication (two 7-sec, 50-W pulses for a 0.5-ml sample) on ice, which extracts proteins from the cytosolic and nuclear fractions, but not from membranes. The extraction by sonication is performed in homogenization buffer without detergents, and allows easy determination of protein concentration.

7. Microcentrifuge the cellular extracts 15 min at $15,000 \times g$, 4°C . Transfer supernatants to fresh, prechilled, and prelabeled microcentrifuge tubes.

The supernatants contain the protein extracts to be examined for phosphorylation.

8. Transfer 5- to 10- μl aliquots of each extract to labeled microcentrifuge tubes for determination of protein concentration, and store the remainder of the extracts on ice until needed.

The protein concentration is determined at this stage so that identical amounts of proteins from the different samples can be compared and the relative amount of phosphoproteins can be determined accurately. Comparing samples based on cell number, rather than protein concentration, can result in differences of up to 20% in the amount of protein; such large differences can cause even larger ones when phosphorylation is assessed immunologically.

Determine protein concentration

9. Add 145 μl kinase buffer to each 5- μl aliquot of cellular extract.

High concentrations of Triton X-100 can interfere with colorimetric measurements of the Coomassie brilliant blue; therefore, dilutions of $\geq 1:20$ are necessary. Similar or higher dilutions are required for sonicated and RIPA extracts as well, because of high detergent or protein concentrations. The Lowry method of protein determination cannot be used because of the dithiothreitol used to prevent degradative oxidation in the extraction buffer.

10. Transfer 10 μl of each of the protein standards into two wells of a 96-well flat-bottomed microtiter plate.

Protein standards should be prepared in the same buffer as was used for cell extraction.

11. Transfer 10 μl of each of the diluted cellular extracts into two wells of the same microtiter plate. Add 200 μl Coomassie protein assay reagent to all wells.
12. Place the microtiter plate in a microtiter plate reader and measure the absorbance of the standards and samples at 595 nm (A_{595}). Use the absorbance of the standards to construct a standard curve (absorbance versus concentration). Calculate the protein concentrations of the samples by comparing the absorbance of the sample with the standard curve.

Analyze samples by SDS-PAGE

13. Based on the calculated protein concentrations, transfer a volume of each cellular extract containing 40 µg protein to a fresh 1.5-ml microcentrifuge tube.
14. Add $\frac{1}{3}$ vol of 4× SDS-PAGE sample buffer to each tube, mix the contents, and boil 3 min.
15. Load samples and prestained protein markers on 12% SDS-polyacrylamide gel.

Load prestained markers into the first or second lane of the gel so the molecular weights of the detected proteins can be determined. These markers will also indicate whether the proteins were completely transferred from the gel onto the nitrocellulose paper during blotting.
16. Place the gel in an electrophoresis apparatus filled with appropriate buffer and run the gel at 150 V (see UNIT 6.1).

Immunoblot protein

17. Soak nitrocellulose membrane in transfer buffer until completely wet.
18. Once the dye front has reached the end of the gel, remove the gel from the electrophoresis apparatus, cut off the stacking (upper) gel, and carefully place the gel in a flat container with transfer buffer.
19. Fill transfer apparatus with transfer buffer. Open the inner transfer apparatus and remove air bubbles from the pads. Make a sandwich of the gel, nitrocellulose membrane, and pads by putting one piece of Whatman 3MM filter paper (wetted with transfer buffer) on the wet pad, the gel on top of the filter paper, the wet nitrocellulose membrane on top of the gel, and the second piece of wet Whatman 3MM paper on top of the nitrocellulose membrane (Fig. 6.2.1).
20. Remove any air bubbles from between the different layers of the transfer sandwich by gently rolling a 10-ml pipet over the sandwich. Place the other wet pad on top of the transfer sandwich.

Make sure air bubbles are not trapped between the gel and the other components.

21. Place the transfer sandwich containing the gel and nitrocellulose membrane into the buffer-filled transfer apparatus, with the nitrocellulose membrane facing the side with the cathode and the gel facing the side with the anode. Connect the apparatus to a power supply and start the current (200-mA constant current, preferably with a cooling device). Run for 2 hr.

The voltage will drop as the transfer progresses and the conductivity increases.

To shorten the time and improve the yield of transfer, methanol or 0.05% SDS is sometimes included in the transfer buffer; their inclusion will allow higher current but will necessitate the use of a cooling device.

22. At the end of the transfer period, turn off the power supply and remove the nitrocellulose membrane from the transfer sandwich. Rinse the nitrocellulose membrane with transfer buffer to remove any adhering pieces of gel and place the membrane in a flat container.

At this stage, the efficiency of protein transfer can be visually monitored by assessing the transfer of prestained protein markers from the gel to the nitrocellulose membrane. The total amount of protein transferred can also be detected by staining the nitrocellulose membrane with Ponceau S (Salinovich and Montelaro, 1986). The total amount of unphosphorylated protein is determined by staining with general antibodies as described later; however, and staining with Ponceau S is not essential.

23. Place nitrocellulose membrane into flat container and incubate in 30 to 50 ml blocking solution for 60 min at room temperature.

This ensures that any free nonspecific protein-binding sites on the membrane are blocked, and will not nonspecifically bind the antibodies used in this protocol.

Incubate nitrocellulose membrane with antibody

24. Dilute primary antibody (e.g., monoclonal anti-active MAP kinase antibody) in TBST according to manufacturer's instructions. Incubate nitrocellulose membrane in a flat container with 15 ml primary antibody overnight at 4°C, 30 min at 37°C, or 1 to 2 hr at room temperature.

Although anti-MAP kinase antibodies are used as an example, other anti-phosphoprotein or anti-PAA antibodies (from, e.g., Transduction Laboratories, Sigma, Santa Cruz Biotechnology, Upstate Biotechnology, New England BioLabs, or Zymed Laboratories) can be used as well, in combination with antibodies directed to the same nonphosphorylated proteins.

25. Wash nitrocellulose membrane with TBST in flat container at least three times, 15 min each time, at room temperature.

26. Dilute secondary antibody in TBST according to manufacturer's instructions. Incubate nitrocellulose membrane with secondary antibody for 45 min at room temperature.

For detection of anti-active MAP kinase antibody; the AP detection system is recommended, because it has a broader linear range than does ECL (see Commentary). Alternatively, HRP-conjugated antibodies can be used as secondary antibodies and can be detected by ECL (see below). If HRP- rather than AP-conjugated antibodies are used at this stage, then AP-conjugated antibodies should be used in step 30.

27. Wash nitrocellulose membrane with TBST at least three times, 10 min each time, at room temperature.

28. Use an AP detection protocol (UNIT 6.2) to detect active MAP kinase.

After detecting active (phosphorylated) MAP kinase, determine whether there is an equal amount of MAP kinase in all lanes by exposing the same nitrocellulose membrane to anti-general MAP kinase antibody and redeveloping it (steps 29 to 31).

29. Incubate the stained nitrocellulose membrane in blocking solution for 30 min at room temperature.

If there is no steric hindrance between the two antibodies, it is not necessary to strip away the antibodies used in steps 24 to 26 because two different types of antibodies are used (mouse and rabbit). To perform the second blotting add the different type of antibody and follow steps 29 to 31. Antibodies from the same species of origin (mouse or rabbit) or antibodies that interfere with each other can be used for both steps, but in that case the membrane must be stripped (UNIT 6.2) before step 29 is performed.

30. Remove blocking solution and incubate nitrocellulose membrane with the new primary antibody (e.g., polyclonal anti-general MAP kinase antibody) as in steps 24 to 25, and use HRP-conjugated goat anti-rabbit antibody as the secondary antibody (step 26).

31. Use an ECL detection protocol (UNIT 6.2) to visualize general MAP kinase staining. Incubate nitrocellulose membrane with ECL solution for 1 min, dry the blot with Whatman 3MM filter paper, wrap the blot in plastic wrap, and expose to X-ray film.

**IMMUNODETECTION OF PROTEIN PHOSPHORYLATION BY
IMMUNOPRECIPITATION FOLLOWED BY IMMUNOBLOTTING**

This protocol, for which reagents are readily available, can be used instead of Basic Protocol 1 when antibodies against phosphorylated sequences (anti-phosphoprotein antibodies) are not available. This protocol involves immunoprecipitation (also see *UNIT 7.2*) using antibodies to the whole protein, followed by immunoblot detection with anti-PAA antibodies, which have been used effectively to detect many tyrosine-phosphorylated adapter molecules in growth factor signaling pathways.

Materials

- Protein A–Sepharose beads
- PBS (*APPENDIX 2A*), room temperature and ice cold
- Antibodies for immunoprecipitation of the desired protein (1 to 5 µg per reaction)
- Homogenization buffer (see recipe), ice cold
- Cellular extract in lysis buffer (see Basic Protocol 1, step 7)
- RIPA buffer (see recipe), ice cold
- 0.5 M LiCl solution, ice cold
- 0.1 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
- 4× SDS-PAGE sample buffer (see recipe)
- 12% SDS-polyacrylamide gel (12% polyacrylamide/0.32% bisacrylamide; *UNIT 6.1*)
- End-over-end rotator
- Microcentrifuge, 4°C
- Additional reagents and equipment for analysis by SDS-PAGE and immunoblotting (see Basic Protocol 1 and *UNIT 6.1*)

Prepare antibody-conjugated protein A beads

1. Place protein A–Sepharose beads (~150 µl) in a 1.5-ml microcentrifuge tube, add 1 ml PBS, and let the beads swell for 10 min at room temperature.

Although protein A–Sepharose is recommended for this method, other commercially available protein A–conjugated resins (e.g., agarose, HiTrap) can be used. Protein G–coupled resins are required to immunoprecipitate certain types of monoclonal antibodies (Table 7.2.1).

Some resins are supplied as ready-to-use solutions and will not require this swelling step.

2. Add 1 ml PBS to swollen beads and microcentrifuge 1 min at 15,000 × g, room temperature. Discard the supernatant. Repeat for a total of three washes.
3. Add 300 µl PBS and 25 µl of the antibody to be conjugated to 250 µl swollen packed beads. Rotate the mixture on an end-over-end rotator for 1 hr at 10 to 30 rounds/min, room temperature to allow antibody to bind to protein A.

These volumes are calculated for ten reactions (usually 10 to 20 µl beads is used per reaction), but because of the density of the beads, this amount will probably only be sufficient for eight reactions. The amounts can be scaled up as long as the proportions are maintained.

For easy handling of the resin, cut the ends of the pipet tips to make the openings larger.

4. Resuspend beads in 1 ml ice-cold PBS, then microcentrifuge 1 min at 15,000 × g, room temperature. Remove supernatant and add 1 ml ice-cold homogenization buffer, then repeat centrifugation two more times with homogenization buffer ending with a final addition of an equal volume of buffer.

Antibody-conjugated beads can be stored in homogenization buffer for ≤2 days at 4°C.

Immunoprecipitate

5. Add 40 μ l antibody-conjugated bead suspension (20 μ l beads and 20 μ l homogenization buffer) to a 300- μ l sample of cellular extract containing 100 to 500 μ g total protein in prechilled 1.5-ml plastic microcentrifuge tubes. Rotate on end-over-end rotator for 2 hr at 10 to 30 rounds/min, 4°C.

See Critical Parameters for the rationale behind this particular method.

6. Microcentrifuge antibody-conjugated beads 1 min at 15,000 \times g, 4°C. Discard supernatant, add 1 ml ice-cold RIPA buffer, and centrifuge again as before. Repeat twice with 1 ml ice-cold 0.5 M LiCl and once with 1 ml ice-cold 0.1 M Tris-Cl, pH 8.0.

These stringent washes will remove most of the phosphorylated proteins that can nonspecifically interact with the protein A beads.

7. Add 30 μ l of 4 \times SDS-PAGE sample buffer to each sample of washed beads, boil 5 min, and centrifuge 1 min at 15,000 \times g, room temperature. Load supernatant and prestained markers on 12% SDS-polyacrylamide gel (see UNIT 6.1).
8. Perform SDS-PAGE and immunoblot analysis (see Basic Protocol 1, steps 16 to 31).

At this stage the immunoprecipitate can also be subjected to a protein kinase assay (see UNIT 14.3).

FLUORESCENT IMMUNOSTAINING OF TISSUE CULTURE CELLS

This protocol uses similar antibody reagents as described in Basic Protocol 1 to allow staining of tissue culture cells, blood cells, thin tissue sections, or lower organisms. It involves fixation of the cells or tissues to be examined, brief permeabilization of the plasma membranes to allow immunological recognition of intracellular components, and fluorescent detection. This method is especially useful to determine localization of signaling events, but is less accurate in determining the level of activations. Recently, Gabay et al. (1997) used this method to identify the MAP kinase pattern of activation in the developing *Drosophila* embryo.

Materials

Rat1 cells
DMEM/10% FBS (APPENDIX 2A)
Starvation medium: DMEM/0.1% FBS
PBS (APPENDIX 2A)
3% (w/v) paraformaldehyde in PBS
Permeabilization buffer: PBS/0.2% (v/v) Triton X-100
Primary antibody
Secondary antibodies: fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit *and* rhodamine-conjugated goat anti-mouse

6-well tissue culture plates
22-mm-square coverslips, sterile
Parafilm, two 10 \times 20-cm pieces
Slide mounting medium (e.g., Polymount, Polyscience)
Confocal or fluorescent microscope

Additional reagents and equipment for tissue culture (UNIT 1.1)

NOTE: All solutions and equipment coming into contact with cells must be aseptic, and proper aseptic technique should be used accordingly.

BASIC PROTOCOL 3

**Signal
Transduction:
Protein
Phosphorylation**

14.2.7

NOTE: All tissue culture incubations should be performed in a humidified 37°C, 5% to 10% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

1. Place one 22-mm-square coverslip in each well of a 6-well tissue culture plate. Seed ~30,000 Rat1 cells in 2 ml DMEM/10% FBS in each well. Incubate 24 hr. Remove medium, replace with starvation medium, and incubate a further 14 hr (see Basic Protocol 1, steps 1 to 2).

Seeding a small number of cells per well allows the cells to be well dispersed as they grow, which usually facilitates good cell staining.

Many cell types other than Rat1 can be used with this protocol.

2. Remove starvation medium and add 2 ml DMEM/10% FBS to each well. Incubate 30 min in incubator.

Serum-induced translocation of MAP kinases into the nucleus is much more pronounced and prolonged than the translocation induced by EGF.

3. Wash wells twice with 5 ml PBS at room temperature. Add 2.5 ml of 3% paraformaldehyde in PBS to each well for 20 min. Remove paraformaldehyde solution and wash three times with 5 ml PBS each time.

Once the samples are fixed by the paraformaldehyde, protein degradation and dephosphorylation are minimized; therefore, the subsequent stages can be performed at room temperature. Leave coverslips in the wells for all washes.

4. Add 2 ml permeabilization buffer to each well for 5 min. Remove permeabilization buffer, add 5 ml PBS to each well, allow to sit for 2 min, then remove. Repeat PBS wash twice.

Other methods of fixation can be used, but paraformaldehyde fixation followed by permeabilization with 0.2% Triton X-100 is a very effective method when antibodies directed against signaling components are used.

5. Stretch one 10 × 20-cm piece of Parafilm on a clean, straight, and stable surface, such as a work bench; fasten it to the surface.

6. Centrifuge primary antibody 5 min at 15,000 × g, room temperature. Dilute primary antibody in PBS as per manufacturer's instructions. Place six 40-μl drops of diluted primary antibody on the stretched piece of Parafilm ~5 cm apart from each other. Remove the fixed and permeabilized coverslips from the wells with fine forceps and place each, cell side down, on top of one of the drops. Incubate 45 min at room temperature.

An antibody dilution of 1:20 to 1:500 is usually used for staining by this method. For costaining with monoclonal and polyclonal antibodies, the 40-μl drops can contain both antibodies. It is important to know which coverslip was exposed to which antibody.

Centrifuging the antibody solution 5 min at 15,000 × g, room temperature, will remove particulate contaminants and antibody aggregates.

7. Remove each coverslip from the drop of primary antibody solution with fine forceps. Holding each coverslip with forceps, tilt it and gently touch its edge to a Kimwipe to remove all antibody solution. Place the coverslips back in the wells of the 6-well plate, cell side up. Wash the wells three times with 2 ml PBS for 10 min each.

8. Centrifuge rhodamine-conjugated secondary antibody 1 min at 15,000 × g, room temperature. Dilute secondary antibody 1:100 to 1:500 in PBS. Prepare a new 10 × 20-cm piece of Parafilm as in step 5 and place 40-μl drops of centrifuged secondary antibody on the Parafilm. Place coverslips on top of the drops, cell side down. Incubate 45 min at room temperature.

The type of secondary antibody will depend on the species in which the primary antibody was generated.

9. Remove coverslips from the drops of antibody and remove any remaining antibody solution with a Kimwipe as in step 7. Place coverslips back in the 6-well plate, cell side up, and wash three times with 2 ml PBS, 10 min each time.
10. Place one drop of mounting medium on each slide. Remove each coverslip from the well with fine forceps and remove remaining PBS by touching its side with a Kimwipe. Place each coverslip on the drop of mounting medium with the cell side facing the mounting medium. Store slides in the dark at least overnight.

Keep stained slides in the dark to minimize decay of the fluorescent dye.

11. Monitor the fluorescent staining by examining the slides with either a normal or confocal fluorescent microscope. Note the amount of staining per cell and the percentage of stained cells per sample.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Enhanced chemiluminescence (ECL) system

Solution A:

2.5 mM luminol (Sigma)
400 mM *p*-coumarin (Sigma)
100 mM Tris·Cl, pH 8.5

(APPENDIX 2A)

Solution B:

5.4 mM H₂O₂
100 mM Tris·Cl, pH 8.5 (*APPENDIX 2A*)

Store solutions A and B up to 3 months at 4°C

Mix equal amounts of solution A and solution B just before use

The ECL system is also commercially available.

Homogenization buffer

50 mM β-glycerophosphate, pH 7.3
1.5 mM EGTA
1.0 mM EDTA
0.1 mM sodium vanadate
1.0 mM benzamidine
10 μg/ml aprotinin
10 μg/ml leupeptin
2.0 μg/ml pepstatin A
1.0 mM DTT (*APPENDIX 2A*)

Store up to 3 months at 4°C without DTT

Add DTT just before use

This buffer is derived from the work of Ahn et al. (1990) and Seger et al. (1994).

Kinase buffer

50 mM β-glycerophosphate, pH 7.3
1.5 mM EGTA
1.0 mM EDTA
0.1 mM sodium vanadate
1.0 mM dithiothreitol (DTT; *APPENDIX 2A*)

Store up to 3 months at 4°C without DTT

Add DTT just before use

This buffer is derived from the work of Ahn et al. (1990).

RadioImmunoPrecipitation Assay (RIPA) buffer

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
137 mM NaCl
10% (v/v) glycerol
0.1% (w/v) SDS (*APPENDIX 2A*)
0.5% (w/v) deoxycholate
1% (v/v) Triton X-100
2.0 mM EDTA
1.0 mM PMSF (*APPENDIX 2A*; add fresh)
20 μM leupeptin
Store up to 3 months at 4°C

SDS-PAGE sample buffer, 4×

200 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
40% (v/v) glycerol
8% (w/v) SDS (*APPENDIX 2A*)
0.2% (w/v) bromphenol blue
8% (v/v) 2-mercaptoethanol
Store up to 12 months at –20°C

TBST (Tris-buffered saline/Tween 20)

20 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
150 mM NaCl
0.05% (v/v) Tween 20
Store up to 3 months at 4°C

COMMENTARY**Background Information**

Protein phosphorylation, the incorporation of phosphates into serine, threonine, and tyrosine acceptors in proteins, is a common means of regulating protein function and hence many cellular processes (Krebs, 1994; Campbell et al., 1995) that has been extensively studied in recent years. More than 30% of cellular proteins are believed to be either constitutively or transiently phosphorylated by ~2000 distinct protein kinases (Hunter, 1994), and the large number of protein kinases and phosphoproteins makes detection of specific phosphorylation sites challenging. Over the years, several methods have been developed to single out and characterize particular phosphoproteins, including the traditional methods of metabolic labeling with inorganic ³²P and in vitro protein phosphorylation (*UNIT 14.1*). Some of these techniques, such as metabolic labeling, phosphorylation by known protein kinases, and purification of phosphorylated proteins, are covered in other units in this chapter.

Another approach is immunological (*UNIT 14.3*); a number of immunological reagents have now been developed that can be used to study many aspects of phosphorylation. Two classes of antibodies have historically been used to

monitor phosphorylation. One class consists of anti-phosphoamino acid (PAA) antibodies, such as anti-phosphotyrosine (PY), -phosphothreonine (PT), and -phosphoserine (PS; see Fig. 14.2.1). The anti-PAA antibodies non-specifically recognize many of the phosphorylated amino acids in most proteins (Fig. 14.2.2). The second class is composed of anti-phosphoprotein antibodies, i.e., antibodies against specific phosphate-containing sequences.

Of the anti-PAA antibodies, the anti-PY antibodies, which can detect membranal signaling in response to extracellular agents, are the most commonly used. Immunoblot analysis with anti-PY antibodies usually detects a dramatic increase in the amount of PY in proteins from stimulated cells, such as epidermal growth factor (EGF)-stimulated A431 cells (Frackelton et al., 1983), and insulin-stimulated Fao hepatoma cells (Pang et al., 1985). A similar growth factor-induced increase in other PAAs is also detected with anti-PT and anti-PS antibodies, but the increases detected with these antibodies are usually less dramatic. Anti-PAA antibodies are, therefore, good probes to detect bulk changes in phosphorylation that accompany signaling processes in a variety of cell types. Changes in the phosphorylation of specific pro-

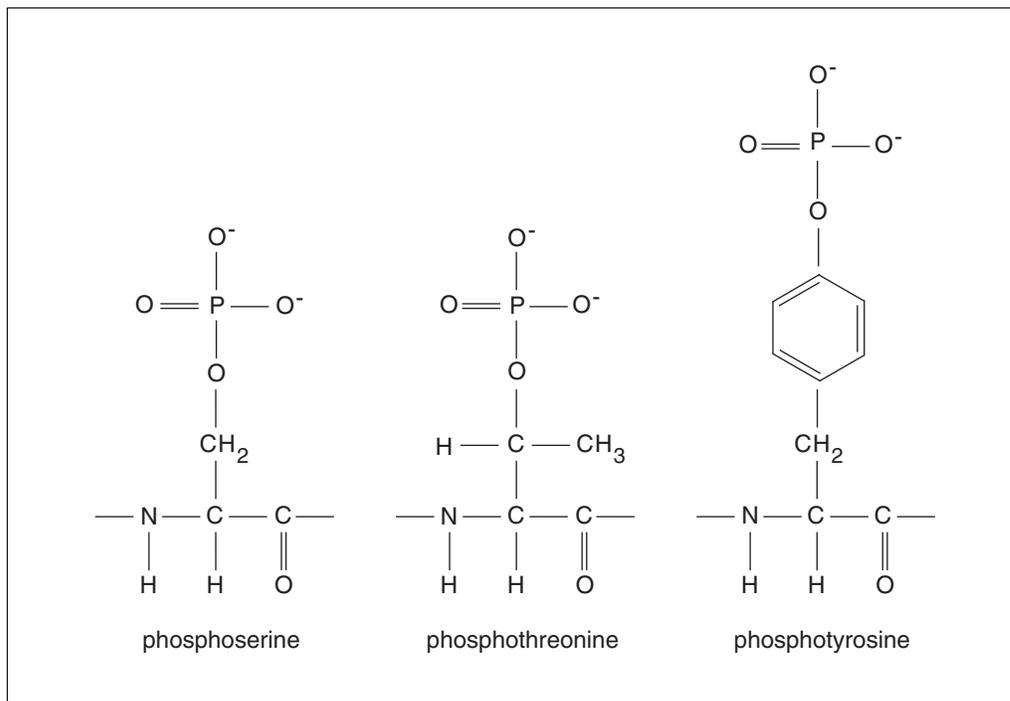


Figure 14.2.1 Schematic representation of phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY).

teins can also be detected by these anti-PAA antibodies if isolated (usually immunoprecipitated) proteins are used (Fig. 14.2.2).

Anti-PAA antibodies are widely used, but they usually do not interact with all the PAAs in a given extract; the anti-PS and anti-PT antibodies appear to recognize only a small percentage of the phosphorylated residues on some proteins. Another disadvantage of using anti-PAA antibodies to monitor phosphorylation is their inability to distinguish between several similar PAAs that may appear simultaneously on a particular protein, which can sometimes preclude detection of activity-modulating site-specific phosphorylation.

Anti-PAA antibodies provide a general and nonspecific tool for monitoring phosphorylation. Anti-phosphoprotein antibodies, those with unique specificity to a certain phosphorylated sequence in a given protein, provide a specific monitoring probe. Probably the best known examples are the anti-active mitogen-activated protein kinase (MAP kinase; Seger and Krebs, 1995) antibodies called anti-diphospho-ERKs (Gabay et al., 1997; Yung et al., 1997), which are now commercially available (Sigma).

In response to mitogen stimulation, the MAP kinases (ERK1 and ERK2) are rapidly phosphorylated at up to six sites (Robbins and Cobb, 1992). Phosphorylation at two of these

sites (Thr 183 and Tyr 185 in ERK2; Payne et al., 1991) can lead to full activation of the enzyme; the effects of phosphorylation at the other sites are not yet known. Antibodies directed towards the activation motif of ERK (PT-glutamic acid-PY) serve as good tools to detect the enzymatic activity of ERK. Anti-PAA antibodies, on the other hand, can detect total phosphorylation, which does not fully correlate with ERK's enzymatic activity.

An important advantage to using anti-phosphoprotein antibodies is that the procedure for their detection is shorter and simpler than that for anti-PAA antibodies, and can be achieved in a single step (immunoblotting or cell staining). Because of the specificity of anti-phosphoprotein antibodies, however, these antibodies do not always provide an overall indication of signaling events, as do anti-PAA antibodies.

Immunological reagents have been extensively used to study the involvement of phosphorylation in growth factor signaling. When growth factors bind to their receptors, the receptor-associated tyrosine kinases are activated to initiate intracellular signaling events (Krebs, 1994). These signals are then transmitted through a series of interacting proteins (Grb2, SOS, and Ras; see UNIT 14.3) that in turn sequentially stimulate the several groups of cytoplasmic protein serine/threonine kinases known as MAP kinase signaling cascades (Seger and

also useful tools for studying cellular signaling. Many of these antibodies are directed against protein kinases that belong to the MAP kinase signaling cascades, and are activated by phosphorylation of distinct amino acids in the kinase domains. For example, antibodies specific for the doubly phosphorylated, activated form of MAP kinases are now extensively used to determine kinase activity, in place of traditional chemical methods (Fig. 14.2.2). As with anti-PY antibodies, anti-MAP kinase antibodies have been used in a variety of methods, including immunoblotting and fluorescence staining of tissue culture cells, tissue sections, and whole tissues. The latter enables the detection of MAP kinase activation in whole or sectioned organisms (see *UNIT 14.3* for more details).

Anti-phosphoprotein antibodies have also been used to efficiently detect the phosphorylation and activation of the transcription factor CREB (Ginty et al., 1993) and the phosphorylation of the brain-specific, microtubule-associated protein Tau (Biernat et al., 1992). The multitude of studies using anti-phosphoprotein antibodies indicate that these reagents are simple and reliable tools to study phosphorylation and that their use will be invaluable for studying a variety of cellular processes that involve phosphorylation and dephosphorylation.

Critical Parameters

The elements most critical to the success of the methods for immunological monitoring of phosphorylation described here are the quality and specificity of the antibodies used. Antibodies should (1) recognize the phosphorylated protein but not its unphosphorylated counterpart and (2) recognize only the desired PAA, not additional phosphorylated sites in either the same or different proteins. In addition, the amount of protein in the different samples subjected to immunoblot analysis and the dilution of antibodies should be optimized to avoid nonspecific recognition of excess proteins.

Successful detection of phosphorylation by immunological or other means also depends in large part on the efficiency of cellular extraction. Since most phosphorylated proteins are localized within cells, cellular membranes must be disrupted to physically access the desired targets. During disruption of these membranes (cellular extraction), most cellular organelles break, exposing phosphoproteins to phosphatases and proteinases (see *UNIT 14.1*), which have detrimental effects on the detection of phosphorylation (Hunter, 1995). The effect of these enzymes can be minimized by adding

specific enzyme inhibitors to extraction buffers and extracting at low temperatures. Phosphatases are usually efficient enzymes, however, and extractions should be performed as fast as possible; if necessary, additional means should be used to secure phosphorylation sites.

Other considerations for the extraction of phosphoproteins are: (1) to ensure that the proteins of interest are indeed presented in the examined fraction and (2) to reduce the number of irrelevant proteins in this fraction. For example, the presence of PY-containing cytosolic proteins might be masked by an excess of activated membranal proteins, and detection of cytoskeletal-associated phosphoproteins might be difficult unless extraction is performed using RadioImmunoPrecipitation Assay (RIPA) buffer. One good cellular extraction method, which can be used for detection of phosphoproteins by either immunoprecipitation or immunoblotting, is described in Basic Protocol 1. In this method, detergent is used to disrupt the cellular membranes, and the resulting extracts should contain membranal, cytosolic, and some nuclear fractions.

Depending on the subcellular localization of the proteins of interest, however, other extraction methods can be used. For example, extracts obtained by sonication usually contain cytosolic and nuclear components, but not membranal ones; those obtained with RIPA buffer should contain most cellular components. Cellular extraction by the addition of hot SDS-PAGE sample buffer to cells is not recommended, because it frees chromatin and results in a gel that is difficult to handle. Extraction by freeze-thawing is also not recommended because molecular degradation can occur during thawing.

Several immunoprecipitation methods have been developed and used over the years (see *UNIT 7.2*). These methods vary in the order in which the antibodies and protein A are added to the cell extracts. In Basic Protocol 2, the antibodies are first conjugated to protein A beads and then added to the cellular extracts. This procedure minimizes the time the samples are incubated with the antibodies, minimizing exposure of the phosphoproteins to phosphatases and proteinases in the extracts. Furthermore, this procedure ensures that only antibodies recognized by protein A will be used for the immunoprecipitation. If the antibodies are added to the lysate prior to precipitation with the beads, antibodies that are not recognized by protein A can bind to the desired antigen, but will not be precipitated when pro-

tein A beads are added, reducing the efficiency of the immunoprecipitation.

The successful use of sequence-specific anti-phosphoprotein antibodies relies on their specificity for the phosphorylated form of the examined protein (see anti-MAP kinase antibodies below). Monoclonal antibodies, which are generally more specific than polyclonal ones, are reliable tools for distinguishing phosphorylated from unphosphorylated forms of proteins; affinity-purified polyclonal antibodies can be used as well. Both monoclonal and polyclonal antibodies can be generated by immunization with whole phosphorylated proteins or with keyhole limpet hemagglutinin (KLH)- or BSA-coupled peptides, followed by standard procedures for the isolation of the monoclonal and polyclonal antibodies. For example, immunization with whole phosphorylated protein was used to prepare monoclonal antibodies against several of the phosphorylated sites of Tau (Biernat et al., 1992). Immunization with KLH-conjugated phosphorylated peptides was used to prepare anti-active MAP kinases (Yung et al., 1997), anti-phosphorylated CREB (Ginty et al., 1993), and other anti-phosphorylated protein antibodies.

Immunofluorescent staining, another immunodetection method, can be performed with intact cells, such as those obtained from blood or tissue cultures, and on thin sections of various tissues. With immunofluorescence, degradation and dephosphorylation is less problematic because of the rapid fixation of the tissues. Although this method is very useful for detecting changes in phosphorylation in intact tissues and for localization studies, it requires highly specific antibodies, which are not always available. When appropriate highly specific antibodies are available, however, this method should be seriously considered.

For accurate comparison of the amounts of phosphoproteins, detection must be performed in the linear range of the detection system. Thus, the amount of protein loaded on the gel, the concentrations of primary and secondary antibodies, and the enhanced chemiluminescence (ECL) exposure time should be optimized in order to attain linearity. Alternatively, a standard curve can be prepared using a range of concentrations of the proteins of interest, and serial dilutions of the cellular extracts of each treatment can be loaded on the SDS-polyacrylamide gel. The blotting detection systems—e.g., ECL-¹²⁵I-, alkaline phosphatase (AP)-, or biotin-conjugated antibody—should be chosen carefully. ECL generally has the narrowest lin-

ear range of these systems, whereas ¹²⁵I-labeled antibodies have a relatively broad range. The AP detection system is convenient to use, has a moderate linear range, and is usually used for the types of experiments described here.

Anticipated Results

The desired phosphoprotein can be selectively detected with immunoblotting. For example, when anti-active MAP kinase antibodies are used (Basic Protocol 1; Yung et al., 1997), two faint bands at molecular weights of 42 and 44 kDa can be detected in the basal, nonstimulated fractions of Rat1 cells (Fig. 14.2.2). These two bands represent the small amount of active MAP kinase (ERK1 and ERK2) present in resting cells. Upon addition of EGF to these cells, the staining intensity of those bands should increase, peak at 30 min after stimulation, and decline thereafter. At later time points after EGF stimulation, a third band, at 46 kDa, usually appears; this may represent another isoform of MAP kinase. The kinetics of detection of the phosphoprotein represents the transient activation of MAP kinase by EGF in these cells. Staining the same blots with anti-general MAP kinase antibodies should result in equal staining of the 42-, 44-, and 46-kDa bands in all lanes of the blot.

Time Considerations

After cell harvesting, Basic Protocol 1 requires extraction (0.5 hr), determination of protein concentration (0.5 hr), SDS-PAGE (2.5 hr), and immunoblot analysis (6 to 8 hr). Since this procedure may take more than a working day, it can be stopped after boiling the samples in sample buffer. Alternatively, the transfer onto the nitrocellulose membrane can be performed at 40 to 50 mA overnight (instead of at 200 mA for 2 hr). Immunoprecipitation and washes (Basic Protocol 2) add ~4 to 5 hr to Basic Protocol 1. Fluorescent staining (Basic Protocol 3) takes ~3 to 4 hr, and up to 24 hr may be required to mount the stained coverslips onto the slides, depending on the mounting medium used.

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Key Reference

Gabay et al., 1997. See above.

Reports the use of anti-active MAP kinase antibodies (anti-diphospho-ERK) to follow MAP kinase (ERK) activation during different stages of Drosophila development, with immunostaining and immunoblotting both used throughout the study.

Contributed by Zhong Yao and Rony Seger
The Weizmann Institute of Science
Rehovot, Israel

Detection of MAP Kinase Signaling

Transmission of extracellular signals to their intracellular targets is mediated by a network of interacting proteins that relay biochemical messages, thereby controlling many cellular processes. Several related intracellular signaling pathways, collectively known as mitogen-activated protein kinase (MAPK) signaling cascades, have been elucidated in the past decade (Seger and Krebs, 1995). Transmission of signals via these cascades is usually initiated by activation of a small G protein (e.g., Ras) and followed by sequential stimulation of several sets of cytosolic protein kinases. Four distinct MAPK cascades, ERK (extracellular signal-related protein kinase), JNK (c-Jun NH₂-terminal kinase), SPK (stress-related protein kinase), and BMK (big MAPK), have been elucidated to date (Fig. 14.3.1). Each is named after the subgroup of its MAPK components and is composed of up to five levels: MAP4K (MAPK kinase kinase kinase), MAP3K (MAPK kinase kinase), MAPKK (MAPK kinase), MAPK, and MAPKAPK (MAPK-activated protein kinase) (Fig. 14.3.1). One or more components in each of these levels phosphorylates and activates components in the next level, until a downstream component phosphorylates a target regulatory molecule. These cascades can cooperate in transmitting signals from most extracellular stimuli and can thus determine a cell's fate in response to the ever-changing environment. For a detailed description of the MAPK cascades, see Background Information.

Since the majority of MAPK cascade components are kinases, the methods used to detect MAPK cascade activation involve determination of protein kinase activities. Immunoprecipitation of desired protein kinases followed by phosphorylation of specific substrates is a convenient and effective way to determine protein kinase activity (see Basic Protocol 1; Fig. 14.3.2). The activity assay is performed while the enzyme is still bound to the beads for immunoprecipitation, and the amount of incorporated phosphate is monitored by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. An alternative method involves affinity purification with a specific substrate of the examined kinase, followed by phosphorylation; this assay is described for JNK (see Basic Protocol 3; Fig. 14.3.2). When the identity of the kinase is not known, or there are no reagents available for its determination, an in-gel kinase assay should be used (see Basic Protocol 2; Fig. 14.3.2). The common procedure in which antibodies directed against the active form of MAPK cascade components are used is described in UNIT 14.2.

CAUTION: Investigators should wear gloves for all procedures involving radioactivity and should be careful not to contaminate themselves and their clothing. When working with ³²P, investigators should frequently check themselves and the working area for radioactivity using a hand-held monitor. Any radioactive contamination should be cleaned up using appropriate procedures. Radioactive waste should be placed in appropriately designated areas for disposal. Follow the guidelines provided by your local radiation safety advisor (also see APPENDIX 1D).

DETERMINATION OF MAP KINASE (ERK) ACTIVITY BY IMMUNOPRECIPITATION

This method describes determination of ERK activity by immunoprecipitating the enzyme with specific antibodies and then performing a phosphorylation reaction in vitro. ERK is used here as an example; with appropriate reagents, this protocol can be performed with most MAPK isoforms and other components of the MAPK cascade. This protocol results in fast and efficient isolation of the desired protein kinase and its reliable quantification by a phosphorylation reaction. Since the solid support (immunoprecipitating beads) may influence the kinase activity, however, the results do not always accurately reflect the specific activity of the examined kinase. As an alternative, it is possible to elute

**BASIC
PROTOCOL 1**

**Signal
Transduction:
Protein
Phosphorylation**

14.3.1

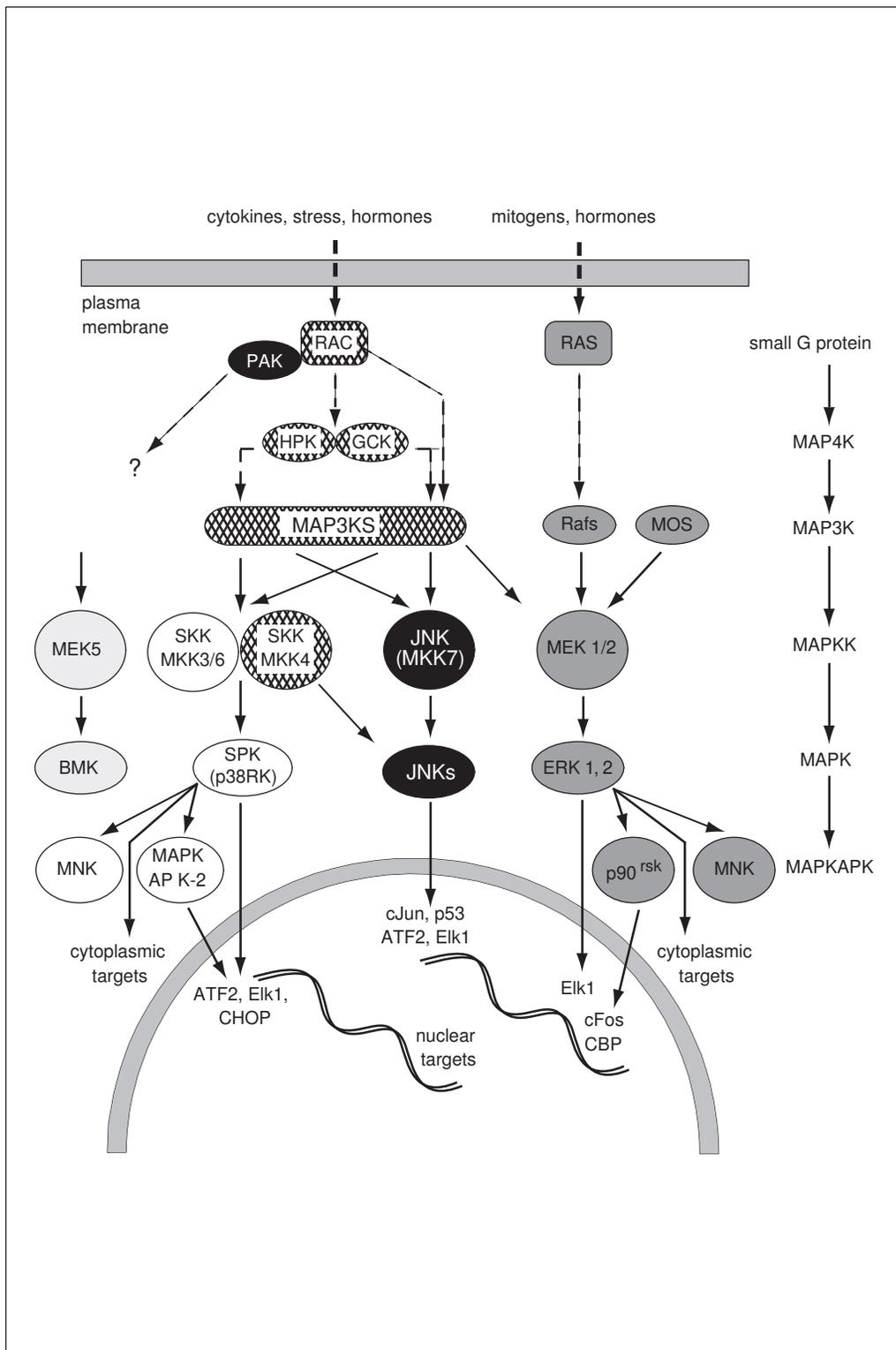


Figure 14.3.1 Schematic representation of mitogen-activated protein kinase (MAPK) cascades. The extracellular signal-related protein kinase (ERK) cascade is represented by light gray shading, the c-Jun NH₂-terminal (JNK) cascade by dark gray shading, the stress-related protein kinase (SPK) cascade by white, and the big MAPK (BMK) cascade by stippled shading. Components that are shared by more than one cascade are indicated by combinations of shading. The connections between components from different levels are shown by arrows; the specifics of these interactions have yet to be defined. Abbreviations: MAP4K, MAPK kinase kinase; MAP3K, MAPK kinase kinase; MAPKK, MAPK kinase; MAPKAPK, MAPK-activated protein kinase.

the kinases of interest from the immunoprecipitating beads (or isolate them by other means) and then determine their activity in solution (Seger et al., 1994). This method can be used with almost all tissue culture cell lines, homogenized animal organs, and even whole lower organisms.

Materials

Six 6-cm tissue culture plates of EGF-stimulated Rat1 cells (*UNIT 14.2*, Basic Protocol 1, steps 1 to 4)
PBS (*APPENDIX 2A*), ice cold
Homogenization buffer (*UNIT 14.2*), ice cold
Kinase buffer (*UNIT 14.2*), ice cold
Coomassie protein assay reagent (Pierce)
Protein standards: 5, 10, 20, 50, 100, and 200 $\mu\text{g/ml}$ BSA in homogenization buffer
Protein A–Sepharose beads, swollen and packed (*UNIT 14.2*, Basic Protocol 2, steps 1 to 2)
Antibody for immunoprecipitation: anti-ERK C-terminal antibody (e.g., Sigma)
0.5 M LiCl solution, ice cold
RadioImmunoPrecipitation Assay (RIPA) buffer (*UNIT 14.2*), ice cold
3 \times reaction mixture (RM \times 3; see recipe)
2 mg/ml myelin basic protein (MBP)
4 \times SDS-PAGE sample buffer (*UNIT 14.2*)
15% SDS-polyacrylamide gel (*UNIT 6.1*)
Prestained protein markers, 16 to 175 kDa (New England Biolabs)
Running buffer: 25 mM Tris·Cl (pH 8.3)/188 mM glycine/0.1% SDS
Staining solution: 40% (v/v) methanol/7% (v/v) acetic acid/25 g/liter Coomassie brilliant blue R-250
Destaining solution: 15% (v/v) isopropanol/7% (v/v) acetic acid in water
X-ray film (not needed if phosphoimager is used)
1.5-ml microcentrifuge tubes, prechilled to 4°C (four sets of six, each labeled 1 to 6)
Stopwatch
1-ml pipet tips, prechilled to 4°C
Microcentrifuge, 4°C
96-well flat-bottomed microtiter plates
Microtiter plate reader, 595 nm wavelength
End-over-end rotator
Shields for radioactive work
Tube heater/shaker (e.g., Eppendorf Thermomixer) *or* water bath, 30°C
Flat container for washing gel
Whatman 3MM filter paper, cut larger than the gel
Film cassette for either X-ray film or phosphoimager
X-ray film developer or phosphoimager
Additional reagents and equipment for preparation of EGF-stimulated Rat1 cells (*UNIT 14.2*, Basic Protocol 1), SDS-PAGE (*UNIT 6.1*), and autoradiography (*UNIT 6.3*)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

Figure 14.3.2 (at right) Detection of mitogen-activated protein kinase (MAPK) activity by the methods described in this unit. **(A)** Detection of extracellular signal-related protein kinase (ERK) activity by immunoprecipitation and myelin basic protein (MBP) phosphorylation. NIH-3T3 cells were grown to subconfluency in 6-cm plates and then starved as described in UNIT 14.2, Basic Protocol 1. Cells were then stimulated with 100 μ M sodium orthovanadate and 200 μ M hydrogen peroxide (VOOH) for 15 min, or 50 ng/ml epidermal growth factor (EGF) for 5 min, or left untreated (basal). Cytosolic extracts were prepared by sonication and the resulting proteins (300 μ g) were incubated with either 30 μ l anti-ERK C-terminal antibody (Santa Cruz Biotechnology)-conjugated protein A-Sepharose beads (+) or with unconjugated protein A-Sepharose beads (-). The phosphorylation reaction on MBP was performed as described in Basic Protocol 1. **(B)** Detection of protein kinase activity by the in-gel kinase assay. MCF7 cells overexpressing the ErbB-2 receptor were stimulated with 50 ng/ml EGF for the indicated times. The in-gel kinase assay was performed as described in Basic Protocol 2. ERK1 and ERK2 bands are indicated. The identity of other bands was not determined. **(C)** Detection of c-Jun NH₂-terminal protein kinase (JNK) activity by an affinity assay. α T3-1 cells were treated with 100 nM D-Trp gonadotropin-releasing hormone for the indicated times. The JNK assay was performed as described in Basic Protocol 3. GST-cJun and a degradation product (p30) are indicated. Abbreviation: GST-Jun, glutathione-S-transferase-conjugated cJun.

Prepare cytosolic extracts

1. Add 350 μ l ice-cold homogenization buffer to each plate of EGF-stimulated Rat1 cells on ice (see also UNIT 14.2), tilt the plate gently, and scrape the cells into the buffer using a plastic scraper or rubber policeman. Using prechilled pipet tips, transfer the cells and buffer to labeled, prechilled 1.5-ml microcentrifuge tubes.

Special consideration should be given to the composition of homogenization buffer. The authors recommend using β -glycerophosphate, which serves both as a buffer and as a general phosphatase inhibitor, rather than Tris or HEPES. Sodium orthovanadate is used to inhibit tyrosine phosphatases, and the mixture of pepstatin-A, aprotinin, leupeptin, and benzamidine is used to inhibit proteinases. Ice-cold homogenization buffer blocks most of the phosphatase and proteinase activities in cell extracts.

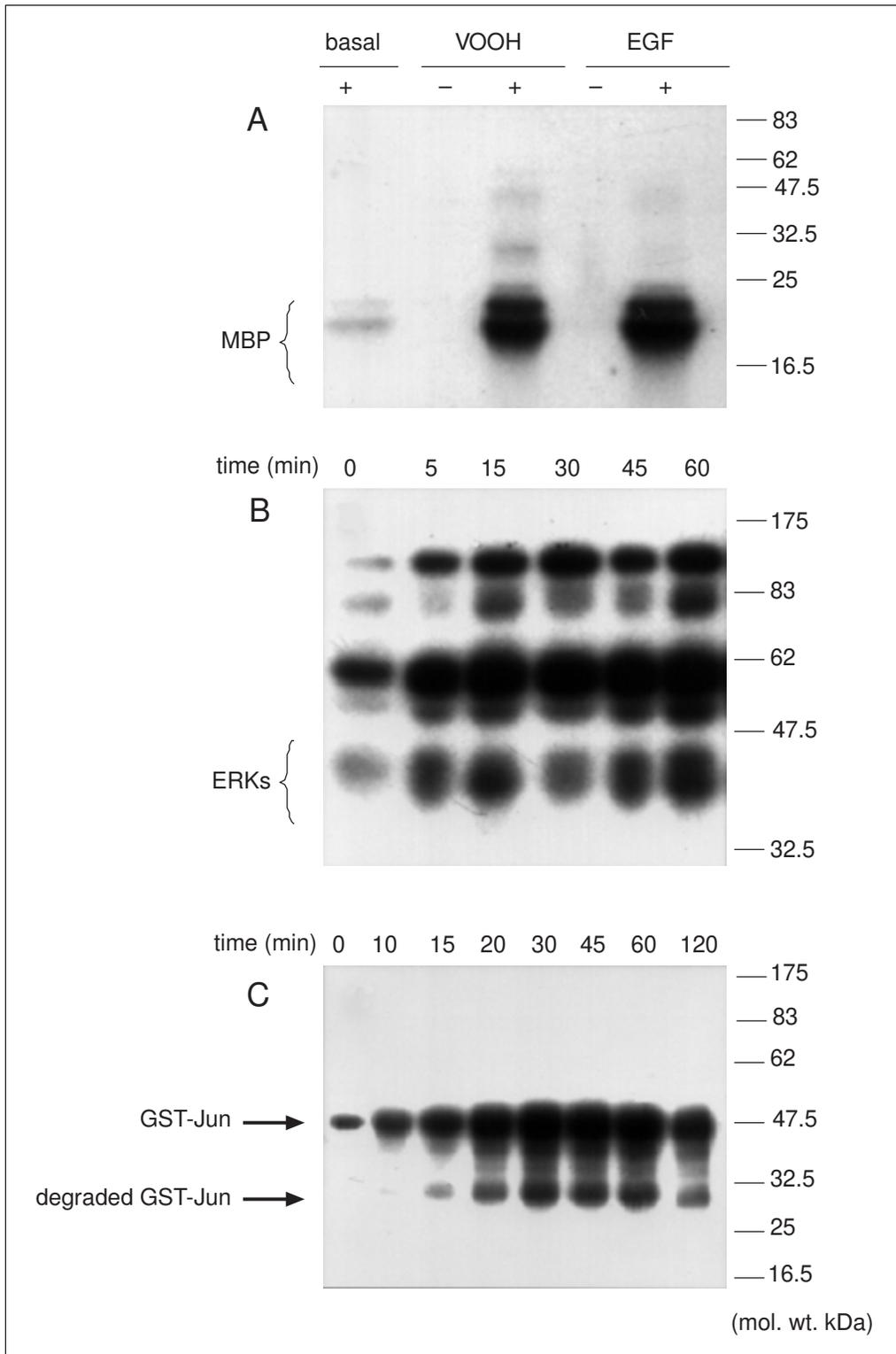
2. Disrupt the cells by sonication (two 7-sec, 50-W pulses per 0.5-ml sample) on ice.

Sonication allows extraction of proteins from the cytosolic and nuclear fractions of the cells but not from the membrane fraction; therefore, the extract is called a cytosolic extract. Cellular extraction with nonionic detergents, which extract proteins from the membrane, cytosolic, and some nuclear fractions of the cell, makes determination of the protein concentration somewhat difficult but is often used (see UNIT 14.2). Extraction with RIPA buffer or by freeze-thawing can be used for some kinases, but these methods are less effective (see Critical Parameters).

3. Microcentrifuge the cytosolic extracts 15 min at 14,000 \times g, 4°C. Transfer supernatants to fresh, prechilled, and prelabeled tubes. Take 5- to 10- μ l aliquots of these extracts for protein determination. Store the remainder of each cytosolic extract on ice until needed.

Determine the protein concentration of each sample at this stage so that identical amounts of proteins from the different samples can be compared and the relative amount of protein kinases in each sample determined accurately. Comparing samples based on cell number, rather than protein concentration, can result in differences of up to 20% in the amount of protein. Such differences can cause even larger ones when phosphorylation is assessed immunologically.

Do not leave samples on ice >45 to 90 min, to avoid unnecessary exposure to phosphatases and proteinases (see Critical Parameters).



Determine protein concentration

4. Add 10 μl of each cytosolic extract to 190 μl kinase buffer in labeled 1.5-ml microcentrifuge tubes.

Dilutions of $\geq 1:20$ are usually necessary to ensure that the sample protein concentrations are in the linear range of the protein determination assay. This dilution is not always necessary with some Coomassie protein assay reagents that have extended ranges.

5. Transfer 10 μl of each of the protein standards into two wells each of a 96-well, flat-bottomed microtiter plate.

Protein standards should be prepared in the same buffer as was used for the cell extraction.

6. Transfer 10 μl of each of the diluted cytosolic extracts into two wells of the same microtiter plate. Add 200 μl Coomassie protein assay reagent to all wells.

7. Place the microtiter plate in a microtiter plate reader and measure the absorbance of the samples at 595 nm (A_{595}). Calculate the protein concentrations of the cytosolic extracts by comparing the absorbance of the sample with the standard curve.

8. Based on the calculated protein concentrations, transfer a volume of each cytosolic extract containing 100 to 500 μg protein into a fresh, prechilled 1.5-ml microcentrifuge tube.

Good results are often obtained with 50 to 500 μg protein; the authors recommend using 300 μg . Since equal amounts of antibodies are used in each of the immunoprecipitation reactions, the protein concentration can vary slightly. To avoid inaccuracy, however, equal amounts of protein should be used in each of the samples to be immunoprecipitated.

Prepare antibody-conjugated protein A–Sepharose beads

9. Place 180 μl swollen, packed protein A–Sepharose beads in a 1.5-ml microcentrifuge tube. Add 320 μl PBS and 25 μl of the antibody to be conjugated, diluted according to the manufacturer's instructions. Rotate the mixture for 1 hr at room temperature on an end-over-end rotator to allow the antibody to bind to the protein A.

This step can be done at 4°C for 16 hr. Ideally, this step should be performed either before or simultaneously with the preparation of cytosolic extracts so that the immunoprecipitation step can proceed without delay.

Anti-C-terminal antibodies are generally used for the determination of kinase activity, because their binding to the kinase does not interfere with its kinase activity. Usually 1 to 5 μg per reaction is sufficient. Polyclonal antibodies are available from a number of suppliers (e.g., Transduction Laboratories, Sigma, Santa Cruz Biotechnology, Upstate Biotechnology, and Zymed Laboratories).

These volumes are calculated for ten reactions (usually 10 to 20 μl beads is used per reaction), but because of the density of the beads they will probably only be sufficient for eight reactions. The amounts can be scaled up as long as the proportions are maintained.

For easy handling of the resin, cut the ends of the pipet tips to enlarge their openings.

10. Resuspend beads in 1 ml ice-cold PBS and microcentrifuge 1 min at 14,000 $\times g$, 4°C. Remove supernatant and add 1 ml ice-cold homogenization buffer. Repeat centrifugation three more times with homogenization buffer, ending with a final addition of an equal volume of ice-cold homogenization buffer.

Antibody-conjugated beads can be stored in homogenization buffer for ≤ 3 days at 4°C.

Immunoprecipitate kinase

11. Add 30 μl antibody-conjugated bead suspension (15 μl beads and 15 μl homogenization buffer) to a 300- μl sample of cytosolic extract containing 50 to 500 μg total

protein in prechilled 1.5-ml microcentrifuge tubes. Rotate on end-over-end rotator for 2 hr at 4°C.

Conjugating the antibodies to protein A–Sepharose beads prior to adding them to the cytosolic extracts minimizes the time the samples are incubated with the antibodies, minimizing exposure of the kinases to phosphatases and proteinases in the extracts. This procedure also ensures that only antibodies recognized by protein A will be used for the immunoprecipitation. If polyclonal antibodies are added to the cytosolic extracts, antibodies that are not recognized by protein A can bind to the desired antigen but will not be precipitated when protein A–Sepharose beads are added, and will reduce the efficiency of immunoprecipitation.

12. Microcentrifuge protein A–Sepharose bead/cytosolic extract mixture 1 min at 14,000 × g, 4°C. Discard supernatant, add 1 ml ice-cold RIPA buffer, and centrifuge as before. Repeat centrifugation twice with 1 ml ice-cold 0.5 M LiCl and twice with 1 ml ice-cold kinase buffer.

These stringent washes remove most of the protein kinases that can nonspecifically interact with the protein A–Sepharose beads.

Perform phosphorylation reaction

13. After the last wash, completely remove kinase buffer from the conjugated beads by removing supernatant, microcentrifuging 1 min at 14,000 × g, 4°C, without adding more buffer, and gently removing residual buffer from above beads.
14. Resuspend beads in 15 μl water. Prepare the laboratory bench for working with small amounts of radioactivity.

Determination of enzymatic activity is not always accurate when enzymes are bound to beads. The kinase(s) of interest can be released from the beads at step 14 or 15 by the addition of excess immunizing peptide, allowing the phosphorylation reaction (steps 15 to 17) to be performed without the interference of the beads. The activity can then be measured as described below (steps 18 to 24) or by paper assay. If by paper assay, terminate the phosphorylation reaction by spotting 20 μl of each cytosolic extract on a phosphocellulose paper square (Whatman P81) and washing immediately with 150 mM phosphoric acid. Measure phosphate incorporation using scintillation cocktail and counter.

15. Add 10 μl RM×3 to each tube.

The most important components of RM×3 are the Mg²⁺ and [γ-³²P]ATP, which are essential for the phosphorylation reaction. The use of 100 μM ATP with ~4000 cpm/pmol [γ-³²P]ATP provides a good linear range and reproducible results. When the enzymatic activity of the kinases is low, which makes detection of phosphorylation difficult, the concentration of unlabeled ATP should be reduced to 10 to 20 μM and the amount of [γ-³²P]ATP increased to 50,000 cpm/pmol. Addition of [γ-³²P]ATP alone is not recommended because this will result in a nanomolar concentration of ATP, which is considerably below the K_M for ATP and may lead to nonspecific phosphorylation.

The β-glycerophosphate in the reaction mixture serves as a buffer, but can also inhibit residual phosphatases that may have nonspecifically bound to the beads. BSA serves as a carrier protein; it can be eliminated if purity is required. EGTA chelates Ca²⁺, which may interfere with some kinase activities, DTT keeps the proteins reduced, and sodium orthovanadate inhibits tyrosine phosphatases.

16. Add 5 μl of 2 mg/ml MBP to each tube and place the mixture in a 30°C Thermomixer or water bath.

MBP is probably not a physiological substrate for any MAPK, but it is a good general substrate for many kinases, including ERKs, in vitro. Substrates should be well phosphorylated by the desired kinases to allow accurate detection of the phosphorylation reaction.

17. Incubate 20 min at 30°C with either constant or frequent shaking.

If a Thermomixer is not available, a water bath or other heating device can be used.

**Signal
Transduction:
Protein
Phosphorylation**

14.3.7

18. Add 10 μ l of 4 \times SDS-PAGE sample buffer to each tube to stop the phosphorylation reaction.

19. Boil for 5 min. Centrifuge for 1 min at 14,000 \times g, room temperature.

Electrophorese the phosphorylation reaction products

20. Prepare a 15% separating gel with a 3% stacking gel (UNIT 6.1).

21. Load samples and prestained protein markers on the gel.

Load prestained markers into the first or second lane of the gel so that the first lane can be located on the dried gel and the molecular weights of the detected proteins determined.

22. Run the gel at 150 V, constant voltage, until the bromphenol blue dye is 0.5 cm from the bottom of the gel (~1 hr).

23. Place the gel in a flat container, add 50 ml staining solution, and let stand for 20 min at room temperature. Remove staining solution and add 50 ml destaining solution for 30 min. Repeat destaining three times with 50 ml destaining solution for 30 min each time.

This extensive destaining removes excess free [γ - 32 P]ATP which would affect the background radioactivity levels. Extensive destaining is not necessary if the phosphorylation of the desired proteins is very high. Alternatively, the proteins can be transferred to a nitrocellulose membrane (UNIT 14.2, Basic Protocol 1, steps 17 to 23) and then exposed as below.

24. Place the gel on Whatman 3MM filter paper, cover with plastic wrap, and dry the gel in a gel dryer for 1.5 hr at 80°C. Expose the gel in a phosphoimager or on X-ray film (UNIT 6.3).

Bands should appear at 16 to 21 kDa, which is the molecular weight of the four MBP isoforms.

BASIC PROTOCOL 2

IN-GEL KINASE ASSAY

If the identity of the kinase is not known or there are no specific antibodies available for the kinase, an in-gel kinase assay can be used in place of immunoprecipitation. This in-gel protocol involves copolymerizing a substrate with SDS and polyacrylamide and running the protein sample on the resulting SDS-polyacrylamide gel. After several rounds of denaturation and renaturation, a phosphorylation reaction is performed on the gel and the phosphorylated bands are visualized by autoradiography or by a phosphoimager (UNIT 6.3). With this method, the molecular weight of the protein kinase is revealed and novel protein kinases can be identified. Not all protein kinases can be renatured under the conditions of this protocol, however, and the linear range of this assay is usually limited; therefore, this method should not be used routinely to monitor and characterize known protein kinases.

Materials

Cell lysates from serum-starved, EGF-stimulated Rat1 cells (UNIT 14.2, Basic Protocol 1, steps 1 to 8) or cytosolic extracts (see Basic Protocol 1, steps 1 to 3)
4 \times SDS-PAGE sample buffer (UNIT 14.2)
2 mg/ml myelin basic protein (MBP)
1.5 M Tris-Cl, pH 8.8
30% acrylamide/0.8% bisacrylamide (UNIT 6.1)
10% ammonium persulfate
Stacking gel (UNIT 6.1)
TEMED
20% isopropanol/50 mM HEPES, pH 7.6
Renaturation buffer: 50 mM HEPES (pH 7.6)/5 mM 2-mercaptoethanol

Renaturation buffer/0.05% Tween 20
6 M urea in kinase buffer (UNIT 14.2)
In-gel kinase buffer: 20 mM HEPES (pH 7.6)/20 mM MgCl₂
In-gel kinase buffer/2 mM DTT/20 μM ATP/100 μCi [γ -³²P]ATP
5% trichloroacetic acid (TCA)/1% sodium pyrophosphate (NaPPi)
Water bath, 30°C, with proper shielding for radioactive work

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and autoradiography
(UNIT 6.3)

Procedure

1. Determine protein concentration of cell lysates (UNIT 14.2, Basic Protocol 1, steps 9 to 12) or cytosolic extracts (see Basic Protocol 1, steps 4 to 8). Based on the calculated protein concentrations, transfer a volume of each cell lysate containing 30 to 80 μg protein to a fresh 1.5-ml microcentrifuge tube.
2. Add ¼ vol of 4× SDS-PAGE sample buffer to each tube and mix thoroughly. Keep at 4°C.

IMPORTANT NOTE: *Do not boil the samples.*

3. Prepare 8 ml of 12% polyacrylamide gel (separating gel) containing MBP by mixing the following:

0.7 ml H₂O
2.0 ml MBP
2.0 ml of 1.5 M Tris·Cl, pH 8.8
3.2 ml 30% acrylamide/0.8% bisacrylamide
100 μl of 10% ammonium persulfate
6 μl TEMED.

After the separating gel polymerizes, add a 3% stacking gel (UNIT 6.1).

4. Load samples and prestained markers onto gel and run at 100 V until the dye front has reached 0.5 cm from the bottom of the gel (~90 min).

The gel should not be heated above 30°C; therefore, the voltage used for electrophoresis should be ≤100 V.

5. Remove the gel from the apparatus, cut off the stacking gel, and carefully place the gel in a flat container. Wash twice with 100 ml of 20% isopropanol/50 mM HEPES, pH 7.6, for 30 min each at room temperature. Repeat washing twice with 100 ml renaturation buffer for 30 min each at room temperature and twice with 100 ml of 6 M urea in kinase buffer for 15 min each at room temperature.

The second wash with 20% isopropanol/50 mM HEPES, pH 7.6, can be done overnight at 4°C.

6. Place the gel in 4°C cold room, remove 50 ml of washing solution, add 50 ml renaturation buffer/0.05% Tween 20, and shake for 15 min. Repeat twice, removing 50 ml of the washing solution, adding 50 ml renaturation buffer/0.05% Tween 20, and shaking for 15 min each time. Wash the gel three times with 100 ml renaturation buffer/0.05% Tween 20 for 15 min each time. Shake the gel overnight in the cold room.

7. Remove washing buffer and incubate the gel in 30 ml in-gel kinase buffer for 30 min at 30°C. Remove the buffer, add 20 ml in-gel kinase buffer/2 mM DTT/20 μM ATP/100 μCi [γ -³²P]ATP, and incubate in 30°C water bath for 2 hr.

The amount of radioactive material is very high at this stage and the reaction should be performed with proper shielding. Make sure that the gel is straight in the flat container.

Signal
Transduction:
Protein
Phosphorylation

14.3.9

Unequal distribution of the phosphorylation buffer can interfere with the phosphorylation reaction.

8. Wash the gel carefully four times with 5% TCA/1% NaPPi for 15 min each at room temperature. If the gel is still very radioactive, continue washing overnight.
9. Dry the gel (see Basic Protocol 1, step 30) and subject to autoradiography.

Bands should appear where kinases phosphorylated the MBP copolymerized in the gel (see Fig. 14.3.2).

BASIC PROTOCOL 3

JNK ASSAY

Protein kinases can sometimes be isolated without using specific antibodies. For example, with JNK it is possible to exploit the tight and specific binding of the two forms of JNK (p46 and p54) to their substrate, Jun (Hibi et al., 1993). Thus, glutathione-*S*-transferase (GST)–Jun bound to glutathione-conjugated beads can be used to specifically isolate the JNKs, while stringent washings remove irrelevant protein kinases. Phosphorylation using [γ -³²P]ATP and Mg²⁺ is then performed with the bead-bound kinase and is detected by autoradiography after SDS-PAGE. This affinity purification method has been successfully used in many recent studies. When GST-Jun on glutathione-conjugated beads is used as the substrate, this method gives more accurate results than the purification of JNKs by immunoprecipitation (Basic Protocol 1). The amount of immobilized Jun is the limiting factor, and the linear range of this assay is limited. Preparation of the GST-Jun beads is described below (see Support Protocol).

Materials

- Cytosolic extracts from serum-starved, EGF-stimulated Rat1 cells (see Basic Protocol 1, steps 1 to 3)
- 10× binding buffer (see recipe)
- GST-Jun beads: GST-Jun(1–91) bound to glutathione-conjugated beads (see Support Protocol)
- HB1B buffer (see recipe), ice cold
- JNK kinase buffer (see recipe), ice cold and room temperature
- 10× ATP mix: 20 mM unlabeled ATP/2 μ Ci [γ -³²P]ATP, freshly prepared
- 4× SDS-PAGE sample buffer (UNIT 14.2)
- 12% SDS-polyacrylamide gel (UNIT 6.1)
- 1.5-ml microcentrifuge tubes, prechilled
- Microcentrifuge, 4°C
- Tube heater/shaker (e.g., Eppendorf Thermomixer) *or* water bath, 30°C
- Additional materials and equipment for SDS-PAGE (UNIT 6.1)

Procedure

1. Determine the protein concentration of cytosolic extracts (see Basic Protocol 1, steps 4 to 8).
2. Place 150 μ l cytosolic extract or cell lysate (50 to 500 μ g protein), 30 μ l of 10× binding buffer, 100 μ l water, and 20 μ l GST-Jun beads in prechilled 1.5-ml microcentrifuge tubes.

The amount of GST-Jun beads can vary according to the amount of the protein conjugated to the beads. Amounts of 2 to 4 μ g protein per 20 μ l beads usually give good results. The truncated form of Jun (residues 1 to 91) is recommended for these experiments, but similar results can be obtained with the full-length or 1-to-74 constructs.

3. Incubate 1 hr at 4°C with constant shaking. Microcentrifuge 2 min at 14,000 × g, 4°C, and discard the supernatant.

The JNKs bind to the GST-Jun on the beads during this incubation.

4. Resuspend pelleted beads in 1 ml ice-cold HB1B buffer and microcentrifuge for 2 min at 14,000 × g, 4°C. Discard supernatants and repeat centrifugation twice with 1 ml ice-cold HB1B buffer each time and once with 1 ml ice-cold JNK kinase buffer.

Remove the supernatant completely after each centrifugation.

5. Add 30 μl JNK kinase buffer at room temperature to the pelleted beads and place the tubes in 30°C Thermomixer or water bath. Add 3 μl of 10× ATP mix. Close the tubes and incubate 20 min with shaking.

Take proper precautions when working with radioactive material.

6. Add 11 μl of 4× SDS-PAGE sample buffer. Boil the samples 5 min.
7. Prepare 12% SDS-polyacrylamide gel with a 3% stacking gel (UNIT 6.1).
8. Load samples and prestained protein markers onto the gel. Run the gel at 150 V, constant voltage, until the bromophenol blue reaches 0.5 cm from the bottom of the gel (~1 hr).
9. Stain, dry, and analyze the gel (see Basic Protocol 1, steps 23 and 24).

A band should be detected at 46 kDa (the molecular weight of the truncated GST-Jun). In many cases, an additional band is observed at 30 kDa, which represents a degradation product of GST-Jun and is a good indication of JNK activity as well.

PREPARATION OF GST-JUN-GLUTATHIONE BEADS

Materials

Bacteria transformed with GST-Jun(1–91)-expressing plasmid (plasmid is available from several investigators; alternatively, similar proteins can be obtained from, e.g., Calbiochem)

LB medium with appropriate antibiotics (LB medium; APPENDIX 2A)

100 mM isopropyl-β-D-thiogalactopyranoside (IPTG; store in aliquots <1 year at –20°C)

PBS/protease inhibitors (see recipe), ice cold

Washed glutathione beads (see recipe)

PBS/protease inhibitors/20% (v/v) glycerol, ice cold

End-over-end rotator

1. Grow bacteria transformed with GST-Jun-expressing plasmid at 30°C in 4 liters LB medium until optical density at 600 nm (OD₆₀₀) is 0.6 (~3 to 4 hr).
2. Add IPTG to 0.4 nM final and grow bacteria an additional 4 hr at 30°C.
3. Centrifuge 10 min at 6000 × g, 4°C.
4. Resuspend pellet in 80 ml ice-cold PBS/protease inhibitors, sonicate, and centrifuge 10 min at 15,000 × g, 4°C. Transfer supernatant to a fresh tube and repeat centrifugation.
5. Discard pellet, add 3 ml washed glutathione beads to supernatant, and rotate end over end 2 hr at 20 rpm, 4°C.

SUPPORT PROTOCOL

Signal
Transduction:
Protein
Phosphorylation

14.3.11

6. Wash beads with ice-cold PBS three times. Add 8 ml PBS/protease inhibitors/20% glycerol.

Beads can be stored 1 to 2 days at 4°C or <4 months at -20°C in ice-cold PBS/protease inhibitors/20% glycerol before being used in Basic Protocol 3.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Binding buffer, 10×

1.5 M NaCl
220 mM HEPES, pH 7.7
20 mM MgCl₂
0.75% (v/v) Triton X-100
200 mM β-glycerophosphate
1 mM EDTA
1 mM sodium orthovanadate
1 mM phenylmethylsulfonyl fluoride (PMSF; APPENDIX 2A)
10 μg/ml aprotinin
10 μg/ml leupeptin
2 μg/ml pepstatin A
Store 2 months at 4°C

This buffer is derived from Hibi et al. (1993).

Glutathione beads, washed

Add 50 ml ice-cold PBS to 3 ml glutathione beads, centrifuge 5 min at 2000 × g, 4°C, and discard supernatant. Repeat centrifugation three times, adding 50 ml ice-cold PBS each time.

HB1B buffer

20 mM HEPES, pH 7.7
50 mM NaCl
0.1 mM EDTA
25 mM MgCl₂
0.05% (v/v) Triton X-100
Store 2 months at 4°C

JNK kinase buffer

20 mM HEPES, pH 7.7
20 mM MgCl₂
20 mM β-glycerophosphate
0.1 mM sodium orthovanadate
2 mM dithiothreitol (DTT; APPENDIX 2A), added fresh
Store without DTT for 2 months at 4°C

PBS/protease inhibitors

PBS containing:

10 μg/ml leupeptin (from 10 mg/ml stock solution)
10 μg/ml aprotinin (from 2 mg/ml stock solution)
2 μg/ml pepstatin A (from 10 mg/ml stock solution)
1 mM phenylmethylsulfonyl fluoride (PMSF; APPENDIX 2A)
Prepare fresh

Leupeptin and aprotinin are prepared in water; pepstatin is prepared in ethanol or methanol. All stock solutions are stored <1 year at -20°C.

Reaction mixture, 3× (RM×3)

75 mM β-glycerophosphate, pH 7.3
30 mM MgCl₂
0.9% (w/v) BSA
3 mM DTT (*APPENDIX 2A*)
3 mM EGTA
0.3 mM sodium orthovanadate
100 μM [γ -³²P]ATP (~4000 cpm/pmol)
Store without [γ -³²P]ATP for 1 year at –20°C

COMMENTARY

Background Information

The MAPK cascades

Sequential activation of kinases (protein kinase cascades) is a common mechanism of signal transduction in many cellular processes (Campbell et al., 1995). Several related intracellular signaling cascades, collectively known as mitogen-activated protein kinase (MAPK) signaling cascades, have been elucidated over the past decade (Davis, 1994; Seger and Krebs, 1995; Kyriakis and Avruch, 1996; Marshall, 1996; Robinson and Cobb, 1997). Each cascade seems to consist of up to five levels of protein kinases that sequentially activate each other by phosphorylation. The similarities between the enzymes that comprise each level in the various cascades make these kinases part of a superfamily of kinases.

The activation of each of these cascades seems to be initiated by a small GTP-binding protein that transmits the signal to protein kinases, commonly referred to as MAPK kinase kinases (MAP4Ks). Then the signal is transmitted downstream through the cascade by enzymes at the next levels that are referred to as MAP3K (MAPK kinase kinase), MAPKK (MAPK kinase), MAPK, and MAPK-activated protein kinases (MAPKAPKs). It should be noted that in some of the cascades, the signals seem to be transmitted from the small GTP-binding protein directly to the MAP3K level of the cascade. The existence of four to five levels in each MAPK cascade allows for signal amplification, determination of specificity, and tight regulation of the transmitted signal.

The four distinct MAPK cascades that are currently known are named according to the subgroup of their MAPK components: the ERK (extracellular signal-related protein kinase), JNK (c-Jun NH₂-terminal kinase), SPK (stress-related protein kinase or p38MAPK), and BMK (big MAPK or ERK 5) cascades. These MAPK cascades cooperate to transmit signals to their

intracellular targets, thereby initiating cellular processes such as proliferation, differentiation, development, stress response, and apoptosis. In this section, the various MAPK cascades will be briefly reviewed.

The ERK cascade, also known as the p42, p44 MAPK cascade, was the first MAPK cascade elucidated (Seger and Krebs, 1995). This cascade is initiated by the small G protein Ras, which upon stimulation causes membrane translocation and activation of the protein serine/threonine kinase Raf1. Once activated, Raf1 continues transmission of the signal by phosphorylating two regulatory serine residues located in the activation loop of MEK, causing its full activation. Other kinases that can also activate MEK are A-Raf, B-Raf, Mos TPL2, and MEKK2 (Seger and Krebs, 1995; Salmeron et al., 1996; Wang et al., 1996), which all seem to phosphorylate the same regulatory residues of MEK. Activated MEK is a dual-specificity protein kinase that appears to be the only kinase capable of specifically phosphorylating and activating the next kinase in this cascade, which is ERK.

ERK activation requires phosphorylation of two regulatory residues, threonine and tyrosine, that reside in a TXY phosphorylation motif (Payne et al., 1991; Canagarajah et al., 1997). Phosphorylation of threonine and tyrosine residues is essential for the activation of all MAPKs. In the other cascades, however, the identity of the middle amino acid in the TXY motif of the MAPK varies, which probably determines the specificity of the signal.

ERK appears to be an important regulatory molecule. ERK, which by itself can phosphorylate regulatory targets in the cytosol such as phospholipase A₂ (PLA₂; Lin et al., 1993), can translocate into and phosphorylate substrates in the nucleus such as ELK1 (Chen et al., 1992; Marais et al., 1993) and can transmit the signal to the MAPKAPK level. The main MAPKAPK of the ERK cascade is RSK, which can also

translocate to the nucleus upon activation and phosphorylate a set of nuclear substrates different from those phosphorylated by ERK. MNK is another MAPKAPK that is activated also by the SPK cascade (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997). Activation of the ERK cascade was initially implicated in the transmission and control of mitogenic signals; this cascade is now known to be important for differentiation, development, stress response, learning and memory, and morphology determination.

ERKs are activated primarily by mitogenic signals, whereas other MAPK cascades are activated mainly by cellular stress, such as heat shock, ischemia, UV irradiation, and cytokines (Woodgett et al., 1996) and are referred to as stress-activated protein kinase (SAPK) cascades (Kyriakis and Avruch, 1996). The SPK (p38 MAPK) cascade consists of MAPKs that contain a glycine residue in their TXY activation motif (TGY; Han et al., 1994).

Many kinases in the MAPKK, MAP3K, and MAP4K levels have been implicated in the SPK cascade (Fig. 14.3.1), but their individual roles are not yet known. GCK1 and HPK1 (Kiefer et al., 1996; Pombo et al., 1995), and probably also PAK1 (Zhang et al., 1995), may belong to the MAP4K level of SPK, although it is not clear whether they are involved in the activation of SAPKs. Twelve distinct kinases have already been implicated in the MAP3K level of this cascade: MEKK1 to 5, MTK1, MLK2, MLK3, ASK1, TPL2, DLK, and TAK1 (Yamaguchi et al., 1995; Blank et al., 1996; Fan et al., 1996; Salmeron et al., 1996; Wang et al., 1996; Deacon and Blank, 1997; Gerwins et al., 1997; Takekawa et al., 1997; Wang et al., 1997). At the MAPKK level, SKK3, SKK6 (MEK6), SKK2 (MKK3), and SKK1 (MKK4, SEK1, JNKK1) seem to play major roles in the activation of all SPKs (Kyriakis and Avruch, 1996).

The MAPK level components of the SPK cascade are p38MAPK (also known as RK, Hog, SAPK2a, and CSBP; Han et al., 1994), SAPK2b, SAPK3, and SAPK4 (Kyriakis et al., 1994; Cuenda et al., 1997; Goedert et al., 1997). Once these SPKs are activated, they either transmit the signal to the MAPKAPK level components MAPKAPK 2 and 3 (Stokoe et al., 1992; McLaughlin et al., 1996) and MNK, or phosphorylate regulatory molecules such as PLA₂ (Kramer et al., 1996) and the transcription factors ATF2, ELK1 (Karin, 1995), CHOP (Wang and Ron, 1996), and MEF2C (Han et al., 1997).

JNKs (also called SAPK1), which comprise a third MAPK subgroup, are also SAPKs. These enzymes are not closely related to the above SPKs, however, mainly because they contain TPY rather than TGY residues in their activation motif. Like the other MAPK cascades, the JNK cascade is triggered by the small GTPases (Crespo et al., 1997) Rac and CDC42. The signals are then transmitted via MAP4K (?) and MAP3K components that are largely shared with the SPK cascades. Since the SPK and JNK cascades are not always simultaneously activated, the signals must be separately regulated to allow separate cascades; the mode of this regulation is unknown as yet.

At the MAPKK level, the JNKs can be activated by at least three dual-specificity enzymes (JNKKs): SEK1 (SKK1, MKK4; Yan et al., 1994), MKK7 (Holland et al., 1997; Tournier et al., 1997), and JNKK2 (which may be an MKK7 isoform; Lu et al., 1997). All three JNKKs seem to be able to activate the components at the MAPK level, JNK1 to 3, SPKs which have molecular weights 46, 54, and 52 kDa, respectively. No enzymes at the MAPK-APK level and no cytosolic targets have been identified for JNKs, but these enzymes appear to be major regulators of nuclear processes, in particular transcription. Shortly after activation, JNKs translocate to the nucleus where they physically associate with and activate their target transcription factors (e.g., c-Jun, ATF, Elk, etc.).

The BMKs comprise another MAPK subgroup (Zhou et al., 1995; Abe et al., 1996) with molecular weights of ~110 kDa. BMK1 (also known as ERK5) and MEK5 are the only known components of this MAPK cascade. Like ERKs, BMKs contain a TEY phosphorylation motif, but they seem to be involved primarily in stress processes, and therefore may belong to the stress-activated subgroup of MAPKs. The C termini of BMKs are unique and appear to be activated by several extracellular stimuli, including osmotic and oxidative stresses.

Kinase cascades other than the MAPK cascades are also activated in response to mitogenic stimulation. These include the NIK- IKK1/2 (DiDonato et al., 1997; Regnier et al., 1997) and PI3K-PDK-AKT-GSK3 (Cohen et al., 1997) Rho-dependent pathways (Leung et al., 1995) and the phosphokinase A (PKA)-phosphorylase kinase pathway (Campbell et al., 1995). Because of their distinct characteristics, these pathways are usually not considered to be genuine MAPK cascades, although

they are involved in transmission of many extracellular signals.

All the pathways mentioned are apparently activated to some extent by distinct extracellular agents and, as a result of their action in an elaborate network, determine the outcome of each stimulation. The full dimensions of this network, the mode of regulation of its components, and the mechanisms by which these cascades determine cell fates in response to various stimuli have yet to be fully elucidated.

Analysis of cascade enzymes

The relative strength and the duration of the signals transmitted in each MAPK cascade are thought to be major determinants of signaling specificity. Accurate detection of the signals transmitted via various MAPK cascades towards target molecules is important, therefore, in the study of intracellular signaling. The activity of one component of the MAPK level of each cascade (e.g., ERK, JNK, SPK) is usually a sufficient indicator of the transmitted signal. Sometimes, however, the activity of additional components at upstream or downstream levels must be determined because of cross-talk between various cascades. For example, SPK (p38MAPK) can be activated by as many as three distinct MAPKKs (MKK3, 4, or 6; Kyriakis and Avruch, 1996), and it is important to determine which of these MAPKKs is the immediate activator in different systems.

Most components of the MAPK cascades belong to the large family of protein kinases, which consists of more than 2000 distinct members (Hunter, 1994). For studying protein kinases in general and MAPK components in particular, specific detection of the activity of the desired protein kinase is essential. The activity of a particular protein kinase can be singled out from a multitude of related activities that might mask its activity in two main ways. One method uses a specific substrate that is recognized only by the desired protein kinase. This method is good for detecting kinases like MEK, which seems to specifically and selectively phosphorylate its downstream component, ERK. The other, and more common, method is to isolate the protein kinase and then use a general substrate as an indicator of its activity. This method has been used successfully in studies of MAPK cascades.

In one of the first methods used for the systematic detection of protein kinases involved in growth factor signaling, protein kinases were isolated using Mono Q fast protein liquid chromatography (FPLC; Ahn et al.,

1990). This method involves stimulating tissue culture cells, fractionating the cytosolic extracts of these cells on a Mono Q column (Pharmacia Biotech), and examining the resulting fractions for protein kinase activity. Since fractionation with Mono Q columns is extremely reproducible, kinases that are activated upon stimulation can be detected by comparing the elution profiles of kinases from activated and unactivated cells. The advantages of this method are (1) the ability to identify novel kinases and measure their activity, (2) the ability to detect the overall activity of many protein kinases, and (3) its good linear range, which allows determination of the ratio between the activities of distinct protein kinases at a given time. The main disadvantage of this method is that separation of various protein kinases is not always complete. In addition, this is a very laborious method and it is difficult to examine more than one sample per day.

Another method that the authors have found useful in detecting novel protein kinases is the in-gel kinase assay (see Basic Protocol 2; Kameshita and Fujisawa, 1989). This technique involves copolymerization of a given substrate in an SDS-polyacrylamide gel, electrophoresis of the samples of interest on the copolymerized gel, and in-gel phosphorylation in the presence of [γ - 32 P]ATP. The advantage of this method is that it reveals the molecular weight of the kinases with the desired specificity, which helps to identify the enzymes of interest. Also, several samples can be examined simultaneously. The main disadvantages are that (1) not all protein kinases can be renatured in the SDS gel, (2) each in-gel assay takes ~3 days, and (3) there is a narrow linear range of protein kinase activities, which can interfere with detection of the increase in induction of protein kinases upon stimulation.

The Mono Q fractionation and in-gel kinase assay methods are mainly used to identify or characterize novel protein kinases. The resolution of these two methods is not always adequate, however, and more specific and convenient methods are recommended for the characterization of a given protein kinase. Such specific methods often require the isolation of the protein kinase of interest, although a specific activator or substrate can sometimes be used (as is the case with PKA or MEK). In studies of MAPKs, the desired protein kinases are often isolated by immunoprecipitation with specific antibodies directed to the C-terminal domain of the kinase or by immunoblot analysis with antibodies to the activated kinase (see

UNIT 14.2). Antibodies can also be used to detect slower migration on SDS-polyacrylamide gel electrophoresis (PAGE) that occurs upon phosphorylation of regulatory residues of some MAPKs. This gel shift does not always correlate with enzymatic activity, however, as was shown for ERK and for Raf1. Methods for affinity purification that do not involve antibodies can sometime be used to isolate given protein kinases (see Basic Protocol 3). Although affinity techniques (including immunoprecipitation) are often used, it should be noted that the attachment to a solid support that occurs in this method can interfere with the accurate detection of the kinase activity.

Critical Parameters

Several points should be considered when using immunoprecipitation followed by phosphorylation. One of the most important parameters for the success of this procedure is the method of protein extraction used. Since MAPKs are localized within cells, the cellular membranes must be disrupted to access the desired targets. The protein kinases of interest must then be obtained and preserved in their active form, and the amount of irrelevant kinases reduced. For example, activated Raf-1 can be present in mitochondrial membranes, which might not be disrupted by some extraction procedures but are disrupted if RadioImmunoPrecipitation Assay (RIPA) buffer is used for extraction. The method for cellular extraction described in Basic Protocol 1 can be effectively used to detect most MAPKs by immunoprecipitation or other methods. Sonication disrupts the plasma membrane but does not solubilize it; therefore the resulting extracts should contain both cytosolic and some nuclear fractions. Depending on the subcellular localization of the proteins of interest, other extraction methods can be used. For example, cellular extracts obtained with detergents such as Triton X-100 usually contain membrane, cytosolic, and some nuclear components. Cellular extracts obtained with RIPA buffer should contain solubilized proteins from most cellular compartments. Cellular extraction by the addition of hot SDS-PAGE sample buffer to cells is not recommended, because it may free chromatin, which causes formation of a gel that is hard to handle. Extraction by freeze/thawing is also not recommended, because molecular degradation can occur during thawing.

Another consideration for successful detection of phosphoproteins is minimization of protein degradation and dephosphorylation. Dur-

ing extraction, most cellular organelles break, exposing phosphoproteins to phosphatases and proteinases (also see UNIT 14.1). To minimize the effects of these enzymes, specific inhibitors of phosphatases and proteinases can be added to the extraction buffers and extraction can be performed at low temperature. Phosphatases are usually efficient enzymes, however, and extractions should be performed as fast as possible even if these precautions are taken.

The success of the immunoprecipitation protocol also depends on the quality and specificity of the antibodies used. The antibodies employed should recognize only the desired protein kinase, not isoforms or irrelevant enzymes. The antibodies should also not interfere with the enzymatic activity of the enzymes tested. In addition, the amount of protein in the different samples and the dilution of antibodies should be optimized to avoid nonspecific recognition of excess proteins. Stringent washing of the immunoprecipitates is necessary to reduce nonspecific precipitation of contaminating kinases. Washing may not completely prevent coimmunoprecipitation of protein kinases other than those desired, however, and these might interfere with the phosphorylation reaction. In this case, it may be necessary to use a specific substrate or direct assaying methods (e.g., in-gel kinase assay).

Other parameters that should be considered for accurate comparison of protein kinases are: (1) starvation of the cells before activation may interfere with activation of the desired protein kinase or may cause activation of some SAPKs; (2) the optimal length of stimulation may vary from cell to cell and from one protein kinase to the other; therefore, appropriate time points for each kinase should be determined; and (3) for accurate comparison of the activities of protein kinases, detection should be performed in the linear range of the phosphorylation reaction. Thus, the amount of protein used for immunoprecipitation, the concentration of antibodies, the length of the phosphorylation reaction, and the exposure to X-ray film or to the phosphoimager should be optimized in order to reach linearity. If necessary, a standard curve with the protein kinases of interest can be made, and serial dilutions of the cytosolic extracts or a time course of the phosphorylation can be used to ensure one is working in a linear range.

Anticipated Results

In Basic Protocol 1, which describes immunoprecipitation followed by phosphorylation, extracts from nonstimulated Rat1 cells contain

very little phosphorylated myelin basic protein (MBP; Fig. 14.3.2), which represents the activity of both ERK1 and ERK2 in these cells. Upon addition of EGF to the cells, the amount of phosphate incorporated into MBP should increase with time, peaking at 30 min after stimulation and declining thereafter. This pattern of phosphorylation represents the transient activation of MAPK by EGF in these cells. When the in-gel assay is used (Basic Protocol 2), both ERK1 and ERK2 should be detected at positions corresponding to molecular weights of 44 and 42 kDa, respectively, and the kinetics of activation of both should be similar to that of the MBP phosphorylation obtained with the immunoprecipitation. JNK activation (Basic Protocol 3) should peak at 30 min and decline thereafter.

Time Considerations

After cell harvesting, the immunoprecipitation protocol requires extraction (0.5 hr), immunoprecipitation and washing (3 to 4 hr), the phosphorylation reaction (0.5 to 1.0 hr), SDS-PAGE (2.5 hr), and processing of the gel (6 to 16 hr). Since this procedure usually takes more than a single working day, it can be interrupted after boiling the samples in sample buffer or at any time during the destaining period. The in-gel kinase assay can take 2 to 3 days and the JNK assay usually takes 24 hr, including SDS-PAGE processing.

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Labeling Cultured Cells with $^{32}\text{P}_i$ and Preparing Cell Lysates for Immunoprecipitation

UNIT 14.4

This unit describes $^{32}\text{P}_i$ labeling and lysis of cultured cells to be used for subsequent immunoprecipitation of proteins. The approach is appropriate, however, for labeling any cellular constituent with ^{32}P . This procedure is suitable for insect, avian, and mammalian cells and can be used with both adherent and nonadherent cultures. The same general approach—biosynthetic labeling with $^{32}\text{P}_i$ in medium containing a reduced concentration of phosphate—can also be applied to bacteria and yeast; however, specific techniques to accomplish this are not presented here. If this approach is used to label cellular constituents other than proteins, it will probably be necessary to modify the Basic Protocol beginning with step 9 (cell lysis).

The first procedure described (see Basic Protocol) is $^{32}\text{P}_i$ labeling of adherent or nonadherent (e.g., hematopoietic) cells with subsequent lysis in a detergent buffer containing either Nonidet P-40 (NP-40), 3[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), or a combination of NP-40, sodium deoxycholate and SDS (RadioImmunoPrecipitation Assay or RIPA buffer). More rigorous lysis conditions to be used for working with proteins that are difficult to solubilize are also described (see Alternate Protocol).

CAUTION: Unshielded ^{32}P will penetrate ~1 cm into flesh. Exposure to the skin and eyes is, therefore, of concern. Gloves and protective eyewear should always be worn when handling significant amounts of ^{32}P . A 1-in.-thick (2.5-cm) Plexiglas shield, tall enough to look through when seated or standing comfortably, should be used when handling samples containing ^{32}P . For more specific precautions, refer to the section on Safe Use of Radioisotopes (*APPENDIX 1D*).

LABELING CULTURED CELLS WITH $^{32}\text{P}_i$ AND LYSIS USING MILD DETERGENT

**BASIC
PROTOCOL**

The first six steps of this protocol describe culturing and labeling procedures for cultures of adherent cells; modifications appropriate for nonadherent cells are described in alternate steps. The remaining steps describe lysis of cells in detergent buffer to prepare the sample for immunoprecipitation. For a more detailed discussion of mammalian cell culture conditions and reagents see *UNITS 1.1* and *1.2*.

Materials

- Cell culture to be labeled
- Labeling medium: phosphate-free tissue culture medium (e.g., DMEM, *APPENDIX 2A*) supplemented with the usual concentration of serum or serum dialyzed against phosphate-free saline, 37°C
- 500 mCi/ml to 1 Ci/ml $\text{H}_3^{32}\text{PO}_4$ in HCl (carrier free ICN)
- Tris-buffered (TBS; see recipe) or Dulbecco's phosphate-buffered saline (DPBS; *APPENDIX 2A*), cold
- Mild lysis buffer or RIPA lysis buffer (see recipes)
- 1-in.-thick Plexiglas shield (*APPENDIX 1D*)
- Plugged, aerosol-resistant pipet tips
- Plexiglas box (*APPENDIX 1D*), warmed to 37°C
- Screw-cap microcentrifuge tubes
- Plugged disposable pipet or disposable one-piece transfer pipet

**Signal
Transduction:
Protein
Phosphorylation**

Rubber policeman

Sorvall refrigerated centrifuge with SM 24 rotor and rubber adaptors, refrigerated microcentrifuge, or equivalent, 4°C

Plexiglas sheet (10 × 10 × ¼-in.) or Plexiglas tube holder, 4°C (*APPENDIX 1D*)

Additional reagents and equipment for cell culture (*UNIT 1.1*) and gel electrophoresis (*UNIT 6.1*) or immunoprecipitation (*UNIT 6.2*)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and appropriate aseptic techniques should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

Label the cell culture

1. Culture the cells to be labeled to an appropriate stage of growth.

Phosphate transport is maximal in rapidly growing cells. Therefore, except in those cases where phosphorylation of a protein in quiescent cells is to be examined, cells to be labeled should be subconfluent (adherent cells) or at less than maximal density (nonadherent cells). It is useful to change the medium to fresh growth medium 3 to 18 hr prior to labeling.

Brief cultivation of cells in low-phosphate medium (to reduce the phosphate pool by starvation prior to labeling) is of minimal value.

For adherent cells

- 2a. Remove growth medium by aspiration. Wash away any residual phosphate-containing medium by adding 37°C labeling medium supplemented with serum, but lacking the label, and removing the wash medium by aspiration.

Phosphate-free DMEM is routinely used for labeling medium because RPMI has a very high concentration of phosphates.

- 3a. Add prewarmed labeling medium to cultures, using 0.5 to 1 ml per 35-mm dish, 1 to 2 ml per 50-mm dish, or 2 to 4 ml per 100-mm dish of adherent cells.

For nonadherent cells

- 2b. Gently centrifuge the culture 1 min at 1800 × g, room temperature, and aspirate the medium away from the cell pellet. Resuspend the cells in labeling medium supplemented with serum, but lacking the label. Centrifuge and remove the medium.

- 3b. Add 2 ml medium per 10⁷ cells and transfer to an appropriate size petri dish.

4. Working behind a Plexiglas shield, use a micropipettor with plugged, aerosol-resistant pipet tips to add ³²P_i to a final concentration of 0.1 to 2 mCi/ml.

Use of plugged, aerosol-resistant pipet tips will minimize contamination of the micropipettor. ³²P_i is usually shipped in HCl and is generally sterile, so it is not necessary to sterilize the labeling medium after adding the radioisotope. Additionally, except in the rare cases when steady-state labeling with ³²P is desired, the labeling interval is usually so short (<6 hr) that microbial growth resulting from added isotope is undetectable. Therefore, addition of ³²P to cells can be performed on the lab bench rather than in a tissue culture hood.

5. Place dishes in a warmed Plexiglas box, and put box in the incubator.

Labeling for 1 to 2 hr is usually sufficient, but cells will tolerate as much as 2 mCi/ml for 6 hr and lower concentrations (0.1 to 0.5 mCi/ml) for 18 hr.

Wash and lyse the cells

6. At the end of the labeling period, carry the labeled cells, still in the Plexiglas box, into the cold room. Place the cells behind a Plexiglas shield.

For adherent cells

- 7a. Take the dish out of the box and remove the labeling medium manually, using either a plugged disposable pipet or a disposable one-piece plastic Pasteur transfer pipet. Discard the medium and pipet as radioactive wastes.

Labeling medium should be removed without using a vacuum aspirator. Vacuum aspiration generates radioactive aerosols and leaves a radioactive film on the equipment.

- 8a. Wash cells once with 2 to 10 ml cold TBS. Remove the wash buffer manually, as in step 7, and discard as radioactive waste.

The uptake of phosphate is efficient and often a majority of the added $^{32}\text{P}_i$ is found within the labeled cells. This necessitates continued shielding of the labeled cells following removal of the labeling medium.

- 9a. Add lysis buffer to cells, using 0.3 ml per 35-mm dish, 0.6 ml per 50-mm dish, or 1.0 ml per 100-mm dish of adherent cells. Dislodge adherent cells by scraping with a rubber policeman, but leave the lysate in the dish. Incubate 20 min at 4°C. With a rubber policeman, scrape the lysate of adherent cells to the side of the dish and transfer lysate to a screw-cap microcentrifuge tube.

If a low background of nonspecific contaminants is critical, use RIPA lysis buffer. If maintenance of enzymatic activity or the structure of protein complexes is critical, use a milder lysis buffer containing either 3[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) or Nonidet P-40 (NP-40) as the only detergent. If complete solubilization of the cells and denaturation of the protein is desired, use SDS for lysis (see Alternate Protocol).

For nonadherent cells

- 7b. Take the dish out of the box, transfer the cells to a screw-cap centrifuge tube, pellet them by centrifugation (1 min at 1800 × g), and remove the medium.

Do not use a vacuum aspirator to remove medium.

- 8b. Resuspend pelleted cells gently in a small volume of cold TBS, transfer to a screw-cap microcentrifuge tube, and pellet the cells by microcentrifuging 1 min at 1800 × g.

Continue to use shielding with cells and lysate.

- 9b. Add 0.5 to 1 ml lysis buffer per 10^7 cells and resuspend the pellet by gentle agitation with a disposable plastic Pasteur pipet. Incubate 20 min at 4°C.

Choose the lysis buffer as in annotation to step 9a.

10. Cap the tube, and clarify the lysate by centrifuging 30 min at 26,000 × g (17,000 rpm in Sorvall SM24 rotor), 4°C.

Use an ice bucket with a sheet of Plexiglas over it, a chilled Plexiglas tube holder, or a prechilled centrifuge rotor to transport the tube of lysate.

It is best to half-fill the tube to prevent spilling. A Sorvall SM24 rotor with rubber adaptors for microcentrifuge tubes is ideal for clarifying lysates. Alternatively, a refrigerated microcentrifuge can be used for 30 min at maximum speed. Do not use a nonrefrigerated microcentrifuge in the cold room because the centrifuge will warm to 20°C during a 30-min spin.

*Lysates prepared with RIPA buffer often become viscous due to lysis of nuclei. If this occurs, increase the time of centrifugation to 90 min, or add 50 μl of fixed *Staphylococcus aureus* bacteria (Pansorbin, Calbiochem) in RIPA buffer to the lysate prior to centrifugation. Either modification will cause the solubilized DNA to pellet.*

11. After centrifugation, transfer the supernatant (lysate) to a new tube and discard the tube and pellet in radioactive waste.

If the pellet is very viscous, so that it is impossible to remove the supernatant cleanly, suck part of the pellet into a micropipet tip, lift the viscous material out of the tube, and discard it in the radioactive waste. The residual liquid in the tube is the supernatant and can be used for immunoprecipitation.

12. Analyze the labeled lysate using gel electrophoresis, immunoprecipitation, or protein purification. Carry out all analytical procedures at 4°C using adequate shielding.

ALTERNATE PROTOCOL

LYSIS OF CELLS BY BOILING IN SDS

Some proteins, such as eukaryotic RNA polymerase II, are difficult to solubilize with mild lysis buffer or RIPA lysis buffer, and some analytical procedures use antibodies that recognize epitopes exposed only in denatured proteins. In these cases, it is useful to solubilize labeled cells completely in SDS and then adjust the composition of the lysate solution to match that of RIPA buffer for immunoprecipitation. To avoid the formation of spurious disulfide bonds, lysis and washing during immunoprecipitation are carried out in the presence of fresh 1 mM dithiothreitol (DTT). The protocol describes the procedure for adherent cells; modifications for working with nonadherent cells are described as alternate steps.

Additional Materials (also see *Basic Protocol*)

SDS lysis buffer (see recipe)
RIPA correction buffer (see recipe)
Immunoprecipitate wash buffer (see recipe)
Fixed *Staphylococcus aureus* bacteria (Pansorbin, Calbiochem; optional)
Boiling water bath

1. Label and wash cells (see *Basic Protocol* steps 1 to 7).
- 2a. *For adherent cells:* Add SDS lysis buffer using 0.1 ml for a 35-mm dish, 0.25 ml for a 50-mm dish, or 0.5 ml for a 100-mm dish. Immediately scrape off the dish with a rubber policeman and transfer the cell lysate to a screw-cap microcentrifuge tube.
- 2b. *For nonadherent cells:* Vortex briefly to loosen the cell pellet, add 1 ml SDS lysis buffer per 5×10^7 cells and vortex again.
3. Boil the samples 2 to 5 min, then add 4 vol RIPA correction buffer and mix well.
4. Clarify the cell lysate by centrifuging 90 min at $26,000 \times g$ (17,000 rpm in Sorvall SM24), 4°C or at maximum speed, 4°C in a refrigerated microcentrifuge.

*Lysate may also be clarified by adding 50 μ l fixed *Staphylococcus aureus* and centrifuging 30 min at $26,000 \times g$, 4°C.*
5. Carry out immunoprecipitation as usual using immunoprecipitate wash buffer for washes.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Immunoprecipitate wash buffer for boiled sample

RIPA lysis buffer (see recipe)
1 mM DTT, added fresh
DTT is added from a 1 M stock solution stored at -20°C.

Mild lysis buffer

10 mM 3[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate
(CHAPS) or 1% (w/w) Nonidet P-40 (NP-40)

0.15 M NaCl
0.01 M sodium phosphate, pH 7.2 (*APPENDIX 2A*)
2 mM EDTA
50 mM sodium fluoride
0.2 mM sodium vanadate added fresh from 0.2 M stock solution
100 U/ml aprotinin (Trasylol, Pentex/Miles)
Store buffer without vanadate at 4°C up to 1 year

CHAPS is a milder detergent than NP-40, but yields precipitates with a higher background and may solubilize some proteins less efficiently.

Sodium vanadate stock solution can be stored in plastic at room temperature.

RIPA (RadioImmunoPrecipitation Assay) correction buffer for boiled sample

1.25% (w/w) Nonidet P-40 (NP-40)
1.25% (w/v) sodium deoxycholate
0.0125 M sodium phosphate, pH 7.2 (*APPENDIX 2A*)
2 mM EDTA
0.2 mM sodium vanadate added fresh from 0.2 M stock solution
50 mM sodium fluoride
100 U/ml aprotinin (Trasylol, Pentex/Miles)
Store buffer without vanadate at 4°C up to 1 year

Sodium vanadate stock solution can be stored in plastic at room temperature.

RIPA (RadioImmunoPrecipitation Assay) lysis buffer

1% (w/w) Nonidet P-40 (NP-40)
1% (w/v) sodium deoxycholate
0.1% (w/v) SDS
0.15 M NaCl
0.01 M sodium phosphate, pH 7.2 (*APPENDIX 2A*)
2 mM EDTA
50 mM sodium fluoride
0.2 mM sodium vanadate added fresh from 0.2 M stock solution
100 U/ml aprotinin (Trasylol, Pentex/Miles)
Store buffer without vanadate at 4°C up to 1 year

Sodium vanadate stock solution can be stored in plastic at room temperature.

SDS lysis buffer

0.5% (w/v) SDS
0.05 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1 mM DTT, added fresh

SDS and Tris·Cl solutions can be made in advance and stored at room temperature. DTT is added from a 1 M stock solutions stored at -20°C.

Tris-buffered saline (TBS)

Dissolve the following salts in 800 to 900 ml H₂O:
8 g sodium chloride (136.8 mM final)
0.38 g potassium chloride (5.0 mM final)
0.1 g calcium chloride (anhydrous; 0.9 mM final)
0.1 g magnesium chloride hexahydrate (0.5 mM final)
0.1 g dibasic sodium phosphate (anhydrous; 0.7 mM final)
Add 25 ml 1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*)
Add H₂O to 1 liter
Dispense 50- or 100-ml aliquots into glass bottles
Autoclave and store indefinitely at room temperature

COMMENTARY

Background Information

The extent to which a protein becomes radiolabeled via biosynthetic labeling with $^{32}\text{P}_i$ depends on the rate of transport of phosphate into the cells being labeled, abundance of the protein, stoichiometry of phosphorylation of the protein, and rate of phosphate turnover in the protein. The rate of turnover of the protein itself is less important because phosphate in protein usually turns over at a much faster rate than does the protein. Most of the phosphate incorporated into cells is not incorporated into phosphoproteins—the vast majority is incorporated into phospholipid, RNA, and DNA. The efficiency of incorporation of $^{32}\text{P}_i$ into proteins is therefore low—only ~1% of incorporated radioactivity is found in phosphoproteins.

An obvious and immediate question is how many cells and how much isotope should be used. The answer is that it depends on the protein being studied. If the protein is abundant (i.e., it constitutes 0.5% to 1% of total cellular protein) and highly phosphorylated (i.e., it contains 1 mole of phosphate per mole of protein), it can be labeled by incubating 10^6 cells with 0.2 to 0.5 mCi $^{32}\text{P}_i$ in 2 ml labeling medium for 1 hr. In contrast, if the protein is rare or non-abundant, it may be necessary to label 10^7 cells with 5 to 10 mCi of $^{32}\text{P}_i$ in 3 to 5 ml labeling medium for 4 to 6 hr. A pilot experiment can be useful: if 50 cpm are recovered in the protein of interest, use more cells and more isotope; if 200,000 cpm are recovered in the protein of interest, use fewer cells with less isotope.

Critical Parameters

Incorporation of $^{32}\text{P}_i$ during biosynthetic labeling is greatest if labeling is done in medium lacking any phosphate except the added radioisotope. Labeling can also be accomplished at a somewhat reduced efficiency in complete growth medium. Phosphate-free medium is commercially available but can also be easily formulated in-house by omitting sodium and potassium phosphate from a recipe and replacing them with either sodium chloride or potassium chloride or both. Serum dialyzed against phosphate-free saline can be used instead of complete serum to further reduce the level of phosphate in labeling medium. Dialysis of serum against phosphate-free saline reduces but never entirely eliminates the phosphate in serum. Washing or rinsing cells in reduced-phosphate medium just prior to labeling significantly increases labeling efficiency, but starva-

tion of cells by incubation in reduced-phosphate medium prior to labeling is of very limited value.

Labeling cells with ^{32}P almost certainly induces radiation damage in the labeled cells and will affect or arrest cell-cycle progression. If phosphorylation of the protein under study varies during the cell cycle, labeling with ^{32}P may alter its phosphorylation.

At the end of the labeling interval, the radioactive medium should be discarded, except in experiments where labeled virions or labeled secreted proteins are being studied. Labeling medium should be removed *without* using a vacuum aspirator. Vacuum aspiration generates radioactive aerosols and leaves a radioactive film inside the aspirator hose. As a result the hose can become an intense source of radiation exposure. It is best to use a plugged disposable pipet or disposable plastic Pasteur pipet for removing labeling medium. The medium and pipet should be discarded immediately as radioactive wastes.

In general, labeled cells are lysed and subjected to gel electrophoresis, immunoprecipitation, or protein purification. If the labeled cells are to be centrifuged, they should be transferred to capped, disposable centrifuge tubes. Tubes should be no more than half full if they are to be spun in a fixed-angle rotor; tubes that are too full may leak radioactive lysate and contaminate the centrifuge. Screw-cap microcentrifuge tubes are ideal for centrifuging lysates. If large volumes are being handled, multiple partially filled tubes are preferable to tubes filled to the top.

An ice bucket can be used for storage and transport of samples to keep the samples cold and provide considerable shielding. Radiation exposure from the tops of the tubes can be minimized by covering the ice bucket with a 1/4-in. Plexiglas sheet.

RadioImmunoPrecipitation Assay (RIPA) buffer (Brugge and Erikson, 1977) is the lysis buffer of choice because it solubilizes proteins well, gives a low background of nonspecific proteins, and is tolerated by most antigens and antibodies. It does however denature some antigens and disrupt some protein:protein complexes. If 1% (w/w) Nonidet P-40 (NP-40) is used as the only detergent, most of these problems are solved without increasing background unacceptably (Sefton et al., 1980). Some workers like to use digitonin as an extremely mild detergent for cell lysis. Digitonin sometimes is

tricky and idiosyncratic, giving erratic results and high backgrounds during immunoprecipitation. 3[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS; Chen et al., 1990) and Brij 96 (Osman et al., 1992) are mild detergents that appear to give more reproducible results.

Besides the usual concern about proteolysis following cell lysis, the cells must be handled carefully to prevent protein phosphorylation and dephosphorylation following lysis of the cells. Both problems can be minimized by proper formulation of the lysis buffer and by keeping the sample at 4°C. Inclusion of 2 mM EDTA in the lysis buffer will minimize phosphorylation in the lysate by chelating both Mg²⁺ and Mn²⁺, which are essential for protein kinase activity. Addition of phosphatase inhibitors to the lysis buffer will reduce dephosphorylation significantly. For example, 50 mM sodium fluoride is used to inhibit serine and threonine phosphatases, and 0.2 mM sodium vanadate is used to inhibit all known tyrosine phosphatases. Sodium vanadate appears to lose its effectiveness if it is stored diluted in lysis buffer, so it should be added fresh each time. The concentrated stock solution should be stored in plastic at room temperature.

Anticipated Results

This protocol can be used to label phosphoproteins or other phosphorylated cellular constituents. Phosphoproteins will contain ~1% of incorporated label.

Time Considerations

Proteins can and should be labeled biosynthetically with ³²P_i and isolated by immunoprecipitation in a single day. As is the case with all ³²P-labeled samples, it is important to work quickly because the specific activity of the sample declines 5% per day. If it is absolutely necessary to stop during the immunoprecipitation, it is best to leave the samples as precipitated pellets after aspiration of the wash buffer. Such samples can be stored at 4°C or -20°C overnight. Freezing the cell lysate tends to increase the background. Frozen lysates should be reclarified by centrifugation prior to immunoprecipitation.

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Phosphoamino Acid Analysis

UNIT 14.5

It is often valuable to identify the phosphorylated residue in a protein. In the case of proteins phosphorylated at serine, threonine, or tyrosine, this is readily accomplished by partial acid hydrolysis in HCl followed by two-dimensional thin-layer electrophoresis of the labeled phosphoamino acid (see Basic Protocol). Phosphothreonine and phosphotyrosine are more stable to hydrolysis in alkali than are RNA and phosphoserine. Therefore, mild alkaline hydrolysis of protein samples can be used to enhance the detection of phosphothreonine and phosphotyrosine (see Alternate Protocol).

Although this procedure can be carried out with a protein eluted from a preparative gel and concentrated by trichloroacetic acid (TCA) or acetone precipitation, it is most easily accomplished by transfer of the protein of interest to a PVDF membrane. This technique is obviously not ideal if the protein being studied does not transfer efficiently.

NOTE: Wear gloves and use blunt-end forceps to handle membranes.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety department (also see *APPENDIX 1D*).

ACID HYDROLYSIS AND TWO-DIMENSIONAL ELECTROPHORETIC ANALYSIS OF PHOSPHOAMINO ACIDS

**BASIC
PROTOCOL**

The protein to be acid hydrolyzed is transferred to a PVDF membrane using the same technique used for immunoblotting (*UNIT 6.2*) or for microsequencing. It is valuable, but not absolutely essential, to keep the filter wet following transfer. Following acid hydrolysis, phosphoamino acids are separated by two-dimensional thin-layer electrophoresis. Because electrophoresis equipment differs considerably in design, the details of the assembly and placement of the plate are not discussed here. It is assumed that a suitable apparatus is available for use by an experienced operator. Electrophoresis conditions are described for using the HTLE 7000 (CBS Scientific). They are almost certainly not correct for other equipment and will need to be altered according to the equipment manufacturer's directions.

Materials

³²P-labeled phosphoprotein (*UNIT 14.4*)

India ink solution: 1 μl/ml India ink in TBS (*UNIT 14.4*)/0.02% (v/v) Tween 20, pH 6.5 (prepare fresh or store indefinitely at room temperature); *or* radioactive or phosphorescent alignment markers

6 M HCl

Phosphoamino acid standards mixture (see recipe)

pH 1.9 electrophoresis buffer (see recipe)

pH 3.5 electrophoresis buffer (see recipe)

0.25% (w/v) ninhydrin in acetone in a freon (aerosol, gas-driven) atomizer/sprayer

PVDF membrane (Immobilon-P, Millipore)

110° oven

Screw-cap microcentrifuge tubes

20 cm × 20 cm × 100 μm glass-backed cellulose thin-layer chromatography plate (EM Sciences)

Large blotter: two 25 × 25-cm layers of Whatman 3MM paper sewn together at the edges, with four 2-cm holes that align with the origins on the TLC plate

**Signal
Transduction:
Protein
Phosphorylation**

Contributed by Bartholomew M. Sefton

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14.5.1

Supplement 3

Glass tray or plastic box
Whatman 3MM paper
Thin-layer electrophoresis apparatus (e.g., HTLE 7000, CBS Scientific)
Fan
Small blotters: 4 × 25-cm, 5 × 25-cm, and 10 × 25-cm pieces of Whatman 3MM paper
50° to 80°C drying oven
Sheets of transparency film for overhead projector
Additional reagents and equipment for SDS-PAGE (UNIT 6.1), immunoblotting (UNIT 6.2), and autoradiography (UNIT 6.3)

Prepare sample

1. Run radiolabeled phosphoprotein on a preparative SDS-polyacrylamide gel (UNIT 6.1).

It is difficult to obtain good results with <100 cpm of labeled protein.

2. Transfer proteins electrophoretically to a PVDF membrane (UNIT 6.2). Wash the membrane several times with water. Do not let the membrane dry.

These washes remove buffer and detergent.

3. Locate the band of interest by staining the filter 5 to 10 min in 30 to 50 ml India ink solution with shaking until bands are detectable, *or* by wrapping the filter in plastic wrap, applying radioactive or phosphorescent alignment markers, and performing autoradiography (UNIT 6.3).

4. Excise the piece of filter containing the band of interest with a clean razor blade. Rewet the piece of filter with methanol for 1 min and then rewet it in >0.5 ml water. Place the piece of filter paper in a screw-cap microcentrifuge tube.

Keep the excised piece as small as possible.

Hydrolyze sample

5. Add enough 6 M HCl to submerge the piece of filter. Screw the cap on the tube tightly and incubate 60 min in 110°C oven.

6. Let cool. Microcentrifuge 2 min at maximum speed, room temperature. Transfer the liquid hydrolysate to a fresh microcentrifuge tube and dry with a Speedvac evaporator.

Drying takes ~2 hr. Simultaneous drying of the hydrolysate and deblocked oligonucleotides in NH₄OH must be avoided, as this will generate a cloud of ammonium chloride that will collect in the centrifuge tube and render the hydrolysate unsuitable for thin-layer electrophoresis.

7. Dissolve the sample in 6 to 10 µl water by vortexing vigorously. Microcentrifuge 5 min at maximum speed.

Prepare plate for first-dimension electrophoresis

8. Spot 25% to 50% of the sample, in 0.25- to 0.50-µl aliquots, on one origin of a 20 cm × 20 cm × 100 µm glass-backed cellulose thin-layer chromatography plate (see Fig. 14.5.1 for arrangement of samples). Between each application, dry the sample spot with compressed air delivered through a Pasteur pipet plugged with cotton.

Use long, thin plastic micropipet loading tips for loading, and do not let the tip touch the plate.

Four samples can be analyzed simultaneously. The complete hydrolysate can be spotted on a single origin, but some streaking in the first dimension may be observed due to overloading. This problem can be avoided by using a fraction of the sample.

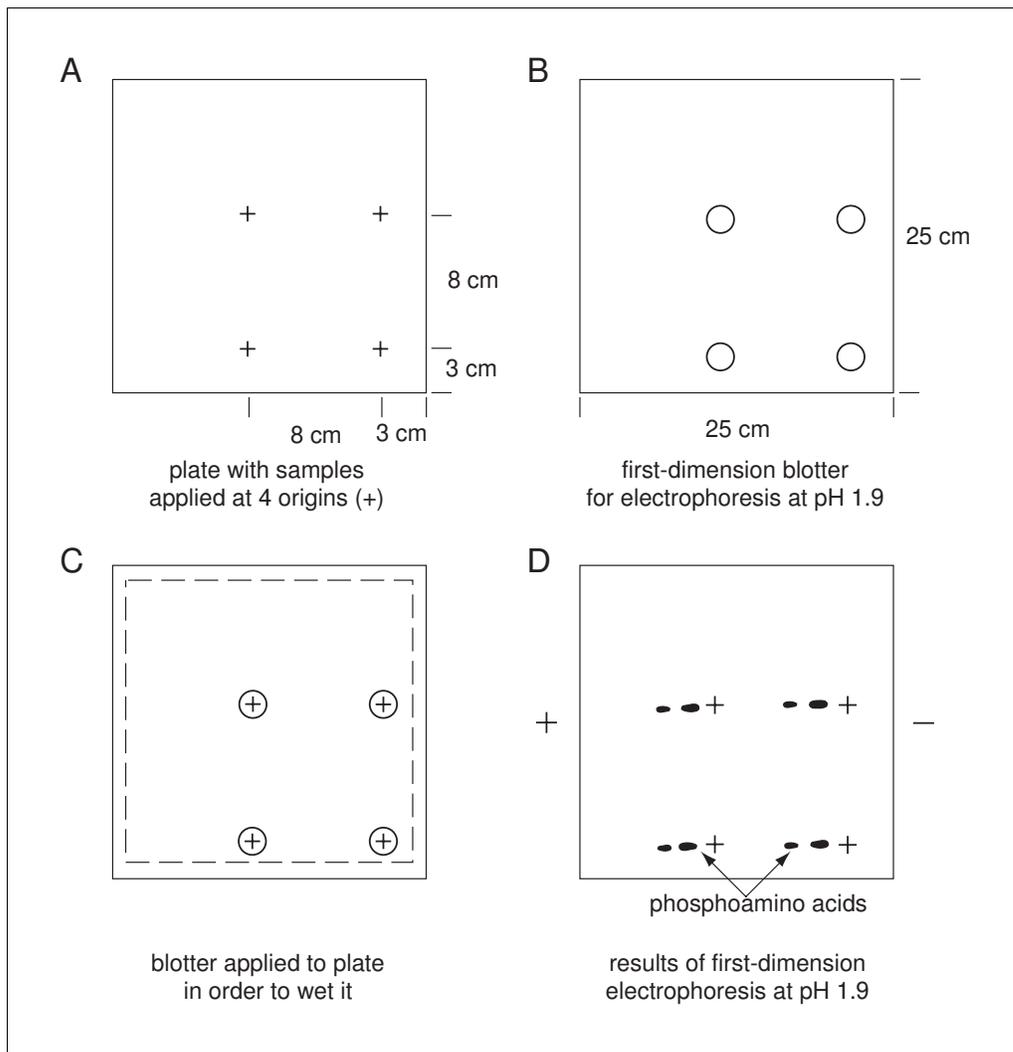


Figure 14.5.1 First-dimension electrophoretic separation of phosphoamino acids at pH 1.9. **(A)** Positions of the four origins on a single 20 × 20-cm plate; **(B)** blotter used for wetting the plate with pH 1.9 electrophoresis buffer; **(C)** placement of the blotter on the plate (underneath; indicated by dashed outline); and **(D)** orientation of the plate between the + and – electrodes with the positions of the phosphoamino acids after electrophoresis.

9. Spot 1 μl nonradioactive phosphoamino acid standards mixture (containing phosphoserine, phosphothreonine, and phosphotyrosine) on top of each sample in 0.25- to 0.50- μl aliquots as above.
10. Wet the large blotter (with four holes) by submerging it in pH 1.9 electrophoresis buffer in a large glass tray or plastic box. Briefly allow the excess buffer to drain off. Lower the wet blotter onto the prespotted plate with the origins on the plate in the centers of the four holes in the blotter (Fig. 14.5.1). Press on the blotter gently to achieve even wetting of the cellulose and concentration of the samples. When the plate is uniformly wet, remove the blotter.

The blotter should be quite damp but not sopping wet. Excess buffer can be wicked off onto filter paper.

Areas of the plate that are too dry can be seen through the blotter and will appear to be whiter than the rest of the plate. If this happens, dab the blotter with a Kimwipe wetted with pH 1.9 electrophoresis buffer. If there are puddles of buffer on the plate, let them dry before carrying out electrophoresis.

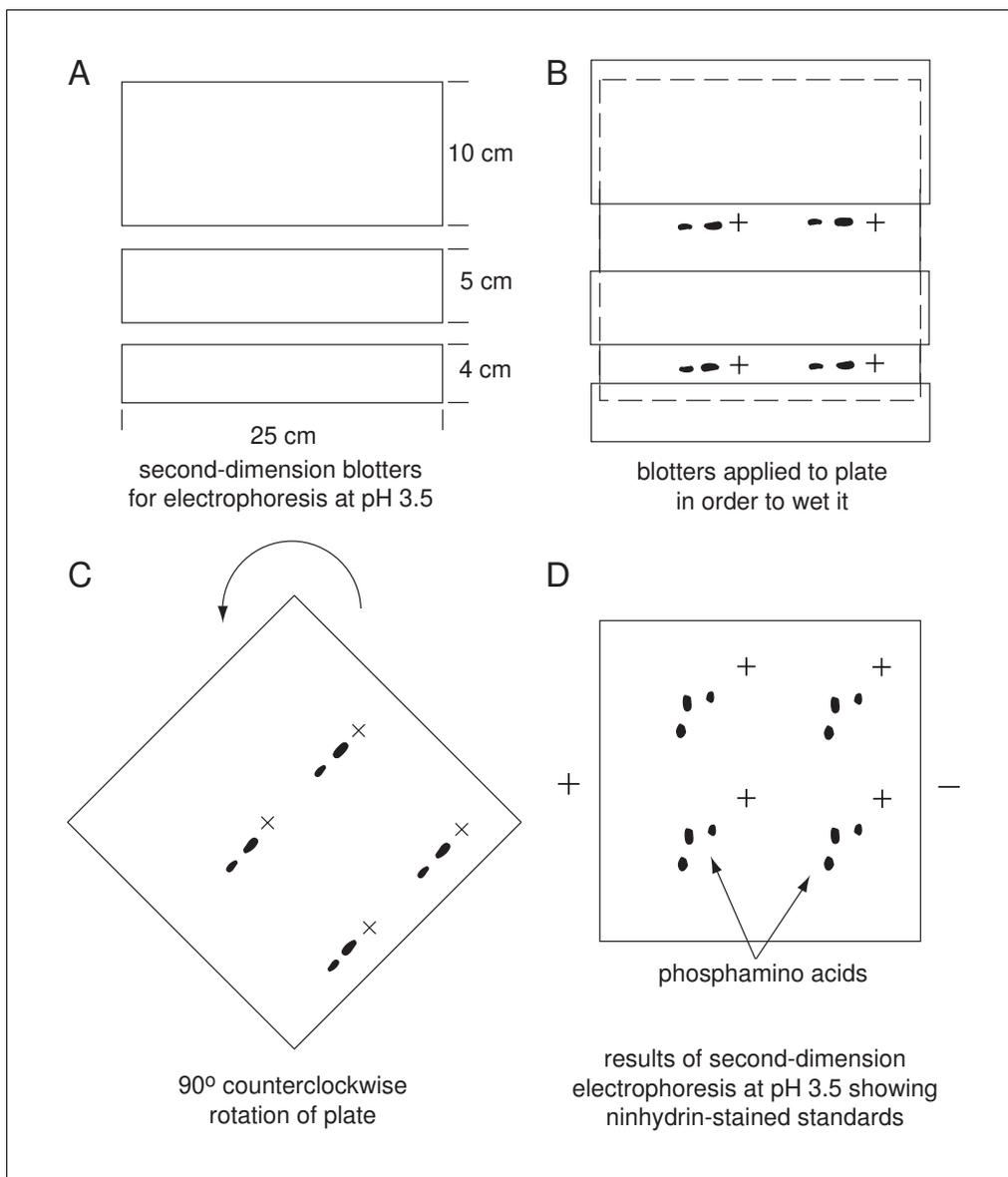


Figure 14.5.2 Second-dimension electrophoretic separation of phosphoamino acids at pH 3.5. **(A)** The three pieces of Whatman 3MM paper used for wetting the plate with pH 3.5 electrophoresis buffer; **(B)** proper placement of the blotters on the plate (underneath; indicated by dashed outline); **(C)** reorientation of the plate for electrophoresis in the second dimension; and **(D)** orientation of the plate between the + and - electrodes with the position of the phosphoamino acids after electrophoresis.

- Place the thin-layer plate in the electrophoresis apparatus and overlap 0.5 cm of the right and left sides of the plate with wicks made of Whatman 3MM paper. If the apparatus has an air bag, be sure to inflate it. Close the cover and start electrophoresis. With an HTLE 7000, double-thickness Whatman 3MM wicks, and a plate with four samples, electrophorese 20 min at 1.5 kV.

For the HTLE 7000 apparatus, use folded-over Whatman 3MM wicks that are 20 cm wide (the same as the plate) and not overly wet. Overly wet wicks will flood the plate and cause sample diffusion.

For other electrophoresis apparatuses the appropriate duration of electrophoresis can be determined empirically by examining the rate of migration of the phosphoamino acid standards.

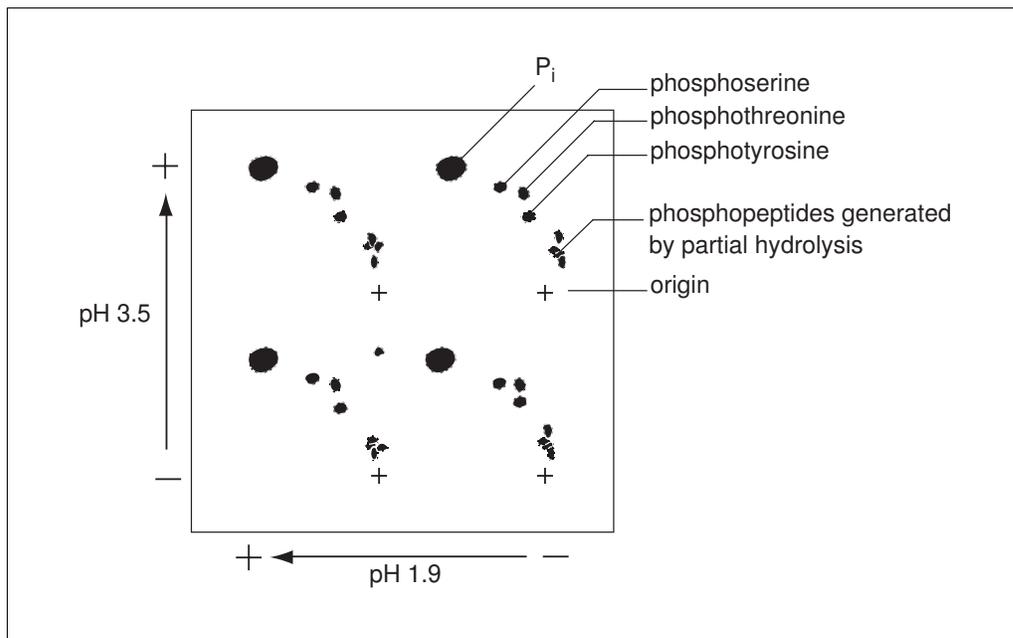


Figure 14.5.3 Hypothetical autoradiogram of a two-dimensional separation. Four samples of acid-hydrolyzed, ^{32}P -labeled proteins are applied at the origins, one in each of the four quadrants. This diagram shows the origins, the directions of electrophoresis, the positions of phosphoserine, phosphothreonine, and phosphotyrosine, the position of P_i , and the position of partially hydrolyzed fragments of the proteins for the upper right-hand sample. Every protein generates different partial hydrolysis peptide fragments.

12. Following electrophoresis, remove the plate and quickly air dry with a fan without heating.

It takes ~20 min to dry the plate.

Perform second-dimension electrophoresis

13. Wet the small blotters in pH 3.5 electrophoresis buffer and use them to wet the plate using the method described in step 10 to achieve even wetting without puddling (Fig. 14.5.2).

After electrophoresis at pH 1.9, phosphoamino acids are present as a streak extending from the origin towards the + electrode. Blotters are not applied directly over the phosphoamino acids to prevent sample blurring or smearing.

14. Remove the blotters, rotate the plate 90° counterclockwise, and electrophorese 16 min at 1.3 kV in pH 3.5 electrophoresis buffer if using the HTLE 7000 apparatus.
15. At the end of the electrophoresis run, remove the plate and dry 20 to 30 min in an oven at 50° to 80°C. When dry, spray with 0.25% ninhydrin in acetone, then reheat in the oven 5 to 10 min to visualize the phosphoamino acid standards.
16. Place radioactive or phosphorescent alignment marks on the plate and autoradiograph with an intensifying screen overnight to 10 days at -70°C.
17. Following autoradiography, trace the alignment markers and the stained phosphoamino acid markers onto a transparent sheet used for overhead projectors. Save this template. Align the film with the plate and identify radioactive phosphoamino acids (Fig. 14.5.3).

Use of fluorography or autoradiography to detect the labeled phosphoamino acids is preferable to use of a phosphorimager. The image on film is precisely the same size as the thin-layer plate, which allows the transparent film to be overlaid on the plate for an unambiguous spot identification. A phosphorimager can subsequently be used for quantification.

**ALKALI TREATMENT TO ENHANCE DETECTION OF TYR- AND
THR-PHOSPHORYLATED PROTEINS BLOTTED ONTO FILTERS**

Phosphothreonine and phosphotyrosine are much more stable to hydrolysis in alkali than RNA or phosphoserine. Detection of proteins containing phosphothreonine and phosphotyrosine in impure samples containing ^{32}P -labeled RNA and serine-phosphorylated proteins can often be enhanced by mild alkaline hydrolysis of gel-fractionated samples. Although this technique was first developed for the treatment of fixed polyacrylamide gels, it is much more easily performed with proteins that have been first transferred to a PVDF membrane.

Alkaline hydrolysis does not preclude subsequent phosphoamino acid analysis. A band from a blot that has been treated with alkali can be excised and subjected to acid hydrolysis as described in the Basic Protocol.

Additional Materials (also see Basic Protocol)

1 M KOH

TN buffer: 10 mM Tris·Cl (pH 7.4 at room temperature)/0.15 M NaCl

1 M Tris·Cl, pH 7.0 at room temperature

Covered plastic container (e.g., Tupperware box)

55°C oven or water bath

1. Run radiolabeled sample on a preparative SDS–polyacrylamide gel and transfer proteins electrophoretically to a PVDF membrane (see Basic Protocol steps 1 and 2).

A band containing as few as 10 cpm is detectable under optimal conditions with this technique.

A nylon membrane may be used in place of a PVDF membrane, but in that case, the bands cannot subsequently be analyzed by acid hydrolysis, as nylon membrane will dissolve in 6 M HCl.

2. Wash membrane thoroughly with water: three 2-min incubations in 1 liter water are sufficient.

These washes remove buffer and detergent.

3. Incubate membrane 120 min at 55°C in an oven or water bath in sufficient 1 M KOH to cover the filter in a covered Tupperware container.

4. Discard KOH. Wash membrane and neutralize remaining KOH by rinsing once for 5 min in 500 ml TN buffer, once for 5 min in 500 ml of 1 M Tris·Cl (pH 7.0), and twice for 5 min in 500 ml water. Wrap the membrane in plastic wrap and autoradiograph (UNIT 6.3) overnight with flashed film and an intensifying screen at -70°C .

Identification of the band of interest is most easily accomplished by coelectrophoresis of a radioactive marker protein of known identity.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

pH 1.9 electrophoresis buffer

50 ml 88% formic acid (0.58 M final)

156 ml glacial acetic acid (1.36 M final)

1794 ml H_2O

Store indefinitely in a sealed bottle at room temperature

pH 3.5 electrophoresis buffer

100 ml glacial acetic acid (0.87 M final)

10 ml pyridine [0.5% (v/v) final]

10 ml 100 mM EDTA (0.5 mM final)

1880 ml H₂O

Store indefinitely in a sealed bottle at room temperature

Phosphoamino acid standards mixture

Prepare a solution of phosphoserine, phosphothreonine, and phosphotyrosine (Sigma) in water at a final concentration of 0.3 mg/ml each. Store in 1-ml aliquots indefinitely at -20°C .

COMMENTARY

Background Information

Phosphoamino acid analysis by the two-dimensional electrophoretic technique described in the basic protocol was first carried out with proteins isolated by elution from unfixed SDS-polyacrylamide gels (Hunter and Sefton, 1980). However, this technique is laborious, especially if it involves grinding up pieces of high-percentage acrylamide gels, and the yields can be disappointing. Additionally, because the eluted protein must be precipitated in the presence of a carrier protein, spotting the whole sample on a single origin usually yields a badly smeared pattern. The grind-and-elute technique is, however, advantageous with proteins that are very refractory to electrophoretic transfer to PVDF membranes.

The alkaline treatment of protein described in the alternate protocol was first developed by Jon Cooper and Tony Hunter, who treated fixed gels with KOH (Cooper and Hunter, 1981). The original technique is tricky because the gel becomes extremely sticky during incubation with KOH and swells. Additionally, the manipulations needed to recover proteins from the gel following treatment are very involved because the proteins are contaminated with products of the hydrolysis of polyacrylamide.

Critical Parameters and Troubleshooting

It is essential to use PVDF membranes to immobilize proteins for acid hydrolysis rather than nylon or nitrocellulose membranes, both of which dissolve in 6 M HCl. Proteins immobilized on either PVDF or nylon membranes may be subjected to alkaline hydrolysis with KOH (Contor et al., 1987), but nitrocellulose membranes are not suitable. Proteins immobilized on nylon cannot subsequently be analyzed by acid hydrolysis because nylon is dissolved by 6 M HCl.

Two-dimensional thin-layer electrophoresis is required for unambiguous identification of phosphorylated residues, as some spots after one-dimensional electrophoresis do not represent pure species. For example, uridine monophosphate, which is generated during acid hydrolysis of RNA (a frequent contaminant of phosphoproteins), comigrates with phosphotyrosine during one-dimensional electrophoresis at pH 3.5.

Streaking of the sample in the first dimension is a symptom of overloading, either with the phosphoprotein itself or with contaminants in the sample. This problem can be corrected by loading less sample. Streaking in the second dimension is usually the result of problems with wetting or running the plate and cannot be corrected by loading less sample.

Some batches of blotting paper contain calcium, which interferes with electrophoresis of phosphoamino acids (probably by precipitating them). In the author's experience Whatman 3MM paper is quite reliable; other blotting papers are probably suitable as well. Inclusion of EDTA in the pH 3.5 buffer alleviates this problem.

This unit calls for glass-backed cellulose thin-layer plates rather than the plastic-backed variety, which are lighter and less expensive. This is because plastic-backed plates can under some circumstances cause sample streaking. They are, however, probably satisfactory for most experiments. If use of plastic-backed plates results in streaking, try glass-backed plates to see if that corrects the problem.

Anticipated Results

To detect a phosphoamino acid by autoradiography, a minimum of 10 cpm must be spotted and the plate exposed for a week with flashed film and an intensifying screen. Only 15% to 20% of the radioactivity in a phospho-

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protein is recovered as phosphoamino acids. The majority is present as $^{32}\text{P}_i$, which is released by dephosphorylation of phosphoamino acids, with the remainder being peptide products resulting from partial acid hydrolysis. As a result, the thin-layer plates will contain a number of radioactive spots that are not phosphoamino acids (see Fig. 14.5.3). Partial hydrolysis products remain near the origin during electrophoresis at pH 1.9, but exhibit some mobility at pH 3.5 (see Fig. 14.5.3). After two-dimensional electrophoresis, they are found above the origin and below the phosphoamino acids. $^{32}\text{P}_i$ has a high mobility at both pH 1.9 and pH 3.5 and is found in the upper left-hand corner of each quadrant of the plate (see Fig. 14.5.3). Because of these additional radioactive spots, it is essential to localize internal phosphoamino acid standards by staining with ninhydrin.

Time Considerations

After the preparative gel has been run and the protein transferred to the membrane, isolation of the membrane fragment containing the protein, followed by acid hydrolysis, takes <2 hr. Two to three hours are required for drying the sample with a Speedvac evaporator. First- and second-dimension electrophoresis and staining the internal phosphoamino acid standards takes no more than ~2 hr. Overnight autoradiographic exposure with flashed film and an intensifying screen is usually sufficient; however, exposures can be carried out for up to 10 days before the background from the screen becomes objectionable.

Detection of phosphorylated protein after alkali treatment, as described in the alternate protocol, is a very quick procedure requiring little more time than it takes to carry out the alkaline hydrolysis. In general, samples of this sort can be detected after overnight exposure with flashed film and an intensifying screen at -70°C .

Literature Cited

- Contor, L., Lamy, F., and Lecocq, R.E. 1987. Use of electroblotting to detect and analyze phosphotyrosine containing peptides separated by two-dimensional gel electrophoresis. *Anal. Biochem.* 160:414-420.
- Cooper, J.A. and Hunter, T. 1981. Four different classes of retroviruses induce phosphorylation of tyrosines present in similar cellular proteins. *Mol. Cell. Biol.* 1:394-407.
- Hunter, T. and Sefton, B.M. 1980. The transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. U.S.A.* 77:1311-1315.

Key Reference

- Kamps, M.P. and Sefton, B.M. 1989. Acid and base hydrolysis of phosphoproteins bound to Immobilon facilitates the analysis of phosphoamino acids in gel-fractionated proteins. *Anal. Biochem.* 176:22-27.

Discusses all of the variables involved in subjecting filter-bound proteins to acid and base hydrolysis.

Contributed by Bartholomew M. Sefton
The Salk Institute
San Diego, California

Determination of Akt/PKB Signaling

UNIT 14.6

Akt—also known as protein kinase B (PKB)—is a central regulator of cell survival (Franke et al., 1997; Hemmings, 1997; Marte and Downward, 1997), and its activity is often used to assess the apoptotic effect of different experimental conditions. This Ser/Thr protein kinase is activated downstream of phosphoinositide 3-kinase (PI3K) in response to a wide variety of growth factors (Fig. 14.6.1). The activated form of the kinase targets a specific set of effector proteins involved in cell-survival signaling. The activation involves membrane translocation and dual phosphorylation on threonine at position 308 and serine at position 473. Because the activation of Akt depends on this phosphorylation, it is possible to assess Akt activity not only with a kinase assay but also by determining its level of phosphorylation.

This unit provides a protocol for assaying the phosphorylation and the dynamics of dephosphorylation of Akt/PKB in cultured cells (see Basic Protocol). A protocol is also provided for assaying membrane translocation in response to Akt activation (see Support Protocol).

DETERMINATION OF Akt/PKB SIGNALING IN CULTURED CELLS

This protocol can be applied to nearly all cultured cell lines with few or no modifications. In this particular example, the chosen cell system involves cells expressing or deficient in the $\beta 1$ integrin because of the links between integrin signaling, Akt activation, and cell survival. This protocol describes PDGF stimulation of GD25 $\beta 1$ integrin-null cells (Fassler et al., 1995) expressing wild-type $\beta 1$ integrin or its mutant variant W/A, which is deficient in Akt signaling (Pankov et al., 2003). Specific treatments and time-course sampling of cultured cells are described; these allow determination of the phosphorylation level of Akt, its sensitivity to growth factor stimulation, and the mode of its inactivation by dephosphorylation. All these results can be obtained in a single nonradioactive experiment by using standard techniques such as immunoblotting (UNIT 6.2) and immunodetection with phosphospecific antibodies (UNIT 14.2). Cell cultures are serum-starved, stimulated with growth factor, and maintained for 2 hr after the stimulation for assessing the dynamics of Akt dephosphorylation. Samples are taken after each treatment, and Akt activity is determined with phosphospecific antibodies.

Materials

- GD25 cells obtained from R. Fassler, Max Planck Institute of Biochemistry, Martinsried, Germany, and expressing wild-type $\beta 1$ integrin ($\beta 1$ cells) or integrin mutant W/A (mutant cells)
- Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (DMEM/10% FBS; APPENDIX 2A)
- Starvation medium: DMEM containing 1% bovine serum albumin (DMEM/1% BSA)
- 0.5 μ M okadaic acid (OA; 1 mM stock solution in DMSO, APPENDIX 1B) in DMEM/1% BSA
- Platelet-derived growth factor-BB (PDGF-BB), freshly prepared at 10 μ g/ml in 10 mM acetic acid
- AG 1433 (e.g., Calbiochem) PDGF kinase inhibitor, 10 mM stock solution in dimethyl sulfoxide (DMSO)
- PBS (APPENDIX 2A), ice cold
- Modified radioimmunoprecipitation assay (mRIPA) buffer (see recipe), ice cold
- 2 \times SDS sample buffer (APPENDIX 2A)
- 10% separating gels with 4% stacking gels (UNIT 6.1)

BASIC
PROTOCOL

Signal
Transduction:
Protein
Phosphorylation

Contributed by Roumen Pankov

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14.6.1

Supplement 22

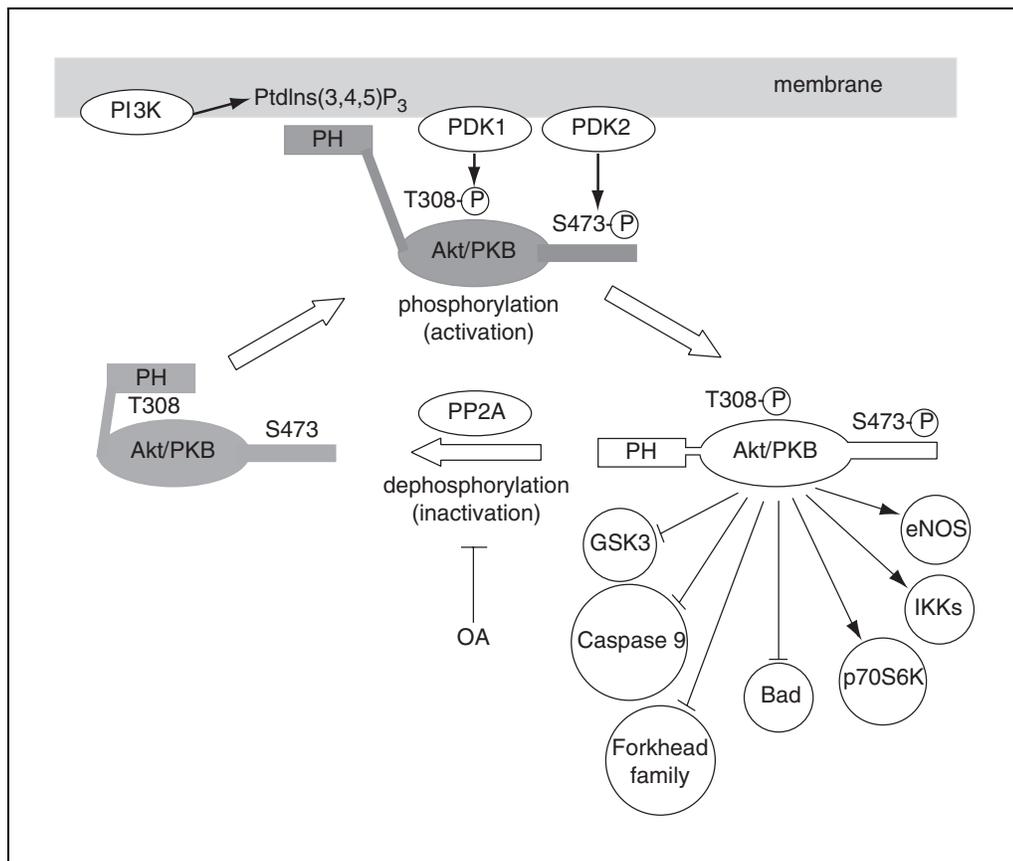


Figure 14.6.1 Activation of Akt/PKB and its effectors. Akt is activated by phosphatidylinositol 3-kinase (PI3K) products PtdIns(3,4,5)P₃ and PtdIns(3,4,)P₂. These phosphoinositides target inactive Akt to the plasma membrane via its pleckstrin homology (PH) domain. At the membrane, Akt is phosphorylated on two residues: threonine 308 (T308) by phosphoinositide-dependent kinase 1 (PDK1) and serine 473 (S473) by an unidentified PDK2. The dual phosphorylation is necessary for full activation of Akt. Activated Akt in turn phosphorylates its targets, thus modulating their function. Akt effectors include glycogen synthase kinase-3 (GSK3), caspase 9, Forkhead family of transcription factors, Bad, p70 ribosomal S6 kinase (p70S6K), IκB kinases (IKKs), and endothelial nitric oxide synthase (eNOS). Akt is inactivated through dephosphorylation by protein phosphatase 2A (PP2A), and this process can be blocked by okadaic acid (OA).

Pre-stained protein standards (e.g., Novex)

Transfer buffer (UNIT 6.2)

Ponceau S solution (UNIT 6.2)

Tris-buffered saline with 0.1% Tween 20 (TTBS, APPENDIX 2A)

Blocking solution: TTBS containing 5% dry nonfat milk (TTBS/milk)

Primary antibodies: polyclonal anti-phospho Akt (Ser 473) (e.g., Cell Signaling, Biosource), monoclonal anti-actin (e.g., Sigma)

Dry nonfat milk

Secondary horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibodies (e.g., Amersham Biosciences)

Enhanced chemiluminescence (ECL) detection reagent (UNIT 14.2)

35-mm tissue culture dishes

Plastic cell scraper (rubber policeman)

1.5-ml microcentrifuge tubes, prechilled

Sonicator/ultrasonic processor

Boiling water bath

Two nitrocellulose membranes cut to gel size

Determination of Akt/PKB Signaling

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Four Whatman 3MM filter papers cut to gel size
SDS-PAGE/transfer apparatus (e.g., Bio-Rad, Novex)
Constant-voltage/current power supply (e.g., Bio-Rad)
Flat containers for washing gels and membranes
Shaker
Heat-sealable plastic bags
Heat sealer
X-ray film (e.g., Hyperfilm; Amersham Biosciences)
Film cassette for X-ray film
X-ray film developer

Additional reagents and equipment for tissue culture (*UNIT 1.1*), SDS-PAGE (*UNIT 6.1*), and immunoblotting (*UNIT 6.2*)

NOTE: All tissue culture incubations should be performed in a humidified 37°C, 10% CO₂ incubator. Use pre-warmed cell culture medium for all treatments.

Treat cells

1. Plate twelve 35-mm tissue culture dishes each of GD25 cells expressing wild-type $\beta 1$ integrin and mutant cells (1.0×10^6 cells/dish) in 2 ml DMEM/10% FBS and allow them to attach and spread for 3 to 4 hr.
2. Wash dishes with 2 ml starvation medium two times, then add 2 ml/dish of the starvation medium and serum-starve the cells overnight.

At this point the dishes should be ~80% confluent. Depending on the cell type being used, adjustment of the initial number of plated cells may be necessary.

3. Treat half of the dishes (six dishes of $\beta 1$ cells and six dishes of mutant cells) with 0.5 μ M okadaic acid (OA) (protein phosphatase inhibitor) in DMEM/1% BSA for 15 min. Label the dishes "OA". Maintain the same concentration of OA in the medium to be used to treat these dishes throughout the entire experiment. To ensure similar treatment, dispense 25 ml of pre-warmed DMEM/1% BSA and add 12.5 μ l of 1 mM okadaic acid stock solution to the 25 ml of medium to be used for the "OA" sets of dishes. Add 12.5 μ l of DMSO to the other 25 ml medium for the untreated sets.

After this treatment, four sets of six samples are formed (two for each cell line). These are untreated cells of each $\beta 1$ cells ($\beta 1$) and W/A mutant cells (mutant), and okadaic acid-treated $\beta 1$ cells ($\beta 1/OA$) and mutant cells (mutant/OA).

4. Aspirate the starvation medium and stimulate cells on five dishes of each set with 20 ng/ml PDGF-BB in 2 ml DMEM/1% BSA for 15 min. Leave four dishes, one from each set ($\beta 1$, $\beta 1/OA$, W/A, and W/A/OA) without stimulation in fresh DMEM/1% BSA and label them as controls. To ensure similar treatment, dispense 40 ml of pre-warmed DMEM/1% BSA and add 80 μ l of 10 μ g/ml stock solution of PDGF-BB, mix well, divide into two 20-ml aliquots, and add 10 μ l of 1 mM okadaic acid stock solution to the 20 ml of medium to be used for the "OA" sets of dishes. Add 10 μ l of DMSO to the other 20 ml of PDGF-containing medium.
5. Rinse all dishes one time with DMEM/1% BSA. Put aside the control dishes and one of each PDGF-stimulated dishes ($\beta 1$, $\beta 1/OA$, W/A, and W/A/OA) for lysis. To the remaining four dishes of each set, add 2 ml/dish of the same medium containing 30 μ M AG 1433 PDGF kinase inhibitor (four of the $\beta 1$ and four of the mutant PDGF-stimulated dishes). Treat the "OA" set of dishes (four of the $\beta 1/OA$ and four of the W/A/OA PDGF-stimulated dishes) with the same AG 1433-containing medium supplemented with 0.5 μ M okadaic acid. Incubate the AG 1433-containing dishes for 30, 60, 90, and 120 min at 37°C. Specifically, dispense 40 ml of pre-warmed

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DMEM/1% BSA and add 120 μ l of 10 mM stock solution of AG 1433, mix well, and divide into 20-ml aliquots and add 10 μ l of 1 mM okadaic acid stock solution to the 20 ml of medium to be used for the “OA” sets of dishes. Add 10 μ l of DMSO to the other 20 ml of AG 1433-containing medium.

AG 1433 is used to eliminate residual receptor kinase activity after the PDGF stimulation and to identify the rate of dephosphorylation.

Prepare cellular lysates

6. Rinse the dishes that were put aside for lysis (control and PDGF-stimulated β 1, β 1/OA, W/A, and W/A/OA) with 2 ml ice-cold PBS two times. Keep the dishes on ice, and use ice-cold buffers to slow down biological processes and prevent protein degradation.
7. Add 100 μ l/dish of ice-cold mRIPA buffer plus inhibitors, scrape the cells with a plastic scraper or rubber policeman, and transfer the lysate into prechilled and prelabeled 1.5-ml microcentrifuge tubes. Incubate the lysates for an additional 10 min on ice.

Prepare gel samples

8. Add 100 μ l/sample of 2 \times SDS sample buffer, sonicate (two 5-sec, 50-W pulses) on ice.

The samples will become quite viscous after the addition of the SDS sample buffer. It is necessary to shear the released DNA by sonication to eliminate the viscosity and to allow correct loading of the samples on SDS-PAGE gels.

9. Boil samples in a water bath for 3 min or heat at 95°C for 5 min on a heating block.
10. Remove the PDGF-stimulated and AG 1433–treated β 1, β 1/OA, W/A, and W/A/OA dishes after 30, 60, 90, and 120 min.
11. Rinse with 2 ml ice-cold PBS two times and repeat steps 7, 8, and 9 for the samples at each timepoint.

The samples can be stored sealed and frozen at least 1 month at –20°C.

Separate samples by SDS-PAGE

12. Cast two 10% separating gels with 4% stacking gels (UNIT 6.1).

Use 15-well combs so that all 24 samples can be loaded on two gels.

13. Load 20 μ l of each sample/gel lane and a separate sample containing prestained protein standards on the gel.

Load each set of samples starting with the control sample, followed by the PDGF-stimulated sample, taken immediately after the stimulation, followed by the samples that have been kept in AG 1433–containing medium for 30, 60, 90, and 120 min for each sample set. Load the prestained protein standards into the first or the last lane so that the orientation of the gel can be identified after it is removed from the apparatus. Fill the empty lanes of the gels with 1 \times SDS sample buffer to prevent distortion of the separation in the adjacent lanes.

14. Run the gels at 150 V until the bromophenol blue dye reaches the bottom of the gel (see UNIT 6.1).

Transfer separated proteins from gel to membrane

15. When the electrophoresis is complete, remove the gels from the gel plates, cut off the stacking gels and incubate the separating portion of the gels in 50 ml transfer buffer for 15 min.

Use gloves to handle the gels and membranes since oils from hands can block the transfer.

16. Assemble the transfer sandwich consisting of pad, Whatman 3MM filter paper, nitrocellulose membrane, equilibrated acrylamide gel, second Whatman 3MM filter paper, and second pad (Fig. 6.2.1).

All pads, filter papers, and nitrocellulose membranes should be handled using gloves and prewetted with transfer buffer. The transfer cassette should be assembled underneath the transfer buffer to avoid trapping air bubbles. Ensure the orientation of the gel (judged by the position of the prestained protein standards) such that the correct order of the samples after transfer onto the nitrocellulose membrane is maintained.

17. Place the transfer sandwich into the electroblotting apparatus filled with transfer buffer with the nitrocellulose membrane on the cathode side of the gel. Connect the apparatus to the power supply and transfer proteins for 1 hr at 100V (constant voltage) with cooling (UNIT 6.2).

Transfer time depends on the size of the proteins, acrylamide percentage, and the thickness of the gel. The completeness of the transfer can be easily judged by the transfer of the prestained protein standards.

18. At the end of the transfer, turn off the power supply and disassemble the apparatus and transfer cassette. Remove the nitrocellulose membranes and stain with 50 ml Ponceau S solution in a flat container for 5 min. Destain the membranes by rinsing several times with distilled water.

Two membranes can be incubated in the same container by orienting them back to back.

Staining with Ponceau S does not interfere with the subsequent antibody reactions and provides a good estimation of the protein loading, separation, and quality of the transfer. The Ponceau S solution can be reused several times.

Probe the membranes with antibodies

19. Rinse the membranes once with TTBS and incubate in 50 ml blocking solution for 30 min at room temperature with gentle shaking.

Milk proteins in the blocking buffer are used to saturate free protein-binding sites and to prevent the nonspecific binding of the antibody. Do not incubate the membrane longer than 1 hr in this buffer since blocking buffer also has a slight stripping effect and may cause detachment of the transferred sample proteins.

20. Dilute primary anti-phospho Akt antibody according to the supplier's instructions in 10 ml TTBS containing 3% dry nonfat milk. Place the membranes in heat-sealable plastic bags, add diluted antibody, and seal the bag with a heat sealer. Incubate the membranes overnight at 4°C with gentle shaking.

Two membranes can be incubated in the same bag by orienting them back to back. Remove all air bubbles from the bag before sealing it.

21. Remove the membranes from the bag and wash them three times, 15 min each with 50 ml TTBS in flat container with vigorous shaking.

Do not allow the membranes to dry out after the incubation with primary antibody.

22. Dilute the secondary antibody in 10 ml TTBS, 3% dry nonfat milk according to the supplier's instructions. Place the membranes in a new heat-sealable bag, add diluted antibody and seal the bag. Incubate for 30 to 45 min at room temperature with gentle shaking.

Either HRP- or AP-conjugated secondary antibodies can be used. HRP-conjugated secondary antibodies can be combined with the high-sensitivity ECL detection system. This system allows detection of signals from weak antibodies although attention should be paid if accurate quantification of the signal is necessary (see Commentary).

23. Repeat step 21.
24. Use the ECL immunodetection protocol (UNIT 6.2) to detect phosphorylated Akt. Incubate the membranes with ECL solution for 1 min, dry the excess fluid by touching the edge of the membrane to a piece of filter paper, wrap the membranes in plastic wrap, and expose to X-ray film for 1 min.
25. Incubate the membranes in 50 ml blocking buffer for 15 min at room temperature with shaking.
26. Repeat steps 20 to 24 with new primary antibody (e.g., anti-actin).

This second reaction is used as an internal control for loading.

If both primary antibodies are generated in the same species (e.g., both are mouse or both are rabbit) and the molecular weights of the antigens are different (e.g., 60 kDa for Akt and 45 kDa for actin) the membranes can be incubated with a mixture of the primary antibodies, and the two signals can be detected simultaneously on the same X-ray film.

SUPPORT PROTOCOL

DETERMINATION OF Akt/PKB TRANSLOCATION TO THE PLASMA MEMBRANE

An important step in Akt activation involves its targeting to the plasma membrane. This translocation is dependent on the pleckstrin homology (PH) domain localized at the N-terminus of the Akt molecule and the presence of phosphatidylinositol 3-kinase products PtdIns(3,4,5)P₃ and PtdIns(3,4,)P₂.

This protocol describes the preparation of membrane and cytosolic fractions from starved and PDGF-stimulated GD25 β 1 integrin-null cells expressing wild-type β 1 integrin or its mutant variant W/A that is deficient in Akt signaling. The distribution of Akt is determined in each fraction by immunoblotting and immunodetection with antibodies. The same protocol can be applied to almost all cultured cell lines with little or no modifications.

Additional Materials (also see *Basic Protocol*)

Cytosolic buffer (see recipe)
Membrane buffer (see recipe)
Dry ice
60-mm dishes

Treat cells

1. Plate two 60-mm dishes each of β 1 and mutant cells (2.5×10^6 cells/dish) in DMEM/10% FBS and allow them to attach and spread for 3 to 4 hr.
2. Wash dishes with 5 ml DMEM/1% BSA (starvation medium) two times, then add 5 ml/dish of DMEM/1% BSA and serum-starve the cells overnight.

At this point the dishes should be ~80% confluent. Depending on the cell type being used, adjustment of the initial number of plated cells may be necessary.

3. Aspirate the starvation medium and stimulate one dish from each cell line with 20 ng/ml PDGF-BB in 5 ml DMEM/1% BSA for 15 min. Leave one β 1 and one mutant dish without stimulation in fresh DMEM/1% BSA and label them as controls. To ensure similar treatment, dispense 10 ml of pre-warmed DMEM/1% BSA and add 20 μ l of 10 μ g/ml stock solution of PDGF-BB, mix well, and add to the dishes to be stimulated.

Prepare cytosol fractions

4. Rinse the control and PDGF-stimulated dishes with 5 ml cytosolic buffer two times. Keep the dishes on ice and use ice-cold buffers to slow down the biological processes and prevent protein degradation.
5. Add 350 μ l/dish of ice-cold cytosolic buffer, scrape the cells with a plastic scraper or rubber policeman, and transfer the lysate into prelabeled 1.5-ml microcentrifuge tubes.
6. Place the tubes on dry ice until frozen. Thaw the cells in a 37°C water bath. Repeat freeze-thaw cycle two additional times.

Freeze-thaw cycles will break plasma membranes and liberate most of the cytosolic proteins.

7. Centrifuge 15 min at 19,000 \times g, 4°C. Transfer the supernatant into prelabeled 1.5-ml microcentrifuge tubes, mix with equal volume of 2 \times SDS sample buffer, and leave on ice.

This supernatant represents the cytosolic fraction.

Prepare membrane fraction

8. Resuspend the pellet in fresh 350 μ l cytosolic buffer, centrifuge as in step 7, and discard the supernatant.

This washing step clears most of the remaining cytosolic proteins from the pellet.

9. Suspend the pellet in 50 μ l membrane buffer and centrifuge 15 min at 10,000 \times g, 4°C.

This step solubilizes cellular membranes. Pipet the pellet up and down through a micropipet tip several times, but avoid excessive foaming.

10. Transfer the supernatant into prelabeled 1.5-ml microcentrifuge tubes and mix with an equal volume of 2 \times SDS sample buffer.

Analyze fractions

11. Boil all the samples (supernatants from steps 7 and 10) in a boiling water bath for 3 min or heat at 95°C for 5 min on a heating block.

The samples can be stored sealed and frozen at least 1 month at -20°C.

12. Proceed with SDS-PAGE and immunoblotting (see Basic Protocol 1, steps 12 through 24). Use anti-Akt antibody to probe the distribution of the total Akt between the cytosolic and membrane fractions.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Cytosolic buffer

20 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
150 mM NaCl
50 μM leupeptin (APPENDIX 1B), add fresh
50 μM pepstatin (APPENDIX 1B), add fresh
1 mM PMSF (APPENDIX 1B), add fresh
1 mM sodium vanadate (APPENDIX 1B), add fresh
50 mM NaF, add fresh
Store up to 3 months at 4°C

The cytosolic buffer is derived from Kobayashi et al. (2001).

Membrane buffer

20 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
150 mM NaCl
1% (v/v) Triton X-100
50 μM leupeptin (APPENDIX 1B), add fresh
50 μM pepstatin (APPENDIX 1B), add fresh
1 mM PMSF (APPENDIX 1B), add fresh
1 mM sodium vanadate (APPENDIX 1B), add fresh
50 mM NaF, add fresh
Store up to 3 months at 4°C

The membrane buffer is derived from Kobayashi et al. (2001).

Modified radioimmunoprecipitation assay (mRIPA) buffer

50 mM HEPES, pH.5
150 mM NaCl
10% (v/v) glycerol
0.1% (w/v) SDS (APPENDIX 2A)
1% (w/v) sodium deoxycholate
1% (v/v) Triton X-100
1.5 mM MgCl₂
1 mM EGTA
50 μM leupeptin (APPENDIX 1B), add fresh
50 μM pepstatin (APPENDIX 1B), add fresh
1 mM PMSF (APPENDIX 1B), add fresh
1 mM sodium vanadate (APPENDIX 1B), add fresh
50 mM NaF, add fresh
Store up to 3 months at 4°C

COMMENTARY

Background Information

Akt (c-Akt) is the cellular homolog of the viral oncoprotein v-Akt. It was first identified as a protein kinase with high homology to protein kinases A and C and was therefore termed PKB (protein kinase B) or RAC (related to A and C). Mammals have three closely related Akt genes that are expressed differentially at both mRNA and protein levels (Datta et al., 1999; Brazil and Hemmings, 2001). The family

of Akt proteins contains a central kinase domain with specificity for serine or threonine residues in substrate proteins and a carboxyl terminus containing a hydrophobic motif (HM) and a proline-rich domain. The N-terminal region of the molecule includes a pleckstrin homology (PH) domain, which together with the HM play important roles in the Akt activation process (Scheid and Woodgett, 2003). The activation mechanism involves direct binding of PI3K

products phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate to the PH domain of Akt (Chan et al., 1999). As a consequence, Akt is localized to the plasma membrane where PI3K-generated 3'-phosphorylated phospholipids reside (Fig. 14.6.1). This translocation is now known to be an important step in Akt activation (Testa and Bellacosa, 1997). Myristylated c-Akt, which is specifically targeted to the plasma membrane, is constitutively active similarly to the oncogenic v-Akt, which is permanently targeted to the plasma membrane by the viral gag sequence, and exhibits constant kinase activity. Membrane-localized Akt undergoes conformational changes leading to the exposure and phosphorylation of two residues—Thr308 in the activation loop, proximal to the catalytic core, and Ser473 in the HM. This dual phosphorylation is necessary for the full activation of Akt. The kinase that phosphorylates Thr308 has been named 3-phosphoinositide-dependent kinase 1 (PDK1) because it also requires lipids for its activity (Vanhaesebroeck and Alessi, 2000). Phosphorylation of Akt on Thr308 causes a charge-induced change in conformation allowing substrate binding and an elevated rate of catalysis. The second important phosphorylation event associated with Akt activation occurs at Ser473. The mechanism of this phosphorylation is not completely understood and may involve autophosphorylation or distinct serine kinases like integrin-linked kinase (ILK; Persad and Dedhar, 2003). The function of phosphorylation at this site is also not fully clarified, though the necessity of this modification for Akt activity is well documented. For example, kinase activity is significantly reduced by mutations of this residue, and a similar effect is observed after ceramide-promoted dephosphorylation at this site. It has been proposed that phosphorylation at Ser473 may change the properties of the hydrophobic motif, shifting it to a docking site for PDK1 (Scheid and Woodgett, 2003). After activation, Akt appears to detach from the inner leaflet of the plasma membrane and to translocate through the cytosol to the nucleus (Andjelkovic et al., 1997; Meier and Hemmings, 1999).

Cells can inhibit or reverse the activation of Akt by several mechanisms (Hill and Hemmings, 2002). One of them includes the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10; Yamada and Araki, 2001), which decreases the levels of phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate within

cells, thus preventing membrane translocation of Akt. Direct inactivation of Akt occurs by dephosphorylation of Thr308 and Ser473. This process is mediated by the ubiquitous Ser/Thr protein phosphatase 2A (PP2A; see Millward et al., 1999) and can be blocked by the phosphatase inhibitor okadaic acid. A negative regulator termed CTMP (carboxy-terminal modulator protein), which attenuates the activation of Akt at plasma membrane, has also been identified (Maira et al., 2001). Thus, the level of Akt activity in steady state is the result of an equilibrium between activation and inhibition events.

Extensive research has clarified the intracellular mechanisms of Akt activation by upstream PI3K and PDK1. Less is known about the means through which Akt regulates cell growth, proliferation, and survival, even though a number of downstream Akt substrates have been identified (Fig. 14.6.1; Chan et al., 1999; Datta et al., 1999; Vanhaesebroeck and Alessi, 2000). While the Akt phosphorylation consensus sequence is defined (RXXXS/THydrophobic), to date only a small number of Akt substrates have been positively or tentatively identified.

Ongoing studies on Akt signaling and the possibility of using this kinase as a drug target for cancer, diabetes, and stroke, make probing for Akt activity a frequently used task. This can be achieved by measuring Akt kinase activity directly or by assaying the level of phosphorylation as described in this unit. Determination of Akt kinase activity follows the general scheme applicable for most kinases (e.g., see UNIT 14.3) and involves immunoprecipitation of the enzyme with antibodies and then performing a phosphorylation reaction *in vitro*. While this approach offers a direct and relatively accurate determination of Akt activity, the method is laborious and often involves use of radioactivity. Determination of Akt activation state by assaying the level of phosphorylation after specific treatments is quicker and employs basic non-radioactive laboratory techniques. Moreover, the same samples (in some cases the same membrane used for immunoblotting with anti-Akt antibodies) can be used for determination of the activity of upstream Akt regulators and the phosphorylation level of downstream Akt substrates, making this method more versatile than the classical kinase assay.

Critical Parameters and Troubleshooting

Several parameters play critical roles for success in determination of Akt activation and

Signal
Transduction:
Protein
Phosphorylation

14.6.9

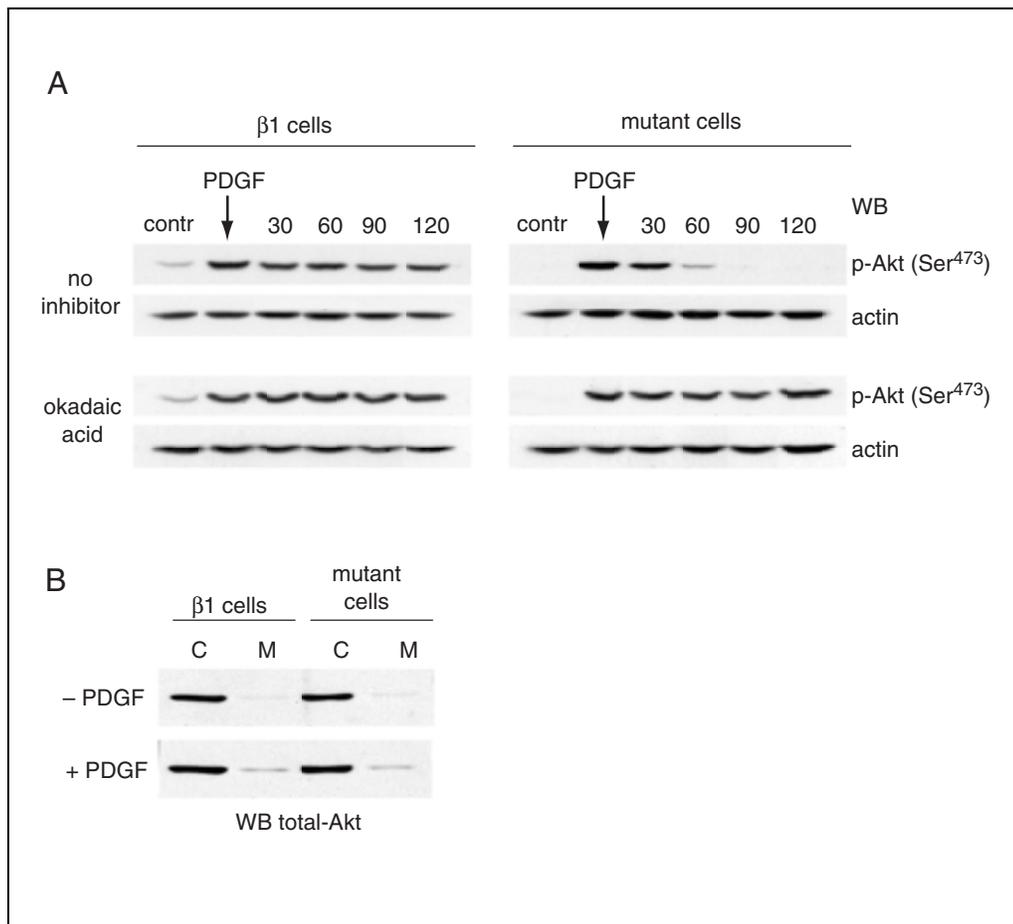


Figure 14.6.2 Probing of Akt signaling by the methods described in this unit. **(A)** Determination of Akt signaling in cultured cells. GD25 cells expressing either wild-type ($\beta 1$ cells) or tryptophan mutant (mutant cells) integrins were cultured overnight in the absence of serum. Cells were then stimulated with 20 ng/ml PDGF-BB for 15 min, the growth factor was washed away, and the cells were maintained in medium without serum supplemented with 30 μ M PDGF kinase inhibitor AG 1433. Samples were taken after starvation (contr), immediately after stimulation (PDGF, \downarrow), and at the indicated time points. The experiment was performed in the absence (no inhibitor) or presence of 0.5 μ M okadaic acid. Lysates from the samples were analyzed by immunoblotting (WB) with anti-phospho Ser⁴⁷³ Akt [p-Akt (Ser⁴⁷³)] or anti-actin (actin) antibodies. Actin was used as an internal control for loading. **(B)** Determination of Akt translocation to the plasma membrane. Cells were starved overnight and stimulated with 20 ng/ml PDGF (+ PDGF) or left without stimulation (-PDGF) and used to prepare cytoplasmic (C) and membrane (M) fractions. Samples from these fractions were analyzed by immunoblotting with anti-total Akt (WB total-Akt) antibodies. Increased amount of Akt is detected in the membrane fraction of the PDGF stimulated cells.

inhibition. Initial reduction of the phosphorylation level of Akt is achieved by a period of starvation, which varies depending on the type of cells and should be determined experimentally. For some cell lines like primary human fibroblasts, withdrawal from serum for 4 hr is sufficient, while for most immortalized cell lines, longer periods of starvation work better. The length of serum starvation of the chosen cell line needs to be such that it will ensure a five-fold or higher increase of Akt phosphorylation after stimulation with growth factor.

The response to PDGF by different cell lines varies significantly. If the increase of Akt phosphorylation after stimulation is not sufficient, activation with another growth factor, e.g., EGF, may be used. If a different growth factor is applied, the corresponding growth factor receptor (GFR) kinase inhibitor should be added to the medium instead of AG 1433 (e.g., AG 1478 if EGF is utilized). Addition of a growth factor kinase inhibitor is necessary to block residual activity of the receptor after withdrawal of the growth factor. This treatment is critical, since residual GFR activity may

mask the Akt inactivation pattern by prolonging the dephosphorylation time. Experimental blocking of dephosphorylation is achieved by treatment with okadaic acid. Incubations with this potent protein phosphatase inhibitor, especially for prolonged times, may cause cell rounding and even detachment from the substrate. Okadaic acid sensitivity should be determined experimentally for each cell type to be used.

Obtaining a high signal-to-noise ratio after immunoblotting is essential for the successful determination of Akt signaling. This can be ensured by: (1) use of specific antibodies that recognize the phosphorylated Akt but not its unphosphorylated form (now offered by several companies like Cell Signaling, Biosource); (2) use of freshly added phosphatase and protease inhibitors added to the lysis buffers to prevent Akt dephosphorylation and degradation after cell disruption; (3) loading sufficient amount of proteins from the cellular lysate that will ensure trouble-free detection of the Akt by the antibodies (this is easily achieved by keeping the samples for SDS-PAGE concentrated—e.g., $>5 \times 10^5$ cells/minigel lane); (4) following the proper techniques for SDS-PAGE and immunoblotting (see *UNITS 6.1 & 6.2*).

Accurate comparison and quantification by densitometry of the amounts of phosphorylated Akt in different samples can be achieved if the detection system is kept in a linear range. The enhanced chemiluminescence (ECL) system should be optimized to obtain linearity by adjustments of the amount of the protein loaded on the gel, concentrations of primary and secondary antibody, and the X-ray film exposure time. If the weakest signal is detectable and the strongest signal is still within the linear range of the film (e.g., not saturated), then the rest of the samples are also in the linear range of the system, which can be used for quantification.

Anticipated Results

Typical results expected after probing for Akt activity (see Basic Protocol) or Akt membrane translocation (see Support Protocol) are presented in Figure 14.6.2A and B, respectively. Comparison between the starved cells (contr) and cells after PDGF stimulation (PDGF) should demonstrate a several-fold increase in the amount of phosphorylated Akt. Attenuation of this response after experimental manipulations of the cell cultures may indicate effects on the upstream pathways leading to Akt activation. Evaluation of the amount of phosphorylated Akt in the samples taken after PDGF

stimulation at different time points provides information about the rate of Akt dephosphorylation (inhibition). While in the samples from $\beta 1$ cells, this decrease is modest, the Akt in the samples from mutant cells is rapidly dephosphorylated. Such a result indicates activation of some of the systems for Akt inhibition (see Background Information). Performing the same experiment in the presence of a PP2A inhibitor, okadaic acid completely reverses this effect, indicating that activation of this phosphatase is involved in the observed increased dephosphorylation rate in W/A mutant cells. If OA is ineffective, additional experiments should be designed to test the role of other Akt inhibitors (see Background Information). Activation of Akt is dependent on its membrane translocation. A typical increase in membrane-bound Akt after growth factor stimulation is presented in Figure 14.6.2B. A modest but detectable increase in total Akt in the membrane fraction is observed in both cell lines after PDGF stimulation. Failure to detect such translocation may indicate defects in the function of the PH domain of Akt or insufficient phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate (see Background Information).

Time Considerations

The entire procedure described in the Basic Protocol can be completed in 3 days. This period includes the time for cell attachment after plating (4 hr); starvation (12 hr); PDGF stimulation and the necessary incubations up to preparation of the SDS-PAGE samples (3 hr); SDS-PAGE and electrotransfer (6.5 hr for mini gels or 9.5 hr for normal size gels); overnight incubation with the primary antibody; and completion of the immunoreactions with ECL processing (4 hr). Since this procedure takes >1 day, it is helpful to use one night for starvation of the cells and the next night for incubation with the primary phosphospecific antibody. There are a number of points where the procedure can be interrupted: (1) after the preparation of the SDS-PAGE samples; (2) after the electrotransfer (membranes can be stored wet or dry in resealable plastic bags at 4°C); and (3) after the completion of the first immunoreaction (membranes can be stored wet in resealable plastic bags at 4°C). A similar timeframe applies for the procedure described in the Support Protocol.

Signal
Transduction:
Protein
Phosphorylation

14.6.11

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CHAPTER 15

Protein Trafficking

INTRODUCTION

As discussed in the introduction to Chapter 3, eukaryotic cells are not simply sacs of amorphous protoplasm but are instead organized into an array of membrane-bound structures known as organelles. Subcellular fractionation (Chapter 3) and microscopic analyses (Chapter 4) have revealed a wondrous diversity of subcellular organelles, including the nucleus, endoplasmic reticulum (ER), Golgi complex, lysosomes, endosomes, secretory granules, plasma membrane, mitochondria, peroxisomes, chloroplasts, and a variety of other structures found in specialized cells. Each of these organelles fulfills a distinct function, which is carried out by a specific set of organellar proteins. Genetic information directing the synthesis of most organellar proteins, as well as cytosolic and extracellular proteins, is encoded by DNA contained within the nucleus. Genomic DNA is transcribed into messenger RNA (see *UNIT 11.6*) and this in turn is translated into protein (see *UNIT 11.1*). It is at the time of their translation that proteins begin a journey that will take them to different locations within the cell, or out to the extracellular space. Some proteins are synthesized on ribosomes that exist free in the cytosol. These proteins either remain soluble in the cytosol, become incorporated into supramolecular structures such as the cytoskeleton, or are imported into the nucleus, mitochondria, peroxisomes, chloroplasts, and in some cases the ER. Other proteins are synthesized on ribosomes that are associated with the cytosolic face of the ER membrane. These proteins are translocated across that membrane and subsequently distributed to different compartments of the secretory and endocytic pathways including the ER proper, the Golgi complex, lysosomes, endosomes, secretory granules, and the plasma membrane; in other cases they are secreted into the extracellular medium. Thus, the interior of the cell is a highly dynamic environment, in which heterogeneous yet precisely controlled protein distributions are achieved via various protein trafficking pathways.

In vitro assays to examine specific protein transport steps and their molecular mechanisms have been described in Chapter 11. However, in vitro assays are valid only if they reflect the transport processes that occur in intact cells. In addition, the itineraries followed by proteins as they traffic through different cellular compartments are often too complex to be reconstituted in vitro and are best studied in intact cells.

Chapter 15 is devoted to biochemical and morphologic assays used to study protein trafficking in vivo. The chapter begins with an overview of protein trafficking in the secretory and endocytic pathways (*UNIT 15.1*), which provides useful background information for some of the succeeding protocol units. Most proteins destined for organelles of the secretory or endocytic pathways, or for secretion into the extracellular milieu, are co-translationally modified by addition of carbohydrate moieties. These moieties are progressively remodeled as the proteins traverse the different compartments of the secretory pathway. The type of carbohydrate present on a protein thus serves as a record of its itinerary through the cell. Gross analyses of the carbohydrate moieties of glycoproteins can be conveniently carried out by treatment of metabolically labeled, immunoprecipitated proteins (see *UNITS 7.1 & 7.2*) with an assortment of specific glycosidases. *UNIT 15.2* presents a comprehensive description of carbohydrate modifications along the secretory pathway, and provides a series of protocols for analysis of the general structure of carbohydrate moieties of glycoproteins labeled in vivo.

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**Protein
Trafficking**

15.0.1

Supplement 22

Even after proteins reach their allotted destinations within the cells, they remain in constant flux. Indeed, most proteins are not fixed at a particular location but attain a particular steady-state distribution as a result of opposing exit and retrieval pathways. This is very neatly exemplified by some plasma membrane proteins, such as endocytic receptors, that are rapidly internalized but return to the cell surface after a sojourn in the endosomal system. *UNIT 15.3* describes a set of biochemical techniques for measuring the steady-state distribution, internalization, and recycling of the transferrin receptor, a typical endocytic receptor, using radioiodinated transferrin as a probe. Protocols are also provided to measure the steady-state distribution, internalization, and recycling of any plasma membrane protein, even those that do not have a physiologic ligand, by using radioiodinated antibodies or Fab fragments. Internalized ligands and antibodies used to tag plasma membrane proteins are often degraded in lysosomes, a process that can be measured using another protocol included in this unit. Finally, the unit also covers methods for measuring fluid-phase uptake from the medium and for inhibiting endocytosis.

Integral membrane proteins expressed at the cell surface are synthesized in the ER and transported to the plasma membrane following the secretory pathway. By the time that these proteins reach the cell surface, almost all post-translational modifications have taken place. For this reason, the carbohydrate analyses described in *UNIT 15.2* cannot be used to determine the kinetics of protein arrival at the cell surface. However, the accessibility of cell surface proteins to externally added reagents has been exploited to develop assays for biosynthetic protein transport to the plasma membrane. *UNIT 15.4* contains several protocols that combine pulse-chase metabolic labeling (see *UNIT 7.1*) with treatments that modify surface-exposed proteins on intact cells: hydrolysis of sialic acid residues with sialidase, biotinylation, protease digestion, and antibody binding. Modified surface-exposed proteins are detected by electrophoretic or immunoprecipitation techniques sensitive to the modifications introduced.

Although one often refers to the plasma membrane as if it were a single compartment, most eukaryotic cells exhibit more than one plasma membrane domain. Polarized epithelial cells are perhaps the best characterized example of cells that have specialized plasma membrane domains. In epithelial cells, these domains are known as apical and basolateral. Protein trafficking to and from these two domains follows distinct pathways, the analysis of which is the focus of *UNIT 15.5*. For analysis of polarized sorting to be possible, these cells need to be grown in special chambers fitted with porous filters. The cells form a tight monolayer in which the apical and basolateral domains are segregated. In the chambers the apical and basolateral media are separately available for addition of reagents or collection of secreted proteins. The unit contains protocols for metabolic labeling of polarized epithelial cells and collection of proteins secreted from the apical and basolateral surfaces, stable transfection and selection of transfected clones, culture of epithelial cells on filters, determination of leakiness of epithelial monolayers, monitoring the arrival of newly synthesized proteins at both plasma membrane domains, and indirect immunofluorescence microscopy of epithelial cells grown as polarized monolayers.

UNIT 15.6 takes us back to early events in the maturation of newly synthesized proteins in the ER. Upon emergence into the lumen of the ER, nascent polypeptide chains undergo a series of post-translational modifications that lead to the development of a mature protein (see *UNIT 15.1*). Among these modifications are glycosylation (*UNIT 15.2*), folding, and disulfide bond formation. *UNIT 15.6* describes a series of protocols for the analysis of protein folding and disulfide bond formation in the ER. In these protocols, newly synthesized proteins are first labeled with radiolabeled amino acids either in intact cells (also see *UNIT 7.1*) or in cell-free (also see *UNIT 11.4*) or semi-permeabilized cell systems.

Protein folding can then be followed by limited proteolytic digestion or immunoprecipitation with conformation-specific antibodies. Disulfide bond formation can be analyzed by monitoring the disappearance of sulfhydryl groups or the appearance of faster-moving species on nonreducing SDS-PAGE (also see *UNIT 6.5*).

The subject of *UNIT 15.7* is phagocytosis—the process by which specialized cells such as macrophages and monocytes ingest particles such as bacteria, yeast, and apoptotic bodies. Because of the size and complexity of the internalized particles, phagocytosis is distinct from the receptor-mediated or fluid-phase endocytosis described in *UNIT 15.3*. The first step in phagocytosis is the recognition of specific molecules on the surface of the particle by the phagocytic cell. These molecules can be intrinsic to the particle or deposited onto the particle by the host (i.e., “opsonization”). The unit starts with microscope-based protocols to measure phagocytosis of red blood cells or latex beads opsonized with immunoglobulin G (IgG), which are internalized upon binding to Fc γ receptors on the phagocytic cells. These protocols are followed by others designed to measure complement-mediated phagocytosis of red blood cells or latex beads opsonized with complement factor C3bi, a process that is mediated by binding to complement receptors. Phagocytosis can be measured more quantitatively by flow cytometry, for which a protocol is also included in this unit. The next protocols deal with events that follow the uptake of particles, such as the acidification and maturation of the phagosome. The final protocol describes a procedure to inhibit phagocytosis with wortmannin or cytochalasin D.

Juan S. Bonifacino

Overview of Protein Trafficking in the Secretory and Endocytic Pathways

COMPARTMENTALIZATION OF EUKARYOTIC CELLS

The interior of eukaryotic cells is organized as an interconnected system of membrane-bounded compartments. The compartments that form part of the secretory and endocytic pathways include the endoplasmic reticulum (ER), the ER-Golgi intermediate compartment (ERGIC), the Golgi complex with its different subcompartments (*cis*, medial, and *trans* cisternae, and the *trans*-Golgi network or TGN), early and late endosomes, lysosomes, regulated secretory granules, the plasma membrane, and various types of intermediary vesicles and tubular-vesicular carriers (Fig. 15.1.1). Each of these compartments contains a characteristic set of resident proteins. Protein localization to these compartments is mediated by specific targeting information present within the proteins (see APPENDIX 1C). This unit is intended as a brief overview of the itinerary followed by proteins as they move through the secretory and endocytic pathways, and of the mechanisms that determine protein sorting within these pathways.

TARGETING AND TRANSLOCATION OF PROTEINS INTO THE ER

Most proteins enter the secretory pathway by translocation from the cytosol into the ER. In mammalian cells, protein translocation in the ER occurs co-translationally as the nascent polypeptide chain emerges from the ribosome. In yeast cells, some proteins utilize this cotranslational mechanism and others are translocated posttranslationally. The steps involved in cotranslational transport are shown in Figure 15.1.2. Early experiments established that the signal for translocation of a protein across the ER membrane lies in the nascent polypeptide rather than in the encoding mRNA (reviewed by Rapoport et al., 1996). As the signal sequence emerges from the ribosome, it binds to the signal-recognition particle (SRP; step 1), a complex composed of six proteins and one 7S RNA molecule. SRP arrests further elongation of the polypeptide on the ribosome and then binds the complex to the SRP receptor on the cytosolic side of the ER membrane (step 2). Both SRP and the SRP receptor bind GTP, which allows transfer of the ribosome to the

translocon. Hydrolysis of GTP induces dissociation of SRP from the SRP receptor (step 3). The nascent polypeptide then moves through the central channel of the translocon, the main component of which is the multi-spanning membrane protein Sec61 α (step 4). The signal peptide is cleaved cotranslationally by signal peptidase. Throughout translocation, the impermeability of the ER membrane is maintained by a complex set of events, involving the ribosome or ER-luminal proteins such as BiP (binding immunoglobulin protein). Translocation is complete either when a stop-transfer sequence in the polypeptide is encountered (step 5), leading to protein integration in the lipid bilayer (step 6), or when a stop codon is reached, in which case the protein is released into the ER lumen.

PROTEIN MODIFICATIONS IN THE ER

Once the nascent polypeptide chains emerge into the ER lumen, they undergo a series of modifications including signal peptide cleavage, glycosylation, folding, and disulfide-bond formation. Amino-terminal signal sequences are cotranslationally cleaved by signal peptidase, an enzyme complex that is closely associated with the translocon (Andrews and Johnson, 1996). In addition to signal peptidase, the oligosaccharyl transferase complex also appears to be in close proximity to the translocon. This complex is responsible for transferring the preassembled core oligosaccharide moieties to the nascent polypeptide chains at Asn-X-Ser/Thr sequences (see UNIT 15.2). The nascent polypeptide chain can be glycosylated when as few as fifteen amino acids have emerged from the translocon.

The nascent polypeptide chain associates with a number of chaperone proteins in the ER, including BiP, GRP94, calnexin, calreticulin, protein disulfide isomerase, and peptidyl proline *cis-trans* isomerase. BiP comprises ~5% of the ER luminal protein content and associates transiently with a number of newly synthesized proteins (Gething and Sambrook, 1992). BiP has been postulated to sequentially bind, release, and rebind newly synthesized proteins in an ATP-dependent manner until the proteins fold correctly and their hydrophobic regions are no longer accessible to BiP. Proteins

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that do not fold correctly remain bound to BiP and are not transported further along the secretory pathway.

Calreticulin and calnexin are carbohydrate-binding chaperones, which are also involved in quality control in the ER (reviewed by Bergeron et al., 1994; Hammond and Helenius, 1995). Calreticulin is a luminal protein, whereas calnexin is an integral membrane protein. Shortly after addition of the core oligosac-

charide complex to the nascent polypeptide, the two terminal glucose residues of the complex are removed by glucosidases I and II (Fig. 15.1.3). This generates a monoglucosylated oligosaccharide to which calnexin or calreticulin binds. Further processing of the oligosaccharide results in the release of the newly synthesized polypeptide from either chaperone. If the protein is not folded correctly, it is re-glucosylated by a UDP-glucosyl transferase, and the

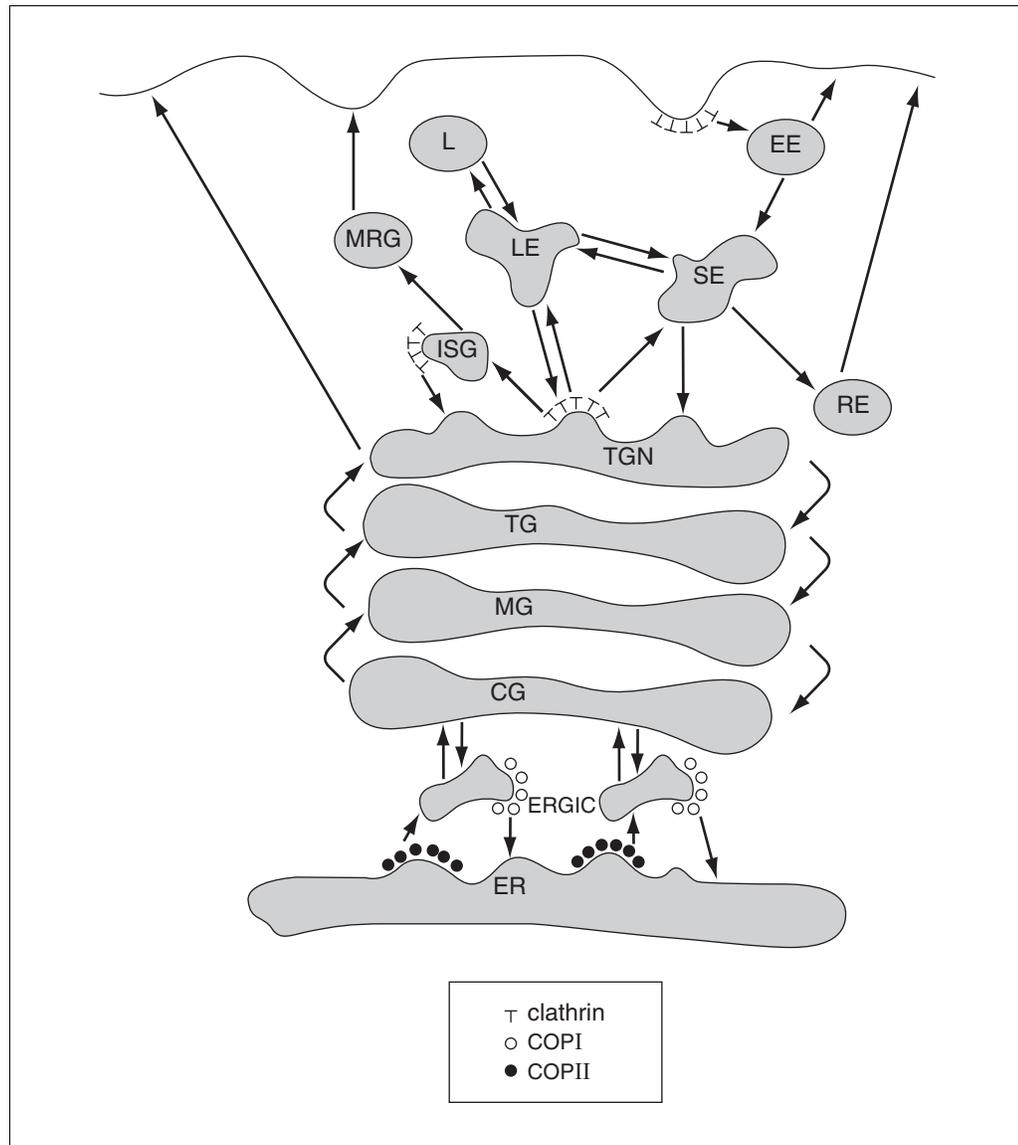


Figure 15.1.1 Compartments of the biosynthetic and endocytic pathways. The known routes in the biosynthetic and endocytic pathways are shown in this schematic diagram. The compartments include: the endoplasmic reticulum (ER), the ER-Golgi intermediate compartment (ERGIC), the *cis*-Golgi (CG), the medial Golgi (MG), the *trans*-Golgi (TG), the *trans*-Golgi network (TGN), the lysosome (L), the late endosome (LE), the sorting endosome (SE), the recycling endosome (RE), the early endosome (EE), the immature secretory granule (ISG), and the mature regulated granule (MRG). The extent to which each of the pathways are utilized is not known. The steps where coat proteins have been identified to be involved in vesicle formation are indicated. See Transit Between the ER and the Golgi Complex for discussion of COPI and COPII.

cycle of calnexin/calreticulin binding is repeated until the protein folds correctly and no longer interacts with the UDP-glucosyl transferase. The lumen of the ER is much more oxidizing than the cytosol, allowing the formation of disulfide bonds in a reaction catalyzed by protein disulfide isomerase.

ER-ASSOCIATED PROTEIN DEGRADATION

Quality control mechanisms of the ER not only prevent misfolded proteins from exiting the ER, but also ensure that abnormal proteins are degraded (Sommer and Wolf, 1997). Recent evidence indicates that misfolded proteins, as well as ER proteins whose levels are subject to

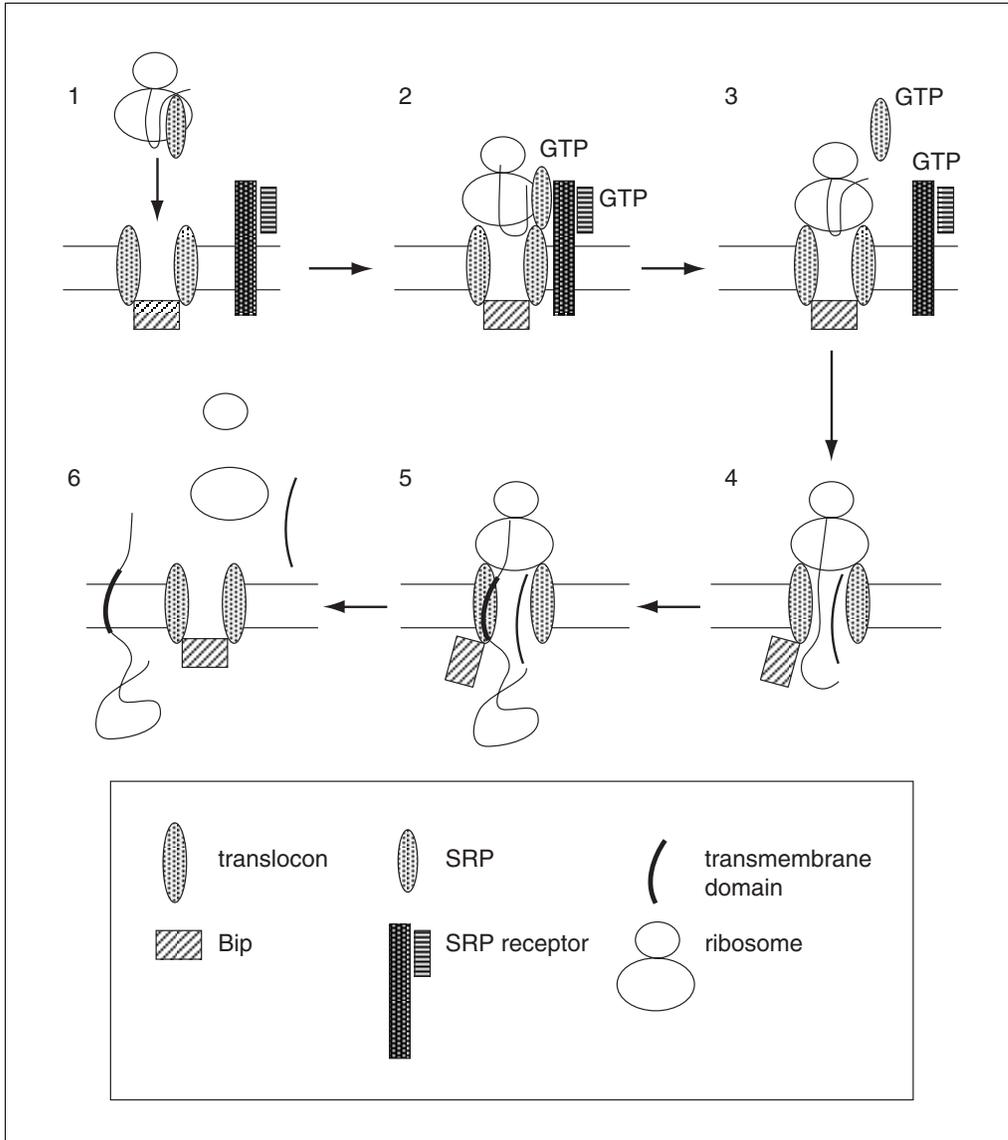


Figure 15.1.2 Cotranslational translocation of a type 1 membrane protein into the ER. (1) When the signal sequence emerges from the ribosome, it binds to the signal-recognition particle (SRP) in the GTP-empty form and translocation is arrested. The pore of the translocon is in the closed position, sealed by binding immunoglobulin protein (Bip). (2) The SRP/signal peptide/ribosome complex binds to the two subunits of the GTP-bound SRP receptor. The ribosome interacts with the translocon. (3) GTP is hydrolyzed, releasing the SRP from the SRP receptor. Translation is continued. (4) The ribosome seals the pore and when the nascent chain reaches ~70 residues Bip is released. The protein is extruded into the lumen of the ER, where the signal peptide is cleaved. (5) The transmembrane sequence emerges from the ribosome and interacts with different proteins in the translocon. (6) The protein is inserted into the bilayer by the completion of translation. The ribosome disassociates and the pore is closed by Bip.

metabolic regulation, are exported from the ER into the cytosol, where they are degraded by the ubiquitin-proteasome pathway (Sommer and Wolf, 1997). A variety of viruses use this degradative pathway to subvert the immune system. For example, viral proteins such as US2 and US11 of human cytomegalovirus appear to

promote ejection from the ER and subsequent proteosomal degradation of the heavy chain of MHC class I complexes (Wiertz et al., 1996). This serves to down-regulate molecules that would otherwise stimulate an immune response against the virus.

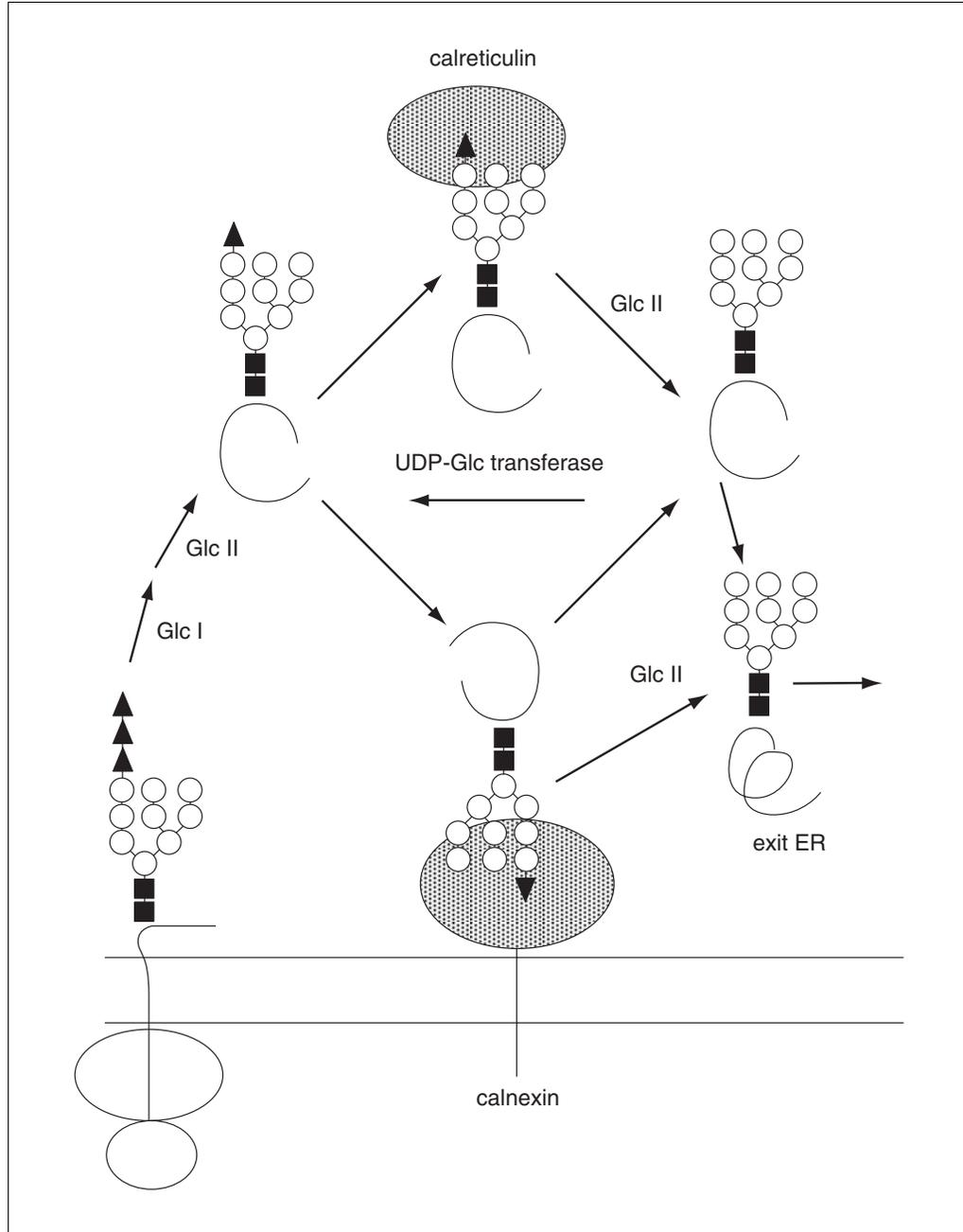


Figure 15.1.3 The calnexin/calreticulin quality control pathway in the ER. Glucosidases I and II (Glc I and Glc II) remove the two outermost glucose residues (black triangles) from the protein and allow interaction of the oligosaccharide with either calnexin or calreticulin. Glucosidase II removes the last glucose residue, and the protein no longer associates with these lectins. If the protein is folded properly, it is free to exit the ER. If it is not folded correctly, it is reglucosylated by the enzyme UDP-glucosyl transferase and rebinds to calnexin or calreticulin. Adapted from Hammond and Helenius (1995). See Figure 15.2.1 for identification of the sugar residues.

TRANSIT BETWEEN THE ER AND THE GOLGI COMPLEX

Vesicle budding in both the secretory and endocytic pathways is mediated by cytosolic proteins that form coated structures on the membranes. Two types of coat proteins—known as COPI and COPII—have been shown to be involved in trafficking of proteins between the ER and the Golgi complex (Cosson and Letourneur, 1997; Kuehn and Schekman, 1997; Fig. 15.1.1). COPII-coated vesicles mediate anterograde ER-to-Golgi transport, whereas COPI-coated vesicles mediate retrograde Golgi-to-ER transport. Controversy still exists as to whether COPI vesicles are also involved in anterograde transport. Vesicles generated from the ER are targeted to and fuse with the ER-Golgi intermediate compartment (ERGIC). ERGIC structures then migrate towards the centrosomal region of the cell and fuse with the Golgi complex.

Newly synthesized proteins were initially proposed to exit the ER via a bulk-flow mechanism that did not involve concentration into transport vesicles. However, recent evidence suggests that newly synthesized proteins destined for transport out of the ER are specifically concentrated at ER exit sites (reviewed by Bannykh et al., 1998). For example, a diacidic (D/EXD/E) sequence close to an internalization signal has been shown to be responsible for the efficient concentration of vesicular stomatitis virus G protein at ER exit sites (Nishimura and Balch, 1997). In yeast, selective packaging of glycosylated pro- α -factor for export from the ER has also been demonstrated (Kuehn and Schekman, 1997). A lectin-like molecule named ERGIC-53 (Emp47p in yeast) is thought to be involved in the transport of some soluble glycoproteins out of the ER after the glucose residues have been trimmed (reviewed by Hammond and Helenius, 1995).

RETENTION IN THE ER AND RETRIEVAL FROM THE GOLGI COMPLEX

Most soluble ER-resident proteins contain a carboxy-terminal tetrapeptide sequence, KDEL (HDEL in *S. cerevisiae*). Addition of this sequence to the carboxy terminus of soluble secreted proteins results in their accumulation in the ER, implying that this motif is sufficient for ER localization. The KDEL sequence is thought to retrieve proteins from the ERGIC or the Golgi complex to the ER. This retrieval is mediated by the membrane-bound receptor protein Erd2p.

Other signals are used to retrieve integral membrane proteins to the ER. Type I ER-resident membrane proteins have cytosolic KKXX or KXXXX (dilysine) motifs at their carboxy termini. Some type II ER-resident membrane proteins have a cytosolic ER localization signal consisting of two arginine residues (RR) located within the first five amino-terminal residues of the protein. Direct binding of COPI to the dilysine motif of ER integral membrane proteins has been implicated in the retrograde transport of these proteins to the ER (reviewed by Cosson and Letourneur, 1997).

Thus, a picture emerges in which anterograde transport of secretory cargo allows newly synthesized proteins to exit the ER selectively, and retrograde transport mediates the retrieval of ER-resident proteins, as well as transport factors necessary for a subsequent round of anterograde transport. The balance of the two transport pathways determines the steady-state distribution of proteins in each compartment.

CARBOHYDRATE MODIFICATIONS IN THE GOLGI COMPLEX

The Golgi complex is a major site of N-linked oligosaccharide modification within the secretory pathway. A highly organized set of glycosyl transferases, glycosidases, and nucleotide- or lipid-linked glycosyl donors and transporters cooperates to produce these modifications. Each component of this protein modification machinery has a characteristic distribution within the Golgi complex. For example, generation of mannose-6-phosphate residues, which serve as a signal for sorting soluble proteins to lysosomes, occurs in the *cis* region of the Golgi complex. In the biosynthesis of complex carbohydrates (UNIT 15.2), mannose residues are removed in the *cis*-medial Golgi complex, and *N*-acetylglucosamine residues are added in the medial Golgi complex, whereas galactose and sialic acid addition occurs in the *trans*-Golgi complex and TGN (Fig. 15.1.4). Sulfation of oligosaccharides and tyrosine residues takes place in the TGN. The extent of glycosylation of proteins traveling through the Golgi complex can be deduced by a variety of methods, including the use of glycosidases specific for certain oligosaccharide structures (see UNIT 15.2). The oligosaccharide composition of a mature glycoprotein can be quite heterogeneous and can depend on the cell type and metabolic state. Moreover, whether a particular consensus sequence is modified and

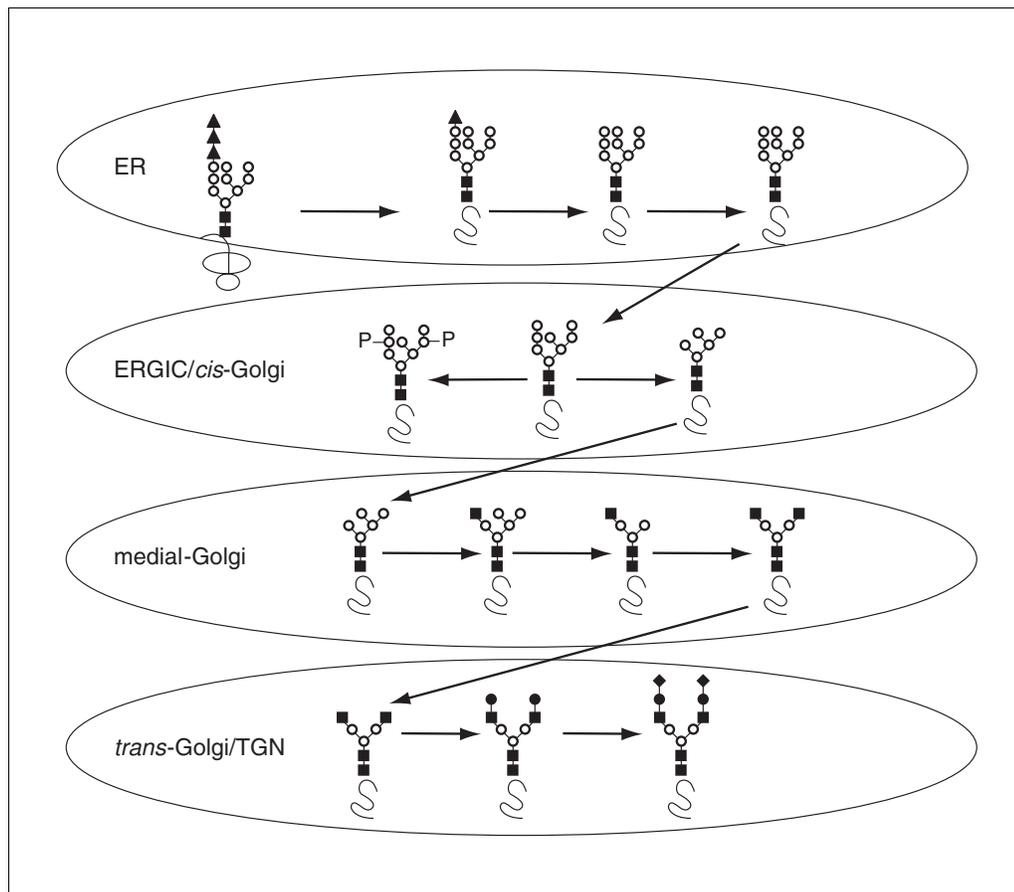


Figure 15.1.4 Processing of oligosaccharides in the Golgi. A schematic diagram of the approximate locations of oligosaccharide modification in the biosynthetic pathway. See Figure 15.2.1 for identification of sugar residues.

to what extent depend on the local conformation of the protein at each glycosylation site (Dwek, 1995).

In contrast to N-linked glycosylation, O-linked glycosylation occurs posttranslationally with the addition of a single *N*-acetylglucosamine residue to a serine/threonine residue. While there is no consensus sequence for O-linked glycosylation, it often occurs in the proximity of proline and close to transmembrane regions (Hounsell et al., 1996; see *UNIT 15.2*).

MODELS OF PROTEIN RETENTION IN AND TRANSPORT THROUGH THE GOLGI COMPLEX

How are secretory proteins transported through the Golgi complex, and how are resident components retained in the Golgi in the face of this continuous transport? At present, no single mechanistic model has been embraced by all cell biologists (Mellman and Simons, 1992; Mironov et al., 1997; Glick and Malhotra, 1998).

A widely accepted model for Golgi protein transport is by vesicular traffic (Rothman, 1994). This model views the Golgi complex as comprised of a series of distinct and stable subcompartments. Secretory cargo moves between subcompartments in a vectorial direction (*cis* to *trans*) by being packaged into transport vesicles that specifically target acceptor membranes. According to this model, membrane association of COPI provides the mechanochemical force for driving vesicle budding from donor membranes. Golgi enzymes are thought to be organized into large, immobile aggregates in Golgi membranes to avoid being packaged into the forward-moving transport vesicles, which deliver secretory cargo by bulk flow. Evidence in favor of this model includes the findings that cisternae exhibit relatively distinct and stable enzymatic composition, that protein transport can be reconstituted *in vitro* between biochemically isolated donor and acceptor Golgi compartments, and that COPI-coated Golgi vesicles accumulate in cells where pro-

tein transport has been inhibited with GTP γ S (a GTP analog that prevents COPI dissociation from membranes; Rothman, 1994).

A second model for Golgi protein transport that is gaining popularity is transport by cisternal maturation (Mironov et al., 1997; Bonfanti et al., 1998; Pelham, 1998). In this model, Golgi cisternae themselves act as carriers through the Golgi stack. The observation that ER-derived vesicles carrying protein into the Golgi complex undergo a process of fusion and maturation into relatively large (200- to 500-nm), pleiomorphic intermediates that translocate into the Golgi region is consistent with this nonvesicular transport model (Presley et al., 1998). According to the maturation hypothesis, as pre-Golgi intermediates arrive at the *cis* face of the Golgi stack, they form a new *cis* cisternae replacing preexisting *cis* cisternae. This replacement of one cisternae for another continues distally through the stack. The size of the stack is kept constant by the continued consumption of *trans* cisternae through the formation of post-Golgi transport intermediates. To account for the observed constancy of cisternal enzyme distribution by this model, the anterograde shift of each cisterna would be coupled with retrograde relocation of its enzymes into the next proximal cisterna. Morphological observations of the transport of supramolecular structures through the Golgi complex—including casein submicelles, apolipoprotein E, and procollagen—are consistent with this possibility (Bonfanti et al., 1998). These structures are detected throughout Golgi cisternae, but are consistently absent from Golgi vesicles. As these secretory products are simply too large to be packaged into transport vesicles, progression of Golgi cisternae seems reasonable. The observation that Golgi enzymes are highly mobile within the Golgi stack (Cole et al., 1996) and undergo continuous recycling (Cole et al., 1998) is also consistent with this model. A problem with this model, however, is that it cannot explain how different cargo passes through the Golgi complex at distinct rates.

A third model for protein transport through the Golgi complex is by directed diffusion within a single compartment that is interconnected by tubules (Mironov et al., 1997). Tubules are a well characterized feature of the Golgi complex, with both the *cis*- and *trans*-most Golgi elements largely tubular in character. Moreover, the central part of the Golgi complex is comprised of stacks of cisternae that are interlinked by tubular-reticular networks. The finding that there is rapid diffusion of

enzymes within the intact Golgi complex of living cells (Cole et al., 1996) is consistent with the possibility that protein transport through the Golgi occurs by directed diffusion within an interconnected structure. Different cargo would move through this system at distinct rates based on their rate of diffusion within the extended membrane system. Directional, *cis*-to-*trans* flow would arise because of continuous membrane addition at the proximal end (*cis* face) of this system, and membrane removal at the distal end (*trans* face). One difficulty with this model is that it requires unknown mechanisms for maintaining distinct membrane domains within an interconnected system. It also requires alternative functions for COPI coat proteins, which previously have been thought of only in the context of vesicle formation.

The above models for Golgi protein transport may not be mutually exclusive; instead, a spectrum of interrelated mechanisms may be involved. For example, recycling of Golgi enzymes may occur by directed diffusion within a continuous bilayer or by vesicles. Intermittent tubular connections might allow fast forward traffic of cargo, while large structures progress by the slower cisternal maturation pathway. Future work in this area will undoubtedly involve more systematic study of key aspects of each of the above models. Distinguishing between these models is nevertheless important for characterizing the exact roles of molecular machinery identified in *in vitro* transport assays and genetic screens (Schekman and Orci, 1996), and for understanding the relationship between protein traffic and Golgi structure/function.

PROTEIN SORTING IN POST-GOLGI COMPARTMENTS

The TGN is the major sorting compartment for newly synthesized proteins destined for endosomes, lysosomes, regulated secretory granules, or different domains of the plasma membrane (Fig. 15.1.1; reviewed by Keller and Simons, 1997; Traub and Kornfeld, 1997). The signals for sorting integral membrane proteins to endosomes, lysosomes, and the basolateral plasma membrane of polarized epithelial cells are found in the cytosolic domain of the proteins (see APPENDIX 1C). Two types of signal, referred to as tyrosine-based and dileucine-based signals, have been shown to play multiple roles in sorting to these compartments (Kirchhausen et al., 1997). Both types of signal interact with the heterotetrameric adaptor protein complexes AP-1, AP-2, and AP-3. AP-1 is com-

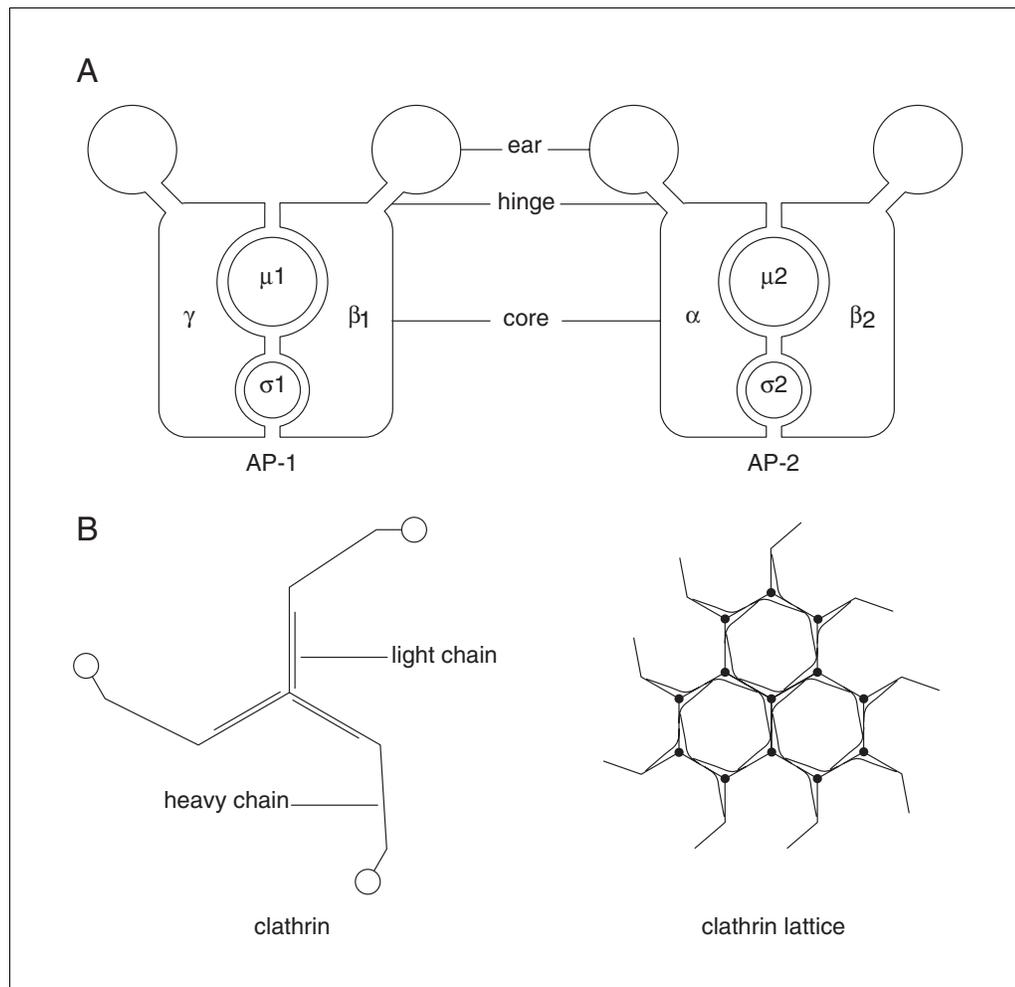


Figure 15.1.5 Clathrin and associated proteins. **(A)** Subunit composition and overall structure of AP-1, which is associated with the TGN, and of AP-2, which is associated with the plasma membrane. **(B)** Clathrin is composed of three heavy chains and three light chains forming a triskelion (left). Clathrin oligomerizes to form two-dimensional lattices (right) or three-dimensional clathrate structures (not pictured).

posed of γ , β_1 , μ_1 , and σ_1 subunits; AP-2 of α , β_2 , μ_2 , and σ_2 subunits; and AP-3 of δ , β_3 , μ_3 , and σ_3 subunits (Fig. 15.1.5A). AP-1 localizes to the TGN and endosomes, whereas AP-2 is found on the plasma membrane. AP-3 has been proposed to exist in association with the TGN and/or endosomes. Both AP-1 and AP-2 associate with clathrin, a protein composed of three heavy chains (~190 kDa) and three light chains (~30 kDa). Electron microscopy of purified clathrin shows a three-legged structure termed a triskelion (Fig. 15.1.5B). The triskelion can self-associate into both planar lattices and three-dimensional clathrate structures. Both AP-1 and AP-2 promote the formation of these structures. AP-3 also interacts with clathrin *in vitro*, although it is currently unclear whether this interaction is required for AP-3 function *in vivo*.

Lysosomal integral membrane proteins, as well as lysosomal luminal proteins bound via mannose-6-phosphate residues to specific receptors, are concentrated into AP-1/clathrin-coated areas of the TGN. The resulting coated vesicles transport these proteins to early or late endosomes, where they are in turn delivered to lysosomes. The cytosolic domain of the proteins targeted to lysosomes by this pathway—e.g., lysosome associated protein 1 (LAMP-1) and the mannose-6-phosphate receptors—appear to interact directly with AP-1. The mannose-6-phosphate receptors are subsequently recycled to the TGN by virtue of sorting information contained within the cytosolic tail. Recent studies suggest that the AP-3 adaptor complex may also play a role in transport of lysosomal membrane proteins to lysosomes.

Transport of proteins to the plasma membrane occurs either by default or by information contained within the cytosolic domains of the proteins. All plasma membrane proteins were originally thought to be targeted directly from the TGN. However, recent evidence suggests that at least a fraction of proteins intersect with the endocytic pathway en route to the cell surface (Fig. 15.1.1). In polarized epithelial cells, proteins can be targeted to either the basolateral or the apical plasma membrane. Basolateral targeting of integral membrane proteins appears to be dominant over apical targeting and depends on information found in the cytosolic domain of the proteins. Truncation of the cytosolic domain results in distribution to both the basolateral and apical membranes or only to the apical membrane. Proteins may be directed to the apical region by concentration into membrane domains rich in sphingolipids and cholesterol, or by interaction of N-linked oligosaccharides with putative sorting lectins in the TGN (Keller and Simons, 1997).

Exocrine, endocrine, and neuronal cells, as well as certain other cell types, have specialized vesicles whose secretion is regulated. Two models have been proposed for the biogenesis of these vesicles. One model proposes that proteins aggregate in the environment of the TGN, and that nonaggregated proteins are sorted from the condensed proteins in the immature vesicles that pinch off from the TGN. A second model proposes that the proteins bind to a putative receptor in a manner similar to the mechanism used by soluble lysosomal proteins (reviewed in Keller and Simons, 1997).

ENDOCYTOSIS

Most plasma membrane proteins are subject to internalization. However, only a subset of plasma membrane proteins are internalized at a very rapid rate. In most cases, rapid internalization occurs at areas of the plasma membrane that are coated with clathrin and the AP-2 adaptor complex (i.e., clathrin-coated pits). Concentration of integral membrane proteins within clathrin-coated pits is mediated by cytosolic tyrosine-based or dileucine-based signals similar to those that mediate sorting at the TGN. These signals interact directly with proteins within the clathrin-coated pits. Rapid internalization of endocytic receptors can occur either constitutively or upon ligand binding. For example, the transferrin receptor and the low-density lipoprotein receptor are internalized by a constitutive mechanism, even in the absence of

ligand. In contrast, the rate of endocytosis of the epidermal growth factor receptor increases several fold upon ligand binding (Wiley, 1988).

Clathrin-coated pits become invaginated and eventually pinch off as clathrin-coated vesicles. Various accessory molecules such as dynamin, amphiphysin, and Eps15 participate in the formation of clathrin-coated vesicles (reviewed in Schmid, 1997). The clathrin-coated vesicles are subsequently uncoated by the HSP70 chaperone in an ATP-dependent reaction and fuse with early endosomes. In this compartment, internalized proteins are either sorted to late endosomes and lysosomes (e.g., activated epidermal growth factor receptors) or recycle to the plasma membrane (e.g., transferrin receptors, low-density lipoprotein receptors).

BIOCHEMICAL ANALYSES OF PROTEIN TRAFFICKING

The localization of proteins to different compartments of the secretory and endocytic pathways described above can be determined by immunofluorescence and immunoelectron microscopy (see Chapter 4). In addition, direct visualization of protein movement in these pathways is now possible through the use of chimeras containing green fluorescent protein (GFP). However, quantitative analyses of protein trafficking in cell populations require the use of biochemical assays. These assays rely on the acquisition of posttranslational modifications (e.g., glycosylation, disulfide-bond formation, proteolytic cleavage), accessibility to externally added reagents (e.g., biotinylating agents, glycosidases), or detection by specific probes (e.g., labeled ligands or antibodies). The following units in this chapter describe a series of protocols based on these principles.

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Use Of Glycosidases To Study Protein Trafficking

Carbohydrate chain modifications are often used to monitor glycoprotein movement through the secretory pathway (UNIT 15.1). This is because stepwise sugar-chain processing is unidirectional and generally corresponds to the forward or anterograde movement of proteins. This unit offers a group of techniques that will help analyze the general structure of carbohydrate chains on a protein and, therefore, oligosaccharide processing mileposts. The minimum requirements are that the protein can be labeled metabolically (UNIT 7.1) and immunoprecipitated (UNIT 7.2) and clearly seen on a gel or blot (UNIT 6.2). The sugar chains themselves are not analyzed, but their presence and structure are inferred from gel mobility differences after one or more enzymatic digestions. This approach is most often used in combination with [³⁵S]Met pulse-chase metabolic labeling protocols, but they can be applied to any suitably labeled protein (e.g., biotinylated or ¹²⁵I-labeled). As the oligosaccharide chains mature, they become either sensitive or resistant to highly specific glycosidases. Some of these enzymes cleave intact oligosaccharide chains from the protein—e.g., endo H, endo F₂, endo F₃, peptide:N-glycosidase F (PNGase F), endo D, and O-glycosidase. Others strip only terminal sugars (e.g., sialidase) or degrade a selected portion of the chain (e.g., endo-β-galactosidase). The techniques can be adapted to count the number of N-linked oligosaccharide chains on a protein. One unusual protease (O-sialoglycoprotease) degrades only proteins containing tight clusters of O-linked sialylated sugar chains. These techniques work best on average size proteins (<100 kDa) that contain a few percent carbohydrate by weight, where a gel shift of 1 kDa can be seen. A summary of the enzymes and their applications is shown in Table 15.2.1.

The ever-changing view of the organization and structure of the secretory pathway, and of protein trafficking through it, presents formidable conceptual challenges, but these will not be covered here. Instead, this unit provides information on how to measure changes in carbohydrate structure and how these changes relate to protein trafficking. Fortunately,

Table 15.2.1 Enzymes Described in This Unit

Enzyme	Indications and uses	Monitors ^a
Endo D	Transient appearance of highly processed, sensitive forms prior to addition of GlcNAc by GlcNAc transferase I	<i>Cis</i> to medial Golgi
Endo F ₂	Presence of biantennary chains ± core fucose	Medial Golgi
Endo F ₃	Presence of core fucosylated biantennary α chains and/or triantennary chains ± core fucosylation	Medial Golgi
Endo-β-galactosidase	Presence of poly lactosamines	<i>Trans</i> -Golgi and TGN
Endo H	Conversion of high mannose to complex type N-linked chains	<i>Cis</i> - to medial Golgi
O-Glycosidase	Presence of Galβ1,3GalNAc-α-Thr/Ser O-linked chains	<i>Cis</i> /medial Golgi
PNGase F	Presence of N-linked chains cleaves; nearly all N-linked chains; only enzyme that cleaves tetrantennary chains	Medial Golgi
Sialidase	Acquisition of sialic acids	<i>Trans</i> -Golgi and TGN
O-Sialoglycoprotease	Presence of mucin-like proteins with cluster of sialylated oligosaccharides	<i>Trans</i> -Golgi and TGN

^aAbbreviation: TGN, *trans*-Golgi network.

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the techniques are independent of mechanistic views, although it should be borne in mind that the organization and distribution of many of these indicator enzymes are cell type dependent.

The starting material for these protocols is assumed to be [³⁵S]Met-labeled, immunoprecipitated protein bound to ~20 μl of protein A–Sepharose beads (as described in UNIT 7.2). The trace amount of protein is eluted by heating in a small volume of 0.1% SDS, diluted in the appropriate buffer and then digested with one or more enzymes in a small volume. The digest is analyzed on an appropriate SDS-PAGE system that can detect a 1- to 2-kDa size change. A change in the mobility of the protein after digestion is evidence that the carbohydrate chain was sensitive to the enzyme and therefore, that the protein had encountered a certain enzyme in the processing pathway. Alternatively, the analysis can be done by two-dimensional isoelectric focusing (IEF)/SDS-PAGE or two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE)/SDS-PAGE (APPENDIX 3) to see the loss of charged sugar residues or of anionic oligosaccharide chains. The same digestions and SDS-PAGE analysis also apply to proteins that are radioiodinated or biotinylated, or to immunoprecipitates derived from subcellular fractions separated on sucrose or Percoll gradients.

It is important to present the glycosylation pathways, as a detailed description of the pathways is needed to appreciate how they will be used in this unit. A single protein can have more than one kind of oligosaccharide (N-linked and O-linked), and each individual N-linked chain can mature into a different final form. The same is true for O-linked chains. Each is described below.

THE N-LINKED PATHWAY

The N-linked oligosaccharide maturation pathway is most frequently used for tracking protein movement through the Golgi complex. A common feature of all N-linked chains is the core region pentasaccharide shown in Figure 15.2.1, which consists of three mannose units and two *N*-acetylglucosamine units. The mannose units comprise the trimannosyl core, and two of these residues are α-linked to the only β-linked mannose in the molecule. The β-linked mannose is bound to one of the two *N*-acetylglucosamines. Because they are β1-4 linked to each other, resembling the polysaccharide chitin, this is called a chitobiose disaccharide. Initially, all *N*-glycosylated proteins begin life when a preformed, lipid-associated oligosaccharide is transferred within the lumen of the endoplasmic reticulum (ER) to Asn of proteins having an Asn-X-Thr/Ser sequence. This precursor oligosaccharide contains three glucose (Glc), nine mannose (Man), and two *N*-acetylglucosamine (GlcNAc) sugar residues, and has the structure shown in Figure 15.2.1. There are several ways to depict this structure. The short-hand symbol method used in Figure 15.2.1 is the most convenient, but be sure to note the linkages of the individual sugars, as they are important. The α and β symbols denote the anomeric configuration of the sugar, and the number indicates which hydroxyl group of the next sugar is involved in the glycosidic linkage. In all cases, the anomeric position is 1, except in sialic acid where it is 2.

The details of the pathway are presented in Figures 15.2.2 and 15.2.3, along with the sensitivity to each endoglycosidase or glycoamidase. The figures show the steps between high-mannose and hybrid types (Fig. 15.2.2) and complex types (Fig. 15.2.3). The three Glc residues (filled triangles) are removed from properly folded proteins within the ER by two different oligosaccharide-processing α-glucosidases. The first α1-2Glc is cleaved by α-glucosidase I, and the next two α1-3Glc residues by α-glucosidase II (Fig. 15.2.2, step 1). An ER-associated α-mannosidase removes one Man residue (open circle; Fig.

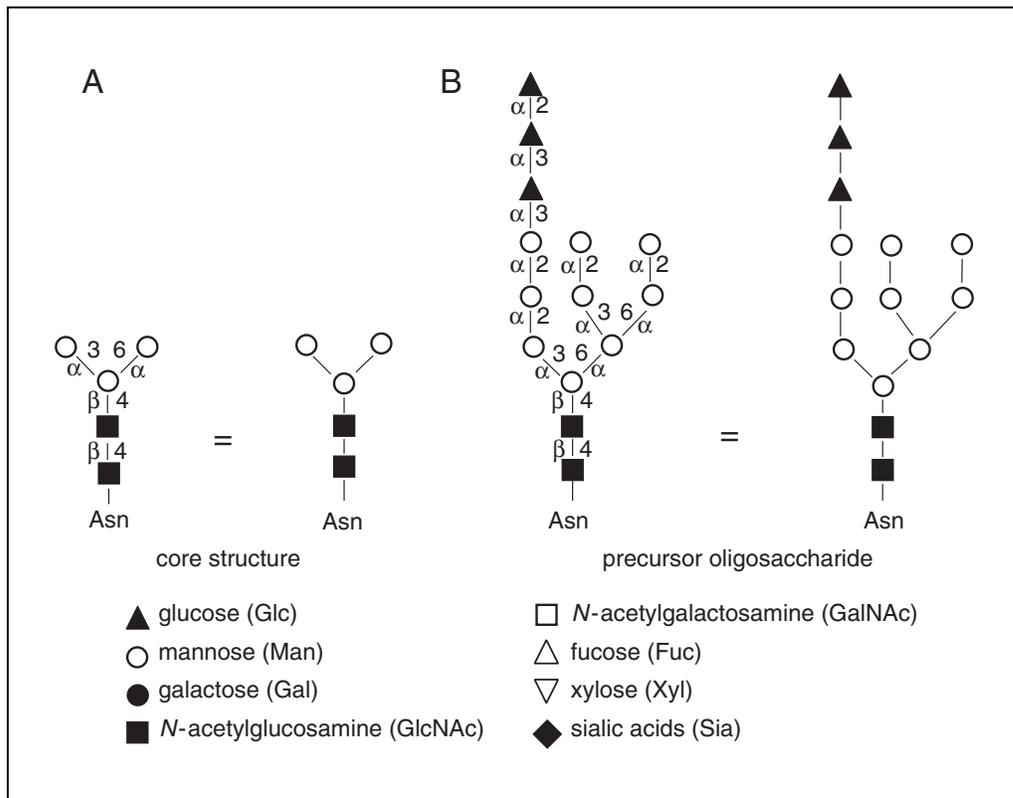


Figure 15.2.1 Symbol structures for the core region and precursor of N-linked sugar chains. Each sugar is given a symbol and abbreviation at the bottom of the figure. Each one except sialic acid uses its anomeric carbon (C-1) for linking to other sugars. Sialic acid uses C-2 for glycosidic linkage to other sugars. Glycosidases and glycosyltransferases are anomeric specific and distinguish α or β configurations of each sugar. The core structure (**A**) is common to all N-linked chains and is composed of three Man and two GlcNAc residues. The α or β configuration of each sugar is indicated, and the OH group to which that sugar is linked is shown on the bar linking the two symbols. Thus, GlcNAc β 1-4GlcNAc β is represented by two filled squares with β and 4 between them. When a structure is first presented, it will have full display such as that on the left side; if it is repeated, only the symbols will be used as shown immediately to the right. The precursor oligosaccharide (**B**) for all N-linked chains is synthesized in the ER and transferred cotranslationally to the peptide containing an available Asn-X-Thr/Ser sequon.

15.2.2, step 2). The protein then moves on to the first step in Golgi-localized processing—the removal of the three remaining α 1-2 Man units by Golgi α -mannosidase I to produce Man₅GlcNAc₂ (Fig. 15.2.2, steps 3 and 4). Many proteins have only high-mannose-type oligosaccharides with five to nine Man residues, and no further processing occurs. Alternatively, one to five GlcNAc residues (filled squares) can be added to the trimannosyl core, and these are usually extended with galactose (Gal; filled circles) and sialic acid (Sia; filled diamonds) residues. These extensions, called antennae, are the hallmarks of complex-type oligosaccharides. The transformation of the precursor sugar chain into various high-mannose or complex types is called oligosaccharide processing (Kornfeld and Kornfeld, 1985).

Man₅GlcNAc₂ is an important intermediate because it can have several fates. The first is the well-established addition of one GlcNAc residue by GlcNAc transferase I (Fig. 15.2.2, step 6). This is the first step toward the formation of complex chains. However, simply adding Gal and Sia to the terminal GlcNAc of this oligosaccharide forms a hybrid structure (Fig. 15.2.2, steps 12 and 13), where the left side of the molecule looks like a complex chain having one antenna, and the right side still resembles a high-mannose chain. The

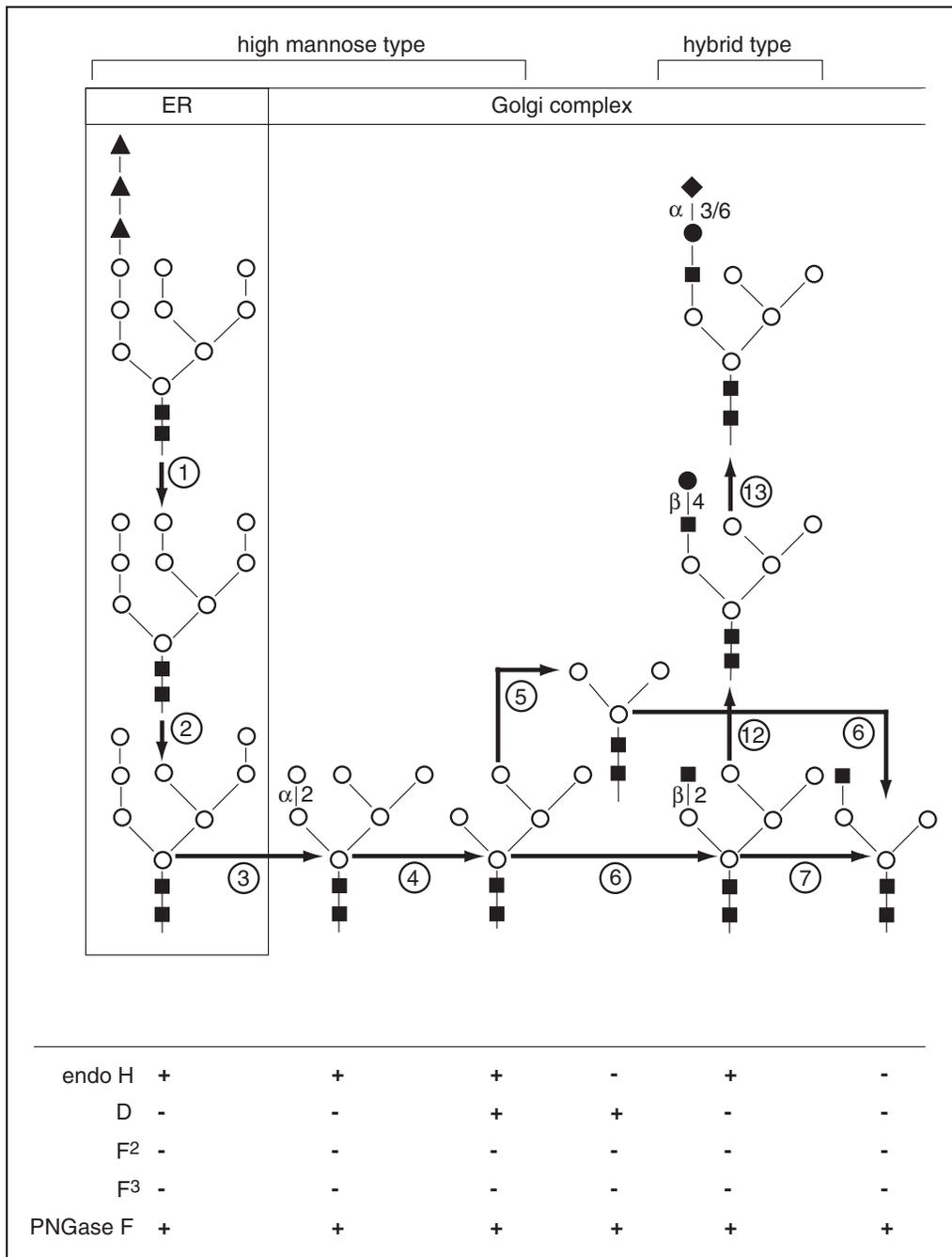


Figure 15.2.2 N-linked oligosaccharide maturation pathway for high-mannose and hybrid types, and sensitivities to various enzymes (see Fig. 15.2.1 for key). Brackets (top) show the structures designated as high-mannose and hybrid chains. The boxes indicate ER or Golgi localization. The pathway begins with the precursor oligosaccharide (see Figure 15.2.1). Each successive numbered step in circles represents a glycosidase or glycosyl transferase that generates a new sugar chain with different sensitivities to the various endoglycosidases or PNGase F. (1) precursor oligosaccharide is trimmed by α -glucosidases I and II, removing three Glc. (2) ER mannosidase removes one Man. (3) α -Mannosidase I in Golgi complex removes two Man to make $\text{Man}_6\text{GlcNAc}_2$, with a single remaining α 1-2Man. (4) The final α 1-2Man is removed by a Golgi complex α -mannosidase I. (5) α -Mannosidase III removes the α 1-3 and α 1-6Man units to make $\text{Man}_3\text{GlcNAc}_2$. (6) GlcNAc transferase I adds GlcNAc to either $\text{Man}_5\text{GlcNAc}_2$ or $\text{Man}_3\text{GlcNAc}_2$. (7) α -Mannosidase II removes the α 1-3 and α 1-6Man units to make $\text{GlcNAc}_1\text{Man}_3\text{GlcNAc}_2$. Sensitivity to various enzymes (bottom) changes when moving from left to right, but remains the same within vertical columns. *NOTE:* This continued maturation to form complex chains is shown in Figure 15.2.3. Additionally, these figures are not comprehensive; many glycosylation steps have not been included, but they do not affect the sensitivities to the enzymes listed.

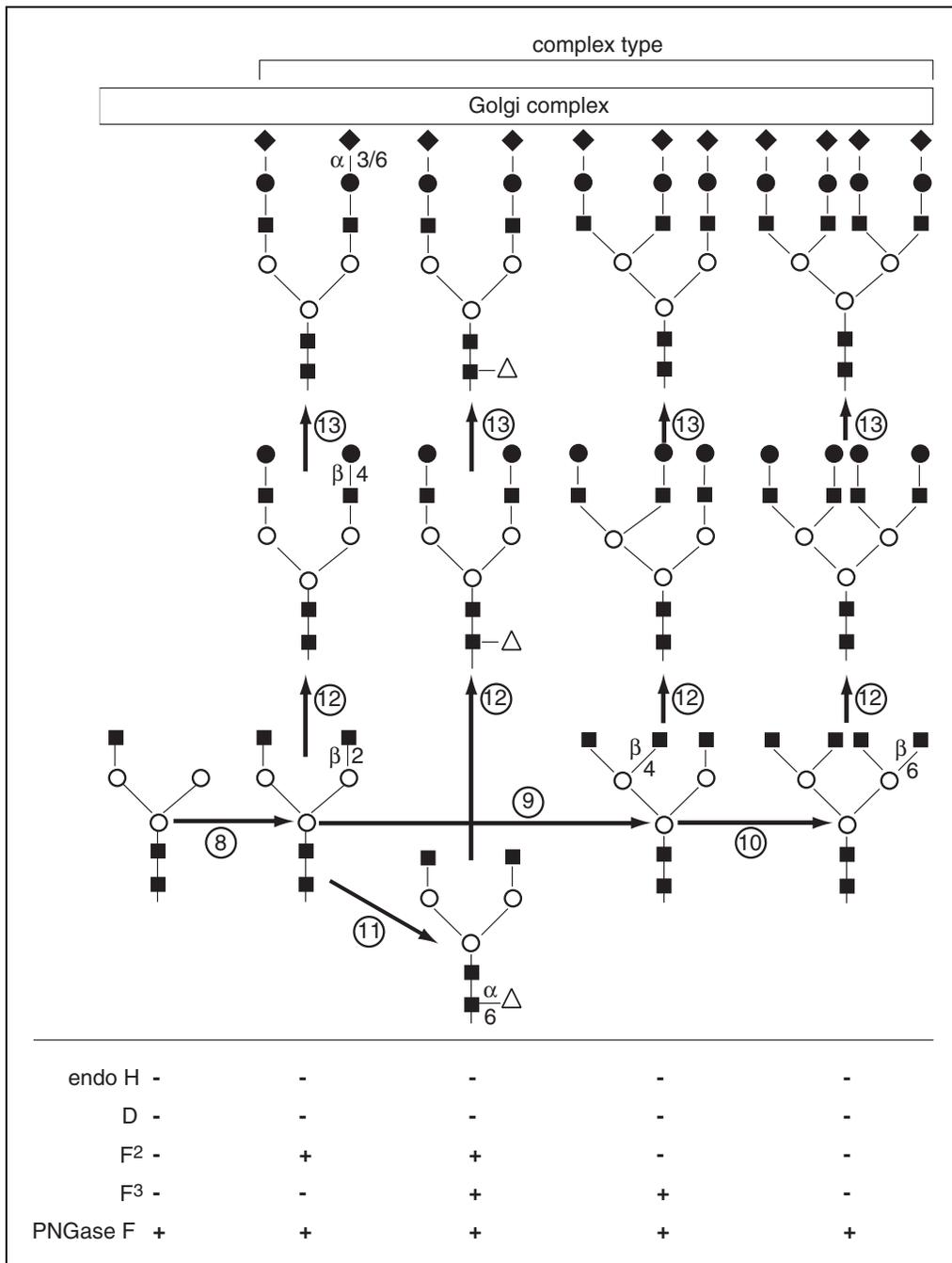


Figure 15.2.3 N-linked oligosaccharide maturation pathway for complex types, and sensitivities to various enzymes (see Fig. 15.2.1 for key; see Fig. 15.2.2 for additional details). **(8)** GlcNAc transferase II adds a second GlcNAc to initiate a biantennary chain. **(9)** GlcNAc transferase IV adds a third GlcNAc to initiate a triantennary chain. **(10)** GlcNAc transferase V adds a fourth GlcNAc to initiate a tetraantennary chain. **(11)** Fucosyltransferase adds α 1-6Fuc to the core region of complex chains. **(12)** β 1-4Gal is added to available GlcNAc residues of hybrid and complex chains. **(13)** α 2-3 or α 2-6Sia is added to Gal residues of hybrid and complex chains.

GlcNAc₁Man₃GlcNAc₂ structure is the *required* substrate for α -mannosidase II, which removes the two terminal Man units from the upper branch of the chain (i.e., the α 1-3Man and α 1-6 Man units; Fig. 15.2.2, step 7). This enzyme only works after the addition of the first GlcNAc.

The other fate for the Man₃GlcNAc₂ chain is to act as a substrate for the newly identified α -mannosidase III (Chui et al., 1997), which removes the same two Man units as α -mannosidase II, but does not require the prior addition of the first GlcNAc (Fig. 15.2.2, step 5). α -Mannosidases II and III show partial overlap in the Golgi complex, but α -mannosidase III may occur preferentially in an earlier compartment. The Man₃GlcNAc₂ product of α -mannosidase III is also a substrate for GlcNAc transferase I. Thus, the same product, GlcNAc₁Man₃GlcNAc₂ can be formed in two ways: first, by the sequential action of α -mannosidase III and GlcNAc transferase I (Fig. 15.2.2, steps 5 and 6) or, second, by GlcNAc transferase I and α -mannosidase II (Fig. 15.2.2, steps 6 and 7). Sensitivity to specific enzyme digestions (endo H and endo D) can distinguish which route was taken (Fig. 15.2.2).

GlcNAc transferase II now adds a second GlcNAc to the α 1-6-linked Man (Fig. 15.2.3, step 8). This molecule can also have several fates. First, fucose (Fuc) can be added to GlcNAc residue linked to the Asn of the protein (Fig. 15.2.3, step 11). Second, one to three more GlcNAc residues can be added to the core mannose residues to initiate tri- and tetraantennary chains (Fig. 15.2.3, steps 9 and 10), and even pentaantennary chains (not shown). GlcNAc additions are considered to occur in the medial Golgi regions. Each GlcNAc-based branch can be individually modified, but they are usually extended by one Gal (Fig. 15.2.3, step 12) and terminated by a Sia (Fig. 15.2.3, step 13). Both of these sugars are usually thought to be added in *trans*-Golgi cisternae or in the *trans*-Golgi network (TGN). Sometimes selected antennae are also fucosylated in the TGN. One or more terminal Gal residues can be extended by variable-length polylactosamines (Gal β 4-GlcNAc repeats) capped by a Sia. GlcNAc and Gal can be sulfated as a late, perhaps even final, step of processing. These extensions/modifications are thought to occur in the late Golgi complex and TGN, but their order and compartmental segregation are not well understood. Other modifications of N-linked sugar chains are known, but there are fewer tools available to analyze their biosynthetic localization.

THE O-LINKED PATHWAY

For practical purposes, only a portion of the O-linked pathway—i.e., the addition of the first few sugars—will be presented. However, it is very important to remember that some of the same outer chain structures such as Sia, polylactosamines, and Fuc residues are common to both N- and O-linked oligosaccharides.

α -*N*-acetylgalactosamine (α -GalNAc; open square) is the lead-off sugar for the O-linked pathway (Fig. 15.2.4; also see Fig. 15.2.1 for symbols). It is added to Ser/Thr residues that occur in the proper configuration, generating a broad variety of acceptor sequences. These sequences often cluster as repeats within mucin-like domains. GalNAc is added in the earliest parts of the Golgi complex, not cotranslationally. GalNAc can be further extended by at least six different sugars. The most common is the addition of a β 1-3Gal (Fig. 15.2.4, step 1), forming a disaccharide that is one of the few O-linked chains that can be diagnosed by enzymatic digestions. This disaccharide is often capped by a Sia (Fig. 15.2.4, step 2). Additional sugars such as Sia (Fig. 15.2.4, step 3) or GlcNAc followed by Gal (Fig. 15.2.4, steps 4 and 5) can be added. Structural analysis can be done by sequential exoglycosidase digestion, but given the complexity and heterogeneity of the sugar chains, such analysis is not a very useful indicator for tracking protein movement through the Golgi complex. Many O-linked chains have terminal Sia residues and, when

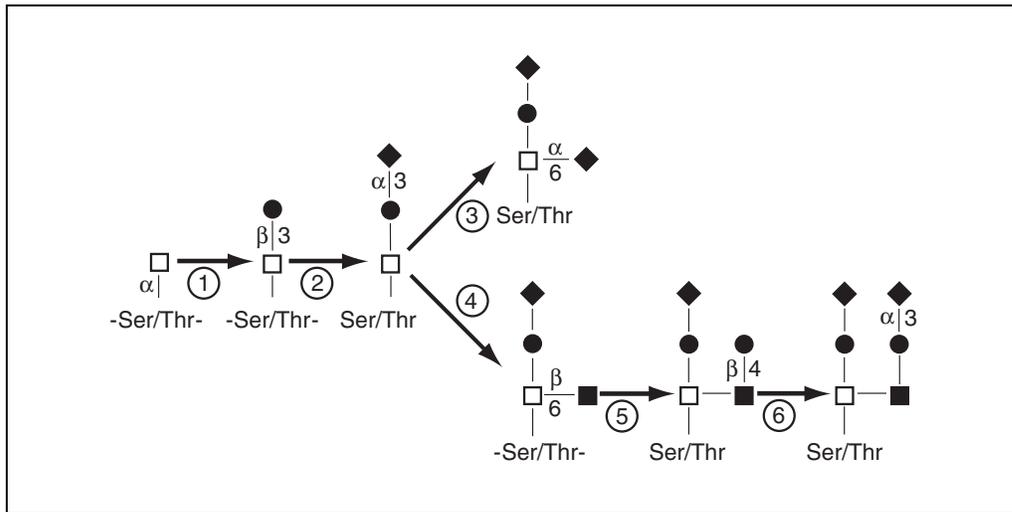


Figure 15.2.4 A small portion of the O-GalNAc pathway (see Fig. 15.2.1 for key). The first step of the O-linked pathway occurs in the early Golgi complex with the addition of α -GalNAc. There are at least six other sugars that can be added at this point in this complex pathway. Often β 1-3Gal is added (1), quickly followed by α 2-3Sia (2). The presence of these structures can be detected with a combination of O-glycosidase and sialidase. Additional sugars can be added as shown. α 2-6Sia (3) or β 1-6GlcNAc (4) followed by β 1-4Gal (5) and α 2-3Sia (6) on Gal. Each of these sugars must be removed before O-glycosidase can cleave the disaccharide.

tightly clustered on Ser/Thr residues, these chains promote proteolysis by O-sialoglycopeptidase regardless of the structure of the underlying sugar chain.

Another type of O-linked glycosylation is the addition of glycosaminoglycan (GAG) chains to form proteoglycans. This occurs by a different pathway than the α -GalNAc linkage. Instead, the chains begin by addition of a β -Xylose (Xyl; open inverted triangle) residue to Ser and are then elongated by two Gal residues and a glucuronic acid (GlcA; half-filled diamond) residue. This core structure can be further elongated by the addition of GlcA β 1-3GalNAc β disaccharides to form the backbone of chondroitin/dermatan sulfate chains, or by GlcA β 1-3GlcNAc α to form the backbone of heparan sulfate chains. Biosynthesis and movement of these proteins have also been followed through the Golgi complex. Initiation begins in late ER/early Golgi complex, and the core tetrasaccharide is probably finished within the medial Golgi, but the addition of chondroitin chains appears to be confined to the TGN. In addition to the well-known O-linked GAG chains, there is clear evidence for the existence of a class of N-linked GAG chains.

ENDOGLYCOSIDASE H DIGESTION

Endoglycosidase H (endo H) cleaves N-linked oligosaccharides between the two *N*-acetylglucosamine (GlcNAc) residues (Fig. 15.2.5) in the core region of the oligosaccharide chain (Fig. 15.2.1) on high-mannose and hybrid, but not complex, oligosaccharides. In this protocol, a fully denatured protein is digested with endo H to obtain complete release of sensitive oligosaccharides.

Materials

- Immunoprecipitated protein of interest (UNIT 7.2)
- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 0.5 M sodium citrate, pH 5.5
- 1% (w/v) phenylmethylsulfonyl fluoride (PMSF) in isopropanol

BASIC PROTOCOL 1

Protein Trafficking

15.2.7

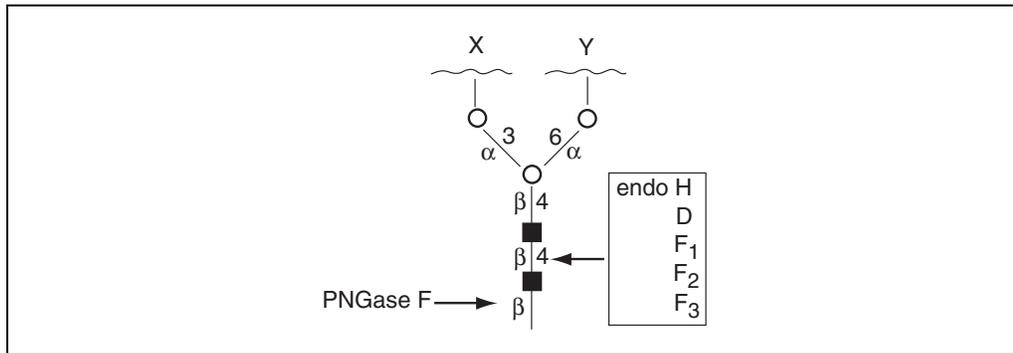


Figure 15.2.5 PNGase F and endoglycosidase-sensitive bonds in the core of N-linked oligosaccharides (see Fig. 15.2.1 for key). PNGase F is a glycoamidase that severs the bond between GlcNAc and Asn, liberating the entire sugar chain and converting Asn into Asp. The endoglycosidases (H, D, and Fs) cleave the bond between the two GlcNAc residues in the core region, leaving one GlcNAc still bound to the protein. The differential specificity of the endoglycosidases is based on the structure of the sugar chain in a fully denatured protein. Incomplete denaturation may not expose all sensitive linkages. X and Y are unspecified sugar residues.

0.5 U/ml endoglycosidase H (endo H; natural or recombinant; Sigma, Glyko, or Boehringer Mannheim)

10× SDS-PAGE sample buffer (APPENDIX 2A)

Water baths, 30° to 37°C and 90°C

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3)

1. Add 20 to 30 μ l of 0.1 M 2-ME/0.1% SDS to immunoprecipitate in a microcentrifuge tube, mix well, and heat denature 3 to 5 min at 90°C.

Use the larger amount of reagent ($\geq 30 \mu$ l) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

Protein solubilization in nonionic detergents such as Triton X-100 or Nonidet P-40 is not always sufficient to completely expose all susceptible cleavage sites. Only strong denaturation with SDS exposes all sites for maximum cleavage.

2. Cool and microcentrifuge for 1 sec at 1000 \times g to collect condensed droplets in the bottom of the tube.
3. Place 10- μ l aliquots of solubilized, denatured protein (supernatant) in each of two clean microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).
4. Add in the following order, mixing after each addition:

6 μ l 0.5 M sodium citrate, pH 5.5

20 μ l H₂O

2 μ l 1% PMSF (in isopropanol)

1 μ l 0.5 U/ml endo H (enzyme digest only; substitute with water in control).

The PMSF prevents proteolysis. Nonionic detergent is not required to prevent inactivation of endo H as long as high-purity SDS is used.

The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate overnight at 30°C to 37°C.

6. Immediately prior to electrophoresis, inactivate endo H by adding 4 μ l of 10 \times SDS-PAGE sample buffer and heating 5 min at 90°C.
7. Analyze protein by one-dimensional SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3).

The presence of high mannose and/or hybrid N-linked oligosaccharide chains will be evidenced by increased mobility of the digested proteins on SDS-PAGE.

ENDOGLYCOSIDASE D DIGESTION

Like endo H, endo D also cleaves between the two GlcNAc residues in the core of the N-linked sugar chains (Fig. 15.2.5). However, its narrow substrate specificity makes it useful for detecting the transient appearance of just a few early processing intermediates. It requires that the 2 position of the α 1-3-linked core Man be unsubstituted. This intermediate arises after processing by either α -mannosidase I or III, but prior to addition of the first GlcNAc or action of α -mannosidase II (see Fig. 15.2.2, steps 3 to 5). Cells with a defect in GlcNAc I transferase (e.g., Lec 1 CHO cells) do not add the first GlcNAc residue (Fig. 15.2.2, step 6), and N-linked oligosaccharides will remain sensitive to endo D because they cannot modify the α 1-3Man residue.

Materials

Immunoprecipitated protein of interest (UNIT 7.2)
0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
0.5 M NaH₂PO₄, pH 6.5
10% (w/v) Triton X-100 (APPENDIX 2A) or Nonidet P-40 (NP-40)
0.5 U/ml endoglycosidase D (endo D; Boehringer Mannheim)
10 \times SDS-PAGE sample buffer (APPENDIX 2A)
Water baths, 37° and 90°C

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3)

1. Denature immunoprecipitated protein in 20 to 30 μ l of 0.1 M 2-ME/0.1% SDS by heating for 3 to 5 min at 90°C.

Use the larger amount of reagent ($\geq 30 \mu$ l) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

2. Cool and microcentrifuge at 1000 \times g for 1 sec to collect condensed droplets in the bottom of the tube.
3. Transfer 10- μ l aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).
4. Add in the following order, mixing after each addition:

2 μ l 10% Triton X-100 or NP-40 (20-fold excess over SDS)
2 μ l 0.5 M NaH₂PO₄, pH 6.5
5 μ l H₂O
1 μ l 1 IU/ml endo D (enzyme digest only; substitute with water in control).

The 20-fold excess of nonionic detergent is essential to prevent inactivation of endo D by SDS.

The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because

they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate at 37°C overnight.
6. Immediately prior to electrophoresis, inactivate by adding 2 µl of 10× SDS-PAGE sample buffer and heating for 5 min at 90°C to 95°C.
7. Analyze protein by one-dimensional SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3).

Endo D sensitivity is detected by increased electrophoretic mobility of the digested proteins on SDS-PAGE.

**BASIC
PROTOCOL 3**

ENDOGLYCOSIDASE F₂ DIGESTION

Endo F₂, like endo H and endo D, cleaves between the two GlcNAc residues in the chitobiose core (Fig. 15.2.5). It preferentially releases biantennary complex-type oligosaccharide chains from glycoproteins, but does not cleave tri- or tetraantennary chains.

Materials

- Immunoprecipitated protein of interest (UNIT 7.2)
- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 0.5 M sodium acetate, pH 4.5
- 10% (w/v) Triton X-100 (APPENDIX 2A) or Nonidet P-40 (NP-40)
- 0.1 M 1,10-phenanthroline in methanol
- 200 mU/ml endoglycosidase F₂ (endo F₂; Glyko)
- 4× SDS-PAGE sample buffer (APPENDIX 2A)
- Water baths, 30° to 37°C and 90°C

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3)

1. Denature immunoprecipitated protein in 20 to 30 µl of 0.1 M 2-ME/0.1% SDS by heating for 3 to 5 min at 90°C.
Use the larger amount of reagent (≥30 µl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.
2. Cool and microcentrifuge at 1000 × g for 1 sec to collect condensed droplets in the bottom of the tube.
3. Transfer 10-µl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).
4. Add in the following order, mixing after each addition:

- 15 µl 0.5 M sodium acetate, pH 4.5
- 3 µl 0.1 M 1,10-phenanthroline in methanol
- 2 µl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
- 1 µl 200 mU/ml endo F₂ (enzyme digest only; substitute 0.5 M sodium acetate in control).

A 10- to 20-fold excess of nonionic detergent is required to stabilize the enzyme.

The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate the mixture at 30°C to 37°C overnight.

Some inactivation of the enzyme occurs at 37°C, even with nonionic detergent present; however, if the enzyme is present in sufficient excess, incubation can generally be carried out successfully at 37°C.

6. Immediately before electrophoresis, inactivate by adding 8 µl of 4× SDS-PAGE sample buffer and heating for 5 min at 90°C to 95°C.
7. Analyze the protein by one-dimensional SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3).

Sensitivity to endo F₂ is detected by increased electrophoretic mobility on SDS-PAGE.

ENDOGLYCOSIDASE F₃ DIGESTION

Endoglycosidase F₃ (endo F₃) is another endoglycosidase with a narrow substrate range and, therefore, high specificity: it cleaves triantennary chains, but not high-mannose, hybrid, nonfucosylated biantennary or tetraantennary chains. A core-fucosylated biantennary chain is the only other demonstrated substrate. When both endo F₃ and endo F₂ digestions are done in parallel on a sample, it can provide evidence for chain branching and core fucosylation. The approach is essentially the same as for the other endoglycosidases.

Materials

Immunoprecipitated protein of interest (UNIT 7.2)

0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)

0.5 M sodium acetate, pH 4.5

10% (w/v) Triton X-100 (APPENDIX 2A) or NP-40

0.1 U/ml endoglycosidase F₃ (endo F₃; Glyko)

10× SDS-PAGE sample buffer (APPENDIX 2A)

Water baths, 37° and 90°C

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3)

1. Denature immunoprecipitated protein in 20 to 30 µl of 0.1 M 2-ME/0.1% SDS and heat denature 3 to 5 min at 90°C.

Use the larger amount of reagent (≥30 µl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

2. Cool and microcentrifuge for 1 sec at 1000 × g to collect condensed droplets at the bottom of the tube.
3. Transfer 10-µl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

4. Add in the following order, mixing after each addition:

2 µl 10% Triton X-100 or NP-40 (20-fold excess over SDS)

4 µl 0.5 M sodium acetate, pH 4.5

5 µl H₂O

1 µl 0.1 U/ml endo F₃ (enzyme digest only; substitute with water in control).

The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate at 37°C overnight.
6. Immediately prior to electrophoresis, inactivate by adding 2 µl of 10× SDS-PAGE sample buffer and heating for 5 min at 90°C to 95°C.
7. Analyze by one-dimensional SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3).
Sensitivity to endo F₃ is detected by increased mobility on SDS-PAGE.

PEPTIDE: N-GLYCOSIDASE F DIGESTION

PNGase F is a glycoamidase that cleaves the bond between the Asp residue of the protein and the GlcNAc residue that joins the carbohydrate to the protein (Fig. 15.2.5). Because it liberates nearly all known N-linked oligosaccharides from glycoproteins, it is the preferred enzyme for complete removal of N-linked chains. It is the only enzyme that releases tetra- and pentaantennary chains. The glycoprotein sample must be denatured and digested with PNGase F to remove N-linked oligosaccharides completely.

Materials

- Immunoprecipitated protein of interest (UNIT 7.2)
- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 0.5 M Tris·Cl, pH 8.6 determined at 37°C (APPENDIX 2A)
- 10% (w/v) Triton X-100 (APPENDIX 2A) or Nonidet P-40 (NP-40)
- 200 to 250 mU/ml peptide:N-glycosidase F (PNGase F; Sigma or Glyko)
- 10× SDS sample buffer (APPENDIX 2A)
- Water baths, 30° to 37°C and 90°C

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3)

1. Denature immunoprecipitated protein in 20 to 30 µl of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 90°C.
Use the larger amount of reagent ≥30 µl for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.
2. Cool and microcentrifuge for 1 sec at 1000 × g to collect condensed droplets in the bottom of the tube.
3. Transfer 10-µl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).
4. Add in the following order, mixing after each addition:
 - 3 µl 0.5 M Tris·Cl, pH 8.6
 - 5 µl H₂O
 - 2 µl 10% NP-40 or Triton X-100
 - 5 µl 200 to 250 mU/ml PNGase F (enzyme digest only; substitute with 0.5 M Tris·Cl in control).

Sodium phosphate or HEPES buffer, pH 7.0, can be used instead of Tris·Cl. Avoid potassium buffers because these may cause precipitation of a potassium SDS salt. Use of a nonionic detergent is essential, because SDS inactivates PNGase F. A 10-fold weight excess of any of the above nonionic detergents over the amount of SDS will stabilize the enzyme.

The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate overnight at 30°C to 37°C.

6. Immediately prior to electrophoresis, inactivate the enzyme by adding 2.5 μ l of 10 \times SDS-PAGE sample buffer and heating 3 to 5 min at 90°C.
7. Analyze the protein by one-dimensional SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3).

The presence of N-linked oligosaccharide chains will be evidenced by increased electrophoretic mobility on SDS-PAGE.

ESTIMATING THE NUMBER OF N-LINKED OLIGOSACCHARIDE CHAINS ON A GLYCOPROTEIN

SUPPORT PROTOCOL

One widely used application of endo H or PNGase F digestion is estimation of the number of N-linked oligosaccharide chains on a given glycoprotein. This is done by creating a ladder of partially digested molecules that each differ by only one N-linked sugar chain. The number of separate bands in a one-dimensional polyacrylamide gel (less one for the totally deglycosylated protein) provides an estimate of the number of N-linked chains. The conditions used to generate partially deglycosylated protein must be determined for each protein studied, because the sensitivity of each chain may be different, even when all of them are completely exposed by denaturation. For this protocol, either the incubation time or the amount of enzyme can be varied to determine the best conditions to produce a ladder of partial digests. Usually five or six points are enough to provide a reasonable estimate (Fig. 15.2.6). Of course, it is important to use enough enzyme to obtain complete deglycosylation. This is best done by monitoring the effects of endo H or PNGase F on newly synthesized [35 S]Met pulse-labeled protein just after synthesis, but before any N-linked oligosaccharide processing has occurred. Pulse labeling of protein for 10 min with [35 S]Met followed by digestion is the best way to be sure that all chains are removed.

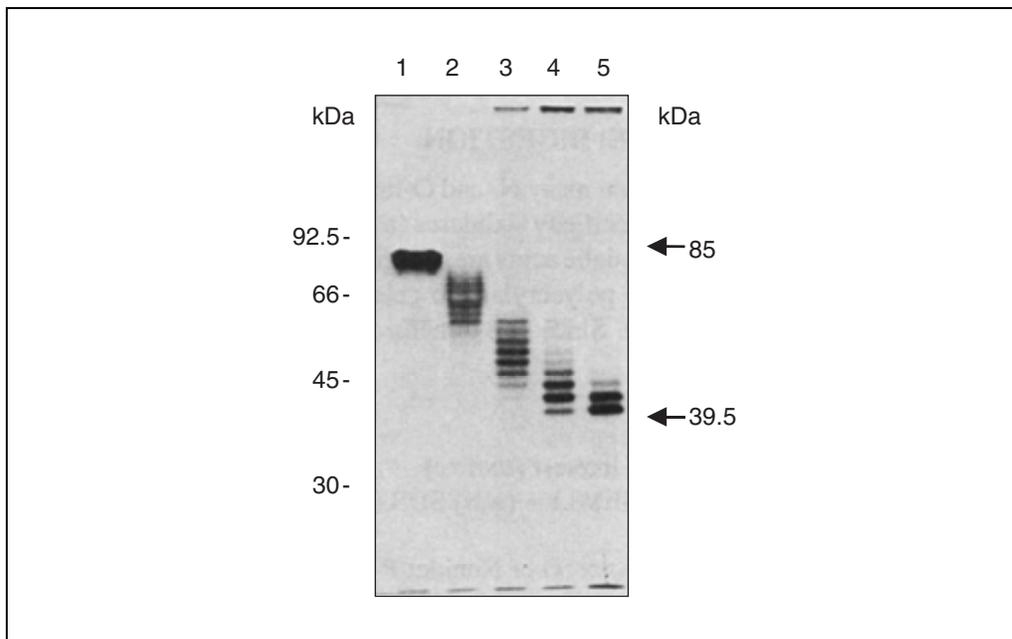


Figure 15.2.6 Data from the estimation of the number of glycosylation sites on lysosome-associated membrane protein 1 (LAMP-1; Viitala et al., 1988). LAMP-1 contains eighteen potential N-linked sites. Graded digestion with increasing amounts of PNGase F was used to generate this ladder of glycoforms. Each band contains at least one less N-linked chain than the band above it. An average N-linked carbohydrate chain has an apparent mass of ~1.5 to 3 kDa. Lysosomal membrane glycoprotein was immunoprecipitated from [35 S]Met-labeled cells and the sample was digested with PNGase F for 0 min (lane 1), 5 min (lane 2), 20 min (lane 3), 45 min (lane 4), and 24 hr (lane 5). Figure courtesy of Dr. Minoru Fukuda.

Protein Trafficking

15.2.13

1. Add 0.1 M 2-ME/0.1% SDS solution to the total volume of immunoprecipitated protein required and heat denature by incubating 3 to 5 min at 90°C.

Each digestion reaction requires 20 μ l of immunoprecipitate. Thus, 120 to 140 μ l is sufficient for one control plus five or six digests.

2. Cool and centrifuge for 1 sec at 1000 \times g to collect condensed droplets at the bottom of the tube.
3. Aliquot 10 μ l supernatant to the number of microcentrifuge tubes required to cover the concentration range (e.g., 0.01 to 1 mU/ml PNGase F) or incubation times (e.g., 5 to 60 min) plus one for an undigested control.
4. Add remaining reagents as specified for endo H (see Basic Protocol 1, step 4) or PNGase F (see Basic Protocol 5, step 4), adjusting the enzyme concentration as desired.
5. Incubate at 30°C for the desired length of time.

High enzyme concentration (10 mU/ml) and prolonged incubation (16 hr) must be among the conditions included, in order to ensure that there is a data point for maximum deglycosylation.

For varying enzyme concentrations, incubate for the same amount of time, but the duration of incubation should be shorter than what would give complete digestion because the goal is to obtain increasing extent of incomplete cleavage.

6. After the desired incubation time, inactivate enzyme by adding 0.1 volume of μ l of 10 \times SDS-PAGE sample buffer and heating 5 min at 90° to 95°C.
7. Analyze the sample from each concentration/time point, including undigested sample, by one-dimensional SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3).

Most newly formed N-linked chains will have a molecular weight in the range of 1500 to 2200, and loss of one chain is sufficient to change the migration of a protein. This procedure has been used to count up to eighteen N-linked sites on one molecule. A sample result is shown in Figure 15.2.6.

BASIC PROTOCOL 6

SIALIDASE (NEURAMINIDASE) DIGESTION

Sialic acids are the terminal sugars on many N- and O-linked oligosaccharides. The great majority are released with broad-specificity sialidases (neuraminidases) such as that from *Arthrobacter ureafaciens*. Because sialic acids are charged, their loss usually changes the mobility on one-dimensional SDS polyacrylamide gels, but it will always change the mobility on a two-dimensional gel. Since one-dimensional analysis is easier, it can be tried first.

Materials

Immunoprecipitated protein of interest (UNIT 7.2)
0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
10% (w/v) Triton X-100 (APPENDIX 2A) or Nonidet P-40 (NP-40)
0.5 M sodium acetate, pH 5.0 (APPENDIX 2A)
1 IU/ml neuraminidase from *Arthrobacter ureafaciens* (Sigma or Glyko)
10 \times SDS sample buffer (APPENDIX 2A)
Water baths, 37° and 90°C

Additional reagents and equipment for SDS-PAGE (UNIT 6.1), IEF/SDS-PAGE, or NEPHGE/SDS-PAGE (APPENDIX 3), and for autoradiography (UNIT 6.3)

1. Denature immunoprecipitated protein in 20 to 30 μl of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 90°C.

Denaturation is less important here, because the sialic acids are exposed at the ends of the sugar chains. In most instances, the denaturation step can probably be omitted and the digestion done while the protein is still bound to the beads.

2. Cool and microcentrifuge for 1 sec at 1000 \times g to collect condensed droplets in the bottom of the tube.
3. Transfer 10- μl aliquots of supernatant to two clean microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).
4. Add in the following order, mixing after each addition:

2 μl 10% Triton X-100 or NP-40 (20-fold excess over SDS)

4 μl 0.5 M sodium acetate, pH 5.0

5 μl H₂O

1 μl 1 IU/ml neuraminidase (enzyme digest only; substitute with water for control).

This amount of neuraminidase should be in great excess. Addition of nonionic detergent is not needed if the digestion is done while the protein was still bound to the beads.

The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate at 37°C overnight.

This time can be shortened to 2 hr, if necessary, but longer incubations are better.

6. Immediately prior to electrophoresis, inactivate the enzyme by adding 2 μl of 10 \times SDS sample buffer and heating 3 to 5 min at 90°C.

If the protein will be analyzed by IEF or NEPHGE, addition of sample buffer is replaced by lysis buffer used for these techniques.

7. Analyze the protein on the appropriate one-dimensional SDS-PAGE system (UNIT 6.1) or by a two-dimensional IEF/SDS-PAGE or NEPHGE/SDS-PAGE system (APPENDIX 3), and detect by autoradiography (UNIT 6.3).

Removal of sialic acids usually results in a decrease in apparent molecular weight on one-dimensional gel analysis, or an increase in the isoelectric point of the protein analyzed by two-dimensional gel analysis.

ENDO- β -GALACTOSIDASE DIGESTION

The endo- β -galactosidase from *Bacillus fragilis* degrades poly-lactosamine chains (Gal β 1-4GlcNAc β 1-3)_n found on both N- and O-linked oligosaccharides. The variable length of these repeating units usually causes the protein to run as a broad band or a smear on the gel. Although not all linkages are equally cleaved by this enzyme (see Fig. 15.2.7), sensitive proteins that often run as broad bands or smears on gels—e.g., lysosome-associated membrane protein 1 (LAMP-1)—produce both sharper bands and lower molecular weight species after digestion.

**BASIC
PROTOCOL 7**

**Protein
Trafficking**

15.2.15

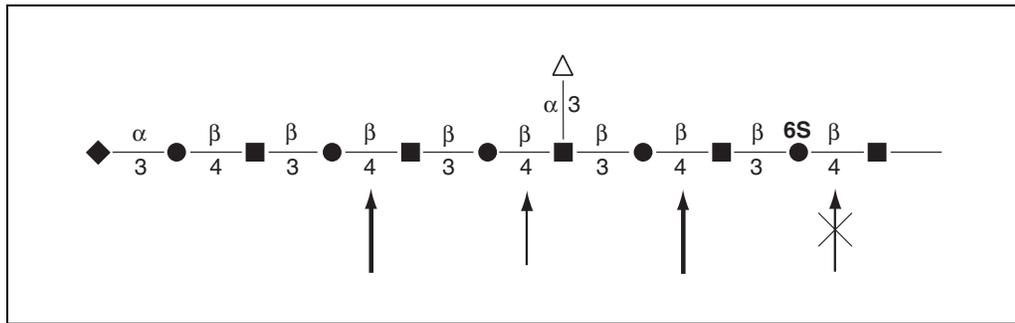


Figure 15.2.7 Endo- β -galactosidase-sensitive linkages in poly-lactosamines (see Fig. 15.2.1 for key). Linear, unsubstituted poly-lactosamine units (GlcNAc β 1-3Gal β 1-4) are sensitive to digestion with endo- β -galactosidase, while substitutions—such as sulfate esters (S) or branches starting with GlcNAc (not shown)—completely block digestion. Substitution of neighboring GlcNAc with Fuc or sulfate esters slows the rate, but does not block cleavage. Sensitive sites are shown with bold arrows, slowly hydrolyzed sites with a lighter arrow, and resistant bonds are struck out. Various substitutions are possible, leading to broad bands on gels. This will create variable sensitivities, but even partial sensitivity should give a sharper, more defined band.

Materials

Immunoprecipitated protein of interest (UNIT 7.2)

0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)

0.5 M sodium acetate buffer, pH 5.8

10% (w/v) Triton X-100 (APPENDIX 2A) or Nonidet P-40 (NP-40)

100 mU/ml endo- β -galactosidase (Boehringer Mannheim, Oxford GlycoSystems, Sigma)

10 \times SDS-PAGE sample buffer (APPENDIX 2A)

Water baths, 37 $^{\circ}$ and 95 $^{\circ}$ C

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3)

- Denature immunoprecipitated protein in 20 to 30 μ l of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 95 $^{\circ}$ C.

Use the larger amount of reagent ($\geq 30 \mu$ l) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

- Cool and microcentrifuge 1 sec at 1000 \times g to collect condensed droplets in the bottom of the tube.
- Transfer 10- μ l aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).

- Add in the following order, mixing after each addition:

2 μ l 10% Triton X-100 or NP-40 (20-fold excess over SDS)

4 μ l 0.5 M sodium acetate, pH 5.8

5 μ l H₂O

1 μ l 100 mU/ml endo- β -galactosidase (enzyme digest only; substitute with water in control).

The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate at 37°C overnight.
6. Immediately prior to electrophoresis, inactivate by adding 3 μ l of 10 \times SDS-PAGE sample buffer and heating for 5 min at 90°C.
7. Analyze protein by one-dimensional SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3).

If the protein has polylactosamine chains, its mobility should increase after digestion.

ENDO- α -N-ACETYLGALACTOSAMINIDASE DIGESTION

This enzyme (also known as O-glycosidase or O-glycanase) has limited utility because it is highly specific for cleaving only one O-linked disaccharide, Gal β 1-3GalNAc α -Ser/Thr. Adding any more sugars, including sialic acid, renders the molecule resistant to cleavage and requires removal of each residue before the enzyme will work. Prior sialidase digestion is sometimes used (see Basic Protocol 6), and this can be done while the protein is still bound to the immunoprecipitation beads.

Materials

- Immunoprecipitated protein of interest (UNIT 7.2)
- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 0.5 M sodium citrate phosphate buffer, pH 6.0, containing 500 μ g/ml BSA (complete buffer supplied with enzyme)
- 10% (w/v) Triton X-100 (APPENDIX 2A) or Nonidet P-40 (NP-40)
- 300 mU/ml endo- α -N-acetylgalactosaminidase (5 \times concentrate from Glyko; use according to directions)
- 10 \times SDS-PAGE sample buffer (APPENDIX 2A)
- Water bath, 37° and 95°C
- Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3)

1. Denature immunoprecipitated protein in 20 to 30 μ l of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 95°C.

Use the larger amount of reagent ($\geq 30 \mu$ l) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.

2. Cool and microcentrifuge 1 sec at 1000 \times g to collect condensed droplets in the bottom of the tube.
3. Transfer 10- μ l aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).
4. Add in the following order, mixing after each addition:

- 2 μ l 10% Triton X-100 or NP-40 (20-fold excess over SDS)
- 4 μ l 0.5 M sodium citrate phosphate buffer, pH 6.0, with 500 μ g/ml BSA
- 3 μ l H₂O
- 1 μ l 300 mU/ml endo- α -N-acetylgalactosidase (enzyme digest only; substitute with water in control).

The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate at 37°C overnight.
6. Immediately prior to electrophoresis, inactivate by adding 2 µl of 10× SDS-PAGE sample buffer and heating for 5 min at 90°C.
7. Analyze protein by one-dimensional SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3).

If the protein contains the disaccharide unit, the mobility of the protein should increase. Presence of only a single unit (mol. wt. ~400 Da) may be difficult to detect unless a high-resolution gel is used.

O-SIALOGLYCOPROTEASE DIGESTION

Digestion with O-sialoglycoprotease requires that the substrate have a tight cluster of sialylated O-linked oligosaccharides. Proteins with a single O-linked chain or a few widely spaced chains will not be cleaved. This property makes the enzyme a valuable diagnostic tool.

Materials

- Immunoprecipitated protein of interest (UNIT 7.2)
- 0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; prepare fresh)
- 0.4 M HEPES buffer, pH 7.4
- 10% (w/v) Triton X-100 (APPENDIX 2A) or Nonidet P-40 (NP-40)
- 2.4 mg/ml O-sialoglycoprotease (O-sialoglycoprotein endoglycoprotease; Accurate Chemical & Scientific; reconstituted according to directions)
- 10× SDS-PAGE sample buffer (APPENDIX 2A)
- Water baths, 37° and 95°C

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3)

1. Denature immunoprecipitated protein in 20 to 30 µl of 0.1 M 2-ME/0.1% SDS by heating 3 to 5 min at 95°C.

Use the larger amount of reagent (≥30 µl) for more complete recovery. This treatment may also release some (unlabeled) antibody molecules.
2. Cool and microcentrifuge 1 sec at 1000×g to collect condensed droplets in the bottom of the tube.
3. Transfer 10-µl aliquots of supernatant into two microcentrifuge tubes, one for a control (no enzyme) and the other to digest (plus enzyme).
4. Add in the following order, mixing after each addition:
 - 2 µl 10% Triton X-100 or NP-40 (20-fold excess over SDS)
 - 4 µl 0.4 M HEPES buffer, pH 7.4
 - 5 µl H₂O
 - 2 µl 2.4 mg/ml O-sialoglycoprotease (enzyme digest only; substitute with water in control).

O-Sialoglycoprotein endopeptidase is a partially purified enzyme, and the specific activity is relatively low. A quantity of 1.0 µg of this enzyme preparation will cleave 5 µg of sensitive substrate per hour at 37°C. Human glycoporphin A can serve as a positive control.

The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but potassium buffers should be avoided because they precipitate SDS as a potassium salt. The reaction volume can be scaled up proportionally if required, but in nearly all cases the enzyme will be in vast excess.

5. Incubate at 37°C overnight.
6. Immediately prior to electrophoresis, inactivate by adding 2.5 μ l of 10 \times SDS-PAGE sample buffer and heating for 5 min at 90°C.
7. Analyze protein by one-dimensional SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3).

If the digestion was successful, the target protein will be undetectable or may be cleaved into small fragments.

COMMENTARY

Background Information

The results of digestion of a hypothetical protein with two N-linked carbohydrate chains as it moves through the ER and Golgi complex with various enzymes are shown in Figure 15.2.8. At 0 min, both N-linked chains are high-mannose type. They have lost their Glc residues and one Man residue in the ER. Both are sensitive to endo H and PNGase F digestion,

yielding a protein with only two remaining GlcNAc residues in the case of endo H digestion, and no carbohydrate at all in the case of PNGase F digestion. These sugar chains are resistant to the other enzyme digestions.

At 45 min, the protein is in the medial Golgi complex and both sugar chains have been processed by Golgi α -mannosidase I. However, one of the chains (left) has been partially processed

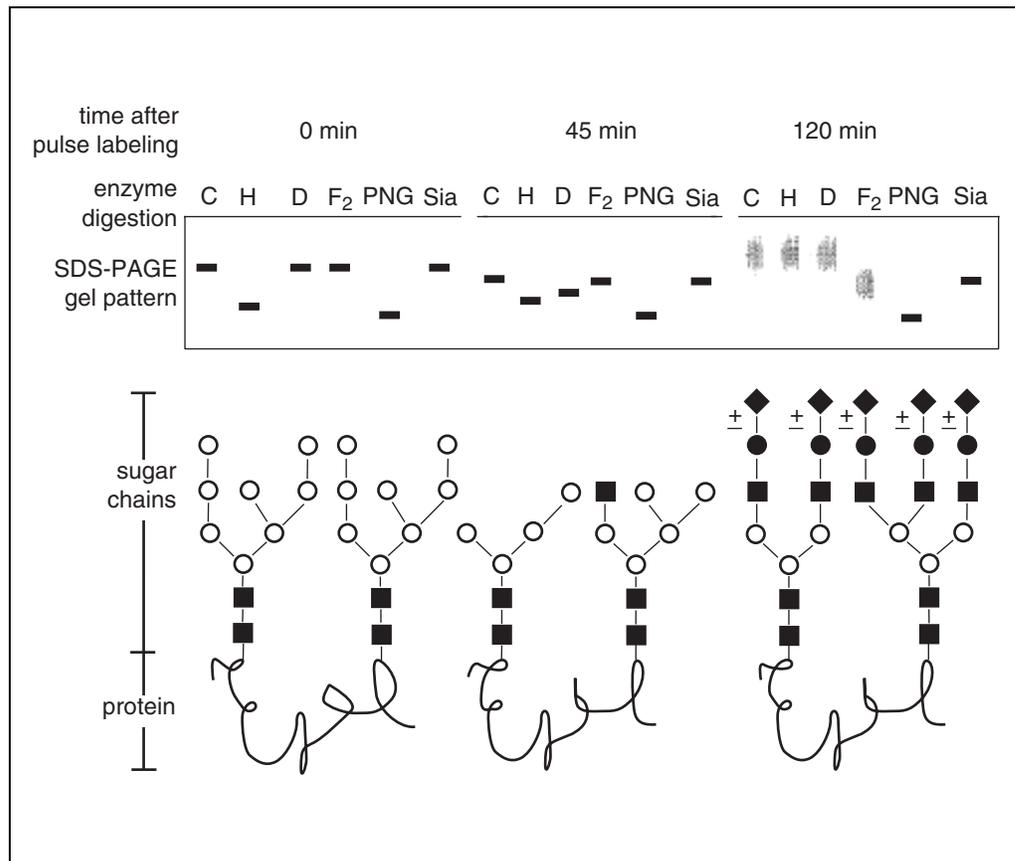


Figure 15.2.8 Schematic diagram showing results that could be obtained for a hypothetical protein with two N-linked glycosylation sites as it moves through the Golgi complex. Assume that the protein has been biosynthetically labeled with an amino acid precursor (such as [35 S]Met) for 10 min and chased in the absence of label for 45 min and 120 min. The protein is then precipitated with a specific antibody. At each time point, equal amounts of the sample are analyzed by fluorography after one-dimensional SDS-PAGE, either without any digestion (control; C) or following digestion with endo H (H), endo D (D), endo F₂ (F₂), PNGase F (PNG), or sialidase (Sia). Oligosaccharide structures consistent with the banding patterns are shown below the schematic gel pattern.

by Golgi α -mannosidase III to an endo H-resistant/endo D-sensitive chain. The other chain (right) has received a GlcNAc residue from GlcNAc transferase I, but has not encountered Golgi α -mannosidase II. This chain is still endo H sensitive and endo D resistant. Both chains are sensitive to PNGase F, but neither is sensitive to endo F₂ or sialidase digestion.

At 120 min, both chains have fully matured to complex-type chains. One is a sialylated biantennary chain and the other is a sialylated triantennary chain. Note that each sialic acid is marked \pm , indicating that not all molecules are fully sialylated, accounting for the broader bands. Neither sugar chain is sensitive to endo H or endo D digestion. The biantennary chain (left) is sensitive to endo F₂ cleavage, leaving one GlcNAc residue on the protein, but the triantennary chain (right) is not sensitive to this enzyme. Both chains are cleaved by PNGase F. All sialic acids are removed by sialidase to produce a sharp band, but the underlying sugar chains remain.

Endoglycosidase H

Endo H from *Streptomyces plicatus* cleaves the bond between the two GlcNAc residues in the core of N-linked oligosaccharides. One GlcNAc residue remains attached to the protein or peptide and the remainder of the chain is released as an intact unit (Tarentino et al., 1989). The oligosaccharide structures cleaved by endo H are shown schematically in Figure 15.2.5. Substrates for endo H include all high-mannose oligosaccharides and certain hybrid types, but not bi-, tri-, tetra- or pentaantennary (complex) chains. Endo H also cleaves oligosaccharides that have α 1-6 fucose residues bound to the reducing (protein to carbohydrate linkage) GlcNAc residue (Tarentino et al., 1989).

Endo H sensitivity is the most common way to trace the movement of newly synthesized glycoproteins from the endoplasmic reticulum (ER) into the Golgi complex. Proteins remain sensitive to endo H while they are in the ER and in early regions of the Golgi complex; they become endo H-resistant after they are processed by enzymes located in the medial Golgi complex. Endo H cleaves the N-linked oligosaccharides from proteins as long as they have not lost the α 1-3Man residue cleaved by Golgi mannosidase II or mannosidase III (Fig. 15.2.2). After removal of that mannose, the oligosaccharide becomes endo H resistant. Endo H is the best enzyme for identifying high-mannose and hybrid chains and their general transition toward complex chains.

Using endo H alone gives an incomplete map of subcellular trafficking. The resolution of this picture can be enhanced by combining it with endo D digestions. In general, N-linked chains become endo H resistant at about the same time they become transiently sensitive to endo D. However, the discovery of the alternative pathway for complex N-glycan processing using α -mannosidase III now gives a different perspective. Both α -mannosidase II and III are expressed in most mouse tissues except for hematopoietic cells, which seem to have only α -mannosidase II. So it is conceivable that some proteins, and even different N-linked chains on a single protein, can be processed by α -mannosidase II and others by α -mannosidase III, yielding complex digestion patterns and kinetics of endo H and endo D sensitivity.

Endoglycosidase D

The narrow substrate specificity of endo D makes it useful for detecting a very restricted set of processing intermediates, particularly for distinguishing α -mannosidase II from α -mannosidase III processing. When the results of endo D digestion are combined with the results of an endo H digestion of the same sample, it can help determine which of the two alternate processing pathways is being used. The key requirement for endo D cleavage is an unsubstituted 2 position on the α 1-3Man that forms the trimannosyl core (Fig. 15.2.1). This window of endo D sensitivity occurs only *after* removal of the α 1-2Man residue by α -mannosidase I and *before* the addition of a GlcNAc residue to this same position by GlcNAc transferase I (Beckers et al., 1987; Davidson and Balch, 1993). The immediate precursor of both pathways is Man₆GlcNAc₂, which is endo D resistant/endo H sensitive. The better-known α -mannosidase II pathway involves the removal of the last α 1-2Man residue by α -mannosidase I (making the oligosaccharide endo D sensitive/endo H sensitive), and then the addition of GlcNAc via GlcNAc transferase I (making it endo D resistant/endo H sensitive). This product, and only this one, is the substrate for α -mannosidase II, which removes two of the remaining five Man residues, specifically the α 1-3Man and α 1-6Man that form the upper branch. Once this occurs, this endo D-resistant molecule also becomes endo H resistant. This structure is also a substrate for GlcNAc transferase II on the way to forming a biantennary chain.

The second pathway involves α -mannosidase III, which was identified as a functionally

important enzyme in nearly all tissues when the α -mannosidase II pathway was ablated in mice (Chui et al., 1997). After α -mannosidase I removes the final α 1-2Man from Man₆GlcNAc₂ to generate Man₅GlcNAc₂, α -mannosidase III cleaves the same two Man residues as α -mannosidase II; however, α -mannosidase III cleavage does not require addition of the first GlcNAc that α -mannosidase II requires. α -Mannosidase III creates the endo D-sensitive/endo H-resistant trimannosyl core. This sugar chain serves as an acceptor for GlcNAc transferase I to form an endo D-resistant/endo H-resistant molecule that can be modified by GlcNAc transferase II, and then on to biantennary chains. As summarized in Figure 15.2.2, using the two endoglycosidases together will distinguish whether the oligosaccharide was processed by α -mannosidase II or α -mannosidase III. This may be important since there is evidence that distribution of the two enzymes in the Golgi complex is different with α -mannosidase III in an earlier compartment. It is important to point out that all chains of even a single protein are not necessarily processed using all the same enzymes.

Mutant cell line CHO Lec1 lacks GlcNAc transferase I activity and cannot synthesize either complex or hybrid chains. These cells can be used to measure the kinetics of acquiring endo D sensitivity (Beckers et al., 1987). This cell line can be obtained through ATCC (CRL-1735). The chains become permanently endo D sensitive, because they lack GlcNAc transferase I and cannot be converted back to an endo D-resistant form. These chains will also remain endo H sensitive, because α -mannosidase II requires GlcNAc transferase I. However, if any chains are processed by α -mannosidase III, they would become endo H resistant. Again, the combination of endo D and endo H digestions can reveal which pathway was used.

CHO Lec1 cells are also useful for tracking the movement of a protein from the ER into the earliest Golgi compartment where α -mannosidase I is located. Acquisition of endo D sensitivity requires the action of this enzyme. The advantage of using Lec1 cells is that the proteins remain permanently endo D sensitive and there is no risk of kinetically missing that small window of sensitivity before the sugar chain might become endo D resistant once again. Even if α -mannosidase III acts on the protein, it would still remain endo D sensitive, and no further processing would occur.

Endoglycosidase F and peptide N-glycosidase F

Elder and Alexander (1982; Alexander and Elder, 1989) made a landmark discovery when they identified an enzyme in culture filtrates of the bacterium *Flavobacterium meningosepticum* that cleaved N-linked oligosaccharides from glycoproteins. This preparation had a broad substrate specificity. The endoglycosidase activity in this preparation (endo F) was actually due to a set of enzymes, each with a more restricted substrate range. Like endo H, the endo F enzymes cleave the sugar chains between the two core GlcNAc residues (Fig. 15.2.5). Endo F was originally thought to be a single enzyme, but it is now known that each of the three enzymes has a distinct specificity (Plummer and Tarentino, 1991). The specificity of endo F₁ is very similar to that of endo H, while endo F₂ prefers biantennary chains, and endo F₃ will cleave core fucosylated biantennary and triantennary, but not tetraantennary, chains (Fig. 15.2.2 and Fig. 15.2.3). All are commercially available from Glyko.

Plummer et al. (1984) carefully analyzed *Flavobacterium* filtrates and found that the very broad substrate range was actually due to a glycoamidase activity rather than an endoglycosidase activity. The glycoamidase releases the entire carbohydrate chain from the protein by cleaving the Asp-GlcNAc bond (Fig. 15.2.5). The enzyme is called by various names, including peptide:N-glycosidase F (PNGase F), glycopeptidase F, and N-glycanase (previously available from Genzyme), but the proper name is peptide N-4(N-acetyl- β -glucosaminyl)asparagine amidase F. PNGase F has the broadest specificity, and it releases most of the N-linked oligosaccharide chains from proteins.

Endo F₂ and Endo F₃

Endo F₂ prefers biantennary chains over high-mannose chains by ~20 fold. Thus, endo H and PNGase F are better choices for broadly distinguishing high-mannose from complex chains as described above in the endo H protocol (Tarentino and Plummer, 1994).

Many proteins have core-fucosylated N-linked glycans, and the addition of fucose can be used as an additional trafficking marker. Endo F₃ will hydrolyze triantennary chains, but endo F₂ will not. Endo F₂ hydrolyzes biantennary chains; however, endo F₃ will also hydrolyze core-fucosylated biantennary chains only a bit more slowly than it does triantennary chains. Thus, if all the chains on a protein are

sensitive to endo F₂ (biantennary) and to endo F₃, this is evidence for the presence of a core fucose on those chains.

The enhancement of endo F₃ activity on biantennary chains with a core α 1-6Fuc points out that some specificities are really a matter of relative rates of cleavage. If both endo F₂ (biantennary) and endo F₃ (triantennary and biantennary with core fucose) cleave the protein, they may be acting on different chains on the same molecule. If there is only a single chain, repeat the experiment under the same conditions using 10- to 20-fold dilutions of each enzyme. If both still cleave the chain about equally, it is evidence for core fucosylation of a biantennary chain.

PNGase F

PNGase F has the broadest specificity of all the enzymes that cleave N-linked oligosaccharides. It is indifferent to all extended structures on the chains, such as sulfate, phosphate, polylactosamines, polysialic acids, and even the occasional glycosaminoglycan chain. Most of the modifications in the Man₃GlcNAc₂ core region also make no difference in chain cleavage. The only oligosaccharide structural feature that confers PNGase F resistance is the presence of an α 1-3Fuc on the GlcNAc bound to Asn (Tretter et al., 1991). This modification is commonly found in plants and in some insect glycoproteins, but it is rare in most mammalian cell lines. However, caution is warranted, as there is evidence that some mammalian cells do have the critical α 1-3Fuc transferase, and some studies show that a majority of N-linked chains of bovine lung are actually PNGase F resistant! It is not known how common this resistance may be. It is thus important to document N-glycosylation with proteins still in the ER (see Support Protocol) before they might be processed to a PNGase F-resistant form.

Sialidase

Sialidases are also called neuraminidases because the most common form of sialic acid is *N*-acetylneuraminic acid. The sialic acids are a family with over forty different members, but fortunately the very great majority of them can be removed from the oligosaccharides by the broad-spectrum sialidase from *Arthrobacter ureafaciens* (AUS). It can even digest polysialic acids, a rare modification found on only a few proteins such as neuronal cell adhesion molecule (NCAM). Sialidases with selected specificities from other sources are available but would not usually be needed. AUS has an op-

imum pH of 5.0, with ~30% of maximum activity at pH 7.

Because sialic acids are charged, they affect gel mobility of proteins more than would be expected from their nominal molecular weight. The magnitude of the gel shift depends on the number of residues. It is difficult to estimate their number by sialidase digestion, but the mobility change is usually sufficient if there are several sialic acid residues. On the other hand, if a protein has only one sialic acid, its presence could be missed using standard one-dimensional SDS-PAGE. To be certain of the effects of sialidase, the sample can be analyzed by a two-dimensional system, using IEF or NEPHGE in the first dimension. The loss of even a single sialic acid will be evident because it changes the isoelectric point.

AUS will remove sialic acids from both N- and O-linked chains, so the type of chain carrying them must be determined independently using PNGase F or possibly O-glycosidase in combination with sialidase. A protein will generally be partially or completely resistant to O-glycosidase because the required disaccharide, Gal β 1-3GalNAc, is usually extended and often sialylated. Until the sialic acid is removed, it will be resistant.

The presence of sialic acid (sialidase sensitivity) is often used as an indication of the transport of a protein into the *trans*-Golgi network (TGN). This may be true in general, but it is important to remember that the distribution of Golgi enzymes is cell type dependent. For instance, α -mannosidases I and II, which are typically considered *cis*/medial Golgi enzymes, are strongly expressed on the brush border of enterocytes—hardly a Golgi compartment. There are other similar examples of various distributions of sialyl transferase. Moreover, there are different sialyl transferases and each may have its own unique distribution. Although one should be cautious, it is probably safe to place sialyl transferase in the late Golgi compartment rather than an early one.

Endo- α -N-acetylgalactosaminidase

This enzyme from *Diplococcus pneumoniae* also goes by various names, including O-glycosidase and O-Glycanase. The last name is a trade name from Genzyme, which no longer sells enzymes; the enzyme is now available from Glyko. This enzyme has a narrow substrate range and cleaves only Gal β 1-3GalNAc α -Ser/Thr. These are only the first two sugars added in the diverse O-linked pathway that can produce glycans with a dozen or more

sugar units. A portion of the pathway is shown in Figure 15.2.4. Fortunately, many, but far from all, O-linked chains have the simple trisaccharide structure and would be sensitive to cleavage after removing the Sia. Thus, sequential individual digestions or mixed digestions can be used. As both Gal and GalNAc (and probably Sia) are added in the early Golgi complex, sensitivity to the enzyme shows that the protein carries O-linked chains, but matching enzyme sensitivity and a Golgi compartment to further chain extension is difficult. Combining a battery of exoglycosidases (sialidase, α -fucosidase, α -*N*-acetylgalactosaminidase, and β -hexosaminidase) with endo- α -*N*-acetylgalactosaminidase will probably remove most O-linked sugar chains, except sulfated ones. The bottom line is that it is easy to use the enzyme in combination with sialidase to show that a protein has simple O-linked chains, but it is difficult to conclude much more concerning either the structure of the sugar chain or intracellular trafficking.

Endo- β -galactosidase

Bacterioides fragilis endo- β -galactosidase is one of several enzymes that specifically degrade polyactosamines by cleaving linear chains of GlcNAc β 1-3Gal β 1-4 repeats at the Gal β 1-4 linkage. Any substitution on the galactose itself blocks cleavage; however, modifications of the neighboring sugars can slow hydrolysis (Fig. 15.2.7). For instance, fucosylation and/or sulfation of nearby GlcNAc slows cleavage, but chain branching or sulfation at Gal block it. Even with these potential complexities, digestion with endo- β -galactosidase will sharpen a broad band even if it does not cleave every linkage. The repeating GlcNAc β 1-3Gal β 1-4 units can be found on both N- and O-linked chains, so sensitivity to PNGase F digestion can potentially distinguish the location. Lysosome-associated membrane protein 1 (LAMP-1) has polyactosamine repeats on N-linked chains. Remember that glycosylation is not template driven, so oligosaccharides often exist as a continuum of different structures on individual proteins. For example, heavily sulfated polyactosamine repeats are also known as keratan sulfate and are degraded by keratanases.

O-Sialoglycoprotease

O-Sialoglycoprotease (also called O-glycoprotease or O-sialoglycoprotein endopeptidase; Mellors and Lo, 1995) is a neutral metalloprotease produced by *Pasteurella*

haemolytica. This enzyme requires clusters of sialylated oligosaccharides on Ser or Thr residues (Norgard et al., 1993). Having a single sialylated O-linked sugar chain on a protein will not lead to degradation, nor will having a nonsialylated sugar chain. Therefore, this enzyme can be used in a typical pulse-chase experiment to indicate whether there are tightly grouped O-linked chains and when they are sialylated. Adding the initial α -GalNAc in the early Golgi complex is insufficient to cause proteolysis; sialylation is specifically required, and this may occur in a later Golgi compartment. Proteolysis can generate smaller fragments of a target protein that are still visible on gels, or the fragments may be so small that they are not even seen on the gels. It depends on the protein. Many leukocyte antigens such as the P-selectin ligand, or others such as CD43, CD44, CD45, and CD34 found on hematopoietic stem cells, are all substrates. As sialic acids are found on both N- and O-linked chains (in clusters and not), sequential digestions using PNGase F, sialidase, and O-sialoglycoprotease endopeptidase in different orders can reveal different kinds of sialylated glycans. If used as part of a pulse-chase protocol, they can reveal different kinetic subcompartments. Not all sialyl transferases are necessarily within the same Golgi compartment.

Critical Parameters

For nearly all of the digestions, complete denaturation of the protein can be important, as maximum deglycosylation occurs only when the sugar chains of glycoproteins are completely exposed. This is not important for sialidase digestions since sialic acids are exposed. Usually endo H digestions will work without full denaturation, but the unprocessed high-mannose chains remain unprocessed because they are often less exposed to the processing enzymes. This may or may not be true of the target protein, but it is better to be safe and denature it completely before digestion. Heating the immunoprecipitate with 0.1 M 2-mercaptoethanol/0.1% SDS is the best way to release the protein in a denatured state. Assuming that the immunoprecipitate contains <10 μ g of protein, 30 μ l of 0.1% SDS still provides a three-fold excess over the protein. Adding SDS presents another problem: free SDS may denature the digesting enzyme before it has a chance to finish its job. For most enzymes, be sure that the free SDS concentration is <0.01%. The best way to do this is to add a 10- to 20-fold weight excess of nonionic detergent with a low critical

micellar concentration (e.g., Triton X-100 or NP-40). These detergents will form mixed micelles with the free SDS and keep it from denaturing the added enzymes.

The amount of enzyme and the incubation time recommended in the protocols are in excess and should be sufficient to cleave any of the sensitive linkages. The incubation times can be shortened, if necessary, but it is better to keep the enzyme concentration as indicated.

Many of the digestions (e.g., sialidase, O-sialoglycoprotease) can be adapted for use on membrane preparations or on live cell surfaces by simply omitting the ionic and nonionic detergents and decreasing the incubation time. The problem is that some linkages may not be exposed and/or sensitive to the digestion. Thus, the usefulness of this approach needs to be determined on a case-by-case basis.

Endo H

Endo H has a broad pH optimum between 5.5 and 6.5, and phosphate or citrate/phosphate buffers can be used in place of citrate. Endo H is very stable to proteases, freezing and thawing, and prolonged incubations. No additives are required for storage of the enzyme. At concentrations below 5 to 10 $\mu\text{g}/\text{ml}$ (200 to 400 mU/ml), endo H will bind to glass, so it should be stored in plastic vials (e.g., screw-cap microcentrifuge tubes).

Endo D

Endo D has a broad pH optimum of 4 to 6.5. One unit of enzyme activity will cleave 1 μmole of a $\text{Man}_5\text{GlcNAc}_3$ glycopeptide per min at 37°C. It is supplied from Boehringer Mannheim as a powder containing 0.1 U of activity. Adding 0.1 ml of water gives a 20 mM phosphate buffer, pH 7, 0.05% sodium azide, and 5 mg/ml BSA. It is stable for 3 months at 4°C or at -20°C, but freezing and thawing should be avoided. There may be a slight contamination (<0.2%) with β -N-acetylglucosaminidase activity.

Endo F

Commercial endo F preparations are mixtures of endo F₁, F₂, and F₃. Endo F preparations should not be used for routine deglycosylation or to draw conclusions about the structure of the released oligosaccharides unless the specificity is clearly defined.

Endo F₂

Endo F₂ has a broad pH optimum of 4 to 6 and retains >50% of its activity at pH 7. The

enzyme is sensitive to SDS, but adding non-ionic detergents prevents denaturation of the enzyme by SDS. Although the enzyme is stable at 4°C for months, it can be frozen in aliquots at -70°C as long as repeated freeze/thaw cycles are avoided. The 1,10-phenanthroline can be used to inhibit a trace of a zinc metalloprotease that may be present.

PNGase F

The pH optimum for PNGase F is 8.6, but 80% of full activity occurs between 7.5 to 9.5 with a range of buffers including phosphate, ammonium bicarbonate, Tris-Cl, and HEPES. Borate buffers inhibit the enzyme. Commercial PNGase F is endo F free and is stable for 6 months at 4°C, or indefinitely at -70°C. However, it should be stored in small aliquots and repeated freeze/thaw cycles should be avoided. PNGase F will bind to glass and plastic surfaces and should not be stored in dilute solutions (<0.1 mU/ml). All of the unit activities of commercial preparations are based on cleavage of dansylated glycopeptides; they are expressed in nmoles/min, which are actually mU, not true International Units (1 International Unit = 1 $\mu\text{mole}/\text{min}$). SDS inactivates PNGase F, but adding a ten-fold weight excess of nonionic detergents protects the enzyme (Tarentino et al., 1989; Tarentino and Plummer, 1994).

Sialidase

AUS is available as a lyophilized powder from typical commercial sources such as Sigma, Boehringer Mannheim, and Glyko. It should be reconstituted in water at 1 to 10 mU/ μl according to manufacturer's directions. It is stable for 6 months at 4°C. Treatment with sialidase is also used in assays of protein transport to the cell surface.

Endo- α -N-acetylgalactosaminidase (O-glycosidase)

Endo- α -N-acetylgalactosaminidase has a pH optimum of 6.0 and has 50% activity at 5.5 to 7.0. The thiol inhibitor parachloromercuric benzoate (PCMB; 1 mM) inactivates the enzyme, and 1 mM EDTA inhibits it (63%), as do Mn^{2+} and Zn^{2+} (50%). Chloride also inhibits the enzyme, so HCl-containing buffers should be avoided. The enzyme will have full access to the sugar chains only after denaturing the protein with SDS, but the excess SDS needs to be removed by forming mixed micelles with nonionic detergents. The enzyme is stable at 4°C and at -20°C, but freeze/thaw cycles should be avoided.

Endo-β-galactosidase

The enzyme is supplied by several commercial sources, including Glyko and Sigma. The enzyme is free of contaminating endo- and exoglycosidases. It has a pH optimum of 5.8 and should be stored at -20°C , but is stable for ≥ 2 months at 4°C . Glyko supplies the enzyme as a lyophilized powder with BSA for stability. EDTA, Ca^{2+} , Mn^{2+} , and Mg^{2+} do not affect stability or activity, but PCMB inactivates it.

O-Sialoglycoprotease

The partially purified enzyme is supplied by Accurate Chemical and Scientific as a lyophilized powder containing nonsubstrate bovine serum proteins and HEPES buffer. The enzyme should be reconstituted according to the manufacturer's instructions, divided into aliquots appropriate for a single use, and stored at -20°C . Freeze/thaw cycles inactivate the enzyme, and it is inhibited by EDTA or 1,10-phenanthroline. It is possible to check the activity with a positive control of glycoporphin A, which is available through Sigma.

Troubleshooting

Most of the procedures should work as described, but there is a chance that the enzyme is inactive because of a variety of factors such as age, poor storage, or excess SDS. To check activity, it is worthwhile to run a positive control digestion using the same solutions including SDS and nonionic detergents as for the samples. Since the positive controls are simply glycoproteins that are visualized by Coomassie or silver staining, this requires running a separate gel for staining. This should not be required on a routine basis if the enzymes are used and stored as directed. The most likely culprit in failed digestions is using SDS solutions that are too old or too impure.

Anticipated Results

If the digestions are effective, the labeled band will usually show increased mobility on the gel. In rare instances, digestions can actually decrease mobility. The amount of change will depend on the contribution of that component to the overall mass of the protein. As mentioned before, a gel system that allows visualization of a 1-kDa change should be used. Proteins that are $>100\text{kDa}$ may cause problems for fine resolution. Here are a few numbers to keep in mind.

1. The smallest N-linked chain ($\text{Man}_3\text{GlcNAc}_2$) will have a mass of $\sim 0.9\text{kDa}$.
2. Two sialic acids on a single N-linked biantennary chain will have a mass of $\sim 0.6\text{kDa}$,

but their loss may appear larger. If they occur on clustered O-linked chains (sialoglycoprotease sensitive), the apparent size difference will be even larger.

3. Most poly-lactosamines are three or more repeats, and therefore their mass would be $\sim 1\text{kDa}$. The protein will probably run as a heterogeneous smear or broad band before digestion.

4. A single O-glycosidase-sensitive disaccharide (0.4kDa) may be below detection limits.

Time Considerations

All digestions can be done overnight for convenience, but the amount of enzymes should be sufficient for complete digestion in less time. The gels are run the next day, but the time needed for the development of autoradiograms will depend on the strength of the signal. A low-abundance protein labeled for 10 to 30 min with $[^{35}\text{S}]\text{Met}$ may give a weak signal and require long exposures (e.g., 2 weeks). Trafficking of abundant glycoproteins such as viral coat proteins require only short exposure times (e.g., a few hours).

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Key References

- Beckers et al., 1987. See above.
Describes the use of Lec 1 CHO cells and endo D to study processing.
- Chui et al., 1997. See above.
Demonstrates the importance of α -mannosidase III.
- Kornfeld and Kornfeld, 1985. See above.
Landmark review of processing.
- Tarentino and Plummer, 1994. See above.
Best and most recent review of the use of these enzymes.

Contributed by Hudson H. Freeze
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Cells internalize a large variety of macromolecules from the extracellular milieu (e.g., growth hormones, nutrients, viruses) by the process of endocytosis (Mellman, 1996; Mukherjee et al., 1997). In addition, endocytic membrane trafficking plays an important role in the constitutive and regulated targeting of proteins among cellular membrane compartments such as the plasma membrane, endosomes, and the *trans*-Golgi network (TGN). Thus, endosomal trafficking includes the internalization of membrane from the cell surface, intracellular traffic among membrane compartments, and the recycling of membrane from endosomes back to the cell surface. This unit describes biochemical methods for analyzing the kinetics of endocytic trafficking. One set of assays describes the use of radio-iodinated transferrin to analyze trafficking of the transferrin receptor. The other set describes the use of radio-iodinated antibody to analyze trafficking of any membrane protein that cycles through the plasma membrane.

The transferrin receptor is the most commonly used probe of endocytic trafficking (Dautry-Varsat et al., 1983; Klausner et al., 1983). Extracellular diferric transferrin binds to transferrin receptor on the cell surface (Fig. 15.3.1). This complex is rapidly internalized through clathrin-coated pits into endosomes. Iron is released from transferrin at the acidic luminal pH of endosomes. The iron-free transferrin remains bound to the receptor because the pH of endocytic recycling compartments is mildly acidic. Iron-free transferrin is recycled back to the plasma membrane with transferrin receptor. At the neutral extracellular pH, iron-free transferrin is released from the receptor. The fact that transferrin remains bound to the receptor for the complete endocytic cycle makes transferrin an ideal ligand for studies of intracellular trafficking and recycling.

Basic Protocol 1 is a method for measuring the steady-state surface-to-internal distribution of the transferrin receptor, and Alternate Protocol 1 is a method for measuring the steady-state surface-to-internal distribution of other proteins that cycle through the plasma membrane. Support Protocol 1 describes how to load transferrin with iron, and Support Protocol 2 is a method for radio-iodinating transferrin. Basic Protocol 2 is a method for measuring the internalization rate of the transferrin receptor, Alternate Protocol 2 measures internalization of other proteins, and Alternate Protocol 3 measures internalization in cells grown in suspension. Basic Protocol 3 is a method for measuring the recycling rate of the transferrin receptor. Basic Protocol 4 is a method for measuring the recycling rate of a membrane protein using an antibody as a probe. Basic Protocol 5 is a method for measuring the proteolysis of internalized proteins. Basic Protocol 6 is a method for measuring fluid-phase uptake. Finally, two methods for inhibiting endocytosis by clathrin-coated pits are described in Support Protocol 3 and Support Protocol 4.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer (also see *APPENDIX ID*).

MEASURING THE STEADY-STATE SURFACE-TO-INTERNAL DISTRIBUTION OF THE TRANSFERRIN RECEPTOR

The steady-state distribution of a membrane protein between the surface and cell interior is dependent on its rate of internalization and its rate of recycling. This distribution is measured for the transferrin receptor by incubating cells with radio-iodinated transferrin and determining the amounts of transferrin on the cell surface and inside cells at steady state. There are two methods for measuring surface-bound label. In one method, transfer-

BASIC PROTOCOL 1

Protein Trafficking

15.3.1

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Supplement 3

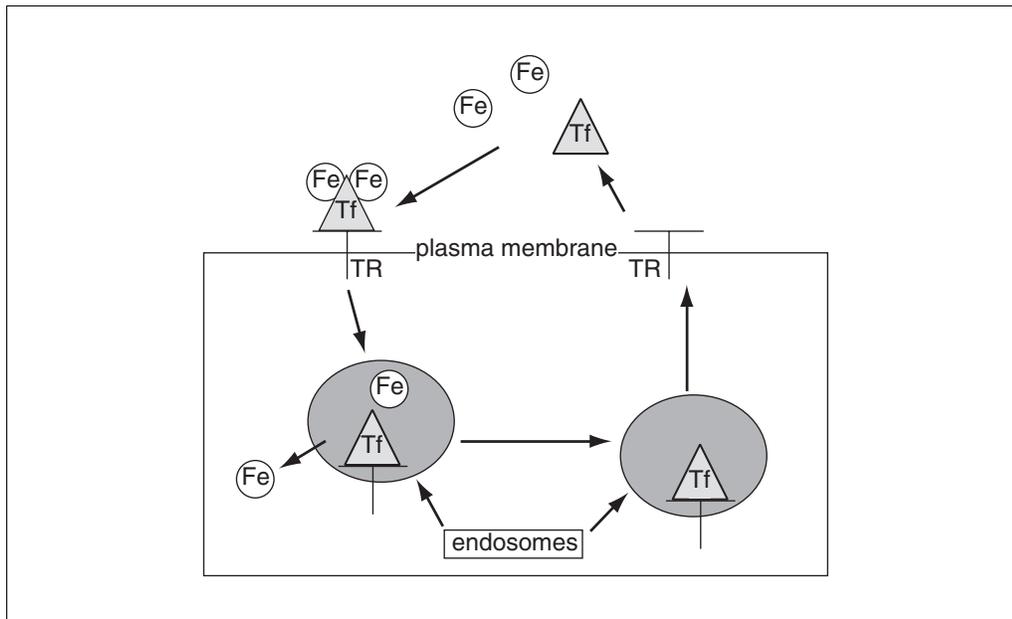


Figure 15.3.1 Transferrin and transferrin receptor cycle. At the near-neutral extracellular pH, diferric transferrin binds to the transferrin receptor with high affinity ($k_d \approx 10^{-9}$), whereas at neutral pH, iron-free transferrin has ~10-fold lower affinity for the receptor. The transferrin receptor–transferrin complex is concentrated in clathrin-coated pits and rapidly internalized into endosomes. At the mildly acidic pH of endosomes, Fe is released from transferrin. However, at this pH iron-free transferrin remains bound to the receptor. Because the endosomal recycling compartments are mildly acidic, the iron-free transferrin remains bound to the receptor until the complex is recycled back to the cell surface. When the iron-free transferrin is exposed to the near-neutral extracellular pH, it is released from the receptor. The unoccupied receptor can bind diferric transferrin and mediate another round of transferrin internalization. The iron-free transferrin binds two atoms of iron, and then binds the transferrin receptor. The transferrin receptor is constitutively cycled regardless of occupancy with ligand. For details see Dautry-Varsat et al. (1983). Tf, transferrin; TR, transferrin receptor.

rin released by an acidic pH wash is measured. In the second method, the amount of transferrin bound to cells at 4°C is measured. Measurement of surface transferrin receptor by incubating cells at 4°C usually gives more consistent results and is the preferred method. Since transferrin does not alter the trafficking of the transferrin receptor, either method can be used. If, however, ligand binding to its receptor changes trafficking of the receptor (e.g., induces internalization), then surface binding should be measured by collecting the acid wash and the two neutral pH washes (step 7).

Materials

- Cells of interest
- SF medium (see recipe)
- ^{125}I -labeled human diferric transferrin (~500 cpm/ng; see Support Protocol 2)
- Unlabeled (nonradioactive) human diferric transferrin
- Neutral pH buffer (see recipe), ice cold
- pH 2.0 buffer (see recipe), ice cold
- Solubilization solution: 1% (w/v) Triton X-100 in 0.1 N NaOH
- 10 mg/ml BSA
- 6-well tissue culture plates
- γ counter and tubes

Prepare cells

1. Plate 1×10^5 cells/well in SF medium in two 6-well tissue culture plates 2 days before the experiment. Allow the cells to reach ~80% confluency ($\sim 5 \times 10^5$ /well) on the day of the experiment.

Bicarbonate-buffered medium is used if the experiment is performed in a CO₂ incubator. HEPES-buffered medium is used if the experiment is performed in air.

Measure intracellular transferrin

2. Wash one 6-well plate of cells with 2 ml/well SF medium.
3. Add 1 ml SF medium containing a saturating concentration (3 $\mu\text{g/ml}$) of ¹²⁵I-labeled transferrin to four wells (total radioactivity wells). Add 1 ml SF medium containing 3 $\mu\text{g/ml}$ ¹²⁵I-labeled transferrin and a 200-fold excess of unlabeled diferric transferrin to the other two wells (nonspecific radioactivity wells).
4. Incubate at 37°C for a sufficient time for all transferrin receptors to be occupied with ¹²⁵I-labeled transferrin.

This time is determined empirically (see Basic Protocol 4). For most cell lines, a 120-min incubation is sufficient.

5. Place plate on ice and wash six times with 2 ml/wash prechilled neutral pH buffer.

Endocytosis is inhibited at 4°C. The neutral pH washes will not remove transferrin bound to the transferrin receptor.

CAUTION: Dispose of radioactive waste appropriately.

6. Incubate cells with 1 ml prechilled pH 2.0 buffer for 5 min at 4°C.

This incubation removes transferrin bound to the cell surface. Internal transferrin is not released from the cells.

7. Remove and discard the pH 2.0 buffer. Wash the cells twice with prechilled neutral pH buffer.
8. Add 1 ml solubilization solution to each well and transfer the solubilized cells to tubes suitable for γ counting. Rinse wells once with 1 ml water, and pool with the solubilization solution.
9. Determine the amount of ¹²⁵I-labeled transferrin per tube using a γ counter.

These values are the amount of intracellular transferrin.

Measure cell-surface transferrin

10. Wash the second 6-well plate with SF medium. Discard wash and incubate in fresh SF medium at 37°C for the same time as in step 4.

This incubation can be performed concurrently with the incubation in step 4.

11. Place cells on ice and wash three times with 2 ml/wash prechilled neutral pH buffer.
12. Add 1 ml of prechilled (4°C) neutral pH buffer containing a saturating concentration (3 $\mu\text{g/ml}$) of ¹²⁵I-labeled transferrin (3 $\mu\text{g/ml}$) and 1 mg/ml BSA to four wells (total radioactivity wells). Add 1 ml of the same solution containing a 200-fold excess of unlabeled transferrin to the other two wells (nonspecific radioactivity wells).
13. Incubate the cells at 4°C for 2 hr.

Endocytosis is blocked at 4°C; therefore, the amount of transferrin bound at 4°C is a measure of the amount of receptor on the cell surface.

- Place plate on ice and wash the cells six times with 2 ml/wash prechilled neutral pH buffer.

CAUTION: *Dispose of radioactive waste appropriately.*

- Solubilize cells and determine the amount of ¹²⁵I-labeled transferrin per tube as described in steps 8 and 9.

These values are a measure of the amount of transferrin receptor on the cell surface at steady state.

Analyze data

- Calculate the specific radioactivity of both intracellular and surface transferrin by subtracting the average of the two nonspecific radioactivity wells from the average of the four total radioactivity wells.

- Present data as the surface-to-internal ratio or as the amount of transferrin on the surface as percent of the total.

In CHO cells, the steady-state surface-to-internal ratio for the transferrin receptor is ~0.5 (~35% on the surface; Johnson et al., 1994).

Another method for determining the surface-to-internal distribution of the transferrin receptor is to collect and count the pH 2.0 buffer and neutral buffer washes of steps 6 and 7. In this method, the surface-to-internal ratio for each well is determined after correcting for nonspecific radioactivity. However, the authors find that the procedure outlined in Basic Protocol 1 yields more reproducible results.

ALTERNATE PROTOCOL 1

MEASURING THE STEADY-STATE SURFACE-TO-INTERNAL DISTRIBUTION OF OTHER MEMBRANE PROTEINS

The surface-to-internal ratio of any membrane protein that constitutively cycles between intracellular compartments and the plasma membrane can be measured using Basic Protocol 1 and a radio-iodinated antibody to the extracellular domain of the protein. Iodinated Fab fragments should be used, because bivalent antibodies cause cross-linking and therefore may modify the protein's trafficking (Weissman et al., 1986). Fab fragments can be prepared using the ImmunoPure Fab preparation kit (Pierce) and iodinated using Support Protocol 2. An excess of unlabeled antibody is used to determine nonspecific binding.

The procedures are the same as described in Basic Protocol 1, except that ¹²⁵I-labeled Fab fragments (2 to 10 μCi/μg) are used in place of ¹²⁵I-labeled transferrin. Some antibodies are not efficiently stripped from the surface with a single incubation in pH 2.0 buffer. If this is the case, several rounds of alternating acid/neutral washes can be used to increase the stripping efficiency. Some antibodies are resistant to the acid stripping. In these cases, incubation with 1 mg/ml proteinase K or pronase at 4°C for 30 min (or more) can be used to release surface-bound antibody.

BASIC PROTOCOL 2

MEASURING THE KINETICS OF TRANSFERRIN INTERNALIZATION

Internalization of the transferrin receptor is measured by monitoring the internalization of ¹²⁵I-labeled transferrin. Cells are washed with an acidic pH buffer to distinguish internalized transferrin from transferrin bound to receptors on the cell surface. The initial transferrin receptor internalization rate constant is the slope of a plot of the ratio of internalized transferrin to surface transferrin versus time.

Materials

Cells of interest
SF medium (see recipe)
 ^{125}I -labeled human diferric transferrin (~500 cpm/ng; see Support Protocol 2)
Unlabeled (nonradioactive) human diferric transferrin
Neutral pH buffer (see recipe), ice cold
pH 2.0 buffer (see recipe), ice cold
Solubilization buffer: 1% (w/v) Triton X-100 in 0.1 N NaOH
10 mg/ml BSA

6-well tissue culture plates
 γ counter and tubes

Prepare cells

1. Plate 1×10^5 cells/well in SF medium in 6-well tissue culture plates 2 days before the experiment. Allow the cells to reach ~80% confluency ($\sim 5 \times 10^5$ /well) on the day of the experiment.

Bicarbonate-buffered medium is used if the experiment is to be performed in a CO_2 incubator; HEPES-buffered medium is used if the experiment is to be performed in air.

One 6-well plate is required for each time point and an additional plate is required to measure steady-state surface transferrin binding. Typically four time points are used, in which case a total of five 6-well plates are required.

Measure internalized transferrin

2. On the day of the experiment, wash the cells from plates for specific time points once with 2 ml SF medium and incubate in SF medium for 1 hr at 37°C.

During this incubation, any cell-associated transferrin from the growth medium will be recycled and dissociated from the cells, ensuring that the receptors will be unoccupied at the beginning of the experiment.

3. Starting with a single 6-well plate, add 1 ml SF medium containing a saturating concentration (3 $\mu\text{g}/\text{ml}$) of ^{125}I -labeled transferrin to four wells (total radioactivity wells) and add 1 ml SF medium containing ^{125}I -labeled transferrin and a 200-fold excess of unlabeled transferrin to the other two wells (nonspecific radioactivity wells).
4. Incubate the plate for the desired time at 37°C.

Incubation time points of <10 min are typically used (e.g., 2, 4, 6, and 8 min). Internalized transferrin is eventually returned to the cell surface; therefore, it is important to use incubation times in which little of the transferrin is recycled.

5. Remove the plate from the incubator, place on ice, and wash six times with 2 ml/wash prechilled neutral pH buffer.

Endocytosis is inhibited at 4°C. The neutral pH washes will remove transferrin not specifically bound to the transferrin receptor.

CAUTION: *Dispose of radioactive waste appropriately.*

6. Incubate the cells with 1 ml/well prechilled pH 2.0 buffer for 5 min at 4°C.

This incubation removes transferrin bound to the cell surface. Internal transferrin is not released from the cells.

7. Remove and discard the pH 2.0 buffer. Wash the wells twice with prechilled (4°) neutral pH buffer.
8. Repeat steps 3 to 7 with the plates for the remaining time points (but not with the plate for steady-state surface binding). Treat each time point individually.

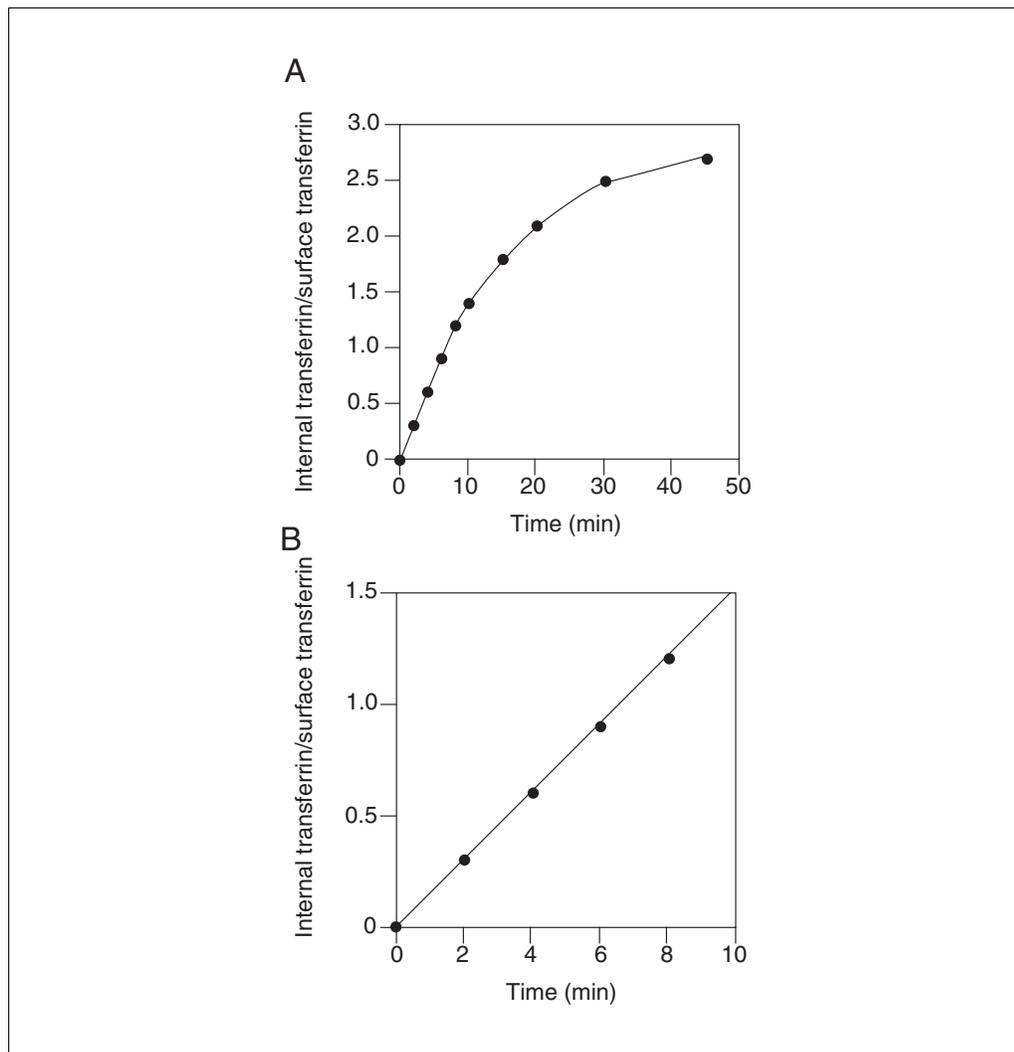


Figure 15.3.2 Data from an idealized transferrin internalization assay are shown. **(A)** Data are shown for an assay carried out for a time sufficient to reach steady state. Data from the early time points are replotted on an expanded time scale in **(B)**. In both graphs the ratios of internal transferrin to steady-state surface transferrin are plotted as a function of time.

9. Add 1 ml of solubilization solution to each well. Remove the solubilized cells by trituration and transfer to tubes suitable for γ counting. Rinse wells once with 1 ml water, and pool with the solubilization solution.
10. Determine the amount of ^{125}I -labeled transferrin per tube using a γ counter.

These values are the amount of transferrin internalized during the incubation at 37°C.

Measure steady-state surface transferrin

11. Wash the steady-state plate with 2 ml SF medium. Discard wash and incubate in fresh SF medium for 1 hr at 37°C.

This incubation can be performed concurrently with the incubations of step 4.

12. Place cells on ice and wash three times with 2 ml/wash prechilled neutral pH buffer.
13. Add 1 ml prechilled neutral pH buffer containing a saturating concentration of ^{125}I -labeled transferrin (3 $\mu\text{g}/\text{ml}$) and 1 mg/ml BSA to four wells (total radioactivity wells). Add 1 ml of the same solution containing a 200-fold excess of unlabeled transferrin to the other two wells (nonspecific radioactivity wells).

14. Incubate the cells at 4°C for 2 hr.

Endocytosis is blocked at 4°C; therefore, the amount of transferrin bound at 4°C is a measure of the receptors on the cell surface.

15. Place the plate on ice and wash the cells six times with 2 ml/well neutral pH buffer.

CAUTION: Dispose of radioactive waste appropriately.

16. Solubilize the cells and quantify the amount of ¹²⁵I-labeled transferrin as in steps 9 and 10.

This value is the amount of transferrin receptor on the cell surface at steady state.

Analyze data

17. Calculate the specific radioactivity of intracellular and surface transferrin by subtracting the average of the two nonspecific radioactivity wells from the average of the four total radioactivity wells.

18. Calculate the internal-to-surface transferrin ratio and plot versus time (see Fig. 15.3.2).

The data should yield a straight line extrapolating through zero. The slope of this line is the internalization rate constant (Wiley and Cunningham, 1982). In CHO cells, the internalization rate constant of the transferrin receptor is $\sim 0.15 \text{ min}^{-1}$ (Johnson et al., 1994). If the slope decreases at the later time points (i.e., if the line flattens), then shorter time points must be used. The internal-to-surface plot will begin to flatten as internalized transferrin is recycled back to the cell surface.

Another method for determining the amount of transferrin bound to the surface is to collect and count the pH 2.0 buffer and neutral buffer washes of steps 7 and 8. For each time point, the total radioactivity removed by the acid wash and the total cell-associated radioactivity are individually corrected for nonspecific radioactivity, and the internal-to-surface ratio is determined for each well. The average of these values for each time point is plotted as a function of time. However, the authors find that the procedure described in Basic Protocol 2 yields more reproducible results.

MEASURING THE KINETICS OF MEMBRANE PROTEIN INTERNALIZATION USING ¹²⁵I-LABELED ANTIBODIES

ALTERNATE PROTOCOL 2

For membrane proteins with no known ligand, antibodies against the extracellular domain can be used to measure internalization. For these experiments an ¹²⁵I-labeled antibody against the extracellular domain is used. Fab fragments should be used, because bivalent antibodies cause cross-linking and therefore may modify the protein's trafficking (Weissman et al., 1986). Fab fragments can be prepared using the ImmunoPure Fab preparation kit (Pierce) and labeled using Support Protocol 2. An excess of unlabeled antibody is used to determine nonspecific binding.

The procedures are the same as that described in Basic Protocol 2, except that iodinated Fab antibody fragments are used instead of ¹²⁵I-labeled transferrin. Some antibodies are not efficiently stripped from the surface with a single incubation in pH 2.0 buffer. If this is the case, several rounds of alternating acid/neutral wash can be used to increase the stripping efficiency. Some antibodies are resistant to the acid stripping. In these cases, incubation with 1 mg/ml proteinase K or pronase at 4°C for 30 min (or more) can be used to release surface-bound antibody.

Protein Trafficking

15.3.7

**MEASURING THE KINETICS OF TRANSFERRIN INTERNALIZATION IN
CELLS GROWN IN SUSPENSION**

For cells grown in suspension (e.g., K562 human erythroleukemia cells), the procedure is basically the same, but the washes are more tedious because they require centrifugation. To avoid handling too many tubes, each time point is a single determination. Nonspecific labeling is determined on two of the time points, and the results are averaged and subtracted from every time point.

Additional Materials (also see *Basic Protocol 2*)

- Suspension culture of interest
- HEPES-buffered SF medium (see recipe)
- pH 2.0 medium, ice cold: medium formulation used to grow cells (e.g., RPMI, DMEM) with 25 mM sodium acetate, adjusted to pH 2.0 with HCl
- pH 11.0 medium, ice cold: medium formulation used to grow cells (e.g., RPMI, DMEM) with 25 mM Tris base, adjusted to pH 11.0 with NaOH

Prepare cells

1. For this assay, $\sim 1 \times 10^5$ cells are used per time point. Transfer the number of cells sufficient for all time points to a 15-ml conical centrifuge tube. Pellet the cells by centrifuging 5 min at $500 \times g$, room temperature.

Typically five time points are used, so a total of 5×10^5 cells would be sufficient.

2. Aspirate medium, wash cells once with 10 ml HEPES-buffered SF medium, and centrifuge 5 min at $500 \times g$, room temperature.
3. Incubate in 10 ml SF medium for 30 min at 37°C .

During this incubation, any cell-associated transferrin from the growth medium will be recycled and released from the cells, ensuring that the receptors will be unoccupied at the beginning of the experiment.

4. Centrifuge cells (5 min at $500 \times g$, room temperature). Resuspend in 0.8 ml SF medium and place at 37°C .

Perform timed internalization

5. Transfer 0.2 ml cell suspension to a microcentrifuge tube and add 0.6 mg/ml unlabeled transferrin to the 0.2-ml aliquot. Place tube in a 37°C water bath.

These cells are for nonspecific labeling (see step 8).

6. For $t = 0$, add $3 \mu\text{g/ml}$ ^{125}I -labeled transferrin to the 15-ml conical tube containing 0.6 ml cell suspension. Place tube at 37°C .

7. At the appropriate time points (e.g., 1, 2, 3, 4, and 5 min), resuspend the cells in the 15-ml tube (containing labeled transferrin only) by gently inverting the tube. Transfer $100 \mu\text{l}$ of cell suspension into a new 1.5-ml tube containing 1 ml prechilled neutral pH buffer on ice. Repeat for each time point.

Diluting the cells in a large volume of cold neutral buffer ensures that endocytosis is stopped at once. Internalized transferrin is eventually returned to the cell surface, therefore it is important to use incubation times in which little of the transferrin is recycled. In K562 cells, incubation times < 5 min are commonly used.

8. Add $3 \mu\text{g/ml}$ of ^{125}I -labeled transferrin to the tube containing 0.2 ml cell suspension and 0.6 mg/ml transferrin (step 5). Place the tube at 37°C . At the appropriate time points (e.g., 2 and 5 min), invert the tube to resuspend the cells and transfer $100 \mu\text{l}$ of the suspension to a new 1.5-ml conical tube containing 1 ml prechilled neutral buffer on ice. Repeat the procedure for the second time point.

These are the nonspecific uptake samples.

9. Wash the time point three times with 1 ml/wash prechilled neutral pH buffer. Centrifuge each wash for 5 min at $500 \times g$, 4°C .

The authors have also successfully used shorter centrifugations at higher speed (e.g., $15,000 \times g$ for 20 sec).

CAUTION: Dispose of radioactive waste appropriately.

10. Incubate cells with 0.2 ml prechilled pH 2.0 medium for 2 min at 4°C .
This incubation removes transferrin bound to the cell surface.
11. Add 0.2 ml prechilled pH 11.0 medium to bring the cell to a neutral pH, and repeat centrifugation.
12. Transfer supernatant to a tube suitable for γ counting (surface radioactivity). Resuspend cell pellet in 200 μl water and transfer to another tube suitable for counting (intracellular radioactivity).
13. Determine the amount of ^{125}I -labeled transferrin in each tube.

Analyze data

14. Calculate the average surface radioactivity of the two tubes containing nonradioactive transferrin (nonspecific surface radioactivity). Subtract this value from the surface radioactivity of each of the sample time points to give the specific surface radioactivity.
15. Similarly, calculate the average nonspecific intracellular radioactivity and subtract this from the intracellular radioactivity of each of the sample time points.
16. Express the results as the ratio of internal to surface label versus time.

MEASURING THE KINETICS OF TRANSFERRIN RECEPTOR RECYCLING

The recycling of the transferrin receptor from endosomes back to the cell surface is examined by measuring the release of transferrin into the medium. For these studies, cells are incubated with ^{125}I -labeled transferrin to achieve steady-state occupancy of the transferrin receptor with transferrin. Transferrin bound to the cell surface is removed, and the release of internal transferrin from cells is then monitored as a function of time. Because transferrin remains bound to the transferrin receptor in intracellular endosomal compartments, the release of previously endocytosed transferrin into the medium reflects the return of transferrin receptors from endosomes to the plasma membrane. The iron-free transferrin returned to the plasma membrane with the recycled transferrin receptor is released from the receptor at the extracellular pH.

Materials

Cells of interest
SF medium (see recipe)
 ^{125}I -labeled human transferrin (see Support Protocol 2)
Unlabeled (nonradioactive) human diferric transferrin
pH 5.0 buffer (see recipe), prewarmed to 37°C
Efflux medium (see recipe), prewarmed to 37°C
Solubilization solution: 1% (w/v) Triton X-100 in 0.1 N NaOH

6-well tissue culture plates
 γ counter and tubes

BASIC PROTOCOL 3

Protein Trafficking

15.3.9

Prepare cells

1. Plate 1.5×10^5 cells/well in SF medium in 6-well tissue culture plates 2 days before the experiment. Prepare one plate for each time point. Allow the cells to reach ~80% confluency on the day of the experiment.

Six time points are typically used. The time points used depend on the rate of recycling of the cell line being studied. In CHO cells, which have a half-time for transferrin receptor recycling of ~2 min, time points of 2.5, 5, 10, 15, 20, and 30 min are used.

Bicarbonate-buffered medium is used if the experiment is to be performed in a CO₂ incubator; HEPES-buffered medium is used if the experiment is to be performed in air.

2. Wash cells with 2 ml SF medium.

Remove surface transferrin

3. Add 1 ml SF medium containing a saturating concentration (3 µg/ml) of ¹²⁵I-labeled transferrin to four wells (total radioactivity wells). Add 1 ml of SF medium containing a saturating concentration of ¹²⁵I-labeled transferrin and a 200-fold excess of unlabeled transferrin to the other two wells (nonspecific wells).
4. Incubate at 37°C for a time sufficient to reach steady-state occupancy of the transferrin receptor with transferrin (~20 min for most cell types).
5. Remove the first plate from the incubator, wash the cells with 2 ml SF medium, and incubate for 2 min at room temperature with 1 ml prewarmed (37°C) pH 5.0 buffer.

Either the citrate- or the MES-based pH 5.0 buffer can be used.

CAUTION: Dispose of radioactive waste appropriately.

6. Discard the pH 5.0 buffer and wash the wells three times over the course of 1 min at room temperature with prewarmed (37°C) efflux medium.

Incubation in pH 5.0 buffer followed by washes with efflux medium promotes the release of transferrin bound to receptors on the cell surface.

Measure recycled transferrin

7. Add 1 ml prewarmed efflux medium to each well and incubate at 37°C for the desired amount of time (see time points in step 1).

The unlabeled transferrin and the iron chelator desferrioxamine in the efflux medium ensure that the released radioactive iron-free transferrin is not reloaded with iron, and therefore cannot rebind the transferrin receptor.

8. Repeat steps 5 through 7 on each six-well plate (i.e., time point).
9. At the appropriate time, remove the plate from the incubator and transfer the efflux medium to a suitable tube for γ counting. Wash the cells (rapidly) with 1 ml efflux medium and add this wash to efflux medium in the tube.
10. Add 1 ml solubilization solution to each well. Remove the solubilized cells by trituration and transfer to tube suitable for γ counting. Rinse the wells once with 1 ml water, and pool with the solubilization solution.
11. Determine the amount of ¹²⁵I-labeled transferrin in each tube of efflux medium and solubilized cells.

Analyze data

12. At each time point, correct the radioactivity of ¹²⁵I-labeled transferrin in each individual vial of efflux medium and solubilization solution for nonspecific binding by subtracting the average of the radioactivity in the two nonspecific radioactivity wells.

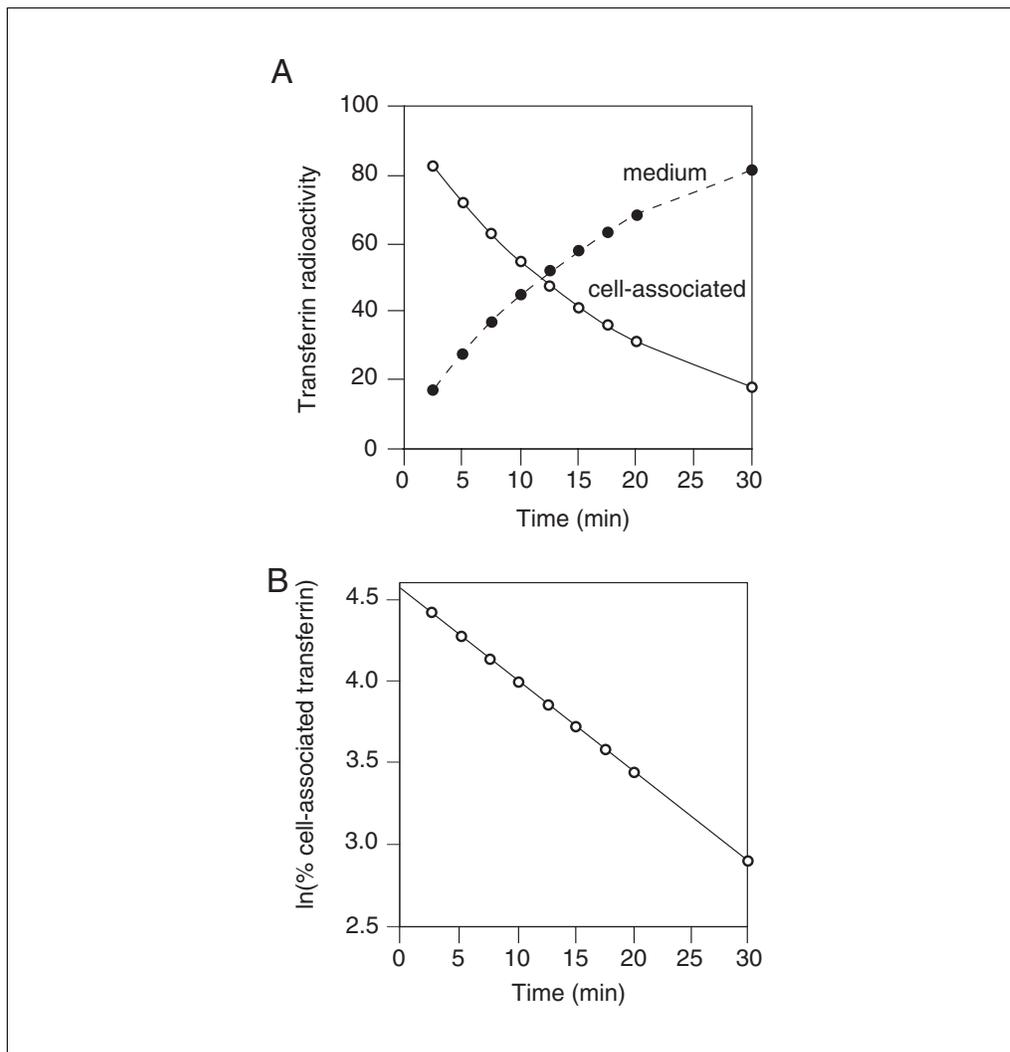


Figure 15.3.3 Transferrin recycling assay. **(A)** Data from an idealized transferrin recycling assay are shown. Over time, the amount of transferrin in the medium increases, while the cell-associated transferrin decreases as iron-free transferrin is released from cells. **(B)** The recycling rate constant is the slope of a graph of the natural logarithm of the percent-cell-associated transferrin versus time.

13. Calculate the percent cell-associated transferrin for each well, and average the four values per time point.
14. Plot percent cell-associated transferrin as a function of time (see Fig. 15.3.3).

In most cell types, transferrin is recycled by an apparent first-order process; therefore, the decrease in cell-associated transferrin should fit an exponential decay. The externalization rate constant can be calculated by determining the slope of a plot of the natural logarithm of the percent cell-associated versus time. In CHO cells, transferrin receptor is recycled at a rate of $\sim 0.06 \text{ min}^{-1}$ (Johnson et al., 1994).

IRON-LOADING TRANSFERRIN

This protocol describes the method for preparing diferric transferrin for use in transferrin recycling experiments (Basic Protocols 1 to 3). Its subsequent radiolabeling is described in Support Protocol 2.

SUPPORT PROTOCOL 1

Protein Trafficking

15.3.11

Materials

Apo-transferrin (iron-free transferrin; available from a number of commercial sources)

PBS (see recipe)

1 mg/ml ferric ammonium citrate solution in 10 mM NaHCO₃/20 mM HEPES acid, pH 7.7

PD-10 columns (pre-packed Sephadex G-25 columns; Pharmacia Biotech) or any other desalting column

0.2- μ m sterile syringe filter

1. Dissolve 200 mg apo-transferrin (iron-free) in 6 ml PBS.

2. Add 4 ml ferric ammonium citrate solution.

The solution will be an ochre color.

3. Incubate at 37°C for 10 min.

The solution will turn a dark rust color.

4. During this incubation, equilibrate five prepacked PD-10 columns with PBS.

Any desalting column can be used.

5. Run 2 ml iron-loaded transferrin over each column.

6. Collect the orange product (iron-loaded transferrin). Pool the orange fractions from each column.

7. Measure the OD of the sample at 280 and 465 nm.

The OD₂₈₀/OD₄₆₅ ratio should be 0.0496 if 100% of the transferrin is loaded with iron.

8. Filter sterilize the iron-loaded transferrin with a 0.2- μ m filter and store at 4°C.

The transferrin is stable for at least 6 months at 4°C.

SUPPORT PROTOCOL 2

RADIO-IODINATION OF DIFERRIC TRANSFERRIN

There are a number of methods for radio-iodinating transferrin. The chloramine T method is described below. This method is suitable for use with any protein that contains tyrosine residues. In addition, it is important to test that the radio-iodination procedure has not grossly altered the affinity of transferrin for the transferrin receptor. This is done by measuring the ability of unlabeled transferrin to compete for the binding of ¹²⁵I-labeled transferrin to cells.

CAUTION: This procedure must be performed in a hood certified for radio-iodination. All personnel performing iodinations or using radioactive materials must be properly trained.

Additional Materials (also see Basic Protocol 1)

PBS (see recipe)

Human diferric transferrin (see Support Protocol 1)

37 MBq/ml Na¹²⁵I (100 mCi/ml; NEN Life Science Products)

4 mg/ml chloramine T in PBS (prepare fresh)

8 mg/ml sodium bisulfite in PBS (prepare fresh)

1 mg/ml BSA

100% (w/v) trichloroacetic acid (TCA)

5 × 10⁵ cells/well plated in 6-well tissue culture plates (see Basic Protocol 1, step 1)

PD-10 column (pre-packed Sephadex G-25 column; Pharmacia Biotech) or any other desalting column

Radiolabel transferrin

1. Equilibrate a PD-10 column with three bed volumes of PBS.
2. Add 2 mg human diferric transferrin in 0.2 ml PBS to a vial containing 1 mCi Na¹²⁵I. Mix by pipetting up and down.
3. Add 25 µl freshly prepared 4 mg/ml chloramine T in PBS. Mix by pipetting up and down. Incubate in the hood for 2 min.
4. To stop the reaction, add 25 µl freshly prepared 8 mg/ml sodium bisulfite in PBS. Mix by pipetting up and down.
5. Transfer solution to the equilibrated PD-10 column and collect 1-ml fractions.

With a PD-10 column, the protein will be in fractions 3 and 4. It is not necessary to collect more than six fractions.

Analyze labeling

6. Quantify radioactivity in 10-µl aliquots of each fraction.
7. To confirm that the counts in the excluded fractions are ¹²⁵I-labeled protein, dilute a 10-µl aliquot of each fraction with 100 µl of 1 mg/ml BSA solution. Add 15 µl of 100% TCA, mix, and incubate at 4°C for 30 min.
8. Microcentrifuge samples at maximum speed for 10 min at room temperature. Quantify radioactivity in the supernatant and pellet separately.

Radioactivity in the pellet is ¹²⁵I-labeled protein, and radioactivity in the supernatant is free ¹²⁵I.

9. Determine the specific activity (radioactivity per ng of protein).

The procedure yields transferrin with a specific activity of 300 to 500 cpm/ng.

CAUTION: Dispose of radioactive waste appropriately.

Test affinity of radiolabeled transferrin

10. Chill 6-well plate of cells (5×10^5 cells/well) to 4°C and wash each well with 2 ml prechilled neutral pH buffer.
11. Add 3 µg/ml ¹²⁵I-labeled transferrin and various amounts of unlabeled transferrin to each sample and incubate for 2 hr at 4°C in SF medium.
12. Wash cells with 2 ml neutral pH buffer (4°C).
13. Solubilize the cells and measure cell-associated radioactivity (see Basic Protocol 1, steps 8 and 9, for details).

A 200-fold excess of unlabeled transferrin reduces ¹²⁵I-labeled transferrin binding to less than 10%.

14. Store radio-iodinated transferrin for up to 1 month at 4°C in a lead container.

TIME COURSE FOR STEADY-STATE OCCUPANCY OF MEMBRANE PROTEIN WITH ANTIBODY

It is not possible to use a modification of Basic Protocol 3 to measure the recycling of a membrane protein using a ¹²⁵I-labeled antibody, because the antibody remains bound to the membrane protein when it is returned to the cell surface, and the methods used to strip antibodies are not compatible with studies in living cells. One method for determining the recycling rate of a membrane protein using an antibody is to incubate cells at 37°C with ¹²⁵I-labeled antibody and monitor the amount of cell-associated antibody as a function of incubation time. The protein on the surface at the start of the experiment will be rapidly bound by the antibody, and will remain bound throughout the course of the

BASIC PROTOCOL 4

Protein Trafficking

15.3.13

experiment (i.e., is it recycles between the cell surface and endosomes). Cell-associated label, however, will increase over time as the pool of protein that was inside the cell at the beginning of the experiment (and therefore unoccupied) is recycled back to the cell surface. Cell-associated label will plateau (reach a steady-state value) when all the cycling protein has bound antibody (i.e., is trafficked to the cell surface). Thus, the rate of approach to the steady-state value is the rate at which the intracellular pool is cycled to the surface. Note that this method for measuring recycling of a membrane protein requires that the antibody remain associated with the protein during its cycle, that the antibody does not modify the trafficking of the protein, and that the amount of protein synthesized over the course of the experiment is negligible compared to the amount of recycled protein.

Materials

Cells of interest
SF medium (see recipe)
 ^{125}I -labeled antibody (Fab fragments; 2 to 10 $\mu\text{Ci}/\mu\text{g}$)
Unlabeled (nonradioactive) antibody
Neutral pH buffer (see recipe), room temperature
Solubilization solution: 1% (w/v) Triton X-100 in 0.1 N NaOH
6-well tissue culture plates
 γ counter and tubes

1. Plate 1×10^5 cells/well in SF medium in 6-well tissue culture plates 2 days before the experiment. Use one plate for each time point. Allow cells to reach ~80% confluency on the day of the experiment.

Eight time points are typically sufficient.

Bicarbonate-buffered medium is used if the experiment is to be performed in a CO_2 incubator. HEPES-buffered medium is used if the experiment is to be performed in air.

2. Wash cells with 2 ml SF medium.
3. Add 1 ml SF medium containing 1 to 5 nM ^{125}I -labeled antibody to four wells (total radioactivity wells). Add 1 ml SF medium containing the same concentration of ^{125}I -labeled antibody and a 200-fold excess of unlabeled antibody (nonspecific radioactivity wells) to the other two wells.
4. Incubate the plates for variable times at 37°C (e.g., 2.5, 5, 10, 15 min).

The time points used are empirically determined.

5. At the appropriate times, remove the plates from the incubator and wash three times with 2 ml neutral pH buffer.

CAUTION: Dispose of radioactive waste appropriately.

6. Add 1 ml solubilization solution to each well. Remove the solubilized cells by trituration and transfer to tubes suitable for γ counting. Rinse the wells once with 1 ml water, and pool with the solubilization solution.
7. Determine the amount of ^{125}I -labeled antibody per tube.
8. Repeat steps 5 to 7 are for each plate after the appropriate incubation time.
9. For each plate (time point), average the values of the four total radioactivity wells and subtract the average of the two nonspecific radioactivity wells.
10. Plot the amount of cell-associated antibody (specific radioactivity) as a function of incubation time.

This curve should asymptotically approach a plateau level. The increase in cell-associated antibody over time reflects the recycling of unoccupied membrane protein receptors from

endosomes to the surface. The recycling rate constant is calculated from this curve (Johnson et al., 1993).

**BASIC
PROTOCOL 5**

DETECTING DEGRADATION OF INTERNALIZED LIGANDS

Most ligands internalized by endocytosis are delivered to late endosomal or lysosomal compartments where they are degraded into peptides. The degradation of ^{125}I -labeled ligand is examined by measuring the release of these peptides into the medium. The medium is collected and proteins are precipitated with trichloroacetic acid (TCA). The radioactivity in the pellet is intact protein, and the radioactivity in the supernatant is degraded ligand. This protocol describes the detection of degraded peptide in the extracellular medium after various times of endocytosis.

Materials

Cells of interest
SF medium (see recipe)
Neutral pH buffer (see recipe), ice cold
 ^{125}I -labeled ligand of interest (Support Protocol 2)
Unlabeled (nonradioactive) ligand
10 mg/ml BSA
100% (w/v) trichloroacetic acid (TCA) solution

6-well tissue culture plate
 γ counter and tubes

Prepare cells

1. Plate 1×10^5 cells/ml in SF medium in three wells of a 6-well tissue culture plate 2 days before the experiment. Prepare one plate (three wells) per time point. Allow cells to reach ~80% confluency on the day of the experiment.

Bicarbonate-buffered medium is used if the experiment is to be performed in a CO_2 incubator. HEPES-buffered medium is used if the experiment is to be performed in air.

2. On the day of the experiment, wash the cells once with 2 ml SF medium and incubate in SF medium for 1 hr at 37°C .

During this incubation, most of the cell-associated ligand from the growth medium will be released from the cells, ensuring that the receptors will be unoccupied at the beginning of the experiment.

3. At the end of this incubation, place the cells on ice and wash two times with 2 ml/well prechilled neutral pH buffer.

Internalize ligand

4. Add 1 ml prechilled (4°C) neutral pH buffer containing a saturating concentration of ^{125}I -labeled ligand and 1 mg/ml BSA to two wells (total radioactivity wells). Add 1 ml of the same solution containing a 200-fold excess of unlabeled ligand to the other well (nonspecific radioactivity well).

The concentration of ligand depends on the ligand used; refer to appropriate literature.

5. Incubate the cells at 4°C for 2 hr.
6. Wash cells four times with 2 ml neutral pH buffer at 4°C .

CAUTION: Dispose of radioactive waste appropriately.

The neutral pH wash removes unbound ligand from the cells.

7. Add 1 ml SF medium to each well and incubate the plate at 37°C .

At 37°C , receptor-bound ligands are internalized.

Collect/precipitate degraded and intact ligand

8. At various time points (e.g., 10, 20, 30, 60, 90, and 120 min) transfer the incubation medium from the three wells into separate microcentrifuge tubes on ice.
9. Solubilize the cells and determine the radioactivity (see Basic Protocol, steps 8 and 9).

This is the cell-associated radioactivity.

10. Add 10 mg/ml BSA to the incubation medium at a final concentration of 2 mg/ml.

Adding BSA will increase the efficiency of precipitation of the intact ligand.

11. Add 100% (w/v) TCA at a final concentration of 15%.
12. Incubate 30 min at 4°C.
13. Centrifuge at 15,000 × g, 4°C, for 20 min.
14. Collect the supernatant and count the radioactivity in each separately.
15. At the same time, test the efficiency of the precipitation procedure by precipitating a 5- μ l aliquot of labeled ligand diluted to 1 ml in SF medium as described in steps 9 to 13.

Typically, more than 90% of the ligand should be precipitable.

Analyze data

16. Correct all the data for nonspecific radioactivity by subtracting the radioactivity in the nonspecific sample from the average of the radioactivity in the other two samples. Determine the radioactivity in the 15% TCA supernatant (ligand released into the medium in degraded form) and in the 15% TCA pellet (ligand released in the undegraded or intact form). Plot the three values—cell-associated radioactivity (step 9), the released degraded ligand, and the released intact ligand—as per cent of the total (the sum of the three values at that time point) versus time.

An alternative protocol is to incubate the cells at 37°C in the presence of ¹²⁵I-labeled ligand (pulse), wash the cells, and measure the appearance of degraded ligand in the medium after various chase times (Brown and Goldstein, 1976), which is more sensitive because it does not rely on a single round of internalization.

**BASIC
PROTOCOL 6**

MEASURING FLUID-PHASE UPTAKE

Any molecule in the extracellular medium can be internalized by bulk fluid uptake inside newly endocytosed vesicles. To measure this non-concentrated uptake, cells are incubated with a protein that does not have a specific membrane receptor and accumulation of this protein inside cells over time is determined.

Materials

Cells of interest
SF medium (see recipe)
5 mg/ml horseradish peroxidase (HRP) in SF medium
Neutral pH buffer (see recipe), ice cold
0.01% (w/v) Triton X-100
6-well tissue culture plates

Additional reagents and equipment for colorimetric assay (see Steinman et al., 1976) and protein assay (*APPENDIX 3*)

1. Plate 1×10^5 cells/well in SF medium in 6-well tissue culture plates 2 days before the experiment. Use three wells for each time point. Allow the cells to reach ~80% confluency on the day of the experiment.

The time points used are determined empirically.

Bicarbonate-buffered medium is used if the experiment is to be performed in a CO₂ incubator. HEPES-buffered medium is used if the experiment is to be performed in air.

2. Wash the cells once with 2 ml SF medium, and incubate the cells in 5 mg/ml HRP in SF medium at 37°C for various times (e.g., 5, 10, 15, 20, 30, 60 min).

¹²⁵I-labeled BSA or other detectable protein can be used instead of HRP.

3. At the appropriate times, remove the plate from the incubator, place on ice, and wash six times with 2 ml/wash prechilled neutral pH buffer.

Endocytosis is inhibited at 4°C. More than six washes may be needed to remove HRP adsorbed to the cell surface. To test this, incubate 6 wells with 5 mg/ml HRP at 4°C for 2 hr. Measure the number of washes necessary to remove essentially all cell-associated HRP activity as determined in step 4. The surface HRP can also be removed by incubation in 0.1% pronase in PBS. This procedure also removes the cells from the plate; the cells are collected by centrifugation 3 min at 15,000 × g.

4. Lyse cells in 1 ml of 0.01% Triton X-100. Take a 100-μl aliquot to measure HRP activity in the lysate using a colorimetric assay (see Steinman et al., 1976). Measure HRP activity of a 5 mg/ml HRP solution as a standard for normalization.

5. Measure protein content in the lysate (APPENDIX 3).

A number of kits are also available to measure protein content.

6. Express the results as the amount of HRP with respect to the total protein content for each well (i.e., ng HRP/mg cell protein). Calculate the average of these values for each time point, and plot as a function of time.

INHIBITION OF CLATHRIN-MEDIATED ENDOCYTOSIS BY POTASSIUM DEPLETION

SUPPORT PROTOCOL 3

All endocytic mechanisms are energy and temperature dependent, and are blocked at 4°C. Clathrin-mediated endocytosis (e.g., transferrin endocytosis) is also inhibited by two other methods: intracellular potassium depletion (Larkin et al., 1983) and cytosol acidification (Sandvig et al., 1987; see Support Protocol 4). The potassium depletion assay is easy to perform, but inhibits clathrin-dependent endocytosis only in certain cell types (e.g., human and chick embryo fibroblasts, Hep 2 cells; Moya et al., 1985). These assays are used to establish that internalization is by clathrin-mediated endocytosis.

Materials

Cells of interest

SF medium (see recipe)

Hypotonic medium: 1:1 (v/v) SF medium/water

K⁺-depleted buffer: 100 mM NaCl, 50 mM HEPES acid, pH 7.4

6-well tissue culture plates

Additional reagents and equipment for measuring internalization (see Basic Protocol 2 or Alternate Protocol 2)

1. Plate 1×10^5 cells/well in SF medium in 6-well tissue culture plates 2 days before the experiment. Allow the cells to reach ~80% confluency ($\sim 5 \times 10^5$ /well) on the day of the experiment. Prepare one plate for each time point and one plate for surface labeling.

**Protein
Trafficking**

15.3.17

Bicarbonate-buffered medium is used if the experiment is to be performed in a CO₂ incubator; HEPES-buffered medium is used if the experiment is to be performed in air.

2. On the day of the experiment, wash the cells once with 2 ml SF medium.
3. Incubate cells for 5 min at 37°C in 2 ml hypotonic medium.
4. Wash cells with 2 ml K⁺-depleted buffer.
5. Incubate cells for 30 min at 37°C in K⁺-depleted buffer.
6. Measure internalization as described (see Basic Protocol 2, steps 3 to 17, or see Alternate Protocol 2), but perform internalization incubations (step 3) in K⁺-depleted buffer.

The inhibition is fully reversible within 15 min of adding back 10 mM KCl in the K⁺-depleted buffer. Incubating cells in hypertonic media (e.g., 0.45 M sucrose) also inhibits clathrin-dependent endocytosis, but may affect other types of endocytosis as well (Sandvig et al., 1989).

SUPPORT PROTOCOL 4

INHIBITION OF CLATHRIN-MEDIATED ENDOCYTOSIS BY CYTOSOL ACIDIFICATION

Only one of a number of methods for acidifying the cytosol are discussed below (Sandvig et al., 1987). Excessive acidification of the cytosol may also inhibit clathrin-independent endocytosis, as measured following fluid-phase endocytosis.

Materials

Cells of interest
SF medium (see recipe)
SF medium supplemented with 25 mM NH₄Cl
140 mM KCl/1 mM amiloride/40 mM HEPES acid, pH 7.0
6-well tissue culture plates

Additional reagents and equipment for measuring internalization (see Basic Protocol 2 or Alternate Protocol 2)

1. Plate 1 × 10⁵ cells/well in SF medium in 6-well tissue culture plates 2 days before the experiment. Allow the cells to reach ~80% confluency (~5 × 10⁵/well) on the day of the experiment. Prepare one plate for each time point and one for surface labeling.

Bicarbonate-buffered medium is used if the experiment is to be performed in a CO₂ incubator; HEPES-buffered medium is used if the experiment is to be performed in air.

2. On the day of the experiment, wash the cells once with 2 ml SF medium.
3. Incubate for 30 min at 37°C in SF medium supplemented with 25 mM NH₄Cl.

The optimal NH₄Cl concentration that inhibits clathrin-mediated entry without affecting other cellular processes may vary with the cell line, and different concentrations (e.g., 10 to 50 mM) should be tested.

4. Incubate cells for 2 min at 37°C in 140 mM KCl/1 mM amiloride/40 mM HEPES acid, pH 7.0.
5. Measure internalization (see Basic Protocol 2, steps 3 to 17, or see Alternate Protocol 2), but perform internalization incubations (step 4) in the amiloride-containing buffer.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

Either concentrated HCl or concentrated NaOH is used to pH all buffers.

Efflux medium

SF medium (see recipe) containing:

0.1 mM desferrioxamine (Sigma; from 10 mM stock in H₂O)

3 µg/ml human diferric transferrin

Prepare fresh

Neutral pH buffer

8.8 g NaCl (final 150 mM)

0.37 g KCl (5 mM)

0.11 g CaCl₂ (1 mM)

0.20 g MgCl₂ (1 mM)

4.77 g HEPES acid (20 mM)

Adjust to 1 liter with distilled water

Adjust pH to 7.4

Store up to 6 months at 4°C

pH 2.0 buffer

29.22 g NaCl (final 500 mM)

28.57 ml glacial acetic acid (0.2 N)

Adjust to 1 liter with distilled water

Adjust pH to 2.0

Store up to 6 months at 4°C

pH 5.0 buffer

8.76 g NaCl (final 150 mM)

9.74 g 2-(*N*-morpholino)ethanesulfonic acid (MES; 50 mM) *or* 5.88 g sodium citrate (20 mM)

Adjust to 1 liter with distilled water

Adjust pH to 5.0

Store up to 6 months at 4°C

Phosphate-buffered saline (PBS)

8 g NaCl (final 137 mM)

0.2 g KCl (2.6 mM)

2.2 g Na₂HPO₄ heptahydrate (8 mM)

0.2 g KH₂PO₄ (1.5 mM)

Adjust to 1 liter with distilled water

Adjust pH to 7.4

Store up to 6 months at room temperature

Serum-free (SF) medium

If the experimental incubations are to be performed in a CO₂ incubator, the SF medium is the medium formulation used to grow cells (e.g., Ham's F12, DMEM, RPMI) supplemented with the amount of sodium bicarbonate recommended for that particular medium formulation, and 10 mM HEPES acid (pH 7.2). A number of manipulations using this medium are performed at room temperature, and the HEPES is included to ensure that the pH of the bicarbonate-buffered medium does not alkalinize during these manipulations. If the experiment is to be performed in an air incubator, omit the sodium bicarbonate from the SF medium.

COMMENTARY

Background Information

The term endocytic trafficking is used to describe the processes of internalization from the cell surface into endosomes, trafficking among various intracellular compartments, and recycling back to the surface. Endocytosis through clathrin-coated pits is the major mechanism for receptor-mediated internalization of extracellular macromolecules. Ligands internalized by endocytosis include peptide hormones (e.g., insulin, epidermal growth factor), nutrient-carrying proteins (e.g., transferrin, low-density lipoprotein), viruses, and both plant and animal toxins. The distribution of receptors between intracellular compartments and the cell surface is determined by the rates of internalization and recycling. Endocytic trafficking also plays a fundamental role in the maintenance of intracellular organelle identity because, in many instances, the localization of membrane proteins to specific compartments involves retrieval and retargeting through the endosomal system. In addition, a number of specialized and regulated trafficking processes overlap, at least in part, with the general endosomal system (e.g., insulin-regulated trafficking of the glucose transporter GLUT4). For reviews of constitutive and regulated endocytic trafficking, see Watts and Marsh (1992), Sorkin and Waters (1993), Holman et al. (1994), Gruenberg and Maxfield (1995), and Mukherjee et al. (1997).

Transferrin receptor trafficking has been extensively studied in many different cell types. There are a number of advantages in using the transferrin receptor to monitor endocytic trafficking. First, transferrin remains bound to its receptor until it is recycled back to the cell surface. Therefore, recycling of the receptor can be followed directly using labeled transferrin. Second, transferrin receptors are expressed in most cell types. Third, transferrin is commercially available and readily labeled for use in biochemistry and microscopy experiments. Fourth, human transferrin has a high affinity for transferrin receptors from other species, making human transferrin a versatile reagent.

Using transferrin as a probe, the kinetics of clathrin-coated-pit-mediated internalization (see Basic Protocol 2) and the kinetics of constitutive recycling (see Basic Protocol 3) can be measured. Since transferrin receptor is constitutively cycled between endosomes and the plasma membrane, the rates of internalization and recycling determine the steady-state sur-

face-to-internal distribution of transferrin receptor (see Basic Protocol 1; Johnson et al., 1994). Thus, when characterizing the behavior of the transferrin receptor, the ratio of the recycling to internalization rate constants should be equal to the measured surface-to-internal ratio. The transferrin receptor assays described in this unit (and slight modifications of these assays) have been used successfully in conjunction with molecular genetic approaches (e.g., expression of dominant negative mutants; Bucci et al., 1992; van der Sluijs et al., 1992) and chemical inhibitors (Schonhorn and Wessling-Resnick, 1994; Martys et al., 1996) to further understand the molecular mechanisms of endocytosis.

The antibody-based trafficking assays described in this unit can be used to monitor the behavior of any membrane protein for which an antibody to the extracellular domain is available (see Alternate Protocols 1 and 2, and Basic Protocol 4). Although many proteins are internalized through clathrin-coated pits, there are other non-clathrin mechanisms for internalization. Methods for inhibiting endocytosis—via potassium depletion (see Support Protocol 3) or cytosol acidification (see Support Protocol 4)—have proven useful in determining the internalization pathways of membrane proteins (e.g., Moya et al., 1985; Sandvig et al., 1987; Subtil et al., 1994). However, conclusions must be drawn cautiously, as these treatments are not specific and the extent of inhibition varies greatly between cell types (Moya et al., 1985).

When characterizing the trafficking of a membrane protein, one can determine whether the protein is recycled or if it trafficks to lysosomes and is degraded (see Basic Protocol 5). If the protein is recycled, then the approach to steady-state occupancy (Basic Protocol 4) can be used to measure the recycling rate. As is the case for the transferrin receptor, if a membrane protein is constitutively recycled, then the ratio of the rate of internalization to the rate of recycling should be equal to the steady-state surface-to-internal distribution. In the case of antibody-based trafficking studies, the internalization rate and the surface-to-internal ratio can be directly measured, and a value for the recycling rate can be determined by multiplying the internalization rate by the surface-to-internal ratio.

In addition to the biochemical assays described in this unit, similar quantitative fluorescence microscopy assays have been used to

characterize endocytic trafficking (e.g., Mayor et al., 1993; Presley et al., 1993). A description of these powerful approaches is beyond the scope of this unit. Quantitative studies can also be performed by flow cytometry using fluorescent ligands or antibodies (Marks et al., 1996).

Critical Parameters

The sensitivity of the biochemical assays depends on the number of receptors (or molecules of membrane protein being studied) per cell and the specific activity of the probe. If a protein expressed by transfection is being studied, highly expressing clones can be used. However, it is imperative to demonstrate that the behavior of the protein is not dependent on the level of expression, as would be the case if a critical trafficking step is saturated at high levels of expression (Marks et al., 1996). Increasing the specific activity of the ^{125}I -labeled probe can compensate for a low number of receptors, but too high incorporation of ^{125}I may result in a loss of specificity (see Troubleshooting).

Another critical parameter is the efficient release of ligand from the cell surface, because this is used to discriminate between surface and internal ligand. The efficiency of the acid stripping is assessed by stripping ligand bound to the surface of cells at 4°C . When it works, acid stripping is the method of choice (see Basic Protocol 1). Several rounds of alternating acid/neutral washes can be performed to increase the stripping efficiency. Some antibodies are resistant to release by incubation in acidic buffers. In those cases, incubation with 1 mg/ml proteinase K or pronase at 4°C for 30 min (or more) can be tried to release surface-associated radioactivity.

To minimize experiment-to-experiment variation, it is important to use cells at the same degree of confluency.

Troubleshooting

The quality of the ^{125}I -labeled ligand (transferrin, other ligand, or antibody) is critical. If the ligand is over-derivatized (too high specific activity), specific binding to receptor/antigen can be compromised, which will result in a reduced signal-to-noise ratio (i.e., the nonspecific radioactivity will be a large percentage of the total radioactivity). In addition, the trafficking of an over-derivatized ligand can be altered. For example, over-derivatized transferrin often aggregates, and these aggregates are trafficked to lysosomes and degraded rather than recycled. On the other hand, if the specific activity is too low, there may not be enough signal. The

optimal specific activity must be determined empirically for each ligand and each cell type. There are a number of different methods for iodinating proteins. If problems are encountered with the degree of derivatization using one method, one of the other methods should be tried.

If the nonspecific radioactivity is a large fraction of the total, the amount of unlabeled ligand used for competition can be increased, and the number of washes can also be increased. Adding 1 mg/ml BSA during the incubation with the radio-iodinated probe can help lower background.

When using an antibody as a probe, verify (if possible) that the antibody does not modify trafficking of the protein (Subtil et al., 1994). Fab fragments should be used because bivalent antibodies cause cross-linking and therefore may modify the protein's trafficking (Weissman et al., 1986). Also, antibodies should be aliquoted and frozen immediately after radio-labeling to avoid freeze/thaw cycles. Aliquots may be kept at 4°C for several days.

Anticipated Results

The methods discussed in this unit (and variations of these methods) have been widely used. Examples of results obtained using these methods can be found in Weissman et al. (1986), Backer et al. (1991), Pelchen-Matthews et al. (1991), Herbst et al. (1994), Johnson et al. (1994), Schonhorn and Wessling-Resnick (1994), and Subtil et al. (1997).

The values for transferrin receptor trafficking in cultured fibroblast cells lines are:

Internalization rate constant: 0.15 to 0.2 min^{-1}

Recycling rate constant: 0.05 to 0.07 min^{-1}

Percent on surface at steady state: 30% to 40%.

Data from an idealized transferrin internalization assay are shown Figure 15.3.2. In panel A, data are shown for an assay carried out for a time sufficient to reach steady-state. During short incubation times, the ratio of the internal transferrin to surface transferrin increases linearly (panel B). The slope of this line is the internalization rate constant. After longer incubations, the rate of increase in ratio of internal-to-surface transferrin slows down as transferrin is recycled. To accurately measure the internalization rate constant it is important to use time points in the linear (early) portion of the curve. Ideally, the line should extrapolate through zero.

Data from an idealized transferrin recycling assay are shown in Figure 15.3.3. At the start

of the experiment, the great majority of transferrin is inside the cells because the pH 5 wash followed by neutral pH washes removes transferrin from the surface of cells. Over time the amount of transferrin in the medium increases and the amount of transferrin inside cells decreases as iron-free transferrin is recycled back to the cell surface. The recycling rate constant is the slope of a plot of the natural logarithm of the percent cell-associated transferrin versus time.

Time Considerations

Each individual experiment is readily performed within a single day.

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Determining Protein Transport to the Plasma Membrane

UNIT 15.4

Many integral membrane proteins, synthesized in the endoplasmic reticulum (ER), ultimately arrive at the cell surface to contact the cell's environment. During export from the ER, and transport through the Golgi subcompartments and *trans*-Golgi network (TGN), proteins may undergo a number of posttranslational modifications that affect the molecular weight and/or charge. These post-translational modifications include N-linked glycan modifications, O-linked glycan modifications, sulfation, phosphorylation, lipid modifications, and proteolytic trimming/cleavage (UNITS 15.1 & 15.2).

Since localization of the enzymes involved in these alterations is well known, posttranslational modifications are the most convenient tool to monitor intracellular transport processes. However, these modifications have gone to completion when the proteins arrive at the plasma membrane. The appearance of a particular protein at the cell surface can then be visualized by pulse-labeling the protein and removing the posttranslational modifications in intact cells using various enzymes (see Basic Protocol 1). Alternatively, cell-surface appearance can be assayed by selectively labeling the cell-surface pool of pulse-labeled proteins using biotin (see Alternate Protocol 1) or antibodies (see Alternate Protocol 3), or by removing the surface-protein pool with proteases (see Alternate Protocol 2). Various techniques for measuring cell-surface appearance based on these principles are also described. Changes in molecular charge can be assessed by one-dimensional isoelectric focusing (IEF; see Support Protocol 2). Fab fragments of antibodies can be biotinylated (see Support Protocol 1) and used to detect surface proteins. It is also possible to measure transport to endosomes before appearance at the plasma membrane (see Basic Protocol 2).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by your local radiation safety officer (also see APPENDIX 1D).

MEASURING ARRIVAL AT THE CELL SURFACE BY DIGESTION WITH SIALIDASE

**BASIC
PROTOCOL 1**

This assay is based on the fact that, in the TGN, glycoproteins acquire one or more sialic-acid residues at the end of their N- and O-linked carbohydrate chains. This sialylation alters not only the total molecular weight (although this will only be observed by SDS-PAGE when many sialic acids are added; see Fig. 15.2.6), but also the charge or isoelectric point of the glycoprotein (UNIT 15.2). Addition of sialic acids can be most readily observed when isolated proteins are separated by isoelectric point, i.e., using one-dimensional IEF (see Support Protocol 2) or two-dimensional IEF/SDS-PAGE (UNIT 6.4). The sialic acids can be easily and quantitatively removed from intact cells by incubation with sialidase, resulting in a more basic isoelectric point and a shift of the glycoprotein to the position on IEF corresponding to that of the glycoprotein prior to sialylation. Cell-surface appearance of glycoproteins can thus be determined by combining a pulse-chase experiment (UNIT 7.1) with incubations of the intact cells in the presence or absence of sialidase (UNIT 15.2). Cell-surface appearance is then determined by comparing the sialic-acid content of a glycoprotein prior to and after sialidase treatment. A glycoprotein has arrived at the plasma membrane when it becomes a substrate for the sialidase treatment of intact cells at low temperature.

**Protein
Trafficking**

15.4.1

Contributed by Jacques Neefjes

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Supplement 5

Materials

Cells

Amino acid-free medium containing 8% to 10% (v/v) FBS

Radiolabeled amino acid

PBS (APPENDIX 2A), ice cold

PBS containing 1 mM CaCl₂

Sialidase Type V from *Clostridium perfringens* (Sigma) dissolved in PBS and stored in 10- μ l 1 to 10 U/ml aliquots up to several years at -20°C

PBS containing 10% (v/v) fetal bovine serum (FBS)

Lysis mixture (see recipe)

Fetuin

Additional reagents and equipment for pulse-labeling (UNIT 7.1), TCA precipitation (UNIT 7.1), immunoprecipitation (UNIT 7.2), SDS-PAGE (UNIT 6.1) or one-dimensional IEF (see Support Protocol 2), and autoradiography (UNIT 6.3)

1. Culture cells in amino acid-free medium containing 8% to 10% FBS. Pulse label $\sim 5 \times 10^6$ cells per sample with a ~ 50 μ Ci radiolabeled amino acid and chase for various times. Chase times can be 0, 1, 2, 3, 5, 10, and 30 min for early events, and 0, 15, 30, 60, 120, and 240 min for following most stages in intracellular transport. Label two sets of cells for each chase time point: one for sialidase treatment and one as a control sample.

Usually cells are labeled with 10 to 50 μ Ci radioactive amino acids (UNIT 7.1), but they can also be labeled with radioactive sugars (Warmerdam et al., 1996).

- 2a. Collect nonadherent cells at each chase point by low-speed centrifugation (3 min at $1500 \times g$, 4°C).
- 2b. Collect adherent cells at each chase point by detaching from the plate with 10 mM EDTA in PBS at <15°C (to stop intracellular transport).

Alternatively, the sialidase digestion can be performed with the cells attached to the plate.

3. Wash cells two times with 1 ml PBS at 4°C. Collect nonadherent or detached cells between washes by centrifuging 3 min at $1500 \times g$, 4°C. Resuspend the pellet by gently tapping the bottom of the tube before adding PBS.
4. Suspend the final pellet from the control sample in 100 μ l PBS containing 1 mM CaCl₂ and the other sample in 100 μ l PBS containing 1 mM CaCl₂ and 0.1 IU sialidase. Incubate cells on ice for 1 hr.

Both the time of sialidase treatment and the concentration of sialidase may be varied to ensure full desialylation.

*Sialidase from other sources, e.g., *Arthrobacter ureafaciens*, can also be used.*

5. Remove sialidase by washing the cells three times with 1 ml PBS containing 10% FBS.
6. Lyse cells in 1 ml lysis mixture containing 1 to 10 mg fetuin to inhibit postlysis desialylation.

It is advisable to confirm the conditions for complete sialic acid removal that may result in postlysis desialylation. This can be done by mixing cells containing radiolabeled surface proteins with unlabeled cells incubated with sialidase prior to lysis.

7. To ensure the isolation of proteins from equal numbers of radioactively labeled cells, determine the amount of incorporated radioactivity per lysate by TCA precipitation of 10 μ l of each cell lysate (see UNIT 7.1).

8. Isolate the proteins from equal amounts of radioactivity by immunoprecipitation (UNIT 7.2).
9. Analyze immunoprecipitates by SDS-PAGE (UNIT 6.1) or one-dimensional IEF (see Support Protocol 2) followed by autoradiography (UNIT 6.3).

Sialic acid addition/removal may sometimes result in an altered apparent molecular weight on SDS-PAGE (UNIT 6.1; Fig. 15.2.6). This can be most easily visualized by loading the control sample next to the sialidase-treated sample. If this does not give proper resolution, however, proteins have to be separated on the basis of isoelectric point, either by one-dimensional IEF (see Support Protocol 2) or two-dimensional IEF/SDS-PAGE (UNIT 6.4). SDS-PAGE as well as one-dimensional IEF allows separation of many samples in a single run and direct side-by-side comparison.

MEASURING ARRIVAL AT THE CELL SURFACE BY BIOTINYLATION OF CELL-SURFACE MOLECULES

ALTERNATE PROTOCOL 1

Biotinylation of surface proteins employs monovalent, water-soluble cross-linkers with an additional biotin group to allow isolation by avidin or streptavidin coupled to Sepharose. Only proteins at the plasma membrane are substrates for this reaction, provided that the reaction is performed at temperatures $<15^{\circ}\text{C}$. Arrival of newly synthesized proteins at the cell surface can be analyzed by performing biotinylation of cells after a pulse-chase protocol (see UNIT 7.1). The experimental design is simple but not quantitative. Generally, between 0% and 25% of the surface-protein pool is biotinylated. As most cross-linkers react with amine groups, including those from serum components, cells should be extensively washed with PBS before biotinylation.

Materials

Cells

Wash buffer: PBS (APPENDIX 2A) containing 1 mM MgCl_2 and 0.1 mM CaCl_2
15 mg/ml sulfo-*N*-hydroxysuccinimide-biotin (sulfo-NHS-biotin; Pierce) in PBS,
prepared fresh prior to use

Wash buffer containing 25 mM lysine monohydrochloride

Lysis buffer containing 50 mM glycine

0.5% (w/v) SDS

50 mM Tris-Cl, pH 7.5 (APPENDIX 2A)

Antibody specific for protein of interest

Avidin- or streptavidin-Sepharose beads

Additional reagents and equipment for pulse labeling (UNIT 7.1),
immunoprecipitation (UNIT 7.2), and SDS-PAGE (UNIT 6.1)

1. Perform a pulse-chase experiment (see UNIT 7.1).

The number of cells required depends on the experiment and expression level of the protein. The cells can be either suspension or adherent cells.

2. At the respective chase times, wash a sample/dish of cells carefully with wash buffer three times.
3. Incubate cells 30 min at 4°C in a small volume of wash buffer (enough to cover the cells) containing sulfo-NHS-biotin to a final concentration of 1 mg/ml.

Other water-soluble, biotin-coupled cross-linkers can be used as well. Since the reagent reacts with amine groups, use of Tris-based buffers should be avoided.

If the biotinylation is inefficient, the concentration of the reagent can be increased up to 10 mg/ml.

Protein Trafficking

15.4.3

4. Wash cells twice with wash buffer containing 25 mM lysine monohydrochloride.
Lysine contains two reactive amino groups for inactivation of the NHS group.
5. Lyse cells in an appropriate lysis buffer containing 50 mM glycine.
Lysis buffer also usually contains Triton X-100; however, when unstable interactions occur use octylglucoside, digitonin, or CHAPS (e.g., see recipe for lysis mixture).
6. Determine the radioactivity, as above (see Basic Protocol 1, step 7). Immunoprecipitate (UNIT 7.2) the protein of interest from equal amounts of TCA-precipitable radioactivity.
7. Split the immunoprecipitate in two equal portions. Analyze one portion by SDS-PAGE (UNIT 6.1) directly after immunoprecipitation.
8. Denature the other portion by boiling 5 min in 100 μ l of 0.5% SDS. Dilute the SDS by adding 900 μ l of 50 mM Tris·Cl, pH 7.5. Centrifuge 5 min at 10,000 \times g, 4°C. Transfer the supernatant to a new microcentrifuge tube. Take the supernatant and reprecipitate the biotinylated pool of immunisolated proteins with ~10 μ l packed avidin- or streptavidin-Sepharose beads. Analyze by SDS-PAGE (UNIT 6.1).
Comparison of the total and biotinylated protein pool (run on the same gel) will show the kinetics of plasma membrane appearance.

ALTERNATE PROTOCOL 2

MEASURING ARRIVAL AT THE CELL SURFACE USING PROTEASE SENSITIVITY

Proteases can also be used to selectively degrade plasma-membrane proteins and thus to measure their arrival at the cell surface. Various proteases can be used depending on the susceptibility of the protein of interest. The optimal concentration of the protease for complete digestion has to be determined first. It should be below protease concentrations that break up cells.

Materials

Cells

PBS (APPENDIX 2A)

Protease: fresh 10 mg/ml stock solutions of either trypsin, chymotrypsin, or pronase in PBS

PBS containing 5% (v/v) fetal bovine serum (FBS)

Protease inhibitors, freshly prepared stocks:

for trypsin: 100 mM (1000 \times) phenylmethylsulfonyl fluoride (PMSF) in methanol and 1 mg/ml (100 \times) trypsin inhibitor in PBS

for chymotrypsin: 100 mM PMSF and 2 mg/ml (1000 \times) aprotinin in PBS

for pronase: 100 mM PMSF and 10 mM (1000 \times) *N*-tosyl-lysine chloromethyl ketone (TLCK) in PBS

Lysis mixture (see recipe)

Antibody specific for protein of interest

Additional reagents and equipment for biotinylation of cell-surface proteins (Alternate Protocol 1), immunoprecipitation (UNIT 7.2), SDS-PAGE (UNIT 6.1), immunoblotting (UNIT 6.2), pulse labeling (UNIT 7.1), and autoradiography (UNIT 6.3)

Determine appropriate protease concentration

1. Label the protein pool at the plasma membrane by biotinylation (see Alternate Protocol 1).
2. Incubate labeled cells (usually one dish per condition or ~ 1 to 5×10^6 cells per condition) 30 min on ice in ~ 5 ml PBS with varying concentrations of proteases (0 to 10 mg/ml).

Note that adherent cells will become detached by the protease treatment.

3. Remove soluble proteases by washing cells at least three times with 1 ml PBS containing 5% FBS each. Collect cells after each wash by centrifuging 3 min at $1500 \times g$, 4°C .

Centrifugation is necessary because the cells will be detached by the protease treatment.

4. After the final wash, resuspend cells in a small volume of FBS (5×10^6 cells in 250 μl) and inactivate the proteases by adding the respective protease inhibitors at the correct $1\times$ concentrations. Incubate the cells 10 min on ice. Centrifuge cells 5 min at $1500 \times g$, 4°C . Remove supernatant.

Pronase should be removed by extensive washing.

Adding protease inhibitors directly from stock solutions involves very small volumes ($\sim 0.25 \mu\text{l}$); it is best if they are serially diluted to allow for more accurate pipetting.

5. Lyse cells with 1 ml lysis mixture containing the appropriate protease inhibitors at indicated $1\times$ concentrations.
6. Isolate the desired protein by immunoprecipitation from equal numbers of cells (*UNIT 7.2*).
7. Analyze samples by SDS-PAGE (*UNITS 6.1*) followed by immunoblotting (*UNIT 6.2*) and incubation of the filters with avidin or streptavidin-coupled to peroxidase.
8. Detect the proteins with peroxidase-catalyzed reactions, preferably by chemiluminescence (e.g., enhanced chemiluminescence; ECL).

This assay will determine the optimal concentration and the appropriate protease that will allow quantitative removal of surface protein without substantial cell destruction.

Measure protein arrival

9. Perform a pulse-chase experiment (*UNIT 7.1*).
10. Incubate cells from every chase time in the presence or absence of proteases under conditions determined above (steps 2 to 4).
11. Lyse cells (step 5), then immunoprecipitate proteins from equal numbers of cells (*UNIT 7.2*).
12. Analyze samples by SDS-PAGE (*UNIT 6.1*).
13. Fix gel and detect surface appearance by autoradiography (*UNIT 6.3*).

Newly arrived protein will be trimmed (migrate faster by SDS-PAGE) or disappear due to protease activity.

MEASURING ARRIVAL AT THE CELL SURFACE USING ANTIBODIES

Cell-surface appearance of proteins can also be analyzed by combining a pulse-chase experiment (UNIT 7.1) with incubation of antibodies with intact cells (Burke et al., 1984). After removal of unbound antibodies, the surface-protein population can be isolated and analyzed. Postlysis binding of intracellular proteins to the antibodies (which have two or more antigen-combining sites) should be prevented, and a control for this should be included in the analysis. To avoid this problem, it is preferable to use biotinylated Fab fragments instead of complete antibodies.

Materials

Cells

PBS (APPENDIX 2A), ice cold

Biotinylated Fab fragments of antibody specific for protein of interest (see Support Protocol 1) or complete antibody

PBS containing 5% (v/v) fetal bovine serum (FBS)

Unlabeled cells or antigen

Protein A–Sephacel or avidin- or streptavidin-coupled beads

Additional reagents and equipment for pulse labeling (UNIT 7.1), immunoprecipitation (UNIT 7.2), SDS-PAGE (UNIT 6.1), and autoradiography (UNIT 6.3)

1. Perform a pulse-chase experiment (UNIT 7.1); include at least one early chase-point at which the protein has not yet reached the plasma membrane (e.g., 10 min).
2. At each chase time, wash cells once with 1 ml ice-cold PBS, suspend cells in a small volume (sufficient to cover the cells) of PBS or culture medium with antibodies or biotinylated Fab fragments, and incubate 30 min on ice.

Antibody or Fab should be added at a saturation concentration as determined by preliminary experiments.

Biotinylated Fab fragments are preferred because they have only one binding site, but complete antibodies can be used. In the latter case, controls for postlysis binding should be performed (see step 4).

3. Remove unbound antibodies/Fab fragments by washing at least three times at 4°C with 1 ml PBS containing 5% FBS.

If using biotinylated Fab fragments, proceed directly to step 5.

4. *If using intact antibodies:* Add a 10-fold excess of unlabeled cells or a 10- to 100-fold excess of antigen before lysis to prevent postlysis binding of intracellular radiolabeled proteins to the antibody.

Most intact antibodies have two antigen-binding sites (up to ten for IgM), of which only one may be occupied by the surface protein. The others may thus capture antigen postlysis. Competition with nonradioactive antigen added exogenously is not necessary when Fab fragments are used.

Control (and exclude) postlysis binding of antigen through the analysis of at least one early chase point (see step 1) that should not give precipitation of radiolabeled surface proteins.

5. Lyse (see Basic Protocol 1, step 6), clear by centrifuging 10 min at 10,000 × g, 4°C, and add 20 μl protein A–Sephacel (when using antibodies) or streptavidin- or avidin-coupled beads (when using biotinylated Fab fragments) to the lysate. Follow a normal immunoprecipitation protocol (UNIT 7.2).

6. Analyze immunoprecipitates by SDS-PAGE (UNIT 6.1) and autoradiography (UNIT 6.3).

PREPARATION OF BIOTINYLATED FAB FRAGMENTS

The production of biotinylated Fab antibody fragments requires some time investment. However, the investment is easily rewarded because they can be used for multiple surface-appearance studies (see Alternate Protocol 3) and internalization experiments. Papain appears to be an ideal protease, cleaving between the Fc and Fab portions of the antibody. Since papain is a cysteine protease, it first has to be activated (by reducing the active-site cysteine) and then titrated to establish the optimal conditions for Ig digestion (also see *UNIT 16.4*).

Materials

23 mg/ml papain in 70% ethanol
25 and 40 mM sodium acetate buffer, pH 4.5 (*APPENDIX 2A*)
2-Mercaptoethanol
100 mM sodium acetate buffer, pH 5.5 (*APPENDIX 2A*)
Protein A- or G-purified antibody (*UNIT 16.3*)
150 mM NaCl/5 mM EDTA/100 mM Tris·Cl, pH 8.0
Dithiothreitol (DTT)
Iodoacetamide (store protected from light and moisture)
50 mM NaHCO₃ buffer, pH 8.5
Sulfo-*N*-hydroxysuccinimidyl-6-biotinamido-hexanoate (sulfo-NHS-SS-biotin; Pierce)
10 mM Na₃PO₄, pH 7.4/150 mM NaCl buffer
Sephadex G-25 column
Centricon-30 (Amicon)
Additional reagents and equipment for dialysis (*APPENDIX 3C*), SDS-PAGE (*UNIT 6.1*), and standard anion or cation exchange chromatography (*UNIT 16.4*)

Activate papain

1. Dilute 23 mg/ml papain stock with 25 mM sodium acetate buffer, pH 4.5, to a final papain concentration of 8 mg/ml.

Ready-to-use kits for activation of papain are available from Pierce.

2. Add 6 μ l of 2-mercaptoethanol to 200 μ l diluted papain and 400 μ l of 40 mM sodium acetate buffer, pH 4.5. Incubate 20 to 30 min at 4°C.

2-ME is added to activate the papain.

3. Equilibrate and elute a 10-ml Sephadex G-25 column with 20 ml of 100 mM sodium acetate buffer, pH 5.5.
4. Run the activated papain solution over the column to remove various impurities from papain. Pool fractions with the highest protein concentration by measuring the absorbance at 280 nm.

Digest antibody

5. Dialyze protein A- or G-purified antibody against three changes of 200 ml of 150 mM NaCl/5 mM EDTA/100 mM Tris·Cl, pH 8.0.
6. Add 1 to 2 mM DTT prior to digestion.
7. Mix antibody and activated papain. Incubate at 4°C or room temperature.

The efficiency of cleavage depends on the antibody isotype. It is thus best to vary the digestion period, the temperature, and the concentration of papain to determine optimal conditions for digesting the antibody of interest.

The ratio of papain to antibody is usually between 1:50 and 1:200 for time points between 1 and 24 hr.

8. Stop reaction by adding iodoacetamide to a final concentration of 2 mM.
9. Check the digestion by analyzing an aliquot 12% SDS-PAGE (UNIT 6.1).

Isolate Fab fragments

10. Separate the Fab fragment from the Fc fragment by standard anion or cation exchange chromatography (UNIT 16.4).

The Fc fragment will be selectively retained by an anion-exchange column, whereas the Fab fragment will be retained by a cation-exchange column and will only elute with high salt.

11. Check separation by SDS-PAGE under reducing and nonreducing conditions (UNIT 6.1).

The Fc fragment contains an intrachain disulfide bridge and contaminations can best be visualized under nonreducing conditions.

Biotinylate Fab fragments

12. Dialyze the Fab fragments with three changes of 200 ml of 50 mM NaHCO₃ buffer, pH 8.5.
13. Add a ten-fold molar excess of sulfo-NHS-SS-biotin and incubate 2 hr at 4°C.
14. Remove unreacted biotin by centrifugation through a Centricon-30 concentrator according to manufacturers' instructions.
15. Dilute the sample in 10 ml of 10 mM Na₃PO₄, pH 7.4/150 mM NaCl buffer and centrifuge two successive times through the Centricon-30 concentrator to remove unreacted biotin.
16. Store labeled Fab at a concentration of 10 mg/ml in 50- to 100- μ l aliquots at -80°C.

BASIC PROTOCOL 2

MEASURING TRANSPORT TO ENDOSOMES BEFORE PLASMA MEMBRANE APPEARANCE

Some proteins, like class II molecules of the major histocompatibility complex, first enter the endocytic pathway before they appear at the plasma membrane (Neeffjes et al., 1990). A variation on the sialidase protection assay, described in Basic Protocol 1, has been used to visualize this on intact cells. Plasma membrane, but not endosomal, glycoproteins can be desialylated by sialidase treatment at 4°C on intact cells, whereas both protein pools are digested by sialidase that has entered the endocytic pathway by fluid-phase endocytosis at 37°C. A comparison of the number of sialic acids on glycoproteins from cells incubated under these conditions will allow determination of the localization of proteins in the ER/Golgi (not sialylated in the control situation), the TGN (sialylated under all conditions), endosomes (desialylated by endocytosed sialidase only), and the plasma membrane (desialylated by sialidase at 4°C and in the medium). Combined with a pulse-chase experiment, this will allow the positioning of the protein in the respective compartments at any time during the chase period. Entry in endosomes *before* arrival at the plasma membrane is indicative of direct transport from TGN to endosomes (see Neeffjes et al., 1990). However, very rapid transit of the protein over the plasma membrane is still possible, and chase points should be selected carefully for validating the direct transport (without intersection with the plasma membrane) from TGN to endosomes.

Determining Protein Transport to the Plasma Membrane

15.4.8

Materials

Cells

Sialidase Type V from *Clostridium perfringens* (Sigma), dissolved in PBS and stored in aliquots at -20°C

Normal culture medium with 0.1% (w/v) BSA and no FBS

PBS (APPENDIX 2A) containing 10% (v/v) fetal bovine serum (FBS), ice-cold

Lysis mixture (see recipe)

Fetuin

Additional reagents and equipment for pulse labeling and TCA precipitation (UNIT 7.1), immunoprecipitation (UNIT 7.2), one-dimensional IEF (see Support Protocol 2), SDS-PAGE (UNIT 6.1), and autoradiography (UNIT 6.3)

1. Perform a pulse-chase experiment (UNIT 7.1); follow a 10- to 15-min labeling period with chases of 0, 15, 30, 60, 120, and 240 min.

In this experiment, three conditions are tested at each chase point: (1) cells will be cultured after the radioactive labeling and analyzed at each chase point without further treatment to determine entry in the TGN; (2) cells will be treated with sialidase at 4°C to show appearance at the plasma membrane; (3) and cells will be cultured after the pulse in the presence of sialidase that will enter the endocytic pathway and thus desialylate endosomal and plasma-membrane proteins.

For adherent cells, label three plates per chase point. Use $\sim 5 \times 10^6$ cells per chase point per condition.

2. Split the cell culture at the beginning of the chase into two aliquots or use different dishes for the various conditions. Culture one set of cells at 37°C in culture medium containing 0.1% BSA and sialidase at a final concentration of 1 IU/ml to allow entry of sialidase in the endocytic route. Culture the other half of the cells in culture medium with 0.1% BSA alone.

The culture medium should not contain fetal bovine serum (which has many sialylated proteins), but should contain 0.1% (w/v) BSA (which is not glycosylated).

Cells like B lymphocytes that endocytose only small volumes may require higher concentrations of sialidase.

3. At every chase point divide the sample of the cells cultured in the medium/BSA alone (step 2) into two equal portions and incubate 1 hr at 4°C (or use two dishes), wash the cells, add PBS/ CaCl_2 with or without 1 IU/ml sialidase. Also incubate the sample that was cultured in the presence of sialidase (step 2) for an additional 1 hr at 4°C .

Include in the assay one sample that is not radiolabeled but is cultured in the presence of sialidase. These cells will be mixed with control radiolabeled cells before washing and lysis to verify complete removal of sialidase activity (because the sialidase should not act postlysis).

4. Remove sialidase by washing the cells three times with 1 ml ice-cold PBS containing 10% FBS.
5. Lyse cells in 1 ml lysis mixture containing 1 to 10 mg/ml fetuin.

Fetuin is highly sialylated (for further inhibition of sialidase) and has a neutral pH. Thus, unlike free sialic acids, it will not increase the background during the immunoprecipitation procedure.

6. Determine the amount of radioactivity incorporated by TCA precipitation of 10 μl of each lysate (UNIT 7.1). Isolate proteins by immunoprecipitation (UNIT 7.2) from lysates containing equal amounts of TCA-precipitable radioactivity.

- Analyze immunoprecipitates by one-dimensional IEF (see Support Protocol 2) or SDS-PAGE (UNIT 6.1).

For best comparison, analyze the three conditions (control, sialidase 4°C, and sialidase 37°C) side by side on the gel. The protein has entered the TGN when the carbohydrate acquires terminal sialic acids that are not removed by the different sialidase treatments. The protein has entered endosomes when desialylated by the sialidase added to the chase medium, but not by the sialidase treatment at 4°C. Finally, the protein has reached the plasma membrane when it is a substrate for sialidase both in the culture medium and at 4°C.

SUPPORT PROTOCOL 2

SEPARATION OF PROTEINS BY CHARGE USING DENATURING ONE-DIMENSIONAL ISOELECTRIC FOCUSING

For one-dimensional IEF a 4% polyacrylamide gel is used to avoid separation by size (Neefjes et al., 1986). This requires careful handling of the gels during the preparation, run, and fixation. This protocol is for gels of ~55 ml.

Materials

- 0.8% (w/v) agarose in water
- Urea
- 10% (v/v) Triton X-100 or Igepal CA-630 (Sigma)
- 30% (w/v) acrylamide/1.6% (w/v) bisacrylamide solution
- Ampholytes (Pharmacia Biotech)
- 10% (w/v) ammonium persulfate
- N,N,N',N'*-tetramethylethylenediamine (TEMED)
- Isoelectric focusing sample buffer (see recipe)
- Immunoprecipitates (see Basic Protocol 1 or see Basic Protocol 2)
- 20 mM H₃PO₄
- Bromphenol blue
- 50 mM NaOH
- 30 × 19-cm glass plates
- 1-mm spacers
- 20-well Teflon comb
- Vertical gel chamber
- Additional reagents and equipment for gel electrophoresis (UNIT 6.1) and autoradiography (UNIT 6.3)

Prepare IEF gel

- Assemble electrophoresis apparatus using 30 × 19-cm glass plates with 1-mm spacers. To prevent the gel from slipping between the glass plates, place spacers slightly tilted towards each other with the narrow side toward the bottom. Apply 0.8% (w/v) agarose outside spacers, between the glass plates, to prevent leakage.
- Prepare 55 ml gel solution by mixing the following reagents. Dissolve urea by gently shaking the solution under hot tap water.

- 30.25 g urea
- 11 ml 10% Triton X-100 or Igepal CA-630
- 8.25 ml 30% acrylamide/1.6% bisacrylamide solution
- 12.1 ml H₂O.

Do not dissolve urea in a microwave or by boiling as this can result in evaporation and crystallization of the urea after polymerization of the gel.

3. Add ampholytes after solubilization of the urea.

The mixture of ampholytes determines the pH gradient over the gel. The author generally uses 2.2 ml of pH 5.7 ampholytes, 550 μ l of pH 3.5 to 10 ampholytes, and 220 μ l of pH 7 to 9 ampholytes.

4. Polymerize the gel by adding 200 μ l of 10% ammonium persulfate and 100 μ l TEMED.
5. Pour gel mixture between the preassembled plates and insert a 20-well Teflon comb. Allow to solidify.

The gel usually takes ~30 min to solidify.

6. Place gel in a vertical gel chamber. Fill lower chamber with 20 mM H₃PO₄.

Prepare samples and run gel

7. Incubate immunoprecipitates \geq 5 min at room temperature with isoelectric focusing sample buffer and load samples.
8. Before addition of the upper buffer, load a small layer of 3-fold diluted isoelectric focusing sample buffer with ~1 (% or mg/ml) bromphenol blue.

This is to prevent hydrolysis of asparagine and glutamine side chains by the NaOH in the upper gel chamber.

The bromphenol blue will indicate the extent of the run but is not strictly necessary.

9. Carefully fill the upper chamber with 50 mM NaOH.
10. Run the gel overnight at 1000 V. Limit power such that the run starts at ~300 to 400 V and increases to a maximum of 1000 V.

This will prevent heating and melting of the gel. The voltage will swiftly rise due to increased resistance. Smaller gels should be run at a lower limiting voltage; e.g., a gel 15 cm wide should be run at ~600 V with the power set such that the run starts at ~200 V.

11. Fix gel and expose for autoradiography (UNIT 6.3).

The gel is very sloppy. Do not take the gel off the glass plates, but rather turn the glass plates around over the fixative. The gel will then fall into the fixative. Perform a similar procedure for transfer of the gel to Whatman 3MM paper prior to drying the gel.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Isoelectric focusing sample buffer

- 9.5 M urea
- 2% (w/v) Triton X-100 or Igepal CA-630 (Sigma)
- 2% (v/v) ampholytes (Pharmacia Biotech), pH 3.5 to 10
- 5% (v/v) 2-mercaptoethanol
- Store 1-ml aliquots at -20°C

Lysis mixture

- 50 mM Tris-Cl, pH 7.5AS (APPENDIX 2A)
- 150 mM NaCl
- 5 mM MgCl₂
- 0.5% (w/v) detergent
- Store at 4°C

The choice of detergent depends on the experiment. For stable protein complexes the author routinely uses 0.5% Igepal CA-630 (Sigma) or Triton X-100. For more unstable protein complexes, 0.5% digitonin or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) should be used.

COMMENTARY

Background Information

Plasma-membrane proteins are synthesized in the ER. Here they have to fold properly and assemble before they are allowed to pass the “quality control system” of the ER and enter the Golgi complex. The folding and assembly steps are rate limiting and can vary considerably between different proteins, although some variability in transport rates through the Golgi complex occurs as well. Consequently, the rate of surface appearance can vary from 10 to 15 min up to many hours, even for proteins belonging to the same family such as MHC class I alleles (Neefjes and Ploegh, 1988). These differences cannot be predicted and can only be experimentally determined.

Many endosomal and lysosomal proteins are transported to their final destination by transient trafficking through the plasma membrane. Because proteins are only briefly at the plasma membrane before endocytosis, it is very hard to distinguish between direct sorting at the TGN for the endocytic pathway and an indirect route involving transport over the plasma membrane. In addition, the two routes are not mutually exclusive. For example, the process of sorting at the TGN appears to be saturable (Marks et al., 1996) and overexpression of endosomal proteins may easily result in transient cell-surface appearance. Transient transport over the plasma membrane can best be visualized by slowing or inhibiting endocytosis in combination with the techniques described in this unit for detection of cell-surface appearance. However, easy and reproducible techniques for selectively inhibiting endocytosis have not been developed yet.

Several different techniques to selectively tag the surface population of glycoproteins have been described in this unit. All of these techniques consist of combinations of pulse-chase experiments and chemical or enzymatic modification on intact cells. These techniques require that the modifications are quantitative and readily inhibitable. In addition, optimal conditions for the modifications should be determined prior to the experiment. Internal controls excluding postlysis modifications should always be included in the experiments. The different experimental proto-

cols all have advantages and disadvantages as discussed below.

Biotinylation of intact cells to tag the cell-surface protein pool is an efficient way to visualize cell-surface arrival (see Alternate Protocol 1). Postlysis biotinylation does not occur. Since the modification is not quantitative, the unmodified pool of proteins contains intracellular as well as cell-surface proteins. The assay employing sialidase to remove the sialic acids at the plasma membrane (see Basic Protocol 1) is quantitative and thus makes it possible to distinguish the intracellular from the cell-surface pool. However, the sialidase treatment protocol is more laborious. Furthermore, the effect of desialylation is not always obvious upon analysis by SDS-PAGE and often requires analysis by one-dimensional IEF or two-dimensional IEF/SDS-PAGE, techniques that are more time consuming.

Protease removal of cell-surface proteins (Alternate Protocol 2) is another alternative, but the usefulness of this technique depends on the sensitivity of the target protein. The optimal concentration and type of protease have to be defined first. If proteolysis requires high concentrations of protease, cell death and DNA release may be observed, which may complicate the interpretation of the experiment. In addition, some remnants of proteolytic activity in the lysate may obscure the results. Unless the protein is sensitive and accessible to a particular protease, this assay will not easily result in quantitative removal of cell-surface proteins.

Finally, biotinylated Fab fragments are an easy tool to follow specifically the surface appearance of proteins by combining a pulse-chase protocol with surface binding (Alternate Protocol 3). However, the generation and biotinylation of Fab fragments is laborious. Complete (bivalent) antibodies may be a more convenient alternative for immunoprecipitation of proteins expressed at the cell surface, but postlysis binding of antigen should be excluded.

Critical Parameters and Troubleshooting

All the protocols presented in this unit require a pulse-chase experiment in combination

with detection of cell-surface appearance. When early events (folding or assembly) are followed, label for 5 min and chase 0, 5, 10, 20, 30, and 60 min. Early folding events may be traced by labeling for 1 min and chasing for 0, 1, 2, 4, 7, and 10 min. For transport processes to the plasma membrane, the author usually labels cells for 15 min followed by chases of 0, 15, 30, 60, 120, and 240 min. Proteins should be sufficiently labeled so as to effectively visualize them after each incubation step. If they are not, use more cells for the pulse labeling, increase the pulse time, and/or add more radioactive amino acid. The concentration of radioactive amino acids during the pulse may be increased by simply performing the pulse in a smaller volume. Starve the cells for 30 to 45 min prior to the pulse in culture medium devoid of the amino acid used for radioactive labeling of the cells. Include 10% fetal bovine serum in this starvation medium; the cells will then be more viable and will incorporate higher amounts of radioactivity. [³⁵S]Methionine and/or cysteine are usually employed for biosynthetic labeling. If proteins are poor in these amino acids, consider pulse labeling the cells with other amino acids (see *UNIT 7.1*).

It is essential to keep the cells viable and intact during the different incubation and washing steps. Gentle centrifugation (e.g., 400 × *g*) during the washing procedures improves the intactness and recovery of the cells. Cells can best be resuspended after removal of the supernatant by simply tapping against the microcentrifuge tube. Addition of 0.1% (w/v) bovine serum albumin or 5% to 10% (v/v) fetal bovine serum to the washing buffer may also increase viability and recovery of the cells.

Biotinylation of intact cells (Alternate Protocol 1) to label surface proteins is a convenient assay although it is not quantitative. However, this protocol is useful for establishing when and at what rate proteins appear at the plasma membrane, provided that identical conditions are used to treat the samples. The decrease in intracellular protein pool cannot be easily determined when, for example, 20% of the surface proteins are biotinylated. Higher concentrations of the biotinylation reagent increase the efficiency of labeling, but such experiments require relatively large amounts of reagent and are very expensive. When biotin labeling is poor, wash cells more carefully to remove competing proteins from the medium.

The sialidase treatment of intact cells (Basic Protocol 1) to detect surface appearance requires establishing the conditions for full desia-

lylation. The conditions for inactivation/removal of sialidase should be internally controlled. The conditions described usually result in quantitative removal of sialic acids on nonadherent cells. Adherent cells can be detached from the culture plates before the sialidase treatment to ensure complete desialylation. Although this is usually not necessary, it ensures complete desialylation. Free sialic acids should not be included in the lysis buffers for inactivation of sialidase because this acidifies the buffer and thus increases the background of the immunoprecipitation. Fetuin (which is a highly sialylated glycoprotein) inhibits sialidase without changing the pH of the lysis buffer.

One-dimensional IEF to separate different sialylated forms of a protein (Support Protocol 2) is a powerful but difficult technique. Always place the spacers on the sides slightly tilted towards each other to prevent the gel from slipping into the lower chamber. Also be careful not to allow direct contact between the lower and upper gel chambers, e.g., along the spacers. If this happens, the gel will become very hot and the glass plates may break. The author often adds a small drop of agarose to the corners of the gel contacting the spacers to prevent leakage and direct contact between the upper and lower buffer. One-dimensional IEF gels are very fragile and can easily break. Handle gels carefully and never pick them up with your hands. Most fixatives will not make the gel stronger. One clear exception is fixation in dimethyl sulfoxide/2,5-diphenyloxazole (DMSO/PPO), but this is no longer regularly used.

The use of proteases (Alternate Protocol 2) also requires titration to determine the optimal conditions. Often, cell destruction is observed and the resulting released DNA interferes with the experiments. This protocol is not suitable for all proteins, as many proteins are very resistant to proteases or are only degraded under conditions that also destroy the intact cell. If so, try to use other proteases that more selectively degrade the surface protein without resulting in significant cell damage. Most commercial proteases have selective and efficient inhibitors. However, this may be a problem for protease cocktails like pronase, which should not be used for cell-surface appearance experiments.

The production of the biotinylated Fab fragments (Support Protocol 1) is somewhat time consuming and requires isolation of reasonably large quantities of antibodies before the papain digestion can be performed. Ready-to-use kits

are available for this. Biotinylated Fab fragments may result in easy isolation of the surface pool of proteins. The unbound fraction may not necessarily be the intracellular fraction unless it is shown that *all* surface proteins were captured by the Fab fragment. To test this, take a late chase point (e.g., 6 hr) and show experimentally that the radioactive protein is quantitatively recovered. It is preferable to not use intact multivalent antibodies because they may induce internalization by cross-linking two or more antigens, and they may easily bind antigens postlysis.

Anticipated Results

Sialidase treatment is the most sensitive and quantitative of the assays described and allows analysis of surface appearance when the chase times are correctly chosen. SDS-PAGE may show a shift in apparent molecular weight. Otherwise, one-dimensional IEF or two-dimensional IEF/SDS-PAGE should be used. Combining a pulse-chase experiment with treatment of cells at 4°C and 37°C (see Basic Protocol 2) allows biochemical detection of the localization of the glycoprotein in pre-Golgi, TGN, endosomes, and plasma membrane.

The biotinylation protocol does not quantitatively modify all proteins at the plasma membrane, but it is still a very practical way to determine cell-surface appearance. Quantitative biotinylation is not required. The isolates can be analyzed by normal SDS-PAGE and surface appearance can be efficiently seen.

Protease treatment of intact cells to remove the plasma-membrane protein pool is possible for protease-sensitive proteins, but it is difficult to verify that the digested protein is obtained from equal numbers of cells/proteins.

Cell-surface immunoisolation by Fab fragments is an efficient way to show cell-surface appearance. The pulse-chase can be analyzed by SDS-PAGE prior to and after Fab isolation and the rate of cell-surface appearance of the protein of interest can be easily determined.

Time Considerations

Most assays described in this unit are relatively laborious. Pulse-chase experiments combined with protease/sialidase/biotin treatment, TCA precipitation for quantitation and immunoprecipitation, followed by SDS-PAGE or one-dimensional IEF take ≥ 3 days before the result can be seen. The preparation of the biotinylated Fab fragments is time consuming (~2 to 3 days) but clearly worth the effort when used for multiple experiments.

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Analysis of Membrane Traffic in Polarized Epithelial Cells

Spatial asymmetry is fundamental to the structure and function of most eukaryotic cells (Drubin and Nelson, 1996). A basic aspect of this polarity is that the cell's plasma membrane is divided into discrete domains. The best studied and simplest example of this occurs in epithelial cells, which line exposed body surfaces (Matter and Mellman, 1994; Mostov et al., 1999, 2000). Epithelial cells have an apical surface facing the outside world and a basolateral surface contacting adjacent cells and the underlying connective tissue. These surfaces have completely different compositions and are separated by tight junctions, which block movement of plasma membrane proteins between the apical and basolateral surfaces and also prevent diffusion of extracellular material between cells. Epithelial cells use two pathways to send proteins to the cell surface. Newly made proteins can travel directly from the trans-Golgi network (TGN) to either the apical or basolateral surface. Alternatively, proteins can be sent to the basolateral surface and then endocytosed and transcytosed to the apical surface. A schematic view of a simple polarized epithelial cell is given in Figure 15.5.1.

Studies on membrane traffic in polarized epithelial cells have been greatly facilitated by the use of cell lines grown as a well-polarized monolayer. The most widely utilized of these is the Madin-Darby canine kidney (MDCK) cell line. Other cell lines that have been used for studying polarized membrane traffic include Caco-2 (human intestinal cancer) and FRTL (Fischer rat thyroid; Weimbs et al., 1997). Table 15.5.1 summarizes the cell lines most commonly used in studies of membrane traffic in polarized epithelial cells. Generally these cell lines are maintained by growing adherent cells in standard cell culture plastic dishes or flasks; however, for the actual experiments in which polarized traffic is assessed, the cells are usually grown on porous filters. Such filters are available mounted in holders sold by several manufacturers, including Corning, Falcon, and Nunc. These devices come in several sizes and are available with pores of different diameters. Most of the work done by the authors has been with 12-mm Corning Transwells with filters containing pores of 0.4- μm diameter. Table 15.5.2 lists the main types of units that are commercially available.

The usual type of study is to examine the biosynthetic delivery of a membrane or secreted protein to the apical or basolateral surface (see Basic Protocol 2). This sort of experiment is quite similar to studying the delivery of proteins to the surface of nonpolarized cells, as described in *UNIT 15.4*, with the principal difference being that delivery to either the apical or basolateral surface is measured, as opposed to the plasma membrane as a whole. Although MDCK cells contain numerous endogenous apical and basolateral plasma membrane proteins, these are usually expressed at very low levels, making it difficult to study the trafficking of any individual protein. Additionally, one often wishes to examine the polarized localization or trafficking of a protein that may not be expressed endogenously in MDCK cells; therefore, one typically begins a project by transfecting cDNA for the protein of interest into MDCK cells in order to overexpress the protein for study (see Basic Protocol 1). Most of the authors' experience is with the calcium phosphate-based transfection system (see Basic Protocol 1; Breitfeld et al., 1989). Cells can be transfected by other methods, including various commercially available lipid-based transfection reagents (e.g., Lipofectamine 2000) or by electroporation. Although transient transfection and expression systems can be used, in many cases the fraction of cells expressing the protein of interest is very low, and the cells that express the transfected protein tend to be less well polarized in part because the process of polarization takes 4

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to 7 days, whereas transient protein expression generally peaks at 48 hr and is often gone by 72 hr; therefore, in most cases it is advisable to generate stable clones (see Support Protocol 1). These clones are then expanded for further use by growing on standard tissue culture dishes. For studies of membrane traffic the cells must be cultured on filter units (see Support Protocol 2). It is important to check that the cells form a tight, well-polarized monolayer on the filter. Several methods are available to do this, and a particularly easy method is presented in this unit (see Support Protocol 3). A cell surface biotinylation assay to measure delivery of proteins is presented (Basic Protocol 3; Luton and Mostov, 1999). As the study of polarized membrane traffic is fundamentally the study of the localization of proteins, it is extremely useful to visualize the location of proteins in the epithelial cell. In addition, a fixation method to use for laser scanning confocal immunofluorescence

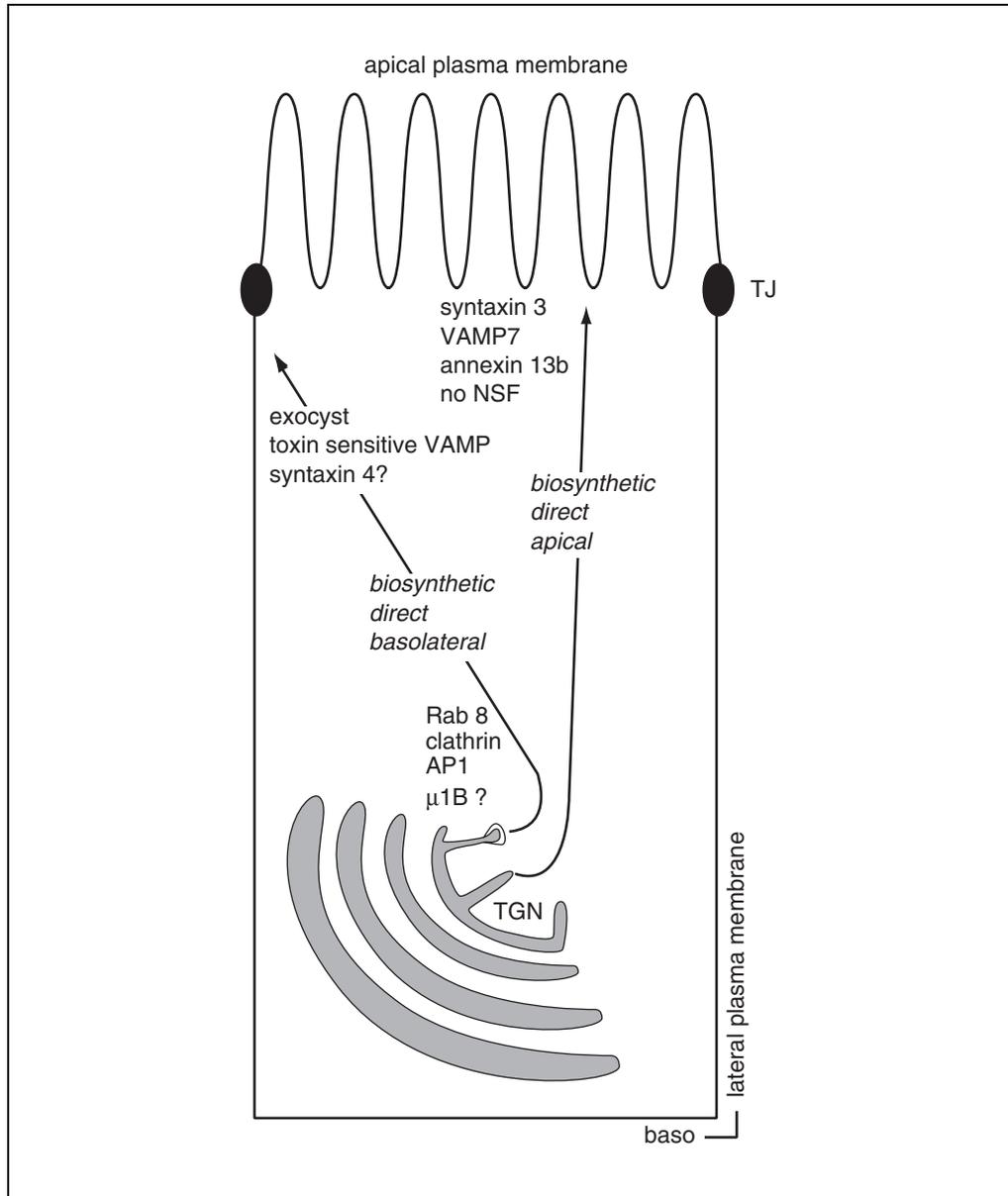


Figure 15.5.1 Schematic diagram of a polarized epithelial cell. Only the major biosynthetic pathways leading directly from the TGN to the apical and basolateral surfaces are shown. Components of the membrane traffic machinery that are likely to be involved in each of these two pathways are also indicated. For further details, see Mostov et al. (2000). Abbreviations: TGN, trans-Golgi network; TJ, tight junction.

Table 15.5.1 Cell Lines Commonly Used to Study Epithelial Membrane Traffic in Polarized Epithelial Cells

Cell line	Origin	Comments	ATCC# ^a
MDCK	Canine kidney	Most widely used. Two major categories, types I and II, though many clones with specific phenotypes.	CCL-34
Caco-2	Human intestine	Although derived from human colon cancer, more closely resembles small intestine. Slow growing.	HTB-37
FRTL	Rat thyroid	Some glycolipids and GPI-anchored proteins are found at the basolateral surface, unlike other epithelial cells.	CRL-1468

^aThe ATCC home page can be reached at <http://www.atcc.org>.

Table 15.5.2 Commercially Available Filters for Growth of Epithelial Cells

Material	Manufacturer	Comments
Polycarbonate	Corning Millipore BD Biosciences Labware Nalge Nunc International	Most widely used
PTFE/FP	Millipore Corning BD Biosciences Labware	Good optics. Coating with extracellular matrix (ECM) is usually required; can be purchased precoated
PET	BD Biosciences Labware	Available in different pore densities, with low density having better optical properties, but lower porosity. Available precoated with several types of ECM
Anopore	Nalge Nunc International	Very low intrinsic fluorescence, well-suited for immunofluorescence microscopy
Cellulose esters	Millipore	High protein binding
Polyester	Corning	Good optics

microscopy of proteins in polarized epithelial cells is also presented (see Basic Protocol 4; Luton and Mostov, 1999).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly (e.g., use of a sterile hood).

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) require altered levels of CO₂ to maintain pH.

**TRANSFECTION OF POLARIZED EPITHELIAL CELLS IN SUSPENSION
AND SELECTION OF RESISTANT CLONES**

The goal of this protocol is to transfect a plasmid containing the gene of interest into mammalian epithelial cells under a suitable promoter (i.e., a CMV promoter). This protocol provides the basis for the other experimental protocols described.

Materials

- Confluent cells grown on 10-cm tissue culture dish
- HEPES buffered saline (HeBS), pH 7.05 (see recipe; also see *APPENDIX 2A*)
- 20 µg plasmid DNA in 1 to 20 µl H₂O
- 2 M CaCl₂: filter sterilize and store up to 6 months at 4°C
- CMF-DPBS (*APPENDIX 2A*)
- Trypsin/EDTA solution—e.g., 0.25% (w/v) trypsin/0.2% (w/v) EDTA (*UNIT 1.1*)
- MEM medium (*APPENDIX 2B*) with and without 5% (v/v) FBS (*APPENDIX 2A*)
- 20 mM chloroquine in water: filter sterilize and store up to 6 months at 4°C
- 15% (w/v) glycerol in HeBS: filter sterilize and store up to 6 months at 4°C
- Selection medium: MEM/5% FBS containing eukaryotic antibiotic (e.g., G418; see recipe)
- Nontransfected cells
- 10-cm dishes

- Additional reagents and equipment for picking clones (see Support Protocol 1)

Transfect cells

1. Split cells grown on a 10-cm dish 1:10 on the day prior to transfection in order to yield ~30% confluency on the day of transfection.
2. To 0.5 ml HEPES buffered saline (HeBS), pH 7.05, add 20 µg plasmid DNA in 1 to 20 µl water, and 31 µl of 2.0 M CaCl₂. Flick the tube for 20 sec.

The pH of the HeBS has to be exact for a good precipitate to form.

This step should be done in a sterile hood.
3. Incubate the plasmid at room temperature for 30 min, avoiding any unnecessary shaking of the sample.
4. Trypsinize one subconfluent (i.e., ~30% confluent or $\sim 1 \times 10^7$ cells) 10-cm dish of cells by adding 1 to 2 ml of trypsin/EDTA solution after washing with CMF-DPBS.

For trypsinization of MDCK cells, use 0.25% (w/v) trypsin/0.2% (w/v) EDTA. The desired concentration of cells is ~ 0.5 to 1×10^6 cells/ml.
5. Centrifuge cells for 3 min at $300 \times g$, room temperature. Resuspend in 5 ml MEM medium with 5% FBS.
6. Transfer 1 ml of the cell suspension into a 10-cm dish.
7. Slowly add 500 µl DNA-Ca²⁺ coprecipitate (step 3) while simultaneously agitating the 10-cm dish. Incubate at room temperature for 15 to 20 min.
8. Add 50 µl of 20 mM chloroquine (200 µM final) to 3.5 ml MEM/5% FBS. Add the MEM/FBS/chloroquine mixture to the dish and agitate to spread cells.
9. Incubate 6 to 18 hr (e.g., overnight) at 37°C in order to allow the cells to attach to the dish.

Plate transfected cells

10. Remove the medium. Wash once with chloroquine-containing medium (see step 8).
11. Add 2 ml of 15% glycerol in HeBS gently. Incubate exactly 1 min at 37°C.
12. Remove glycerol by washing gently twice with MEM without FBS, and then adding 10 ml MEM/5% FBS.
13. Allow cells to grow 2 to 3 days at 37°C, until dish is confluent.

Don't allow the cells to grow >3 days, even if the dish is not yet confluent as this will result in an overgrowth of nontransfected cells. The important thing is to allow the cells to divide several times.

14. Trypsinize the cells (step 4), and resuspend in 10 ml selection medium. As a control, use nontransfected cells.

Clone cells

15. Split the cells into 7 different dilutions in 10-cm dishes:

Cell suspension (ml)	0.025	0.05	0.1	0.2	0.4	0.8
Selection medium (ml)	9.975	9.95	9.9	9.8	9.6	9.2

Shake to distribute cells evenly.

Select stably transfected cells

16. Incubate cells at 37°C for 16 to 21 days (i.e., until the colonies are big enough to pick), adding fresh selection medium every 4 to 6 days.

Depending on the stability of the selection agent, it may not be necessary to change the medium.

Cells will start to die after 3 to 5 days and colonies should be seen within 10 to 14 days, but will not be big enough to pick until after 16 to 21 days.

17. Clones should generally be picked from the dishes with the highest dilution possible (see Support Protocol 1).

Dilutions in which 0.4 or 0.8 ml cells were used can usually serve as the pooled clones.

PICKING STABLY TRANSFECTED CLONES

The goal in this protocol is to identify and stably propagate epithelial cell clones that express the gene of interest.

Additional Materials (also see *Basic Protocol 1*)

~16-day-old 10-cm dishes containing diluted transfected and nontransfected cells in selection medium (see *Basic Protocol 1*)

Calcium- and magnesium-free DPBS (CMF-DPBS; *APPENDIX 2A*)

Medium-sized glass cloning ring, sterile (Bellco Glass)

0.5% (w/v) SDS lysis buffer

12% (w/v) slurry of CL-2B beads

12-well tissue culture plates

1. Examine ~16-day-old 10-cm dishes containing diluted transfected and nontransfected cells in selection medium, looking for single colonies. With a marker, draw a circle around the single colonies on the outside bottom of the dishes.

SUPPORT PROTOCOL 1

Protein Trafficking

15.5.5

If the original 10-cm dishes (i.e., the one which was split into seven different dilutions; see Basic Protocol 1, step 15) was ~30% confluent, single colonies are generally found in the dilutions in which 0.025 to 0.2 ml cells were used.

2. Wash dishes with 10 ml CMF-DPBS.
3. Use a Pasteur pipet attached to a vacuum device to aspirate a “dry” ring (i.e., devoid of liquid) around a single colony.

The “dry” ring will prevent trypsin from leaking under the cloning ring in step 4. Do this procedure for each colony individually, as the cells will dry out if more than one colony is harvested at a time.

4. Place a medium-sized sterile glass cloning ring around the colony. Add trypsin/EDTA solution until the cloning ring is filled. Repeat steps 3 and 4 for all colonies to be picked.

For epithelial cells (e.g., MDCK), use 0.25% trypsin and 0.2% EDTA.

At least 15 clones should be picked, though more is preferable (i.e., 50 if possible).

5. After all the cloning rings have been placed, incubate for ~20 min at room temperature in a sterile hood.
6. Remove the trypsin containing the loose cells and add to the well (i.e., 1 colony per well) of a 12-well tissue culture plate containing 2 ml selection medium. Repeat for all colonies.
7. Resuspend cells by pipetting up and down several times. Transfer 1 ml from each well to the corresponding well of a new 12-well plate (one plate will be for protein collection and the other for colony expansion).
8. Incubate to confluency (i.e., ~5 to 7 days) at 37°C.

It is generally not necessary to change the medium if the cells are harvested as soon as they are confluent.

The length of time to confluency depends on how many loose cells are transferred in good condition to the wells. This can vary significantly from well to well.

9. Add 500 μ l 0.5% SDS lysis buffer, boil the samples 5 min at 100°C. Vortex samples 10 min. Preclear the samples by adding 20 μ l of a 12% slurry of CL-2B beads. Mix several times. Microcentrifuge briefly at maximum speed. Collect supernatant and use an aliquot for immunoblotting (UNIT 6.2).
10. For the colonies that show expression, use the second identical well to expand the cells.

The authors recommend keeping the cells under selection while expanding the colony size.

SUPPORT PROTOCOL 2

CULTURE OF EPITHELIAL CELLS ON FILTERS

The goal in this protocol is to allow epithelial cells to become fully polarized. This is accomplished by culturing epithelial cells on porous filters that allow the cells to access medium both apically and basolaterally.

Additional Materials (also see Basic Protocol 1 and Support Protocol 1)

- 10-cm dish of confluent epithelial cells (Table 15.5.1)
- 12-mm Transwell filters and appropriate dishes
- IEC clinical centrifuge with 12 \times 15 rotor

Additional reagents and equipment for determining the tightness of epithelial monolayers (see Support Protocol 3)

1. Wash a 10-cm dish of confluent epithelial cells with 10 ml CMF-PBS.
2. Add 3 ml trypsin/EDTA solution (e.g., 0.25% trypsin/0.2% EDTA for MDCK cells) and incubate at 37°C for 15 min.
3. Tap dish to agitate cells. When all cells are detached, add 8 ml MEM/5% FBS.

This step is done in order to neutralize the trypsin.

4. Transfer to an appropriate tube and centrifuge cells 3 min at $300 \times g$ (e.g., 1000 rpm in an IEC 12 \times 15 rotor), room temperature. Discard the supernatant.
5. Resuspend cells in 10 ml MEM/5% FBS and mix thoroughly by pipetting up and down \sim 10 times. Add 0.5 ml of this solution to the apical chamber (i.e., inside) of a 12-mm Transwell filter. Then add an additional 0.4 ml MEM/5% FBS to the apical chamber for a total of 0.9 ml.

This corresponds to $\sim 2.5 \times 10^5$ cells/well.

6. Add 1 ml MEM/FBS to the basal chamber (i.e., area surrounding the Transwell filter).
7. Incubate at 37°C, replacing 1 ml apical and 1 ml basolateral medium every day for 3 to 5 days until cells form a tight monolayer (see Support Protocol 3).

IMPORTANT NOTE: *It is important to first remove the basal medium and then remove the apical medium, and to replace in the opposite order. If the basal medium is present and the apical medium is absent then the cells can be pushed off the filter.*

The cells are fully polarized and ready to examine after 4 to 7 days of growth on the filter.

DETERMINING THE LEAKINESS OF A MONOLAYER OF CELLS GROWN ON A FILTER

**SUPPORT
PROTOCOL 3**

When grown on a Transwell or similar filter support, epithelial cells should form a confluent monolayer; however, with most types of filter materials, it is difficult or impossible to clearly visualize the cells themselves. Hence, it is not easy to determine if they have formed a confluent tight monolayer. Several methods have been devised to measure how intact the monolayer is. Classically, electrical resistance across the monolayer is measured; however, type II MDCK cells typically have a rather low electrical resistance, on the order of 100 ohms/cm², and so electrical resistance is rather insensitive to defects in the monolayer. Thus, the authors devised an extremely simple method to monitor the integrity of the monolayer. Although it is qualitative, it has been found to be a very reliable predictor of the integrity of the monolayer and the success of the experiment in the authors' laboratory.

Additional Materials (also see Support Protocol 2)

Pasteur pipet connected to a vacuum system

1. Culture cells on filters, as described (see Support Protocol 2).
2. The day before the experiment (i.e., typically after 3 to 6 days of growth on the filter), add normal cell culture medium to the apical well to fill it to overflowing.

This support protocol is usually performed as part of the daily replacement of apical and basolateral media.

**Protein
Trafficking**

15.5.7

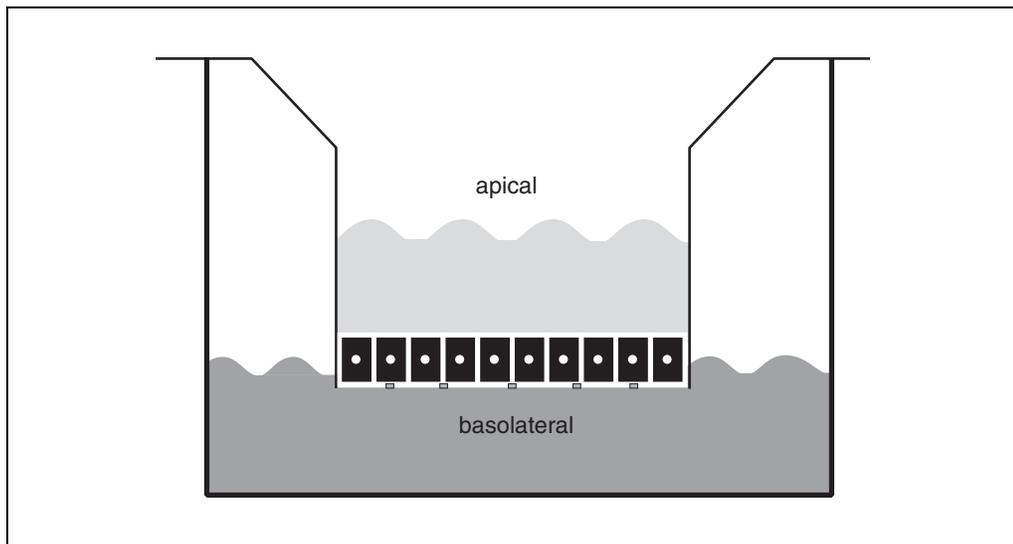


Figure 15.5.2 Schematic diagram of polarized epithelial cells growing in a Transwell. Cells are black rectangles with small white circles indicating nuclei. The apical and basolateral medium are gray. The fluid level of the apical medium is higher than the basolateral medium, as occurs during Support Protocol 3.

3. Use a sterile Pasteur pipet connected to a vacuum system to aspirate most of the medium from the basolateral chamber, leaving just enough medium to contact the entire basal surface of the filter.
4. Incubate the Transwell filter, still in the multiwell plate, at 37°C overnight (i.e., typically 8 to 16 hr).
5. Determine the tightness of the monolayer by assessing the fluid level of the apical chamber.

If the cell monolayer is intact, the fluid level inside the apical chamber will not go down overnight—i.e., the apical chamber will remain filled to the brim (Figure 15.5.2). If, on the other hand, the fluid level leaks, the monolayer is not tight and the cells should be discarded.

BASIC PROTOCOL 2

PULSE-CHASE EXPERIMENTS IN POLARIZED EPITHELIAL CELLS

The goal of this protocol is to study the trafficking of apical and basolateral secreted proteins in transfected polarized mammalian epithelial cells. This is accomplished utilizing a radioactive amino acid as a tracer molecule (Lipshutz et al., 2000).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1D*).

Materials

- 4- to 7-day-old epithelial cell cultures growing on 12-mm Transwell filters (see Support Protocol 2)
- Dulbecco's phosphate-buffered saline (DPBS; *APPENDIX 2A*)
- Starvation medium: MEM medium (*APPENDIX 2B*) lacking the radioactive tracer amino acid
- Radioactive amino acid (e.g., 1175 Ci/mmol [³⁵S]methionine)
- MEM medium
- 0.5% (w/v) SDS lysis buffer (see recipe)

Parafilm

Humid box: plastic box with 2 charcoal bags and a piece of wet Whatman filter paper

12-well Transwell tissue culture plate (Table 15.5.2)

Phosphorimager

Scalpel

Additional reagents and equipment for gel electrophoresis (Chapter 6)

Label cells

1. Obtain 4- to 7-day-old epithelial cell cultures growing on 12-mm Transwell filters.
2. Wash cells three times with 37°C Dulbecco's phosphate-buffered saline (DPBS).
3. Add 500 µl starvation medium apically and basolaterally. Incubate at 37°C, 5% CO₂ for 15 min.

For example, if [³⁵S]methionine is to be used as the radioactive amino acid, then the starvation medium is medium lacking methionine.

4. Place 25-µl spots containing radioactive amino acid (e.g., 4 µl of 1175 Ci/mmol [³⁵S]methionine and 21 µl starvation medium) on a Parafilm sheet inside a humid box.

The humid box is a plastic box to which two bags containing charcoal are attached to the top inside surface of the box in order to trap the aerosolized [³⁵S]methionine. A piece of Whatman filter paper is placed on the bottom of the plastic box and soaked with water in order to prevent the cells from drying out. Finally, a piece of Parafilm is placed on top of the damp Whatman filter paper.

5. Remove the basal medium while leaving the apical medium in place. Carefully, set one filter on top of each spot for basolateral exposure of cells to the radioactive amino acid.
6. Incubate (i.e., allow labeling to occur) for 15 to 20 min at 37°C. Analyze labeled protein.

If one is interested in analyzing the synthesis of secretory proteins, carry out steps 11 and 12 at this point.

7. Prepare a new 12-well plate containing fresh MEM medium. Transfer transwells to the new plate and wash cells three times apically and basolaterally with 37°C DPBS.
8. Add 300 µl MEM medium apically and 500 µl basolaterally. Incubate for the desired amount of time (e.g., 1 hr) at 37°C.

In the case of incubation in an environment without CO₂, 20 mM HEPES is added to the medium as a buffer.

9. Collect apical medium. Transfer transwells to a new 12-well plate, and collect the basolateral medium.
10. Analyze aliquots of the secreted apical and basolateral medium on an appropriate gel by electrophoresis (Chapter 6), and determine amounts of radioactivity using a phosphorimager.

Immunoprecipitation (UNIT 7.2) of specific secretory proteins can be done before gel electrophoresis.

11. Cut out the filters with a scalpel and collect the cells. Lyse cells by placing the filters in 500 µl of 0.5% SDS-lysis buffer.
12. Determine the amounts of intracellular protein radioactivity by analyzing aliquots on an appropriate gel (UNIT 6.1) followed by phosphorimaging (UNIT 6.3).

Again, immunoprecipitation (UNIT 7.2) can be performed before gel electrophoresis.

**BIOTINYLATION OF NEWLY SYNTHESIZED EPITHELIAL CELL
SURFACE PROTEINS**

The goal in this protocol is to study the trafficking of newly synthesized apical and basolateral epithelial cell surface proteins using a combination of a radioactive amino acid tracer molecule and sulfo-NHS-biotin, an exogenous cell surface marker. Sulfo-NHS-biotin is used to label all cell surface proteins. Individual surface proteins are then isolated by immunoprecipitation (*UNIT 7.2*). This method is adapted from one developed by E. Rodriguez-Boulan and colleagues (LeBivic et al., 1989).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1D*).

Materials

- Radioactively labeled amino acid
- 4-day-old epithelial cultures growing on 12-mm Transwell filters (see Support Protocol 2)
- Hanks' balanced salt solution (HBSS; *APPENDIX 2A*), 4°C
- 20 mg/ml sulfo-NHS-biotin (Pierce) in anhydrous DMSO: prepare just before use
- 10 mM Tris buffered saline, pH 7.4 (TBS; *APPENDIX 2A*)
- 0.5% (v/v) SDS lysis buffer (see recipe)
- 2.5% (v/v) Triton dilution buffer (see recipe)
- Protein A–Sepharose beads without and with rabbit antimouse and the monoclonal antibody of choice, or rabbit polyclonal antibodies
- Streptavidin beads (e.g., Pierce)
- Mixed micelle wash buffer (see recipe)
- Final wash buffer (see recipe)
- 5% (w/v) SDS
- 2× loading buffer (see recipe)
- Platform rocker
- Orbital shaker (e.g., Bellco)
- Phosphorimager
- Additional equipment and reagents for radioactively labeling epithelial cultures (see Basic Protocol 2, steps 1 to 6) and denaturing (SDS) gel electrophoresis (*UNIT 6.1*)

Label cells

1. Add radioactively labeled amino acid to 4-day-old epithelial cultures growing on 12-mm Transwell filters as described for pulse-chase experiments (see Basic Protocol 2, steps 1 to 6), labeling for 15 min.
2. Remove medium and wash cells three times with cold HBSS.
3. Just before using prepare 20 mg/ml sulfo-NHS-biotin in anhydrous DMSO and then dilute it 1:100 with HBSS to a final concentration of 200 µg/ml. Add either 400 µl apically or 800 µl basolaterally to cells.

Sulfo-NHS-biotin binds tightly to primary amines on cell surface proteins via the NHS ester.

4. Incubate 30 min at 4°C on a platform rocker.
5. Remove biotin solution and wash five times with 10 mM Tris buffered saline.

Prepare lysate

6. Cut out filters and place in 500 μ l of 0.5% SDS lysis buffer in a microcentrifuge tube. Boil 5 min, vortex 15 min, and microcentrifuge 5 min at 13,000 rpm. Transfer supernatant to new microcentrifuge tube.

The filters are most easily cut out of the plastic ring with a surgical scalpel (i.e., Bard-Parker no. 11 blade).

7. Add an equal volume of 2.5% Triton dilution buffer to give final concentrations of 0.25% SDS lysis buffer/1.25% Triton dilution buffer.

Immunoprecipitate target protein

8. Preclear the lysate by incubating with 4 μ l protein A–Sepharose beads free of antibody on an orbital shaker for 30 min at 4°C. Microcentrifuge 20 min at 13,000 rpm, 4°C. Transfer supernatant to a new microcentrifuge tube.

9. Add protein A–Sepharose precoated with rabbit antimouse and the monoclonal antibody of choice, or rabbit polyclonal antibodies, to 10 mg/ml. Incubate 12 hr at 4°C.

10. Block streptavidin beads by incubating in equal amounts of 0.5% SDS and 2.5% Triton dilution buffer for 1 to 12 hr.

This mixture of SDS and Triton buffers is also called lysis buffer.

11. Wash protein A–Sepharose-antibody complexes three times with mixed micelle wash buffer and one time with final wash buffer by diluting with buffer and then microcentrifuging.

12. To recover immunoprecipitated biotinylated antigens, boil the beads in 40 μ l of 5% (w/v) SDS for 5 min.

Boiling in 5% (w/v) SDS will separate the biotinylated antigens from the antibody-protein A complex. The biotin, however, will remain attached to the antigen (surface protein of interest).

13. Dilute with 460 μ l of 0.5% SDS/2.5% Triton dilution buffer and microcentrifuge 1 min at 13,000 rpm. Transfer the supernatant to a fresh microcentrifuge tube.

14. Add 13 μ l streptavidin beads (i.e., 13 μ l per filter) and immunoprecipitate overnight on an orbital shaker at 4°C.

15. Wash streptavidin-bead-antibody complex three times with mixed micelle wash buffer and one time with final wash buffer as described above (step 11).

16. Add 8 μ l of 2 \times loading buffer, boil 5 min, and analyze by denaturing SDS gel electrophoresis (UNIT 6.1).

The DTT (dithiothreitol; present in the loading buffer) is a reducing agent that will disrupt the S-S bond in the sulfo-NHS-biotin allowing for recovery of the protein.

17. Determine phosphorimager counts according to the manufacturer's instructions.

This procedure identifies newly synthesized proteins of interest that reach the apical or basolateral surface, depending on which was exposed to biotin.

**INDIRECT IMMUNOFLUORESCENCE OF PROTEINS IN POLARIZED
EPITHELIAL CELLS**

The goal of this protocol is to identify the location of proteins in polarized epithelial cells. This is accomplished using a primary antibody directed against the protein of interest and a secondary antibody containing an immunofluorescent marker directed against the species in which the primary antibody was made.

Materials

- 5- to 7-day-old cells growing on 12-mm Transwell filters (see Support Protocol 2)
- DPBS (APPENDIX 2A), ice cold
- 16% or 40% (v/v) paraformaldehyde
- Quenching solution, fresh (see recipe)
- Permeabilizing solution, fresh (see recipe)
- Primary antibody
- Secondary antibody coupled to fluorophore
- DPBS/0.1% (v/v) Triton: add 250 μ l of 20% (v/v) Triton X-100 to 49.75 ml DPBS (APPENDIX 2A), store up to 6 months at 4°C
- Antifade mounting solution
- Metal board
- Orbital shaker (e.g., Bellco)
- Humid box: plastic box with a piece of damp Whatman filter paper
- Aluminum foil
- Scalpel
- Inverted fluorescent microscope with appropriate glass slide and cover slip

1. Cool 5- to 7-day-old cells growing on 12-mm Transwell filters to 4°C by placing on a metal board on ice.
2. Prepare 4% (v/v) paraformaldehyde in ice-cold DPBS from either a 16% or 40% paraformaldehyde stock.
3. Wash Transwell filters twice with 1 ml ice-cold DPBS, both apically and basally.
4. Fix cells with 4% paraformaldehyde in DPBS by adding 0.5 ml to the basal and apical surfaces. Allow cells to fix by incubating 20 min on a slowly rotating orbital shaker (e.g., setting 3 on a Bellco Glass orbital shaker) in a container on ice.
5. Wash Transwell filters three times with 1 ml DPBS to remove paraformaldehyde.
6. Add 1 ml fresh quenching solution both apically and basally, and rotate slowly for 10 min at room temperature.

The glycine in the quenching solution will bind to the remaining paraformaldehyde.

7. Wash once with 1 ml DPBS.
8. Add 1 ml fresh permeabilizing solution both apically and basally. Incubate 15 min in a 37°C water bath.
9. Dilute primary antibody in permeabilizing solution as appropriate (i.e., 1:100) in 150 μ l. Place 40 μ l as a drop on a square of Parafilm inside a humid box and place the Transwell filter on the drop (i.e., add basally) and add 110 μ l to the apical surface of the Transwell filter.

The concentration of primary antibody depends on the properties of each individual antibody. A rough rule is that this technique will require five to ten times the amount required for immunoblotting.

10. Incubate 1 hr at 37°C on an orbital shaker.
11. Transfer Transwell filter back to the plate. Wash four times with 1 ml permeabilizing solution, incubating on an orbital shaker for 5 min each time.
12. Dilute secondary antibody coupled to fluorophore 1:100 (generally) with permeabilizing solution. Add to the filter as described for the primary antibody solution (see step 9). Wrap the humid box in aluminum foil and incubate on a platform rocker 30 min at 37°C.

The samples in the following steps should be protected from light as much as possible.

13. Wash four times with 1 ml permeabilizing solution for 5 min each on an orbital shaker.
14. Rinse once with 1 ml DPBS.
15. Wash twice for 3 min each with 1 ml DPBS/0.1% Triton.
16. Rinse once with 1 ml DPBS.
17. Post-fix with 4% paraformaldehyde in DPBS for 15 min at room temperature (see step 4).
18. Rinse once with 1 ml DPBS.
19. Cut out filter with a scalpel and place on slide containing a drop of antifade mounting solution. Add a coverslip and dry overnight at room temperature in the dark.

Mount the filter cell-side-up so that the coverslip can be placed directly on an inverted fluorescent microscope.

20. Analyze on an inverted fluorescent or confocal microscope to determine the location of the protein of interest, or store in the dark at -20°C to prevent fading of the fluorophore.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Final wash buffer

- 37.5 ml 4 M NaCl (150 mM final; APPENDIX 2A)
- 20 ml 1 M triethanolamine chloride (TEA·Cl), pH 8.6 (20 mM final)
- 10 ml 0.5 M EDTA, pH 8.0 (5 mM final; APPENDIX 2A)
- 1.0 ml Trasylol (0.001% v/v final)
- 2.0 ml 10% NaN₃ (0.02% w/v final)
- Adjust volume to 1 liter with H₂O
- Store up to 6 months at 4°C

Geneticin (G418)

Prepare G418 as a 100 mg/ml stock in 200 mM HEPES (sodium salt), pH 7.9. Filter sterilize and store at -20°C or -80°C. For every new batch (new lot number of G418 powder), determine the effective G418 concentration which kills cells by performing killing curves (i.e., expose the cells to different G418 concentrations and determine the minimal drug concentration needed to kill the entire cell population in the dish in a 14-day test).

For more information concerning geneticin, see APPENDIX 1B.

HEPES buffered saline (HeBS), pH 7.05

For 500 ml HeBS, add 2.5 g HEPES (final concentration 20 mM) to DPBS and adjust the pH to 7.05 with 5 M NaOH (*APPENDIX 2A*).

Loading buffer, 2×

0.3 ml 1 M Tris·Cl, pH 6.8 (15 mM; *APPENDIX 2A*)
2.0 ml 10% SDS (1% w/v; *APPENDIX 2A*)
0.05 ml 100 mM EDTA, pH 7.0 (25 mM; *APPENDIX 2A*)
0.2 g DTT (65 mM)
0.5 ml 1% bromophenol blue dye (0.025% w/v)
1.2 ml 100% glycerol or sucrose (6% w/v)
5.75 ml H₂O
10 ml total volume
Store up to 6 months at 4°C

Concentrations in parentheses are for a 1× solution.

Mixed micelle wash buffer

37.5 ml 4 M NaCl (150 mM final; *APPENDIX 2A*)
20 ml 1 M TEA·Cl, pH 8.6 (20 mM final)
10 ml 0.5 M EDTA, pH 8.0 (5 mM final; *APPENDIX 2A*)
1.0 ml Trasylol (0.001% v/v final)
2.0 ml 10% NaN₃ (0.02% w/v final)
60 ml 65% (w/w), 85% (w/v), or 39 g sucrose
942 ml H₂O
50 ml 20% Triton X-100 (1% v/v final)
20 ml 10% SDS (0.2% v/v final; *APPENDIX 2A*)
Total volume 1 liter
Store up to 6 months at 4°C

Permeabilizing solution

3.5 g of cold-water fish skin gelatin (0.7% w/v final; Sigma-Aldrich)
0.8 ml 10% (w/v) saponin in H₂O (0.016% final)
Bring to 500 ml with PBS (*APPENDIX 2A*)
Prepare fresh on the day of the experiment; do not store

This is a sufficient amount of solution for the preparation of twelve 12-mm Transwell filters.

Quenching solution

1.5 ml 1 M NH₄Cl (75 mM final)
0.4 ml 1 M glycine (20 mM final)
18.1 ml PBS (*APPENDIX 2A*)
20 ml total volume
Prepare fresh on the day of the experiment; do not store

SDS lysis buffer, 0.5% (w/v)

12.5 ml 4 M NaCl (100 mM final; *APPENDIX 2A*)
25 ml 1 M TEA·Cl, pH 8.1 (50 mM final; *APPENDIX 2A*)
5 ml 0.5 M EDTA, pH 8.0 (5 mM final; *APPENDIX 2A*)
1.0 ml Trasylol (0.2% v/v final)
1.0 ml 10% NaN₃ (0.02% w/v final)
25 ml 10% SDS (0.5% w/v final; *APPENDIX 2A*)
475 ml H₂O
500 ml total volume
Store up to 6 months at room temperature

Triton dilution buffer, 2.5% (v/v)

12.5 ml 4 M NaCl (100 mM final; *APPENDIX 2A*)
50 ml 1 M TEA·Cl, pH 8.6 (100 mM final; *APPENDIX 2A*)
5 ml 0.5 M EDTA, pH 8.0 (5 mM final; *APPENDIX 2A*)
5.0 ml Trasylol (1% v/v final)
1.0 ml 10% NaN₃ (0.02% w/v final)
Add H₂O to 436 ml
Add 62.5 ml of 20% Triton X-100 (2.5% v/v final)
Total volume 500 ml
Store up to 6 months at 4°C

COMMENTARY

Background Information

Growing cells on porous (i.e., Transwell) filters has two major advantages. First, polarized epithelial cells usually obtain most of their nutrients from their basolateral surface, which in vivo is exposed to the blood. When grown on a typical impermeable plastic culture dish, the cells cannot obtain nutrients from the overlying culture medium in contact with the apical surface because the tight junctions prevent nutrients from reaching the basolateral surface. This forces the cells to become partially depolarized in order to survive. In contrast, when grown on permeable filters, the cells can obtain nutrients from the culture medium that underlies the filter, which is in contact with the basolateral surface via the pores in the filter.

Second, the cells form a continuous monolayer covering the filter, and the tight junctions between cells largely prevent diffusion between them. Thus, the culture medium in contact with the apical surface of the cells cannot exchange with medium in contact with the basolateral surface. In the Transwell or similar device, the filter is mounted at the end of a short plastic tube, which is suspended in the well of a multiwell tissue culture plate. This effectively provides two chambers holding two potentially different pools of culture medium: an apical chamber overlying the apical surface of the cells on top of the monolayer, and a basolateral chamber underlying the filter and in contact with the basolateral surface of the cells.

Many early studies on biosynthetic protein sorting utilized viral membrane proteins. Typically in these studies, cells were infected with a virus and the transport of the envelope glycoproteins to the surface was studied. This provided a large amount of one or two viral glycoproteins, which could easily be followed. The most widely used examples were the hemagglutinin (HA) protein of influenza, which goes apical and the G protein of vesicular stomatitis

virus (VSG-G) which goes basolateral (Brewer and Roth, 1991); however, infection with these and other viruses has significant toxic repercussions to the cell. Thus, expression of these proteins from the actual virus has largely been supplanted by expression from transfected vectors (e.g., Brewer and Roth, 1991). VSV-G has the added advantage that a temperature sensitive form (i.e., ts-045) accumulates in the rough endoplasmic reticulum (RER) at 40°C, but is transported through the biosynthetic pathway at ≤30°C. Additionally, green fluorescent protein (GFP)–fusions with this and other proteins are being used to follow transport in live cells.

This unit focuses on biosynthetic pathways leading to the cell surface. One can also study endocytosis from either the apical or basolateral surface. The approaches used are similar to those presented in *UNIT 15.4*. Additionally, one can study the fate of material endocytosed from either surface (*UNIT 15.3*). This can include recycling to the original surface from which the material was endocytosed, transcytosis to the opposite surface, or degradation. One difficulty is that there are relatively few endogenous receptors in Madin-Darby canine kidney (MDCK) cells that can be conveniently utilized to follow receptor-mediated endocytosis. When confluent and well polarized, MDCK cells express relatively few receptors for many nutrients (e.g., transferrin or low-density lipoprotein). The endogenous transferrin receptors work very poorly with human transferrin, although they do work considerably better with canine transferrin, which is available commercially from Sigma-Aldrich. Most endogenous endocytotic receptors are located on the basolateral surface, so finding a receptor system for studying apical endocytosis is more difficult. Most studies on endocytosis and post-endocytic sorting in epithelial cells have utilized transfected exogenous receptors, expressed at a high level. For instance, the authors' labora-

tory has primarily utilized the transfected polymeric immunoglobulin receptor.

Critical Parameters and Troubleshooting

Clonal variation of MDCK cells

The original MDCK cell line, available from the American Type Culture collection (<http://www.atcc.org>; ATCC #CCL-34), is quite heterogeneous. Several investigators have isolated individual clonal lines from this heterogeneous population. Clones of MDCK cells can be broadly divided into two categories, type I and type II. Generally, type I have a high electrical resistance (i.e., >1000 ohms/cm²), whereas type II have a low electrical resistance (~100 ohms/cm²). In reality, MDCK clones can be thought of as forming a nearly continuous spectrum in terms of their electrical resistance as well as other properties. Almost all of the work done by the authors has utilized a nominally type II clonal line originally isolated by Daniel Louvard at the European Molecular Biology Laboratory (EMBL) in Heidelberg, which is sometimes referred to as Heidelberg MDCK (Louvard, 1980).

When transfected cDNA is expressed in MDCK cells, and a stable expressing clone is isolated, the cell line is effectively subcloned. The authors have found that each subclone of MDCK can have unique and quite distinct properties; therefore, it is important to characterize each one. For example, an easy test for the ability of a subclone to form a tight monolayer is described (see Support Protocol 3). Moreover, the authors have found that the polarized trafficking of any particular protein can vary considerably among subclones. For this reason, when studying the trafficking of a newly transfected protein, the authors generally examine at least three independent subclones (Aroeti et al., 1993). If clones show different trafficking properties it is likely due to clonal variation and not a direct result of the exogenous protein being expressed. Only if three separate clones give the same result can one assume that the effect is due to the truncated gene/protein.

In addition to MDCK cells, several other epithelial cell lines have often been used for studies of polarized membrane traffic, including the major ones listed in Table 15.5.1.

Instability and heterogeneity of expression of exogenous proteins

In many cases, expression of an exogenous protein in MDCK cells appears to be detrimen-

tal to the long-term growth of the cell. In severe cases, the protein can be so toxic that it is difficult or impossible to recover clones that express a detectable level of the exogenous protein. In less severe cases, expression of the protein is lost during successive passages of the clonal cell line (Barth et al., 1997). Presumably, this is due to a growth advantage of spontaneously arising cell variants, which do not express the protein. Moreover, when the expression of the exogenous protein is observed in a population of MDCK cells by immunofluorescence microscopy, the level of expression can appear to vary from cell to cell. One strategy to deal with these problems is to freeze numerous vials of early passage cells, which are then thawed at frequent intervals and used for only a limited period before the cells are discarded. In some cases, the newly thawed cells are used for only one experiment. This helps to ensure that the cells used in every experiment are consistent.

Another approach is to repeatedly subclone the cells. In principle this should yield a clone with uniform expression of the exogenous protein; however, it is often observed that despite repeated subcloning, expression of exogenous proteins is not uniform. This suggests that variation in expression is an inherent feature of MDCK cells.

Type of filter material and support unit

Most of the authors' experience is with polycarbonate filters containing 0.4- μ m nominal size pores, as part of Transwell units from Corning. Filters are available in a number of materials and support units, from several manufacturers (Table 15.5.2). Some filters are available in pore sizes >1 μ m; however, these are not recommended for growth of polarized epithelial monolayers as the cells can migrate into and through the pores. Indeed, filters with pores of 3 μ m or larger are used for cell migration studies. In general, filters that have more pores and/or larger pores will have optical transmission properties which are more poor, though it is not necessary to be able to visualize live cells on the filter for most experiments.

Anticipated Results

Ideally, one will find that the protein of interest is delivered directly to one surface of the cell or the other and resides at that surface. In this case, there will be a clear-cut agreement between the steady-state localization determined by immunofluorescence microscopy, and the site of delivery, as measured by the pulse-chase and biotinylation protocols. An ex-

ample of results from immunofluorescence microscopy is presented in Figure 15.5.3; however, it should be kept in mind that no protein is ever delivered 100% to only one surface. Even in the best cases, typically only ~90% of a protein is delivered to one surface, with the balance reaching the other surface. Furthermore, proteins can differ in the stability at the surface. Some, such as Na-K-ATPase, can reside at the basolateral surface for hours. Others, such as the polymeric immunoglobulin receptor, are delivered to the basolateral surface, but are then endocytosed and transcytosed to the apical surface within minutes.

Time Considerations

It takes ~4 hr to carry out a pulse-chase labeling experiment to assess trafficking. Transfection of the cells requires ~2 hr plus 2 to 3 weeks to select clones. Picking clones takes ~2 hr. It takes 7 to 14 days to expand the clones. Plating cells on Transwell filters requires ~1 hr, but 4 to 7 days are required to grow confluent monolayers of polarized cells. It takes one complete day to assess a culture for leakiness. Biotinylation of newly synthesized proteins and their analysis requires ~8 hr. Immunofluorescence experiments require ~6 hr to complete.

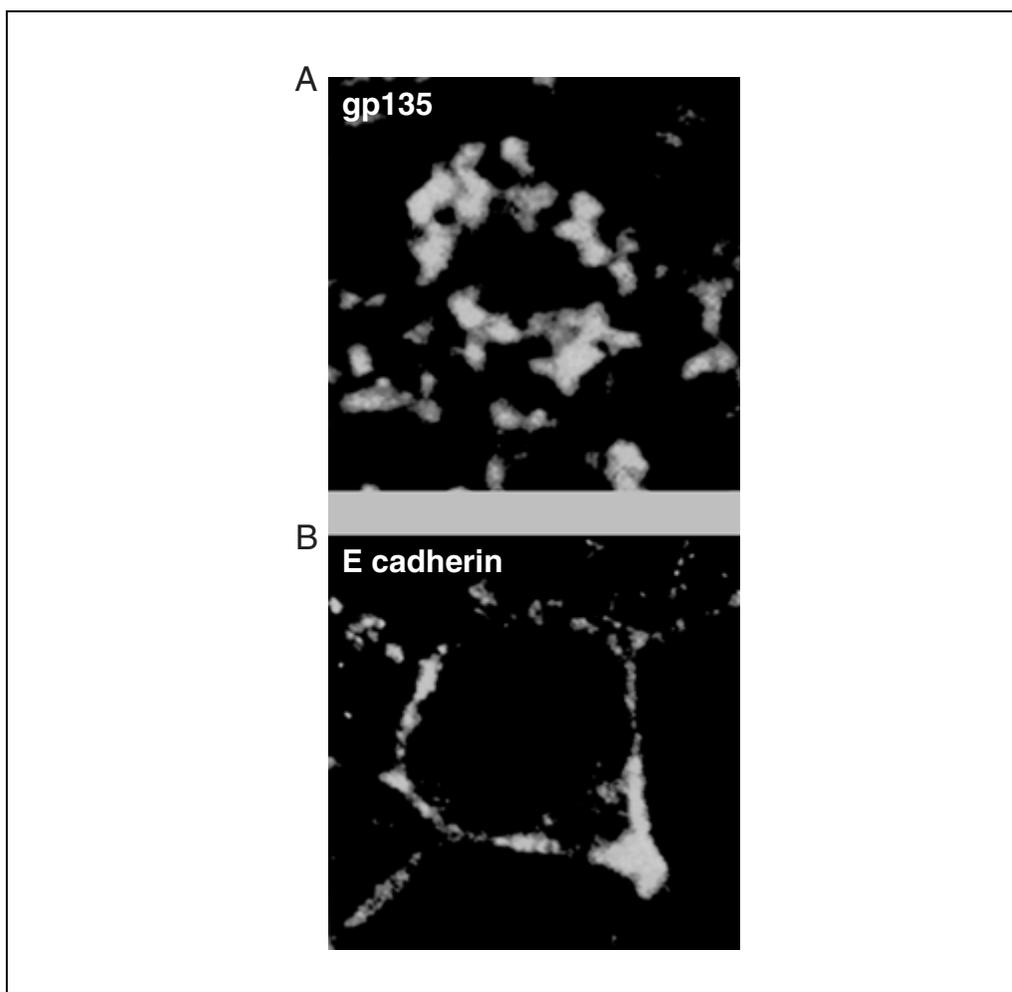


Figure 15.5.3 Immunofluorescence micrographs of polarized epithelial cells grown on a Transwell. Samples were prepared as in Basic Protocol 4, and then viewed with a Bio-Rad 1024 confocal microscope. **(A)** Staining using a monoclonal antibody directed against an apical plasma membrane protein, gp135 (kindly provided by George Ojakian, SUNY Downstate Medical Center, Brooklyn, NY), as a primary antibody. The micrograph was taken at the level of the apical surface. Staining appears irregular, due to microvilli and other irregularities of the apical surface. It is not easy in this view to discern the outline of individual cells. **(B)** A sample prepared using a primary antibody against a basolateral surface marker, E-cadherin (Chapter 9). The micrograph was taken at a level below the apical surface, approximately midway between the apical and basal poles of the cell. The E-cadherin largely outlines the lateral borders of the cell, though some intracellular staining is also seen.

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Analysis of Protein Folding and Oxidation in the Endoplasmic Reticulum

UNIT 15.6

Proteins that traverse the eukaryotic secretory pathway are co-translationally inserted through the endoplasmic reticulum (ER) membrane into the lumen. Upon emergence of the protein into the lumen, a series of important maturation steps begin co-translationally and finish post-translationally before the protein exits from the ER. Among these processes are protein folding and the formation of disulfide bonds, which is supported by the oxidizing ER environment. This unit describes protocols for analyzing the folding and oxidation of proteins in the ER.

Protein oxidation can be analyzed either co-translationally (Basic Protocol 1) or post-translationally (Alternate Protocol 1) by monitoring the disappearance of free cysteine thiols or appearance of faster-migrating oxidative forms on nonreducing SDS-polyacrylamide gels. Protein folding can be followed by analyzing a protein's increased resistance to proteolysis during the course of maturation (Basic Protocol 2) or by immunoprecipitation with conformation-specific antibodies (Basic Protocol 4). The transient association of folding or oxidative intermediates with chaperone proteins can also be studied using a co-immunoprecipitation procedure (Basic Protocol 3 and Alternate Protocol 2). These procedures can be applied to study protein maturation in the ER using a variety of experimental systems including: a cell-free system composed of rough ER-derived microsomes and an *in vitro* translation system (Basic Protocol 1), adherent cell monolayers (Basic Protocol 5), cells in suspension (Alternate Protocol 3), and an *in vitro* translation system that targets proteins to intact ER from semipermeabilized (SP) cells (Support Protocol). To choose the most effective system(s) for a given study, the inherent advantages and disadvantages of each system must be weighed.

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter, instruments, and surroundings. Carry out the experiments and the disposal of the wastes in the appropriately designated area, following the guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*). In addition, some extra precautions should be taken when using ³⁵S-labeled amino acids (*UNIT 7.1*).

ANALYSIS OF DISULFIDE BOND FORMATION IN ROUGH ENDOPLASMIC RETICULUM-DERIVED MICROSOMES BY ALKYLATION AND NONREDUCING SDS-PAGE

**BASIC
PROTOCOL 1**

Disulfide bond formation can be followed by translating proteins under oxidizing conditions in the presence of rough ER-derived microsomes. The polypeptide chain is co-translationally translocated across the ER membrane into the lumen where disulfide bond formation occurs. Because free cysteine thiols form disulfide bonds with the aid of an oxidizing agent, the disappearance of these free thiols can be used to monitor disulfide bond formation. The modification of free thiols with an alkylating agent can be used to trap oxidative intermediates and cause a mobility shift proportional to the number of free thiols modified (Fig. 15.6.1). Common alkylating reagents for this technique are *N*-ethylmaleimide (NEM; 125 Da) and 4-acetamido-4'-maleimidylstilbene-2, 2'-disulfonic acid disodium salt (AMS; 536 Da).

As proteins fold and form disulfide bonds, they become more compact. This generally causes the protein to migrate faster by nonreducing SDS-PAGE compared with its fully reduced form (Fig. 15.6.1). Therefore, a mobility comparison of the reduced and oxidized

**Protein
Trafficking**

15.6.1

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Supplement 14

protein on nonreducing SDS-PAGE can be used to monitor the formation of disulfide bonds. These studies can also be used in some cases to identify oxidative intermediates. In this protocol, a time course for protein oxidation is performed and the immunoprecipitates or lysates are analyzed by nonreducing SDS-PAGE to detect changes in mobility over time due to disulfide bond formation.

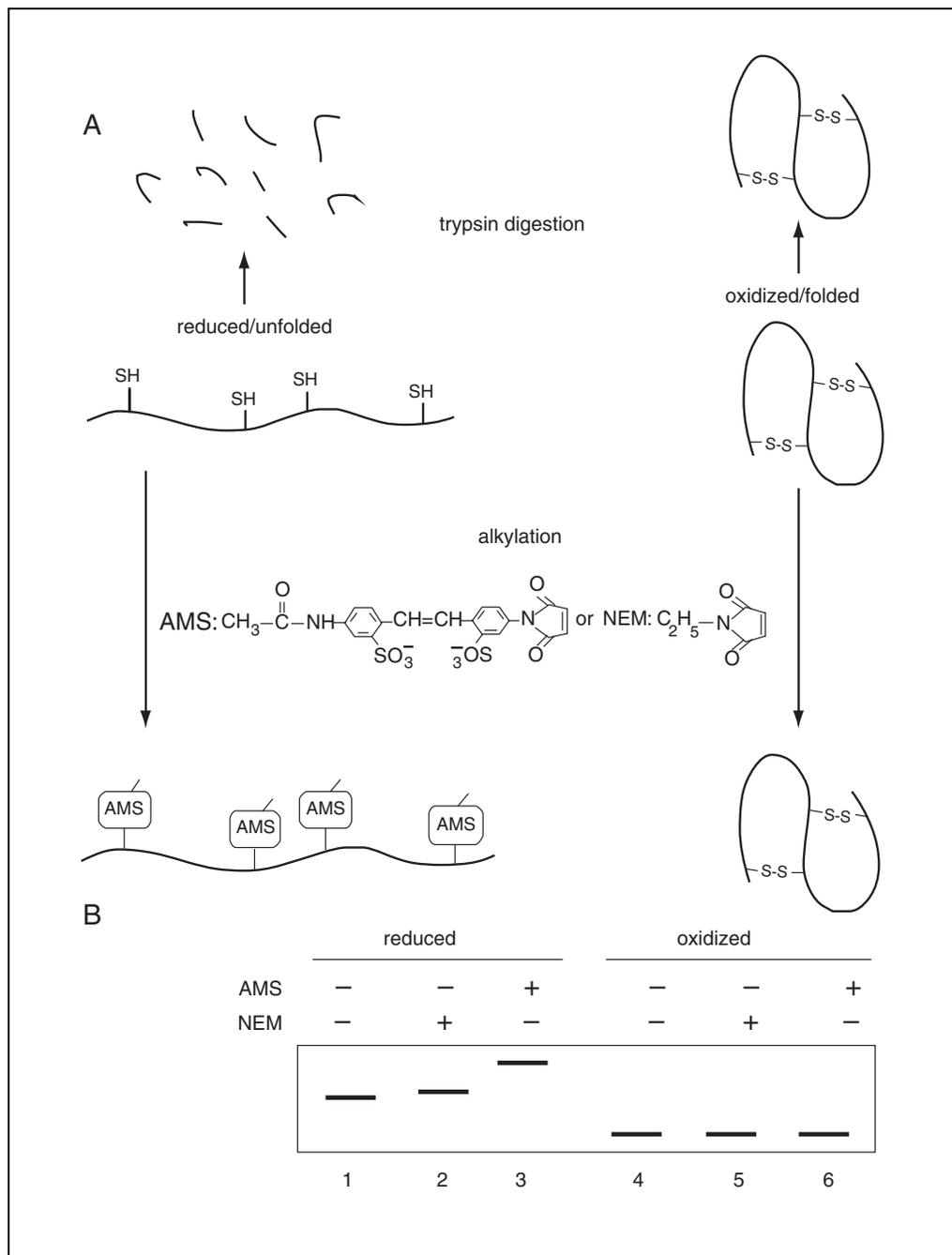


Figure 15.6.1 The effects of disulfide bond formation in proteins. **(A)** Schematic demonstration of the sensitivity of a reduced and oxidized polypeptide chain to protease digestion (top) and alkylation (bottom). **(B)** Resulting mobility changes visualized by SDS-PAGE. When a reduced or unfolded polypeptide containing free thiols is alkylated with 4-acetamido-4'-maleimidylstilbene-2, 2'-disulfonic acid disodium salt (AMS), a bulk alkylating agent, or *N*-ethylmaleimide (NEM), a shift in protein mobility on an SDS-polyacrylamide gel is observed (compare lanes 2 and 3 to lane 1). The oxidized (disulfide bonded) or folded protein is not sensitive to alkylation with either AMS or NEM as fewer or no free cysteines are available (lanes 4 to 6). The reduced or unfolded protein is more accessible to proteolytic digestion with trypsin than the oxidized or folded protein.

This protocol uses ^{35}S -labeled proteins generated by translating an mRNA (*UNIT 11.2*) for the protein of interest with a rabbit reticulocyte lysate translation system along with [^{35}S]methionine and [^{35}S]cysteine in the presence of rough ER–derived microsomes. This system is advantageous for studying protein maturation in the ER as it provides optimum flexibility in the control and manipulation of experimental conditions. One manipulation is the addition of the oxidizing agents oxidized glutathione (GSSG) or flavin adenine dinucleotide (FAD) during translation. Both FAD and GSSG provide conditions that mimic the normal physiological environment under which proteins acquire their native disulfide bonds in the cell. Therefore, these reagents, when present during translation, permit disulfide bond formation to commence co-translationally or vectorially from the N terminus to C terminus of the protein and to be completed post-translationally.

Materials

In vitro translation reagents, including:

- 1 equivalent/ μl nuclease-treated canine pancreatic microsomes (*UNIT 11.4*) or 1×10^5 cells/ μl semipermeabilized (SP) cells (see Support Protocol)
- Rabbit reticulocyte lysate treated with ATP-regenerating system and nucleases (e.g., Promega)
- 1 mM amino acid mixture lacking methionine and cysteine
- 11 mCi/ml [^{35}S]methionine and [^{35}S]cysteine (1175 Ci/mmol; e.g., PE Biosystems)
- 40 U/ μl RNase inhibitor (e.g., RNasin; Promega)
- 100 mM GSSG (see recipe) or 2.5 mM FAD (see recipe)
- 100 mM dithiothreitol (DTT; e.g., Promega or *APPENDIX 2A*)
- 2.5 M KCl
- 1 $\mu\text{g}/\mu\text{l}$ mRNA for the protein of interest (e.g., *UNIT 11.2*)
- Nuclease-free H_2O
- 50 mM cycloheximide (see recipe)
- 120 mM *N*-ethylmaleimide (NEM) (see recipe)
- 20 mM AMS, prepared fresh (see recipe)
- 0.5% and 2% (w/v)
 - 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (CHAPS) in HeBS (CHAPS/HeBS, see recipe), ice cold
- 2 \times nonreducing sample buffer (see recipe)
- 10% (w/v) protein A–Sepharose (see recipe)
- Antibodies raised against the protein of interest
- PBS (*APPENDIX 2A*), optional
- 2% (w/v) salicylate (see recipe)
- 27°C water bath
- 1.5-ml microcentrifuge tubes, RNase free
- Tube rotator (capable of end-over-end inversions), 4°C
- Microcentrifuge with a fixed-angle rotor (Eppendorf 5415C or equivalent), 4°C and room temperature
- Vacuum aspirator
- Microcentrifuge tube rack vortex mixer (e.g., Tommy MT-360; Tomy Tech USA Inc.), 4°C
- 95°C heating block
- Whatman 3MM filter paper
- Additional reagents and equipment for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) minigel with Laemmli buffers (*UNIT 6.1*), Coomassie blue staining and destaining (*UNIT 6.6*), and gel autoradiography (*UNIT 6.3*)

NOTE: It is important to avoid contamination by RNases that degrade the mRNA during in vitro translations. Wear gloves throughout the experiment. Treat water and salt solutions with diethylpyrocarbonate (DEPC) to chemically inactivate RNases. Treat all glass and plasticware with DEPC-treated water or otherwise to remove RNase activity.

Carry out in vitro translation

1. Thaw in vitro translation reagents rapidly in a 27°C water bath and then place on ice.

Canine pancreatic microsomes should be stored in aliquots of working volumes (multiples of 6 μ l). Freeze-thawing of rabbit reticulocyte lysate more than twice should be avoided as each freeze-thaw cycle results in a decrease in translation and translocation efficiency. All other reagents should be frozen rapidly in liquid nitrogen after use and stored at -70°C.

2. Assemble reaction mixture in a 1.5-ml RNase-free microcentrifuge tube at room temperature (~92.0 μ l total volume) as follows:

6 μ l of 1 equivalent/ μ l nuclease-treated canine pancreatic microsomes or 12 μ l of 1×10^5 cells/ μ l SP cells (1.3×10^4 cells/ μ l final)
52 μ l treated rabbit reticulocyte lysate
2 μ l of 1 mM amino acid mixture lacking methionine and cysteine
8 μ l of 11 mCi/ml [³⁵S]methionine and [³⁵S]cysteine
4 μ l of 40 U/ μ l RNase inhibitor
3.7 μ l of 100 mM GSSG (4 mM final) or 2.5 mM FAD (100 μ M final concentration)
3 μ l of 100 mM DTT
3.2 μ l of 2.5 M KCl
4 μ l of 1 μ g/ μ l mRNA for the protein of interest
6.1 μ l nuclease-free H₂O.

This reaction mix is sufficient for nine samples (i.e., three time points) and should be adjusted proportionately as needed.

One equivalent per microliter of microsomes is equal to 50 A₂₈₀ U/ml. A range of 6 to 12 μ l of 1 eq/ μ l of microsomes or 0.5 to 2.5×10^4 cells/ μ l of SP-cells provides optimum translocation for a 92- μ l translation mix.

The authors have found that ~4.0 μ g mRNA per 92- μ l reaction is optimal for their transcripts, but this is transcript dependent and should be optimized individually. The KCl and microsome concentrations in the translation also have to be optimized for individual mRNAs. A final KCl concentration range of 30 to 90 mM is a good starting point. In general, increasing the amount of microsomes in the reaction increases the proportion of polypeptides that are processed, but it may also reduce the amount of total polypeptide synthesized.

For oxidation, usually 4 mM GSSG or 100 μ M FAD with 1.1 mM DTT can provide optimal redox conditions, but a titration may be required to provide maximal results. If more than one mRNA is used or conditions are varied, it is advantageous to aliquot the mix and make the adjustments prior to mRNA addition.

3. Mix the lysate gently but thoroughly by pipeting up and down a few times and incubate 1 hr at 27°C.

To avoid air bubbles during mixing, the pipettor should be set to less than the total volume of the mixture. If necessary, the sample should be centrifuged briefly to bring the mixture to the bottom of the tube.

4. Add 2 μ l of 50 mM cycloheximide (1 mM final) and pipet up and down to stop the translation reaction.

Cycloheximide is a protein synthesis inhibitor.

Alkylate proteins

5. Transfer 10 μl lysate into each of three 1.5-ml microcentrifuge tubes. Return the remaining lysate to the 27°C water bath for further oxidation and maturation.

These samples represent the first time point. For each time point, three fractions will be generated: control, no alkylation; alkylation with NEM; and alkylation with AMS.

6. Set one of the three tubes aside on ice for the control, nonalkylated sample.
7. To the second tube, add 2.0 μl of 120 mM NEM (20 mM final) to alkylate free thiols. Place on ice for 10 min.
8. To the third tube, add 25 μl of 20 mM AMS (14 mM final) to alkylate free thiols and incubate 1 hr at room temperature in the dark.

Because AMS is a membrane-impermeable alkylating agent, the membranes have to be lysed. Therefore, the solution containing AMS also contains SDS for membrane solubilization. AMS is sensitive to light; the bottle containing the solution should be wrapped with aluminum foil, and a freshly prepared stock solution should be used.

9. After appropriate incubation times, repeat steps 5 to 8 for remaining time points.
- 10a. *For immunoprecipitation:* Add 500 μl ice-cold 2% CHAPS/HeBS to each sample and continue with step 11.
- 10b. *For direct analysis of total proteins translated:* Add 2 \times nonreducing sample buffer to the three samples as follows and continue with step 19:

10 μl to nonalkylated sample
12 μl to NEM-treated sample
35 μl to AMS-treated sample.

Adjust the sample volume with 1 \times nonreducing sample buffer to 70 μl to keep the protein concentration constant for quantification purposes.

Immunoprecipitate proteins

11. Add 100 μl of 10% protein A–Sepharose to each tube to preclear immunoprecipitation samples. Rotate samples end over end on a tube rotator for 1 hr at 4°C.

Preclearing with protein A–Sepharose should decrease the amount of background associated with the use of protein A. More details on immunoprecipitation can be found in UNIT 7.2.

12. Microcentrifuge samples 5 min at 2900 $\times g$ (6000 rpm), 4°C, and transfer the supernatant to a new 1.5-ml microcentrifuge tube containing 100 μl of 10% protein A–Sepharose and the appropriate amount of antibodies raised against the protein of interest.

A titration with antiserum may be required to obtain optimal results. Excess antibodies may give nonspecific binding or saturate the protein A–Sepharose.

13. Rotate samples end over end for 4 to 8 hr or overnight at 4°C.
14. Microcentrifuge samples 5 min at 2900 $\times g$, 4°C, to pellet the protein-antibody immune complexes.
15. Place samples on ice, and use a vacuum aspirator to remove supernatant.
16. Add 500 μl of 0.5% CHAPS/HeBS and vortex in a microcentrifuge tube rack vortex mixer at medium speed for 10 min at 4°C, to wash pellet.

For in vitro translated proteins, only a single wash is needed. However, cellular studies (see Basic Protocol 5 and see Alternate Protocol 3) may require multiple washes to reduce background.

17. Microcentrifuge samples 5 min at $2900 \times g$, 4°C , and aspirate supernatant.
18. Resuspend samples in $40 \mu\text{l}$ of $2\times$ nonreducing sample buffer by vortexing for 5 min.

Electrophoresis proteins

19. Heat samples 5 min in a 95°C heating block.

A boiling water bath may be used instead of the heating block.

20. Microcentrifuge samples 5 min at $16,000 \times g$ ($14,000 \text{ rpm}$), room temperature, to pellet the Sepharose beads prior to loading on the gel.

Take care not to clog the pipet tip by pipetting beads.

21. For reduced samples, place $13.5 \mu\text{l}$ nonreduced sample into a fresh 1.5-ml microcentrifuge tube. Add $1.5 \mu\text{l}$ of 1 M DTT (100 mM final) and heat 5 min at 95°C . Centrifuge briefly to concentrate sample at the bottom of the tube.

Reducing and nonreducing conditions are used to identify the disulfide bonded forms of the protein that migrate faster in the nonreducing gel compared to the reducing gel.

22. Prepare a 1- or 0.75-mm -thick SDS-polyacrylamide separating and stacking minigel with a 10- or 15-well comb (UNIT 6.1).

Separating-gel acrylamide concentration depends on the molecular weight of the proteins to be resolved.

23. Load $10 \mu\text{l}$ nonreduced sample or $11.1 \mu\text{l}$ reduced sample into each well and bring all lanes, including the empty ones, up to $11.1 \mu\text{l}$ with $1\times$ sample buffer.

Each well must be intact and straight. If nonreduced and reduced samples are to be placed on the same gel, care must be taken to ensure that the DTT from the reduced samples does not reduce the nonreduced samples in the gel. This can be avoided by leaving two empty lanes between nonreduced and reduced samples and filling these lanes with nonreducing sample buffer. If nonreduced and reduced samples need to be loaded next to each other, samples should be cooled, and NEM added to a final concentration of 100 mM to both sets of samples to alkylate excess DTT. Samples should be loaded carefully using a gel loading pipet tip.

24. Run the gel for $\sim 2 \text{ hr}$ at 20 to 25 mA until the dye front is at the bottom of the gel.
25. Stain and destain the gel, including stacking gel, with Coomassie blue stain and destain (UNIT 6.6)

Stacking gels often contain aggregated proteins.

26. Wash gel with distilled water or PBS by rocking gently. Repeat two or three times. Check that the pH has reached neutrality with pH paper.

Salicylate (step 27) is inactivated by acidic pH.

27. Incubate gel 10 min with gentle agitation in 2% salicylate.

Salicylate is used to enhance the radioactive signal 3- to 5-fold (UNIT 6.3).

28. Dry gel onto Whatman 3MM filter paper and expose gel to radiographic film for autoradiography (UNIT 6.3).

An exposure of 6 to 12 hr is generally sufficient for total protein lysate samples. Immunoprecipitated samples may require longer exposure time.

**ANALYSIS OF POST-TRANSLATIONAL DISULFIDE BOND FORMATION
IN ROUGH ENDOPLASMIC RETICULUM-DERIVED MICROSOMES**

The formation of disulfide bonds can be inhibited by the addition of a powerful reducing agent such as dithiothreitol (DTT) while protein synthesis, translocation, signal sequence cleavage, and glycosylation continue. Here, disulfide bond formation is initiated post-translationally by the removal of the reducing agent or the addition of oxidizing agents. This post-translational method allows for synchronous initiation of disulfide bond formation. In addition, the isolation of the oxidation and folding steps in the post-translational procedure permits the characterization of factors required in this process independent of other processes involved in protein maturation such as translocation and glycosylation (Braakman et al., 1992).

This protocol is similar to Basic Protocol 1 (and uses the same list of materials) with the exception that the radiolabeled protein is synthesized under reducing conditions to inhibit disulfide bond formation (Fig. 15.6.2). Synthesis is then terminated and oxidizing conditions are restored by adding oxidizing agents such as oxidized glutathione (GSSG) or flavin adenine dinucleotide (FAD) to initiate disulfide bond formation. An intact endoplasmic reticulum (ER) source can also be used here by substituting semipermeabilized (SP) cells (see Support Protocol) for the microsomes. For cellular studies, the reducing agent (5 mM DTT final concentration) is added during the radioactive pulse and removed to initiate oxidation because intraluminal oxidizing conditions will be rapidly restored upon returning the cells to medium lacking reducing agents (Braakman et al., 1992).

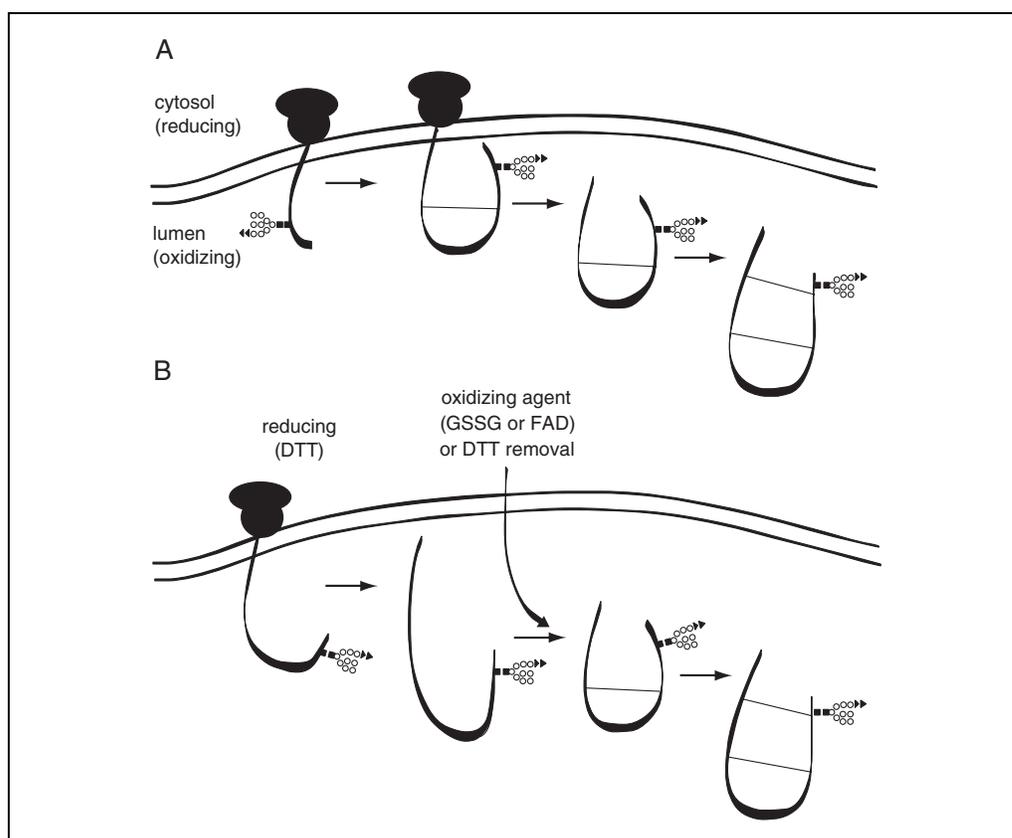


Figure 15.6.2 Formation of disulfide bonds. **(A)** Co-translational bond formation. **(B)** Post-translational bond formation. Under normal cellular redox conditions, the cytosol is reducing and the endoplasmic reticulum (ER) lumen is oxidizing. Disulfide bond formation and glycan addition can take place co-translationally (A) as the C terminus of the protein is being completed by the ribosome. Synchronous oxidation of the completed glycosylated and reduced chain can be initiated post-translationally (B) by synthesizing the protein under reducing conditions (e.g., DTT), inhibiting protein synthesis, and then adding oxidizing agents, such as oxidized glutathione (GSSG) or flavin adenine dinucleotide (FAD), post-translationally.

NOTE: It is important to avoid contamination by RNases that degrade the mRNA during *in vitro* translations. Wear gloves throughout the experiment. Treat water and salt solutions with diethylpyrocarbonate (DEPC) to chemically inactivate RNases. Treat all glass and plasticware with DEPC-treated water or otherwise to remove RNase activity.

1. Carry out *in vitro* translation using either canine pancreatic microsomes or SP cells as described (see Basic Protocol 1, steps 1 to 4) with the omission of the oxidizing agent GSSG or FAD in step 2. Adjust the reaction volume with nuclease-free water accordingly.
2. Add 3.7 μ l of 100 mM GSSG (4 mM final) or 3.7 μ l of 2.5 mM FAD (100 μ M final) to initiate oxidation. Mix and incubate for an appropriate length of time at 27°C.

The incubation time(s) will depend on the experimental design.

3. Alkylate the remaining free cysteines with NEM and/or AMS to arrest further oxidation, and analyze proteins as described (see Basic Protocol 1, steps 5 to 28).

BASIC PROTOCOL 2

ANALYSIS OF PROTEIN FOLDING BY PROTEOLYTIC SENSITIVITY

The folded state and stability of proteins synthesized *in vitro* and *in vivo* can be analyzed by proteolysis. Limited proteolysis is a powerful tool for probing the globular structure of folded proteins. While a variety of promiscuous proteases can be employed for these studies, the most commonly used is trypsin. Trypsin cleaves the peptide bonds C-terminal to the basic amino acids lysine and arginine. Peptide regions buried in the protein core and in the secondary structural elements are less accessible to the enzyme (usually in the folded conformation), therefore they are not cleaved as quickly (Fig. 15.6.1). Here, *in vitro* translated, ^{35}S -labeled protein is allowed to fold under oxidizing conditions in the presence of canine pancreatic microsomes (see Basic Protocol 1). Microsomal membranes containing the ^{35}S -labeled protein are isolated from the lysate and solubilized in a digestion buffer. The folded state of the protein is analyzed by trypsin digestion. Early oxidation or folding forms will be easily digested by the protease while mature forms will be more protease resistant. The digested samples are analyzed directly by reducing SDS-PAGE and autoradiography. This technique can also be used with semipermeabilized (SP) cells (see Support Protocol), or it can easily be adapted for intact cell studies (see Basic Protocol 5 and see Alternate Protocol 3).

Materials

- 1 \times sucrose cushion (see recipe)
- 1% and 10% digestion buffer: 1% and 10% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (CHAPS) in HeBS (see recipe for HeBS)
- 50 μ g/ml trypsin (prepared from 1 mg/ml stock; stored at 4°C up to 6 months)
- 50 μ g/ml soybean trypsin inhibitor (prepared from 1 mg/ml stock; stored at 4°C up to 6 months)
- 150 mM PMSF (see recipe)
- 2 \times reducing sample buffer: 10% (v/v) 1 M DTT (*APPENDIX 2A*; 100 mM final) in 2 \times nonreducing sample buffer (see recipe)
- Beckman Airfuge, 4°C, and 5 \times 20-mm ultraclear centrifuge tubes (Beckman)

Additional reagents and equipment for *in vitro* translation using either canine pancreatic microsomes or SP cells, analyzing proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and gel autoradiography (see Basic Protocol 1)

NOTE: It is important to avoid contamination by RNases that degrade the mRNA during *in vitro* translations. Wear gloves throughout the experiment. Treat water and salt solutions

with diethylpyrocarbonate (DEPC) to chemically inactivate RNases. Treat all glass and plasticware with DEPC-treated water or otherwise to remove RNase activity.

Carry out *in vitro* translation

1. *In vitro* translate the protein of interest as described (see Basic Protocol 1, steps 1 to 3) in the presence of GSSG or FAD.

The number of samples should be determined and the translation reaction scaled up accordingly.

2. Add 50 mM cycloheximide to a final concentration of 1 mM to stop the translation reaction.

For a 92- μ l reaction, 2 μ l of 50 mM cycloheximide is added.

Alkylate proteins

3. Transfer 10 μ l lysate to a new 1.5-ml microcentrifuge tube containing 2 μ l of 120 mM NEM (20 mM final) to alkylate free cysteines, and place on ice for 10 min.
4. Return remaining lysate to the 27°C water bath for further oxidation and maturation.
5. After appropriate incubation times, repeat steps 3 and 4 for remaining time points.

Isolate membranes

6. Add 50 μ l of 1 \times sucrose cushion to the bottom of a 5 \times 20-mm ultraclear centrifuge tube. Overlay 12 μ l NEM-alkylated lysate on the top of the sucrose cushion and centrifuge 10 min in a Beckman Airfuge at 20 psi, 4°C.
7. Discard supernatant and resuspend pellet containing the ³⁵S-labeled protein in 20 μ l of 1% digestion buffer.

Adherent cells or cells in suspension can be applied to this assay after this step. For adherent cells perform steps 1 to 10, Basic Protocol 5, or for cells in suspension perform steps 1 to 11, Alternate Protocol 3. Then immunoprecipitate the protein (UNIT 7.2) and resuspend the pellet in 20 μ l of 1% digestion buffer and continue with step 8.

Care must be taken not to create air bubbles while resuspending the pellet. Make sure that no pellet clumps are left undissolved.

8. Transfer 1 μ l sample to pH paper to check the pH. If necessary, add 2 to 3 μ l of 10% digestion buffer to increase the buffer strength and recheck the pH.

The optimal pH range for trypsin is 7.5 to 8.0.

Digest with trypsin

9. Add 0.4 to 0.8 μ l of 50 μ g/ml trypsin (1 to 2 μ g/ml final) to the sample (step 7) and incubate 30 min at 27°C.

Control samples without added enzyme should be set aside for all time points. Set aside half of the sample (~10 μ l) from step 8 for a control. Trypsin concentration may need to be titrated for optimal digestion. The incubation time and temperature can also be varied for optimal digestion.

10. Add 2 μ l of 50 μ g/ml trypsin inhibitor and 1 μ l of 150 mM PMSF to stop the digestion. Mix thoroughly by pipetting up and down. Leave the sample at room temperature for 2 min.

**SUPPORT
PROTOCOL**

Electrophoresis proteins

11. Add 25 μ l of 2 \times reducing sample buffer and mix thoroughly. Immediately heat 5 min at 95°C and microcentrifuge 5 min at 16,000 \times g (14,000 rpm), room temperature.
12. Prepare and run an SDS-polyacrylamide gel and analyze proteins by gel autoradiography as described (see Basic Protocol 1, steps 22 to 28), but load 10 μ l sample in step 23.

The remaining sample can be stored up to 2 months at -20°C.

**ISOLATION OF SEMIPERMEABILIZED CELLS FOR ANALYSIS OF
PROTEIN FOLDING**

To study the oxidation of a protein in an intact cellular ER while maintaining the manipulative advantage of the in vitro translation system, proteins translated in vitro can be translocated into the ER of semipermeabilized cells (SP cells). Here, essentially any eukaryotic cell type can be used as the ER source. The plasma membrane is permeabilized with the detergent digitonin while the endomembranes remain intact. The SP-cell membranes can then be substituted for the microsomal membranes as discussed in Basic Protocol 1.

Materials

- Cells in 75-cm² tissue culture flask in appropriate medium
- PBS (APPENDIX 2A)
- Trypsin/EDTA solution (see recipe), room temperature
- KHM-STI buffer (see recipe), ice cold
- 0.4% (w/v) trypan blue solution (Sigma-Aldrich)
- KHM buffer (see recipe), ice cold
- 20 mg/ml digitonin in dimethyl sulfoxide (DMSO), stored in 1-ml aliquots up to 2 years at -20°C
- HEPES/potassium acetate buffer (see recipe), ice cold
- 100 mM CaCl₂ (APPENDIX 2A)
- 15,000 U/ml micrococcal nuclease, from *Staphylococcal aureus* (Boehringer Mannheim), stored in working-volume aliquots (e.g., 5 μ l or multiples of 5 μ l) at -80°C
- 250 mM ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), adjusted to pH 7.4 with NaOH (stored up to 2 years at -20°C)
- Vacuum aspirator
- 15-ml sterile polystyrene centrifuge tubes, prechilled on ice
- Beckman GPR centrifuge (or equivalent), 4°C
- Microcentrifuge, 4°C
- Additional reagents and equipment for counting cells (UNIT 1.1)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

Isolate cells

1. Grow cells in 75-cm² tissue culture flask in appropriate medium to ~80% confluency.

Chinese Hamster Ovary (CHO) cells commonly yield 0.6 to 2 \times 10⁶ SP-cells from a single 75-cm² flask. For the 12 μ l of SP-cells used in the 92 μ l translation mix, two flasks are sufficient, scale up the number of flasks with respect to the size of the experiment.

2. Remove medium from flask, rinse twice with ~10 ml PBS, and use a vacuum aspirator to remove the PBS.
3. Add 4 ml trypsin/EDTA solution, prewarmed at room temperature. Quickly tilt flask back and forth so that the whole flask is covered. Remove trypsin solution immediately and incubate flask 5 min at room temperature.
4. Slap the bottom of the flask to loosen cells, add 6 ml ice-cold KHM-STI buffer, and suspend cells by pipetting up and down until there are no visible cell clumps. Place flask on ice.

NOTE: Cells should be kept on ice for all remaining steps except where indicated.

5. Rinse flask with an additional 2 ml KHM-STI buffer for a total volume of 8 ml.
6. Transfer 10 μ l suspended cells to a 1.5-ml microcentrifuge tube and add 10 μ l of 0.4% trypan blue solution. Mix thoroughly and count cells. Note percent of dyed cells at this stage (*UNIT 1.1*).
7. Transfer cell suspension (step 5) to a prechilled 15-ml sterile polystyrene centrifuge tube ($\leq 5 \times 10^7$ cells/tube) and place on ice.

The cell suspension should be divided among multiple tubes as needed.

8. Centrifuge 5 min at $250 \times g$ (1200 rpm), 4°C, in a Beckman GPR centrifuge, to pellet cells.

Permeabilize cells

9. Remove supernatant and add 100 μ l ice-cold KHM buffer at the bottom of the tube and tap the bottom of the tube to resuspend cells. Add 5.9 ml ice-cold KHM buffer and invert tube two to three times.
10. Add 6 μ l of 20 mg/ml digitonin in DMSO (20 μ g/ml final) to each tube and mix immediately. Incubate 5 min on ice.
11. Add 8 ml ice-cold KHM buffer and mix by inverting two or three times. Centrifuge 5 min at $250 \times g$, 4°C. Discard supernatant.
12. Resuspend cells in 14 ml ice-cold HEPES/potassium acetate buffer and incubate 10 min on ice. Centrifuge 5 min at $250 \times g$, 4°C. Discard supernatant.
13. Resuspend cells in 1 ml ice-cold KHM buffer and transfer cells into a 1.5-ml microcentrifuge tube.

All cell pellets from different tubes ($\leq 4 \times 10^7$ cells) should be pooled in 1 ml KHM buffer.

14. Transfer 10 μ l cell suspension to a 1.5-ml microcentrifuge tube and dilute 1:5 in ice-cold KHM buffer. Transfer 10 μ l of this dilution to a new 1.5-ml tube and count cells and test for permeabilization efficiency with trypan blue (step 6).

At this step, there are usually about half of the total number of cells that were present in step 6.

15. Microcentrifuge cells (step 13) 2 min at $5,200 \times g$ (8,000 rpm), 4°C. Aspirate supernatant and resuspend cells in 100 μ l ice-cold KHM buffer (for $2\text{--}4 \times 10^6$ cells).

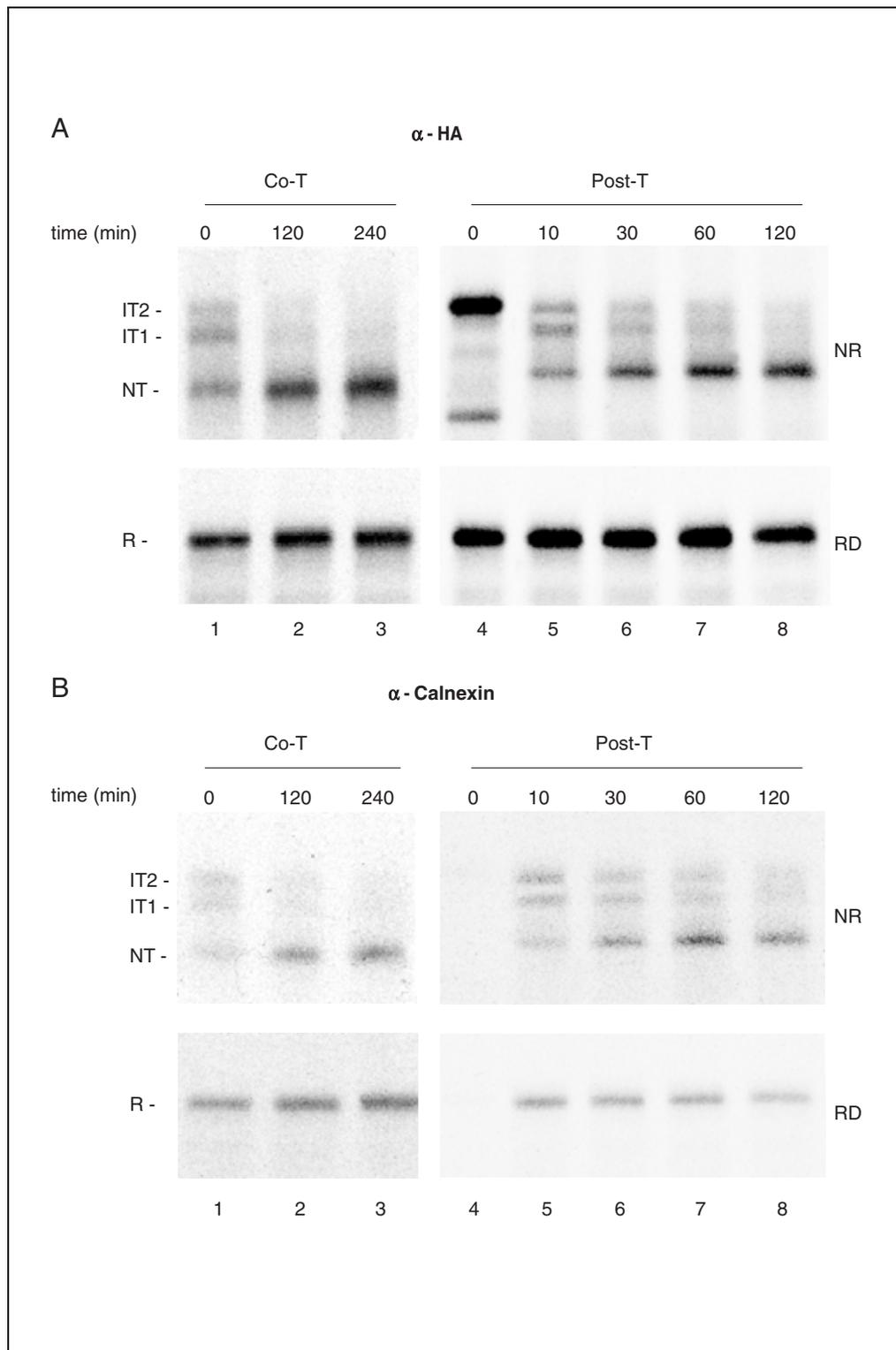


Figure 15.6.3 Disulfide bond formation and folding of hemagglutinin (HA). (A) Co-translational and post-translational formation of disulfide bonds. (B) Calnexin binding. ^{35}S -labeled HA was in vitro translated with rabbit reticulocyte lysate at 27°C for 1 hr in the presence of canine pancreatic endoplasmic reticulum (ER)-derived microsomes under oxidizing (co-translational; Co-T) or reducing (post-translational; Post-T) conditions. Cycloheximide was added to inhibit protein synthesis and protein samples were removed after the indicated chase times and subjected to immunoprecipitation with antisera raised against the whole influenza virus (A) and resolved by nonreducing (NR) and reducing (RD) SDS-PAGE. For post-translational analysis, oxidation was initiated at time zero and followed. The same samples were used to identify interactions with calnexin by the co-immunoprecipitation of ^{35}S -labeled HA with anti-calnexin antiserum (B). Protein forms are as follows: IT1, intermediate 1; IT2, intermediate 2; NT, native; R, reduced.

Treat with nuclease

16. Add 1 μl of 100 mM CaCl_2 (1 mM final) and 2 μl of 15,000 U/ml (300 U/ml final) micrococcal nuclease. Mix and incubate 12 min at room temperature.

Micrococcal nuclease is a calcium-dependent nuclease that will digest endogenous mRNA.

17. Add 1.6 μl of 250 mM EGTA (4 mM final) to chelate calcium and inactivate the nuclease.

18. Microcentrifuge cells 2 min at $5,200 \times g$, 4°C . Aspirate supernatant and resuspend SP-cell pellet with ice-cold KHM buffer to a final concentration of 1×10^5 cells/ μl .

SP cells should be used as soon as they are prepared.

ANALYSIS OF FOLDING-INTERMEDIATE BINDING TO MOLECULAR CHAPERONES IN ROUGH ENDOPLASMIC RETICULUM-DERIVED MICROSOMES

Protein folding in the cell is a highly assisted process. Molecular chaperones and foldases play important roles in aiding the folding or stabilization of nascent proteins. In the endoplasmic reticulum (ER), proteins that help in the folding process include BiP (GRP78; an hsp70 family member), GRP94 (an hsp90 family member), the lectin chaperones calnexin and calreticulin, and the protein disulfide isomerase family members PDI, ERp57, and ERp72. The transient binding of these folding factors to the nascent chain can be studied through a co-immunoprecipitation procedure if the interactions persist during the solubilization and immunoprecipitation protocol.

In this protocol, a co-immunoprecipitation procedure is described that permits the characterization of transient interactions of folding factors. Co-immunoprecipitations (Fig. 15.6.3) require modifications to the general immunoprecipitation protocol described previously (see Basic Protocol 1). First, the detergent used in cell or microsomal lysis must enable the persistence of the native interaction of the chaperones with the substrate. Common nondenaturing detergents used for this purpose include Triton X-100, digitonin, and CHAPS (UNIT 7.2). In addition, antibodies against the chaperones or folding factors are required. Finally, if folding-factor binding is regulated by ATP binding (as found with BiP), it may be necessary to deplete ATP during the solubilization step to trap the interaction with the nascent chain. Chemical cross-linking can also be performed to provide a covalent bond to trap interactions between the chaperone and its substrate prior to lysis (see Alternate Protocol 2).

Materials

Antiserum raised against protein of interest
Antiserum raised against chaperone of interest

Additional reagents and equipment for in vitro translation using canine pancreatic microsomes or SP cells, immunoprecipitation, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and gel autoradiography (see Basic Protocol 1)

NOTE: It is important to avoid contamination by RNases that degrade the mRNA during in vitro translations. Wear gloves throughout the experiment. Treat water and salt solutions with diethylpyrocarbonate (DEPC) to chemically inactivate RNases. Treat all glass and plasticware with DEPC-treated water or otherwise to remove RNase activity.

1. In vitro translate the protein of interest as described (see Basic Protocol 1, steps 1 to 3, when using microsomes or Support Protocol, steps 1 to 18 for SP-cells), adjusting the volume of any control reactions with nuclease-free water.

The number of time points should be determined and the translation reaction scaled up accordingly. For each time point, three samples will be needed: lysate (for total protein analysis), immunoprecipitation with antibodies directed against the maturing protein, and immunoprecipitation with antibodies directed against the chaperone(s). A reaction mixture of 30 μ l is appropriate for each time point when only one chaperone immunoprecipitation is performed. For each additional chaperone immunoprecipitation increase the reaction mixture by 10 μ l. Keeping the DTT concentration constant (1.1 mM) for these reactions, titrate GSSG from 2 to 6 mM or FAD from 50 to 200 μ M to determine optimum oxidation conditions.

A control reaction without microsomes or SP-cells should be performed to eliminate the possibility that non-specific binding is occurring in the chaperone binding assays.

2. Transfer 30 μ l lysate into a 1.5-ml microcentrifuge tube. Return the remaining lysate to the 27°C water bath for further oxidation and maturation.
3. Add 6 μ l of 120 mM NEM to 30- μ l sample to arrest oxidation and incubate 10 min on ice to stop the translation.

Cycloheximide at a final concentration of 1 mM can also be included to stop the translation if further maturation is to be followed in the absence of protein synthesis.

4. Divide the sample into three 12- μ l aliquots.
5. To the first, add 12 μ l of 2 \times nonreducing sample buffer and 16 μ l of 1 \times nonreducing sample buffer, store samples at -20°C until all samples are prepared. To prepare the samples heat 5 min at 92°C and analyze total proteins (see Basic Protocol 1, steps 22 to 28).
6. To the remaining two aliquots, add 500 μ l ice-cold 2% CHAPS/HeBS to lyse the samples.
7. Immunoprecipitate both samples as described (see Basic Protocol 1, steps 11 to 18), using an antibody raised against the protein of interest in one sample to measure total protein level and an antibody raised against the interacting molecular chaperone in the other.
8. Electrophorese and analyze proteins by SDS-PAGE and gel autoradiography as described (see Basic Protocol 1, steps 19 to 28).

Table 15.6.1 is a list of chaperones and their molecular weights; this can be used to calculate the molecular weight change in complexes.

Table 15.6.1 Approximate Molecular Weights of Endoplasmic Reticulum Chaperones

Chaperone ^a	Molecular weight (kDa)
BiP (GRP78)	75
CNX	57 ^b ; 90 ^c
CRT	48 ^b ; 60 ^c
ERp57	57
ERp72	72
GRP94	94
PDI	55

^aAbbreviations: CNX, calnexin; CRT, calreticulin; ER, endoplasmic reticulum; GRP, glucose-regulated protein; PDI, protein disulfide isomerase.

^bCalculated molecular weight.

MONITORING TRANSIENT CHAPERONE INTERACTIONS USING CROSS-LINKING AND DENATURING IMMUNOPRECIPITATIONS

In some cases, the interactions between maturing proteins and molecular chaperones may be weak, therefore disruption can occur during the immunoprecipitation procedure. These types of interactions may require the additional stability offered through chemical cross-linking. Several types of membrane-permeable chemical cross-linkers are commercially available with reactivity towards free sulfhydryls, amines, carboxyls, or other functional groups, while others are UV excitable and can trap a broader range of functional groups (Table 15.6.2).

Once a cross-link is recognized by a molecular weight shift following SDS-PAGE, it can be identified using denaturing immunoprecipitation with antiserum raised against the molecular chaperone adduct. To minimize background cross-linking, the microsomes are isolated from the translation system by centrifugation and resuspended in an isotonic resuspension buffer prior to cross-linking.

Additional Materials (also see *Basic Protocol 3*)

Isotonic sucrose cushion (see recipe)

Isotonic resuspension buffer (see recipe)

10 mM BMH (see recipe) or alternative cross-linker

Quenching solution: 100 mM 2-mercaptoethanol (stored up to 1 month at 4°C)

20% (w/v) SDS (APPENDIX 2A)

Denaturing immunoprecipitation buffer (see recipe)

Beckman Optima TLX ultracentrifuge and TLA 120.2 rotor, 4°C, and 7 × 20-mm ultracentrifuge tubes *or* Beckman Airfuge, 4°C, and 5 × 20-mm ultraclear centrifuge tubes

NOTE: It is important to avoid contamination by RNases that degrade the mRNA during *in vitro* translations. Wear gloves throughout the experiment. Treat water and salt solutions with diethylpyrocarbonate (DEPC) to chemically inactivate RNases. Treat all glass and plasticware with DEPC-treated water or otherwise to remove RNase activity.

1. *In vitro* translate the protein of interest as described (see *Basic Protocol 3* steps 1 to 3), but when using a thiol-reactive cross-linker, such as BMH (step 4), do not add NEM. Adjust samples to 1 mM cycloheximide and incubate 10 min on ice to terminate translation.

NEM will block reactive groups on thiol-reactive cross-linkers (e.g., BMH).

Table 15.6.2 Commonly Used Cross-Linkers for Monitoring Chaperone Interactions

Cross-linker ^a	Reactivity
BASED	Nonselective, photoreactive
BMH	Sulfhydryls
DSP	Amines
EDC	Amines and carboxyls
SMCC	Amines and sulfhydryls

^aAvailable from Pierce Chemical. Abbreviations: BASED, bis[β-(4-azidosalicylamido)ethyl] disulfide; BMH, bismaleimidohexane; DSP, dithiobis(succinimidyl propionate); EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; SMCC, succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate.

For interactions with some folding factors, it is essential that the translation be performed under oxidizing conditions, as this will enable the chaperones to remain in their native oxidized state. Nonnative reducing conditions may reduce a critical disulfide bond in the chaperone thereby destroying its ability to interact with other substrates (for example, calnexin and calreticulin's lectin-binding properties are diminished under reducing conditions; Fig. 15.6.3B, lane 4).

- Carefully layer a 10- μ l sample on a 30- μ l isotonic sucrose cushion (1:3 sample/cushion ratio) within a 7 \times 20-mm ultracentrifuge tube or a 5 \times 20-mm ultraclear centrifuge tube, and centrifuge 10 min either at 157,000 \times g (60,000 rpm), 4°C, using a Beckman Optima TLX ultracentrifuge with a TLA 120.2 rotor or at 20 psi, 4°C, using a Beckman Airfuge, respectively.
- Keeping samples on ice, aspirate the supernatant. Resuspend pellet by pipetting up and down in 10 μ l isotonic resuspension buffer, and transfer to a new 1.5-ml microcentrifuge tube.

It is important that the microsomal pellet is fully resuspended and the samples are kept on ice.

Removal of the reticulocyte lysate by centrifugation ensures that cross-links will not occur with reticulocyte lysate proteins.

- Add 1.1 μ l of 10 mM BMH (1 mM final) and incubate 10 min at 27°C.

It may be necessary to increase or decrease the cross-linker concentration to obtain the optimal results.

- Add 1.2 μ l quenching solution and place samples on ice for 10 min.

While the authors describe a protocol for BMH, a thiol-reactive cross-linker that is quenched with alkylating agents, a variety of cross-linkers (Table 15.6.2) should be tested for optimal cross-linking. The quenching agent employed will depend on the cross-linker chemistry.

- Add 0.65 μ l of 20% SDS to a 12.2 μ l sample (1% final) and heat 5 min at 95°C.

This will denature all noncovalent interactions and ensure that only cross-linked adducts are visualized,

- Add 500 μ l denaturing immunoprecipitation buffer to quench SDS.

- Complete immunoprecipitation as described (see Basic Protocol 3, steps 7 to 8), but use 500 μ l denaturing immunoprecipitation buffer in place of 500 μ l of 0.5% CHAPS/HeBS for the wash step (see Basic Protocol 1, step 16).

Antibodies to predicted adducts can be used here (Table 15.6.2; Affinity BioReagents, Stressgen). Once adducts are observed, their molecular weights can be estimated by reducing SDS-PAGE. Antibodies to molecular chaperones with similar molecular weights (Table 15.6.1) can then be used to identify protein adducts. StressGen Biotechnologies and Affinity BioReagents are excellent sources for antibodies against ER folding factors.

BASIC PROTOCOL 4

MONITORING PROTEIN FOLDING USING CONFORMATION-SPECIFIC ANTIBODIES

The recognition of protein determinants (epitopes) by antibodies can be conformation dependent. This enables the use of conformation-specific antibodies as a powerful tool for the characterization of the folding process (Goldberg, 1991). The acquisition or disappearance of specific epitopes can be used as a probe to monitor the folding reaction. On occasion, monospecific antibodies that recognize a single epitope can be obtained from polyclonal sources by generating antipeptide antibodies or by antibody purification. However, more commonly, monoclonal antibodies (mAbs) have been found to be the most effective for these studies.

This protocol describes the use of conformation-specific antibodies to characterize the folding process in rough endoplasmic reticulum (ER)-derived microsomes. It is similar to Basic Protocol 3, which explains the analysis of chaperone binding with the use of co-immunoprecipitation studies. Here, it is also important to lyse the microsomes and perform the antibody binding and washing under gentle nondenaturing conditions to ensure that the protein conformation is not disrupted. The antibodies employed are mAbs raised against the folding substrate that recognize individual epitopes. In short, the folding reaction is initiated with the radiolabeled protein as in Basic Protocol 1 (co-translationally) or Alternate Protocol 1 (post-translationally), with samples being removed at various times and analyzed by immunoprecipitation with the mAbs, and then the radiolabeled precipitate is resolved by SDS-PAGE and autoradiography.

Materials

50% (w/v) protein G-Sepharose or protein A-Sepharose bead (Sigma-Aldrich) slurry in PBS/0.1% (w/v) BSA/0.01% (w/v) sodium azide

Conformation-specific monoclonal antibody (mAb) against protein of interest
Control antibodies (preimmune control and antibody control that recognizes all conformations of the protein of interest)

Additional reagents and equipment for in vitro translation using canine pancreatic microsomes, immunoprecipitation, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and gel autoradiography (see Basic Protocol 1)

CAUTION: Sodium azide is poisonous; follow appropriate precautions for handling, storage, and disposal.

NOTE: It is important to avoid contamination by RNases that degrade the mRNA during in vitro translations. Wear gloves throughout the experiment. Treat water and salt solutions with diethylpyrocarbonate (DEPC) to chemically inactivate RNases. Treat all glass and plasticware with DEPC-treated water or otherwise to remove RNase activity.

NOTE: All solutions should be ice cold and procedures should be carried out at 4°C or on ice unless otherwise indicated.

1. In vitro translate the protein of interest as described (for cotranslational analysis see Basic Protocol 1, steps 1 to 3; for post-translational analysis see Alternate Protocol 1, steps 1 to 3).

The number of time points should be determined and the translation reaction scaled up accordingly. For each time point three 12- μ l samples will be needed: lysate (for total protein analysis), immunoprecipitate with antibody that is not conformation dependent, and immunoprecipitate with antibody that is conformation dependent.

2. Transfer 30 μ l lysate into a 1.5-ml microcentrifuge tube. Return the remaining lysate to the 27°C water bath for further oxidation and maturation.
3. Add 6 μ l of 120 mM NEM to 30- μ l sample and incubate on ice 10 min to alkylate proteins and arrest disulfide bond formation.
4. Add 500 μ l ice-cold 2% CHAPS/HeBS and vortex in a microcentrifuge tube rack vortex mixer at medium speed for 10 min at 4°C to lyse ER microsomes.
5. Add 100 μ l of 50% protein G (or A)-Sepharose bead slurry and rotate end over end on a tube rotator for 1 hr at 4°C, to preclear sample.

For a discussion and chart on the use of protein A versus protein G, see UNIT 7.2 (Table 7.2.1).

6. Centrifuge sample 5 min at $2900 \times g$, 4°C , and divide sample among three 1.5-ml microcentrifuge tubes containing 100 μl of 50% protein G (or A)–Sepharose and 2 to 10 μl of either a conformation-specific mAb against the protein of interest, a preimmune control antibody, or a control antibody that recognizes all conformations of the protein of interest.

A titration with antiserum may be required to obtain optimal results. Excess antibodies may give nonspecific binding or saturate the protein G (or A)–Sepharose.

7. Immunoprecipitate and analyze the proteins using nonreducing and reducing SDS-PAGE and gel autoradiography as described (see Basic Protocol 1, steps 13 to 28).

BASIC PROTOCOL 5

ANALYSIS OF PROTEIN FOLDING AND DISULFIDE BOND FORMATION IN CELLS GROWN IN INTACT MONOLAYERS (ADHERENT CELLS)

Co-translational and post-translational folding of a protein can be followed in cells grown in monolayers in tissue culture dishes (Braakman et al., 1991). This procedure allows multiple rapid wash steps, facilitating experiments that require short pulse-chase times. Adherent-cell studies are also ideal for experiments requiring frequent changes of medium. The disadvantage of these studies is that large volumes of medium can be required. When low total volume of medium is desired (e.g., when expensive reagents are needed for the experiment), the analysis can be performed with cells in suspension (see Alternate Protocol 3).

For a detailed explanation of metabolic labeling procedures, see *UNIT 7.1*. In brief, cellular proteins are metabolically labeled in tissue culture dishes with [^{35}S]methionine and [^{35}S]cysteine (the pulse). The labeling medium is removed by aspiration and replaced by medium containing cold (i.e., unlabeled) amino acids, and the cells are incubated (the chase). Samples are removed at various times and oxidative intermediates trapped with chilled buffer containing alkylating agents. Because all cellular proteins are labeled by this procedure, analysis of a specific protein requires isolation by immunoprecipitation (*UNIT 7.2* or see Basic Protocol 1). The isolated protein can then be analyzed for disulfide bond formation (Basic Protocol 1), protease sensitivity (Basic Protocol 2), chaperone binding (Basic Protocol 3), or folding with conformation-specific antibodies (Basic Protocol 4).

Materials

Adherent cells grown to 80% to 90% confluency in a 60-mm tissue culture dish

Depletion medium: cysteine- and methionine-free tissue culture medium, 37°C

Labeling medium: depletion medium containing 0.125 to 0.75 mCi/ml

[^{35}S]methionine and [^{35}S]cysteine (prepared fresh), 37°C

Chase medium (see recipe), 37°C

Stop buffer: 20 mM NEM (see recipe for 1 M stock) in PBS, prepared just before use and kept on ice

Lysis buffer (see recipe), ice cold

Aspirator

Cell scraper

Additional reagents and equipment for immunoprecipitation (*UNIT 7.2* or see Basic Protocol 1) and analysis of disulfide bond formation (see Basic Protocol 1), protein folding by proteolytic sensitivity (see Basic Protocol 2), chaperone binding (see Basic Protocol 3), or protein folding with conformation-specific antibodies (see Basic Protocol 4)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

NOTE: For the analysis of co-translational protein folding no reducing agents are added to either the pulse or chase medium. Chase medium should contain 1 mM cycloheximide in order to stop protein synthesis. In the case of post-translational protein folding and disulfide bond formation, reducing agent (5 mM DTT final) is added to the pulse medium. When the reducing agent is removed after pulse labeling, redox conditions are restored that support the formation of disulfide bonds.

NOTE: Separate cell culture dishes will be required for each condition and time point.

Perform metabolic labeling

1. Use an aspirator to remove culture medium from adherent cells grown to 80% to 90% confluency in a 60-mm tissue culture dish, and wash dish twice by gently swirling with 10 ml prewarmed depletion medium, aspirating medium after each wash.

Prior to the experiment, the number of chase time points should be determined. Depending on the cell type, a confluent 60-mm dish will contain $1.8-7 \times 10^6$ cells, or enough for approximately one time point. Other dish sizes can be used. The volumes should be adjusted accordingly based on the surface area of the dish.

2. Add 5 ml prewarmed depletion medium and incubate 15 min in a humidified 37°C, 5% CO₂ incubator to deplete intracellular pools of methionine and cysteine.
3. Remove medium from cells, add 450 µl labeling medium, and incubate 10 to 60 min in a CO₂ incubator.

It is critical that the plates sit on a perfectly horizontal surface during incubation to avoid drying of the cell monolayer.

Mammalian translation rates are on the order of five amino acids per second. Labeling time will depend on the length and the expression level of the protein of interest, as well as the number of methionine and cysteine residues in the protein.

4. Remove and discard labeling medium.

Perform chase

- 5a. *For 0-min chase:* Add 2 ml chase medium and gently rock solution over cells. Rapidly aspirate chase medium, immediately add 2.5 ml ice-cold stop buffer, and place dish on ice.

The chase medium contains the protein synthesis inhibitor cycloheximide (1 mM final).

- 5b. *For other chase times:* Add 2 ml chase medium and gently rock solution over cells. Aspirate medium, replace with 2 ml fresh chase medium, and incubate for desired times in the CO₂ incubator. Rapidly aspirate chase medium, immediately add 2.5 ml ice-cold stop buffer, and place dish on ice.

Chase times are dependent upon the time it takes the protein of interest to oxidize, fold, and exit the endoplasmic reticulum (ER).

Prepare lysate

6. Remove stop buffer and add 2.5 ml fresh stop buffer. Aspirate dish dry.
7. Add 600 µl ice-cold lysis buffer.

8. Scrape off cells using a cell scraper and collect in a 1.5-ml microcentrifuge tube.
9. Microcentrifuge 5 min at $11,800 \times g$ (12,000 rpm), 4°C , to pellet nuclei.
10. Transfer post-nuclear supernatant to a new 1.5-ml microcentrifuge tube.
11. Analyze disulfide bond formation (see Basic Protocol 1, steps 5 to 28), protein folding by proteolytic sensitivity (see Basic Protocol 2, steps 7 to 12), chaperone binding (see Basic Protocol 3, steps 7 to 8), or protein folding with conformation-specific antibodies (see Basic Protocol 4, steps 5 to 7). Immunoprecipitate the protein of interest (*UNIT 7.2*) if needed.

To analyze the disulfide bond formation (Basic Protocol 1) use ice-cold PBS without NEM for the stop buffer (steps 5b and 6 above). Divide the sample appropriately after step 10 and alkylate with either NEM or AMS. Keep one sample that is not alkylated on ice as a control.

When analyzing for proteolytic sensitivity immunoprecipitate (UNIT 7.2) the 600 μl of post nuclear lysate from step 10 prior to performing steps 7 to 12 of Basic Protocol 2.

ALTERNATE PROTOCOL 3

ANALYSIS OF PROTEIN FOLDING AND DISULFIDE BOND FORMATION IN SUSPENDED (NONADHERENT) CELLS

When cells do not adhere adequately or when it is desirable to use small volumes of reagents for analysis (i.e., when expensive drugs are used), cells in suspension may be used to analyze protein folding and disulfide bond formation. The advantage with this approach is that samples at different chase intervals can be divided from the same population of ^{35}S -labeled cells. Cells are collected in 15- or 50-ml polystyrene conical centrifuge tubes and the pulse-chase is performed in the tube. However, the exchange of media and buffers requires centrifugation of cells. Each chase time point should have $\sim 1 \times 10^6$ cells. Co-translational and post-translational folding can be followed using appropriate reducing and oxidizing conditions in the pulse and chase medium (see Alternate Protocol 1) and analyzed for disulfide bond formation (Basic Protocol 1), protease sensitivity (Basic Protocol 2), chaperone binding (Basic Protocol 3), or protein folding with conformation-specific antibodies (Basic Protocol 4).

Additional Materials (also see Basic Protocol 5)

Cell suspension

Suspension labeling medium: depletion medium containing 25 to 50 $\mu\text{Ci/ml}$ [^{35}S]methionine and [^{35}S]cysteine (prepared fresh), 37°C

15-ml sterile polystyrene conical centrifuge tubes

Beckman GPR centrifuge or equivalent

37°C water bath

Additional reagents and equipment for immunoprecipitation (*UNIT 7.2* or see Basic Protocol 1) and for analysis of disulfide bond formation (see Basic Protocol 1), protein folding by proteolytic sensitivity (see Basic Protocol 2), chaperone binding (see Basic Protocol 3), or protein folding with conformation-specific antibodies (see Basic Protocol 4)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C , 5% CO_2 incubator unless otherwise specified.

NOTE: Keep cells in suspension by periodically swirling the tube during incubations.

Collect cells

1. Collect cell suspension in a 15-ml sterile polystyrene conical centrifuge tube.

Prior to the experiment, the minimum volume in which the cells are to be suspended, the number of chase time points, and the desired sample volume should be determined. Approximately 1×10^6 cells should be used per time point. The volumes should be scaled up accordingly; a 50-ml conical centrifuge tube should be used for larger volumes.

The cells can also be collected by treating adherent cells with sterile 0.25% (w/v) trypsin/0.2% (w/v) EDTA at room temperature and resuspending in regular culture medium. (Some cell types require EDTA for detachment while others do not. This must be determined in each case.) Cells are resuspended in normal growth medium by pipetting up and down two to three times.

2. Centrifuge cell suspension 5 min at $250 \times g$ (1200 rpm), room temperature, in a Beckman GPR centrifuge and discard the medium.

The pelleting efficiency may vary with different cell types.

3. Resuspend cells in 0.5 ml per time point depletion medium and centrifuge 5 min at $250 \times g$, room temperature.

For resuspending cells, a minimal volume of depletion medium should be used.

4. Discard supernatant and resuspend cells in 0.5 ml per time point depletion medium and incubate 30 to 60 min in a 37°C water bath to deplete intracellular methionine and cysteine. Resuspend the cells periodically by gently swirling the tube.

5. Centrifuge cells 5 min at $250 \times g$, room temperature, and aspirate supernatant.

Perform pulse-chase labeling

6. Resuspend cells in 2 ml suspension labeling medium. Cap tube tightly and incubate for the desired pulse time at 37°C . Resuspend the cells periodically by swirling the tube.

A 15- to 30-min pulse is usually sufficient. However, labeling time will depend on the length and expression level of the protein of interest, as well as the number of methionine and cysteine residues in the protein.

7. Centrifuge cells 5 min at $250 \times g$, room temperature, and aspirate supernatant. Resuspend cells in 0.5 ml per time point chase medium and centrifuge again. Aspirate supernatant and resuspend cells in 0.5 ml per time point chase medium.

Chase medium contains a protein synthesis inhibitor (1 mM cycloheximide).

For experiments with multiple time points, a single population of cells is labeled, and then aliquots (each containing $\sim 1 \times 10^6$ cells) are removed at appropriate chase time points. Because the cells settle to the bottom of the tube during the pulse and chase intervals, the tube should be swirled gently at regular intervals and the cells mixed thoroughly by pipeting up and down several times before dividing.

- 8a. *For 0-min chase:* Immediately take an aliquot of cells ($\sim 10^6$ cells) and add an equal volume of ice-cold lysis buffer to the aliquot, mix well, and place on ice.

Alternatively, 14 mM 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid disodium salt (AMS) can be used instead of NEM in the lysis buffer. When analyzing disulfide bond formation AMS provides a larger molecular weight shift than NEM.

- 8b. *For other chase times:* Incubate cell suspension for the desired chase time at 37°C . At the end of each of the appropriate chase times, take an aliquot of $\sim 10^6$ cells and add an equal volume ice-cold lysis buffer, mix well, and place on ice.

Alternatively, 14 mM 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid disodium salt (AMS) can be used instead of NEM in the lysis buffer.

Prepare lysate

9. Transfer sample to a 1.5-ml microcentrifuge tube, and vortex sample 10 min in a microcentrifuge tube rack vortex mixer at full speed, 4°C, to ensure complete cell lysis.
10. Microcentrifuge 10 min at 11,800 × g, 4°C.
11. Discard nuclear pellet. Transfer postnuclear supernatant to a new 1.5-ml microcentrifuge tube.
12. Analyze disulfide bond formation (see Basic Protocol 1, steps 5 to 28), protein folding by proteolytic sensitivity (see Basic Protocol 2, steps 7 to 12), chaperone binding (see Basic Protocol 3, steps 7 to 8), or protein folding with conformation-specific antibodies (see Basic Protocol 4, steps 5 to 7). Immunoprecipitate the protein of interest (see *UNIT 7.2*) if needed.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

4-Acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid disodium salt (AMS), 20 mM

5.36 mg AMS (Molecular Probes; 20 mM final)
40 μl 1 M Tris·Cl, pH 6.8 (*APPENDIX 2A*; 80 mM final)
25 μl 20% (w/v) SDS (*APPENDIX 2A*; 1% final)
H₂O to 0.5 ml
Prepare fresh daily, keep on ice, and avoid exposure to light
AMS is sensitive to light and hydrolysis.

Bismaleimidohexane (BMH), 100 mM

2.76 mg bis-maleimidohexane (BMH; Pierce Chemical)
100 μl 50% (v/v) dimethyl sulfoxide (DMSO)
Store up to 6 months at –20°C
Dilute as needed in 50% (v/v) DMSO

Chase medium

2 ml 1 M HEPES buffer (see recipe), pH 7.3 (20 mM final)
0.5 ml 1 M methionine (see recipe; 5 mM final)
0.5 ml 1 M cysteine (see recipe; 5 mM final)
2 ml 50 mM cycloheximide (see recipe; 1 mM final)
Tissue culture medium to 100 ml
Prepare fresh daily
The tissue culture medium should be appropriate for the cells of interest.

Cycloheximide, 50 mM

0.28 g cycloheximide
H₂O to 10 ml
Store 1-ml aliquots up to 2 years at –20°C

Cysteine, 1 M

1.212 g L-cysteine
H₂O to 10 ml
Store 1-ml aliquots up to 2 years at –20°C

Denaturing immunoprecipitation buffer

5 ml 1 M Tris·Cl, pH 7.6 (*APPENDIX 2A*; 10 mM final)
70 ml 1 M NaCl (140 mM final)
2 ml 250 mM EDTA, pH 8.0 (*APPENDIX 2A*; 1 mM final)
5 ml Triton X-100 (1% final)
H₂O to 500 ml
Store up to 6 months at 4°C

Flavin adenine dinucleotide (FAD), 25 mM

0.207 g FAD
H₂O to 10 ml
Store in 1-ml aliquots up to 2 years at -20°C

Glutathione (GSSG), 100 mM, pH 7.0

0.656 g oxidized GSSG
9 ml H₂O
Adjust pH to 7.0 with 1 N NaOH
H₂O to 10 ml
Store 1-ml aliquots up to 2 years at -20°C

NaOH is used here because K⁺ concentrations are more critical in maintaining the fidelity of the translation system.

HEPES buffer

260.3 g *N*-2-hydroxyethylpiperazine-(*N'*2)-2-ethanesulfonic acid (HEPES), sodium salt
H₂O to 800 ml
Adjust pH to 7.2, 7.3, or 7.5 with 10 N KOH
H₂O to 1 liter
Store up to 6 months at 4°C

HEPES-buffered saline (HeBS)

50 ml 1 M HEPES buffer (see recipe), pH 7.5 (50 mM final)
11.69 g NaCl (200 mM final)
H₂O to 1 liter
Store up to 6 months at 4°C

HEPES/potassium acetate buffer

18 ml 1 M HEPES buffer (see recipe), pH 7.2 (90 mM final)
10 ml 1 M potassium acetate, pH 7.2 (50 mM final)
H₂O to 200 ml
Store up to 6 months at 4°C

Isotonic resuspension buffer

2.5 ml 1 M sucrose (250 mM final)
1 ml 1 M potassium acetate (100 mM final)
50 µl 1 M magnesium acetate (5 mM final)
0.5 ml 1 M HEPES buffer (see recipe), pH 7.5 (50 mM final)
H₂O to 10 ml
Store up to 6 months at 4°C

Isotonic sucrose cushion

2.5 ml 1 M sucrose (250 mM final)
0.5 ml 1 M potassium acetate (500 mM final)
50 µl 1 M magnesium acetate (5 mM final)
0.5 ml 1 M HEPES buffer (see recipe), pH 7.5 (50 mM final)
H₂O to 10 ml
Store up to 6 months at 4°C

KHM buffer

55 ml 1 M potassium acetate (110 mM final)
1 ml 1 M magnesium acetate (2 mM final)
10 ml 1 M HEPES buffer, pH 7.2 (see recipe; 20 mM final)
H₂O to 500 ml
Store up to 6 months at 4°C

KHM-STI buffer

100 ml KHM buffer (see recipe)
200 µl 50 mg/ml soybean trypsin inhibitor
Prepare fresh daily and store at 4°C

Lysis buffer

0.2 ml 10% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in HeBS (see recipe; 2% final)
26.7 µl 150 mM PMSF (see recipe; 0.4 mM final)
10 µl 10 mg/ml leupeptin (10 µg/ml final)
80 µl 1.25 mg/ml aprotinin (10 µg/ml final)
0.2 ml 1 M NEM (see recipe; 20 mM final)
PBS or similar buffer to 10 ml
Add PMSF, leupeptin, aprotinin, and NEM from concentrated stocks just before use

Methionine, 1 M

1.49 g L-methionine
H₂O to 10 ml
Store 1-ml aliquots up to 2 years at -20°C

N-ethylmaleimide (NEM), 1 M

1.25 g NEM
10 ml 100% (v/v) ethanol
Store in 1-ml aliquots up to 2 years at -20°C protected from light
Dilute as needed with 100% (v/v) ethanol

NEM is sensitive to hydrolysis in water and light. The number of freeze thaws should be limited as the NEM will precipitate out and the solution will turn yellow.

Nonreducing sample buffer, 2×

4.5 g SDS (9% final)
7.5 ml glycerol (15% final)
1.5 ml 1 M Tris-Cl, pH 7.8 (APPENDIX 2A; 30 mM final)
25 mg bromphenol blue (0.05% final)
H₂O to 50 ml
Store up to 1 year at room temperature

Phenylmethylsulfonyl fluoride (PMSF), 150 mM

2.613 g PMSF
10 ml DMSO
Store in 500-µl aliquots up to 2 years at -20°C

PMSF is highly unstable in water: the half life is ~30 min at 37°C and a few hours on ice.

Protein A-Sepharose, 10%

0.5 g protein A-Sepharose
20 ml 0.5% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in HeBS (see recipe for HeBS)
Store up to 6 months at 4°C

Salicylate, 2%

2 g sodium salicylate

30 ml methanol

H₂O to 100 ml

Cover the bottle with aluminum foil and store up to 6 to 12 months at room temperature

CAUTION: Gloves should be worn at all times; sodium salicylate can elicit allergic reactions and is readily absorbed through the skin.

Sucrose cushion, 1×

5 ml 1 M sucrose (0.5 M final)

76.1 μ l 98% (w/v) triethanolamine (50 mM final)

H₂O to 10 ml

Store up to 6 months at 4°C

Trypsin/EDTA solution

90 ml 1× S-MEM (from 10× S-MEM; Life Technologies) containing 0.23% (w/v) NaCO₃ mix and adjust to pH 4.1 to 4.2

10 ml 2.5% trypsin/2% EDTA (Life Technologies; 0.25% final trypsin and 0.2% final EDTA)

Prepare fresh daily

COMMENTARY

Background Information

The early maturation events for a protein in the secretory pathway involve protein folding and oxidation in the endoplasmic reticulum (ER). The folding process commences co-translationally as the protein is vectorially inserted into the ER lumen and is generally completed post-translationally once the protein is released from the ribosome (Braakman et al., 1991; Chen et al., 1995; Fig. 15.6.2). Protein folding can be monitored by using antibodies for immunoprecipitation that recognize specific epitopes that appear or disappear during the folding process (see Basic Protocol 4). In addition, as a protein folds and stabilizes, it becomes more resistant to protease digestion. Domains that are cleaved by the protease in the unfolded state become concealed within the protein core making them inaccessible to proteases (see Basic Protocol 2).

The ER lumen provides an intracellular compartment that is topologically equivalent to the extracellular space. This allows the preparation of proteins that will be secreted and will reside outside the cell. The ER lumen is an oxidizing environment that supports the formation of disulfide bonds. Disulfide bonds can form within a protein (intramolecular) or between two different polypeptide chains (intermolecular). Intramolecular disulfide bond formation can begin co-translationally and is often completed post-translationally. Usually, disul-

fide bond formation results in a more compact structure that can be resolved following nonreducing SDS-PAGE as an increase in protein mobility (see Basic Protocol 1). As a general rule, intermolecular disulfides form after the folding and oxidation of individual polypeptides. Once this occurs, the two subunits can come together to form an intermolecular covalent bond causing a decrease in protein mobility on nonreducing SDS-polyacrylamide gels.

Besides providing an environment for disulfide bond formation, the ER also contains a variety of resident proteins that assist in the protein maturation process. These proteins include covalent modifiers that add glycans, remove carbohydrates or signal sequences, and catalyze the formation of disulfide bonds as well as molecular chaperones that assist in translocation, protect vulnerable nascent chains from aggregation, and retain misfolded proteins. These proteins play a central role in defining the ER, retaining misfolded or misassembled proteins so they do not travel through the secretory pathway and ensuring the high success rates of nascent protein maturation observed in the cell.

Pulse-chase experiments in live cells permit the biogenesis of a protein to be followed after sufficient radiolabel has been incorporated for visualization (see Critical Parameters, discussion of protein radiolabeling). Currently this methodology provides the most sensitive meth-

Table 15.6.3 Troubleshooting Guide for Protein Folding Analyses^a

Problem	Possible causes	Solutions
Little or no protein translation	Ionic strength not optimized for transcript	Perform a K ⁺ titration, or purify mRNA to remove residual buffer salts and then perform K ⁺ titration
	Oxidation conditions are inhibitory	Perform a titration with GSSG or FAD to determine best oxidation and translation conditions
	Microsome or SP-cell concentrations are inhibitory (i.e., too high)	Perform a microsomal or SP-cell titration to optimize translation and translocation
	mRNA degradation	Check mRNA on agarose gel for degradation; monitor working environment and supplies more carefully to prevent RNase degradation while performing a new transcription
Little or no translocation	Transcript too large	Translate individual domains of the protein
	Microsome or SP-cell concentrations not optimized	Perform a microsomal or SP-cell titration
	SP cells not permeabilized	Make sure cells are not overconfluent. Use an appropriate amount of digitonin after counting cells for optimum permeabilization
	Poor signal sequence	Replace old signal sequence with a powerful ER-targeting signal sequence
No post-translational oxidation	SRP degraded or absent	If using wheat germ, switch to reticulocyte lysate or add purified SRP
	Too much oxidizing agent	Check nonreducing gel for aggregates at the top and within the stacker; if there are a lot, decrease oxidizing agent concentration
Inability to co-immunoprecipitate protein	Insufficient oxidizing agent	Perform titration with GSSG or FAD
	Detergent is denaturing	Use less or change detergent in the lysis buffer to a more native condition (e.g., digitonin, Triton X-100)
	Not enough antibody Binding too transient	Perform antibody titration Cross-link prior to immunoprecipitation Deplete ATP with apyrase before adding antibody
Trypsin overdigestion	Too much trypsin	Perform a trypsin titration
	Incubation too long	Incubate at 4°C for shorter time Pick the best concentration and perform a time course for digestion

continued

Table 15.6.3 Troubleshooting Guide for Protein Folding Analyses^a, *continued*

Problem	Possible causes	Solutions
No trypsin digestion	Incubation temperature too high or low	Pick the best digestion time and perform a digestion at various temperatures
	Microsomes or SP cells not lysed	Use a higher concentration of lysis buffer
	Incubation time too short	Pick the best concentration and perform a time course for digestion
	Trypsin not the appropriate protease for the protein	Try proteinase K, chymotrypsin, or another protease
Inability to cross-link	No reactive groups available in the protein	Check protein sequence, or structure if available, to determine the best cross-linker Try a nonspecific broad-range UV cross-linker
	Too much cross-linking	Check stacker and top of gel for aggregates; if present, try decreasing the amount of cross-linker
Nonreducing gel is blurry	pH of resolving buffer incorrect	Test pH of the resolving buffer to make sure it is 8.8
	Stacking gel too small	Increase size of the stacker and decrease current when running sample through the stacker
	Gel too thick	Decrease thickness of the gel

^aAbbreviations: ER, endoplasmic reticulum; FAD, flavin adenine dinucleotide; GSSG, oxidized glutathione; SP, semipermeabilized.

ods to monitor the synchronous maturation of nascent proteins in the complexity of the live cell. The pulse-chase approach can also be used with an *in vitro* translation system coupled with canine pancreatic microsomes to study the maturation of proteins that can be controlled and manipulated to a higher degree than intact cells. Originally, this system was used to study the translocation of secretory-pathway proteins across the ER membrane (Blobel and Dobberstein, 1975). More recently, it has been shown to support the maturation of proteins from their earliest folding intermediate to the mature oligomer through careful control of the redox conditions (Hebert et al., 1996). The *in vitro* translation system can also be coupled with semipermeabilized (SP) cells to combine the facilitated production of radiolabeled proteins with the maturation in an intact ER (Wilson et al., 1995). Together, these various biological systems can be combined with the protocols described to dissect the protein folding and oxidation pathway in the ER.

Critical Parameters

Protein radiolabeling

All the methods described in this unit involve the generation of a radiolabeled protein, which permits the monitoring of its maturation. In choosing a labeling reagent, the amino acid composition of the protein should be considered so that an adequate number of radiolabeled sites will be incorporated. Most commonly, a mixture of ³⁵S-labeled methionine and cysteine is used, but other radiolabeled amino acids are also available for use with proteins lacking an adequate number of cysteines or methionines. The number of radiolabeled sites, the length of the protein, and the level of protein expression will all be important for determining the length of the radioactive pulse. Generally with cells, a 15- to 30-min radioactive pulse is sufficient for visualization of a moderately expressed protein by autoradiography. *In vitro* translation studies require a longer pulse period because the translation rate is three to five times slower and the optimal temperature for translation is lower at ~27°C. A pulse time of 1 hr is sufficient for most transcripts. Chase times are highly

protein dependent, and a variety of durations (1 to 5 hr) should be explored.

In vitro translation systems

Success with the *in vitro* translation systems used in Basic Protocols 1 to 4, and Alternate Protocols 1 and 2 is dependent on optimizing the protein translation, translocation, and maturation processes. For specific details for the optimization of each step, see Table 15.6.3. In short, translation levels rely on the integrity of the transcript used, as well as salt concentrations. It is also common for different reticulocyte lysate or wheat germ preparations (*UNIT 11.2*) to translate a given transcript with varying levels of success. The efficiency of protein targeting to and translocation into the ER membrane source (ER-derived microsomes or SP cells) is highly dependent on the membrane concentration. Care must also be taken to ensure that a luminal redox environment that supports disulfide bond formation is maintained using the oxidizing agents GSSG or FAD. However, the extralumenal environment cannot be overly oxidizing as this will inhibit translation. Therefore, a thorough titration with the oxidizing agent is needed, and in many cases this has to be repeated for every batch of reticulocyte lysate or wheat germ or membrane preparation employed. When SP cells are used as membranes, the cell concentration may be varied for efficient translation and translocation.

Cell studies

Because pulse-chase experiments in cells involve the production of a lysate containing thousands of radiolabeled proteins, an immunoprecipitation procedure is essential to monitor a single individual protein. This often results in a high background. To optimize the signal-to-background ratio, a number of wash buffers at various temperatures and different numbers of wash steps should be tested (*UNIT 7.2*).

Care must also be taken to ensure that the adherent cells remain attached to the cell dish during the many wash steps performed with medium changes (see Basic Protocol 5). The presence of Ca^{2+} and Mg^{2+} in the wash buffer aids in cell adherence.

Monitoring protein maturation

To characterize the protein folding and oxidation process, the maturation process has to be stopped and the protein conformation maintained during the extraction and analysis procedures. An alkylating agent such as NEM, AMS, iodoacetamide, or iodoacetic acid can be

used to prevent further formation of disulfide bonds. To preserve the protein's conformation during the extraction procedure, nondenaturing detergents such as CHAPS, Triton X-100, digitonin, Nonidet P-40, or octyl glucoside should be used for membrane and protein solubilization (*UNIT 5.1*). In some cases, these gentle detergents may also preserve the interactions with ER resident molecular chaperones and therefore can be used to monitor these interactions.

Anticipated Results

Regardless of the system used for the studies (adherent, suspended, or SP cells or ER-derived microsomes), a similar maturation pathway should be identified for a given protein. However, because the rate of translation with the *in vitro* translation system is 3- to 5-fold slower than in live cells, the time frame for maturation may vary. As a protein is oxidized, it will generally migrate faster on nonreducing SDS-polyacrylamide gels (Fig. 15.6.1). For the influenza glycoprotein hemagglutinin (HA), two folding intermediates have been identified that are designated intermediate 1 and 2 (IT1 and IT2; Fig. 15.6.3). The native (NT) protein, with all its disulfides in place, migrates at an even faster rate. Overall, a shift that corresponds to ~15 kDa is observed between the reduced (R) and completely oxidized (NT) protein on non-reducing SDS-polyacrylamide gels. On a reducing gel, all oxidative forms migrate with a similar mobility, indicating that the molecular mass of the oxidative form is the same and that the differences in mobility are due solely to disulfide bond formation. Oxidation can be followed under normal physiological conditions (co-translationally; Figs. 15.6.2 and 15.6.3), or synchronous oxidation can be monitored by accumulating the protein under reducing conditions and initiating oxidation post-translationally (Figs. 15.6.2 and 15.6.3).

The alkylation of free thiols with alkylating agents can also be used to accentuate mobility shifts observed for oxidative forms of a protein by SDS-PAGE. The larger the number of free thiols, the more alkylating groups should attach to the polypeptide, producing an enhanced shift on reducing SDS-polyacrylamide gels (Fig. 15.6.1).

Chaperone binding to the oxidative intermediates can be probed by co-immunoprecipitation studies. Interactions between the lectin chaperone calnexin and HA were analyzed by immunoprecipitating with anti-calnexin antibodies (Fig. 15.6.3B). Calnexin binds to proteins possessing monoglucosylated glycans

(UNIT 15.1; Fig. 15.1.3). Calnexin bound to all oxidative forms of HA under oxidizing conditions. However, under reducing conditions, the lectin-binding properties of calnexin were inhibited by the reduction of a calnexin intramolecular disulfide bond (Fig. 15.6.3B, compare lanes 4 and 5; Hebert et al., 1995). In the microsomal system at 27°C (Fig. 15.6.3), binding to calnexin persists even after 120 min of post-translational oxidation. Release from the chaperone and oligomerization can be observed if the chase temperature is increased to 32°C (Hebert et al., 1996).

Under reducing conditions, one protein band should be observed with cell studies; however, with systems that use in vitro translations, two bands should be generated for glycosylated substrates. Because protein targeting and translocation are not 100% efficient with the in vitro translation systems, a common artifact is the production of an untranslocated product. This protein retains its signal sequence and does not receive glycosylations. Therefore it migrates faster than the glycosylated and translocated form (Fig. 15.6.3).

Time Considerations

In vitro translation experiments require 1 hr for translation with chase times varying depending on the protein. For cell studies, the cells are starved for 2 hr prior to the pulse-chase experiment, which can take an additional 1 to 8 hr depending upon the chase times. SP-cell preparation requires 2 to 3 hrs. Processing of samples using both systems requires 90 min followed by an overnight (or 4 to 6 hr) incubation with antibodies for immunoprecipitations. Sample preparation, including cross-linking and protease sensitivity studies, requires ≥90 min after the antibody binding step. Immuno-

precipitates can then be analyzed immediately by SDS-PAGE or frozen for future analysis.

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Measurements of Phagocytosis and Phagosomal Maturation

Invading microorganisms and other foreign particles, as well as apoptotic bodies, are eliminated in the organism by a process known as phagocytosis (Aderem and Underhill, 1999; Greenberg and Grinstein, 2002). The elimination process can be conceptually divided into two stages: an initial uptake phase that involves ingestion of the target particle into a plasma membrane–derived vacuole, known as the phagosome, and a secondary phase in which the particle is degraded and the debris are cleared. The nascent phagosome, which is generated by invagination of the plasmalemma, lacks the machinery required for particle degradation and clearance. These capabilities are acquired subsequently by an exquisitely coordinated sequence of membrane fusion and fission events collectively termed phagosomal maturation (Vieira et al., 2002). The objective of this unit is to briefly overview the processes of phagosome formation and maturation, and to provide protocols to assess and quantify their occurrence.

Phagocytosis is initiated by the interaction of receptors on the plasma membrane of the effector cell, or phagocyte, with their cognate ligands on the surface of the target particle. The ligands can be endogenous components of the target particle, such as lipopolysaccharides of the bacterial wall, or the phosphatidylserine that becomes exposed on the outer monolayer of apoptotic cells. However, particles lacking intrinsic ligands can also be targeted for phagocytosis by a process called opsonization, which entails coating of their surface with host-derived proteins that are themselves recognized by phagocytic receptors. Such coating host proteins, termed opsonins, include immunoglobulins, components of the complement cascade, and other serum proteins such as thrombospondin. Different receptors are responsible for the recognition of these opsonins, and they, in turn, differ from the receptors that mediate nonopsonic phagocytosis. The responses triggered by these different receptors are not identical, and one must therefore appreciate that phagocytosis is not a single phenomenon, but a term of convenience used to encompass a collection of different, yet related, processes.

At the molecular level, the sequence of events that result in particle engulfment has only been studied in some detail for a couple of receptor types. Briefly, engagement by their ligands on the particle surface induces the clustering and activation of the receptors. Though none of the phagocytic receptors studied to date possess intrinsic kinase activity, phosphorylation has been identified as the most proximal response to receptor clustering. Tyrosine kinases of the Src family are an integral part of these early events, which are rapidly followed by extensive remodeling of phospholipids in the immediate vicinity of the phagocytic cup. Kinases and lipases that target phosphoinositides are activated during the early stages of phagocytosis, as are phospholipases D and A2. Jointly, the products of tyrosine phosphorylation and lipid metabolism recruit and activate other effectors that direct two principal responses: the extensive remodeling of the actin cytoskeleton and the focal fusion of endomembranes with the membrane of nascent and early phagosomes. The cytoskeletal reorganization is complex and incompletely understood, but early activation of Rho-family GTPases is believed to be essential. In contrast, little is known about the determinants of membrane fusion and fission that initiate phagosomal maturation.

Phagosomal maturation resembles, yet is not identical to, the progression of internalized fluid or solutes along the endocytic pathway. Both processes involve sequential fusion with components of the early and late endosomal compartments and culminate in coalescence with lysosomes. It is thought that the lysosomal hydrolases play a central

role in microbial killing and in the degradation and disposal of foreign particles in general. The lumen of the hybrid organelles, formed as the phagosome merges with components of the endocytic pathway, undergoes a gradual acidification that is similarly important for the microbicidal and degradative functions of the phagosome.

Simple protocols are included for the assessment of Fc γ R-mediated phagocytosis (see Basic Protocol 1, Alternate Protocol 1, and Support Protocol 1), for the assessment of complement-mediated phagocytosis (see Basic Protocol 2, Alternate Protocol 2, and Alternate Protocol 3), and for flow cytometric analysis of phagocytosis (see Basic Protocol 3). These protocols include steps for opsonization of target particles and for the assessment of the efficiency of phagocytosis. Additional protocols are provided for assessment of phagosomal acidification (see Basic Protocol 4 and Alternate Protocol 4) and maturation (see Basic Protocol 5, Alternate Protocol 5, and Alternate Protocol 6). Finally, a protocol is provided for assessment of inhibition of phagocytosis (see Basic Protocol 6). Phagocytosis assays are illustrated schematically in Figure 15.7.1.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

ASSESSMENT OF Fc γ RECEPTOR-MEDIATED PHAGOCYTOSIS

Particles opsonized by immunoglobulins are recognized by Fc γ receptors (Fc γ R), that specifically interact with the Fc portion of IgG (McKenzie and Schreiber, 1998; Heyman, 2000). Focal clustering of Fc γ R upon binding to IgG-opsonized materials results in receptor activation and initiation of phagocytosis. There are three general classes of Fc γ R (Fc γ RI, II, and III) that vary in their molecular structure, affinity for different IgG isotypes, and signaling ability. It is noteworthy that not all Fc γ R are equally capable of eliciting phagocytosis. A subgroup of Fc receptors, typified by Fc γ RIIB, are unable to support phagocytosis and in fact generate inhibitory signals.

The protocols below detail several different methods to opsonize particles with IgG and to estimate Fc γ R-mediated phagocytosis. Two types of model particles, namely latex beads and red blood cells (RBC), have been extensively used to assess phagocytosis. The advantages and limitations of these systems are described below. Labeling of the particles with fluorescent probes is recommended in some of the protocols listed below to locate the particles and to quantify their uptake. A wide range of fluorophores is available for these purposes; consult the manufacturers' handbooks to select the probes with the appropriate spectral features that will best suit the particular microscopic setup and experimental design (also see *APPENDIX 1E*).

BASIC PROTOCOL 1

Assessment of Fc γ R-Mediated Phagocytosis Using IgG-Opsonized Red Blood Cells (RBCs)

This section describes a method to coat RBCs with IgG and to assess phagocytosis of the resulting opsonized particles. A procedure for labeling such IgG-coated RBCs by conjugation with an amine-reactive fluorescent dye to facilitate detection is included. The protocol is written for assessment of phagocytosis of the opsonized particles by adherent cells, but it can easily be adapted for use with suspended phagocytic cells.

RBCs are very well suited for the study of phagocytosis, for several reasons: (1) their size is remarkably homogeneous; (2) normal, unopsonized RBCs are rarely internalized by

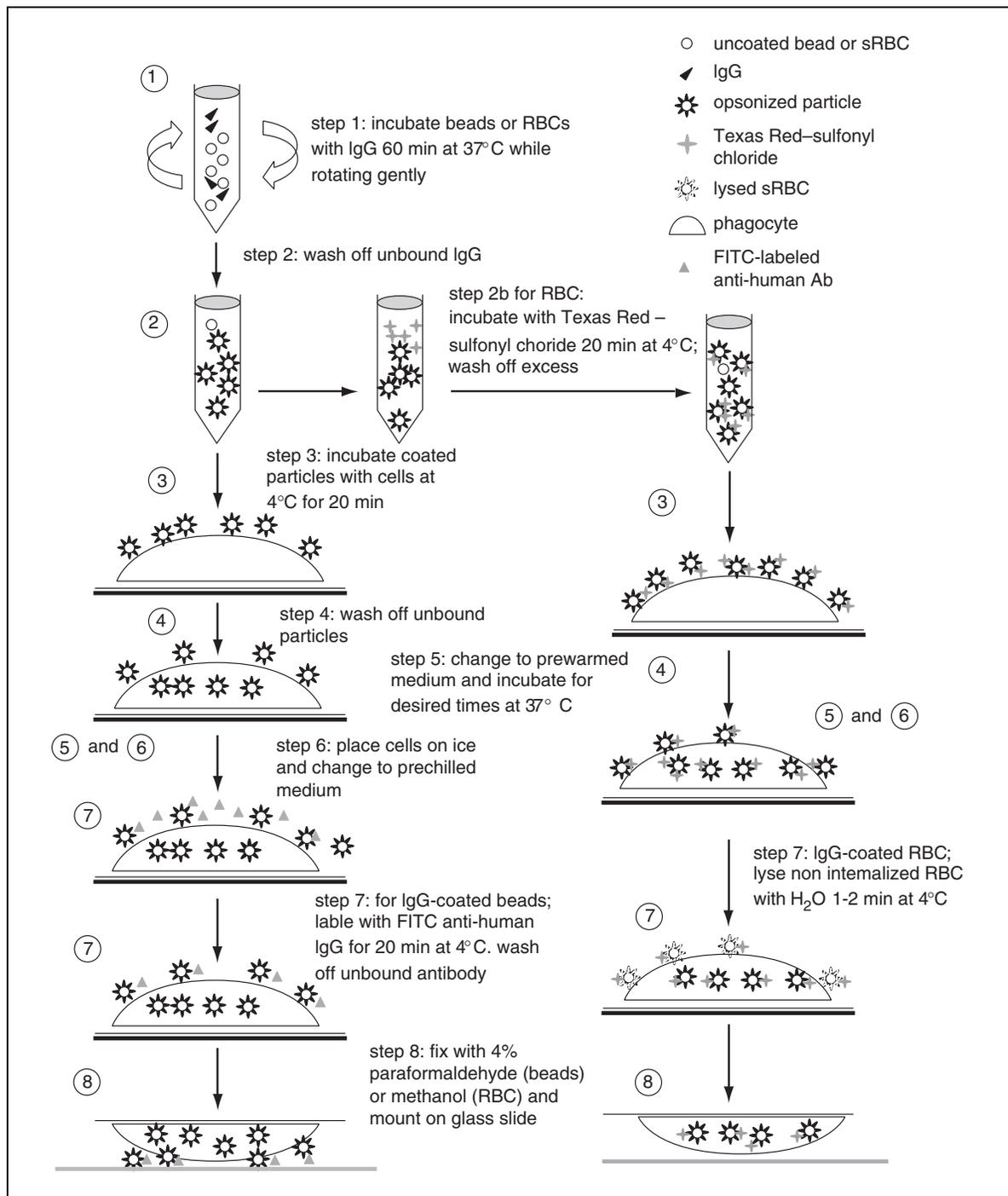


Figure 15.7.1 Schematic representation of the protocol for phagocytosis of IgG-opsonized particles. The procedure outlines the steps involved in the preparation of IgG-coated red cells or latex beads, for the identification of intracellular versus extracellular particles or for elimination of the latter prior to analysis. Note that Texas-Red labeled red cell ghosts that remain adherent (extracellular) after hypotonic lysis will be observable by fluorescence microscopy but not by bright field because they have lost hemoglobin.

phagocytosis, contributing a very low background; (3) the light absorbance of hemoglobin facilitates their detection by light microscopy; and (4) adherent, noninternalized RBCs are susceptible to osmotic lysis, while those protected within phagosomes are not. This provides a simple means of identifying internalized particles. On the other hand, RBCs are malleable and can undergo constriction and fragmentation during phagocytosis, which may lead to an underestimation of the number of phagocytic events.

Materials

Primary monocytes or macrophages (*UNIT 2.2*), or monocytic cell lines (e.g., RAW 264.7 cells ATCC #TIB-71 or J774 cells, ATCC #HB-197).

Culture medium: e.g., DMEM with or without 10% FBS (*APPENDIX 2B*); nominally bicarbonate-free, HEPES-buffered media can be used to obviate the need for a CO₂ atmosphere

Sheep red blood cells (sRBCs; 10% v/v suspension, ICN Biomedicals)

Phosphate-buffered saline (PBS; without Ca²⁺ or Mg²⁺; see recipe)

4% (v/v) paraformaldehyde in PBS (optional)

PBS (see recipe) containing 100 mM glycine

Rabbit IgG stock: reconstitute rabbit antibody (IgG fraction) to sRBC (ICN Biomedicals) with Milli-Q water to 5 mg/ml (store in aliquots at -20°C; store thawed aliquots up to 1 month at 4°C)

HEPES-buffered RPMI 1640 (e.g., Life Technologies)

Texas Red-sulfonyl chloride (Molecular Probes, 1 mg per ampule)

Methanol, prechilled to -20°C

Fluorescence mounting medium (Dako)

6-well tissue culture plates containing sterile glass coverslips (see recipe)

End-over-end rotator

Glass microscope slides

Epifluorescence microscope with bright-field capabilities and appropriate filters (see *UNITS 4.1 & 4.2* and *APPENDIX 1E*)

Additional reagents and equipment for cell culture and counting cells (*UNIT 1.1*) and microscopy (*UNITS 4.1 & 4.2*)

Plate phagocytes (day 1)

1. Grow cells of choice in DMEM with 10% FBS (*UNIT 1.1*). At a point 24 to 48 hr prior to the experiment, passage cells onto sterile glass coverslips contained in 6-well plates (prepared as described in Reagents and Solutions) so that they will reach ~75% confluence at the time of the experiment.

The phagocytic index and rate are easier to quantitate when cells are subconfluent.

Opsonize sRBCs with IgG (day 2)

2. Prewarm the end-over-end rotator within an incubator to 37°C. Keep all reagents on ice until step 8.
3. Place a 200- μ l aliquot of 10% sRBC suspension into a 1.5-ml microcentrifuge tube.
4. Sediment the sRBCs by microcentrifuging 5 to 30 sec at maximum speed.
5. Aspirate supernatant and resuspend cells in 1 ml ice-cold PBS.
6. Wash two more times with ice-cold PBS as in steps 4 to 5.
7. *Optional:* Fix sRBCs with 4% paraformaldehyde prior to opsonization.

This has been found empirically to enhance phagocytosis, most likely by exposing ligands to receptors other than Fc γ R.

- a. Resuspend the sRBCs in 1 ml of 4% paraformaldehyde in PBS.
- b. Incubate overnight at 4°C in end-over-end rotor.
- c. Sediment and wash fixed cells as in steps 4 to 5. Repeat two additional times.
- d. Resuspend the fixed sRBCs in 1 ml PBS containing 100 mM glycine and incubate 20 to 30 min at 4°C to quench free aldehyde groups.
- e. Sediment fixed cells using the microcentrifuge. Aspirate supernatant. Wash twice with 1 ml PBS as in steps 4 to 5.

8. Resuspend the washed sRBCs in 200 μ l PBS.
9. Dilute 1 μ l of rabbit IgG stock into 50 μ l of PBS. Add 4 μ l of the diluted rabbit IgG to the washed sRBCs.

The protocol has been optimized for the sRBCs and rabbit IgG from ICN Biomedicals. However, for other suppliers of sRBCs or IgG it may be necessary to optimize the opsonization ratio by varying the dilution of IgG.

10. Incubate sRBC-rabbit IgG mixture at 37°C for 1 to 1.5 hr in the prewarmed end-over-end rotator (see step 2).

To perform phagocytosis assay using unlabeled opsonized sRBCs

- 11a. Wash opsonized sRBCs from step 10 three times with ice-cold PBS as in steps 4 to 5, and resuspend in 500 μ l ice-cold PBS. Keep on ice until use.
- 12a. Count the opsonized sRBCs using a Coulter counter or hemocytometer (*UNIT 1.1*) and resuspend in cold HEPES-buffered DMEM at $5\text{--}7 \times 10^8$ cells/ml.
- 13a. Proceed to phagocytosis assay (step 14).

To perform phagocytosis assay using opsonized sRBCs labeled with Texas Red-sulfonyl chloride

- 11b. Take one ampule of Texas Red-sulfonyl chloride and dissolve in 0.5 ml ice-cold PBS in a 1.5-ml microcentrifuge tube.

Covered with aluminum foil and stored at 4°C, this stock solution will keep for a several days.

- 12b. Add 100 μ l of the diluted Texas Red-sulfonyl chloride to 0.5 ml of opsonized sRBCs (from step 10). Incubate on ice for 20 min. Mix by inverting the tube every 5 min.

Texas Red-sulfonyl chloride is an amine-reactive dye that labels proteins covalently. As an alternative, IgG prelabeled with the fluorescent dye of choice can be used for opsonization.

- 13b. Wash the IgG-sRBC-dye mixture three times with 1 ml cold PBS, or until the supernatant is clear, using the technique described in step 4 to 5. Resuspend in 0.6 ml cold HEPES-buffered DMEM as in step 12a. Proceed to phagocytosis assay (step 14).

Perform phagocytosis assay (day 2)

14. Place the tissue culture plates with the prepared cells (from step 1) on ice.

Optionally, replace the culture medium with 1 ml fresh, prechilled medium to accelerate the cooling process.

15. For each well to be assayed, prewarm 2 ml of HEPES-buffered RPMI 1640 medium to 37°C.

16. Add 50 to 100 μ l of prepared IgG-sRBCs (from step 13a or b) to each well and incubate on ice for 10 to 20 min.

Ideally, the ratio of IgG-sRBC to phagocytic cells should be between 5:1 and 10:1.

Phagocytosis does not proceed at 4°C. This step will therefore standardize and synchronize the binding of IgG-sRBC to the cells and allow for comparison of different treatment conditions or at different time points.

17. Wash cells gently once with cold medium to remove unbound IgG-sRBC.

18. To initiate phagocytosis, remove cells from ice, aspirate the cold medium, and add 2 ml prewarmed (37°C) DMEM medium per well. Place in 37°C incubator.

The duration of incubation at 37°C will depend on the stage of phagocytosis and phagosomal maturation of interest. For assessment of phagocytic efficiency, 30 min is a convenient point for evaluation.

19. Stop phagocytosis by placing the cells back on ice. Wash with ice-cold PBS.
20. *Optional:* To remove the IgG-sRBCs that have not been internalized, lyse the sRBCs by incubating with 1 ml of water for 2 min at 4°C.

Depending on the cell type being assessed, incubation with water may affect cell morphology and/or integrity. When evaluating different phagocytes, a preliminary time course experiment may be necessary to optimize the duration of hypotonic lysis of sRBC.

21. Wash again with cold PBS. Keep on ice.

Wet mounts of the preparation can be analyzed directly or the cells can be fixed for subsequent analysis. For direct analysis proceed to step 23. For fixation see step 22.

Fix cells (days 2 to 3)

22. Aspirate PBS and add 1 ml per well of prechilled methanol. Incubate on ice 20 min. Wash twice with PBS.

It is important to aspirate gently and add the methanol slowly to the side of the well to avoid dislodging any cells. The authors recommend prechilling the methanol to -20°C. An alternative to fixation with methanol is to fix with 1% to 2% glutaraldehyde. When using sRBCs, do not use paraformaldehyde for fixation because retention of hemoglobin is poor.

Mount coverslips and score phagocytosis

23. Mount coverslips containing cells onto slides with Dako fluorescence mounting medium. Allow to dry at room temperature for several hours prior to visualization.

It is preferable to dry the slides overnight before analysis.

Dab the coverslip on tissue wipe to remove excess fluid before mounting. This will decrease the time required for the slide to dry. To minimize air bubbles in the mounted slide, place a drop of the mounting medium on the glass slide first, then slowly ease the coverslip onto the slide. Slides can be stored for months at -20°C with little loss of fluorescence.

24. Score phagocytosis under microscope.

Only the internalized sRBC remain intact and can be readily identified by bright-field (phase or differential interference contrast) microscopy. Typically, phagocytic efficiency is scored as the number of sRBC internalized per 100 phagocytic cells counted.

Fluorescence microscopy and automated image analysis can be used if the sRBC are coupled to Texas-Red.

ALTERNATE PROTOCOL 1

Assessment of FcγR-Mediated Phagocytosis Using IgG-Opsonized Latex Particles

A second approach to evaluating FcγR-mediated phagocytosis utilizes inert latex beads opsonized with IgG. The advantages of beads over sRBC include their uniform (spherical) shape, which allows for ready identification of phagosomes on visual inspection or when using automated image analysis programs, and the availability of varying bead sizes. However, uniform-size latex beads that are appropriate for phagocytosis experiments are considerably more expensive than RBCs. Furthermore, unlike sRBCs, extracellular beads are not amenable to hypotonic lysis for elimination. Therefore, if it is necessary to distinguish internalized versus noninternalized adherent beads by alternative means, the experiment will consume additional time and reagents. Lastly, plain, unopsonized latex beads can be recognized and engulfed by phagocytes at significant rates, complicating the analysis of the contribution of FcγR.

Additional Materials (also see *Basic Protocol 1*)

- 10% slurry of latex beads, ~3- μ m diameter (Bangs Laboratories cat no. PS05N or equivalent from other manufacturers)
- Human IgG stock solution: reconstitute IgG from human serum (Sigma) to 50 mg/ml in PBS (see recipe) or Milli-Q purified H₂O (store in aliquots at -20°C; store thawed aliquots up to 1 month at 4°C)
- Fluorescently labeled anti-human IgG secondary antibody (e.g., Cy3- or FITC-labeled anti-human IgG F(ab)₂, Jackson Laboratories), diluted 1/500 in PBS (see recipe) containing 1% (w/v) BSA and 5% (v/v) goat serum
- 15-ml polystyrene tubes, sterile
- End-over-end rotator
- Tabletop centrifuge

Prepare cells (day 1)

1. Grow cells of choice in DMEM with 10% FBS (*UNIT 1.1*). At a point 24 to 48 hr prior to the experiment, passage cells onto sterile glass coverslips contained in 6-well plates (prepared as described in Reagents and Solutions) so that they will reach ~75% confluence at the time of the experiment.

Opsonize beads with IgG (day 2)

2. Place the following in a sterile 15-ml polystyrene tube:

- 3 ml PBS
- 60 μ l bead suspension
- 60 μ l human IgG stock solution.

The volumes given are sufficient for an experiment using one 6-well plate. They can be scaled up or down depending on the number of wells being assessed.

3. Incubate 60 min at 37°C, rotating gently in an end-over-end rotator.
4. Sediment beads by centrifuging 10 min at 750 \times g (2500 rpm), 4°C, in a tabletop centrifuge. Aspirate supernatant, being careful not to aspirate the beads.
5. Resuspend beads in 5 ml cold PBS, centrifuge again as in step 4, and aspirate supernatant. Repeat this washing procedure a second time.
6. Resuspend beads in 3 ml cold HEPES-buffered DMEM. Keep on ice until ready to use.

Perform phagocytosis assay (day 2)

7. Prewarm culture medium at 37°C (to be used in step 12).
8. Put prepared plate of cells (from step 1) on ice.

One may wish to exchange the culture medium with prechilled medium just prior to this step.

9. Add 0.5 ml of the IgG-opsonized bead suspension (from step 6) per well.
10. Incubate on ice for 20 min to allow beads to bind.
11. Wash off excess beads with cold PBS.

The excess beads can be washed three times with PBS and reused. When stored at 4°C with 0.01% sodium azide, the beads can be used for 2 to 3 months.

12. Remove the plate from ice. Aspirate medium and add 2 ml of 37°C prewarmed culture medium per well.
13. Incubate at 37°C for the desired time periods.

14. Stop phagocytosis by placing plates on ice and changing to ice-cold medium or PBS.

Label extracellular opsonized beads

15. To label beads that are adherent to the cells but not internalized, incubate 20 min on ice with a fluorescently labeled anti-human IgG secondary antibody.

Cover the plate with foil to minimize bleaching of the fluorophore during the incubation procedure.

The authors typically use Cy3- or FITC-labeled anti-human IgG F(ab)₂ (Jackson Laboratories). Dilute antibody 1:500 in PBS (see recipe) containing 1% (w/v) BSA and 5% (v/v) goat serum.

Fix cells (days 2 to 3)

16. Wash three times with PBS.
17. Fix the cells by covering them with 4% paraformaldehyde in PBS. Incubate 20 min at room temperature. Wash three times with PBS.
18. *Optional:* Quench free aldehyde groups with PBS containing 100 mM glycine in PBS for 20 min. Wash three times with PBS.
19. Mount coverslips containing cells onto slides with Dako fluorescence mounting reagent. Allow to dry at room temperature for several hours prior to visualization.

Slides can be stored at -20°C in the dark for months with little loss of fluorescence intensity.

20. Score phagocytosis under microscope.

The phagocytic index is generally defined as the number of particles internalized per 100 cells located.

**SUPPORT
PROTOCOL 1**

Assessment of Surface Expression and Binding Capacity of Fcγ

The surface expression and the binding affinity of the FcγR for their ligands will determine the efficiency and rate of phagocytosis. For many experiments (e.g., comparison of phagocytic rates under different treatment conditions or in phagocytes with different genotypes), it is important to determine the surface expression and binding capacity of the FcγR in order to draw valid conclusions. Receptor density and phagocytic efficiency can then be directly compared.

Additional Materials (also see *Basic Protocol 1* and *Alternate Protocol 1*)

FcγR-specific antibody: e.g., rat anti-mouse CD 16/CD32 (BD Bioscience)
Fluorescently labeled secondary antibody (Jackson Immunoresearch Labs)

To assess Fcγ receptor expression and binding using IgG-opsonized sRBC

- 1a. Follow steps 1 to 16 of Basic Protocol 1.
- 2a. After the 20-min incubation on ice, wash wells twice with ice-cold PBS.
- 3a. Fix with methanol and mount as in Basic Protocol 1, step 22-24.
- 4a. Score the number of bound sRBCs per phagocytic cell.

To assess Fcγ receptor expression and binding using IgG-opsonized beads

- 1b. Follow steps 1 to 12 of Alternate Protocol 1.
- 2b. Fix with 4% paraformaldehyde as in Alternate Protocol 1, steps 17 to 19.
- 3b. Score the number of bound beads per cell.

To assess Fcγ receptor expression and binding using FcγR-specific antibodies

- 1c. Label surface receptors by incubating 60 min at 4°C with 0.5 ml of FcγR-specific antibody diluted 1:10 PBS containing 1% BSA.

Antibodies directed against several classes of human as well as murine FcγR are commercially available and can be used to assess surface expression of these receptors. If using these antibodies to label surface receptors, perform the incubations at 4°C to prevent internalization of the receptors by endocytosis.

- 2c. Wash twice with PBS.
- 3c. Mount using Dako fluorescent mounting medium and evaluate expression by counting the fraction of fluorescently labeled cells using microscopy or flow cytometry.

ASSESSMENT OF COMPLEMENT-MEDIATED PHAGOCYTOSIS

Activation of the complement cascade leads to cleavage of the normally inactive complement proteins into several functional fragments. Some of these complement fragments function as opsonins which attach to microorganisms or particles, promoting their internalization via complement receptor (CR)-mediated phagocytosis. Complement-dependent phagocytosis is mediated primarily by the complement receptor CR3 (also known as CD11b/CD18 or Mac1). Unlike FcγR-mediated phagocytosis, where binding of the ligand to its cognate receptor is sufficient to initiate phagocytosis, CR3-mediated phagocytosis requires an additional stimulus to render the CR competent for phagocytosis (Newman et al., 1985; Aderem and Underhill, 1999). Success of experiments involving complement-opsonized particles depends on the optimal deposition of the C3bi component of complement onto the particles and on suitable priming of the phagocytic cells to capacitate the CR.

Assessment of Complement-Mediated Phagocytosis Using C3bi-Opsonized Erythrocytes

The protocol below describes a method to attach C3bi to RBCs. This method relies on binding of IgM to activate the complement cascade on the particle surface by the classical pathway. Under the conditions described, C3b fragments are rapidly deposited onto RBCs and are almost completely converted to C3bi (Newman and Mikus, 1985).

Materials

- Primary monocytes or macrophages (UNIT 2.2) or monocytic cell lines (e.g., RAW 264.7 cells ATCC #TIB-71 or J774 cells, ATCC #HB-197)
- Culture medium: e.g., DMEM with or without 10% FBS (APPENDIX 2B); nominally bicarbonate-free, HEPES-buffered media can be used to obviate the need for a CO₂ atmosphere
- Sheep red blood cells (sRBCs; 10% v/v suspension, ICN Biomedicals)
- Phosphate-buffered saline (without Ca²⁺ or Mg²⁺; see recipe)
- Gelatin veronal buffer (GVB; Sigma)
- Rabbit anti-sheep erythrocyte IgM (Cedarlane or Accurate Chemical): reconstitute lyophilized antibody in 1 ml H₂O (final concentration 1 mg/ml) and store aliquots at -20°C (avoid repeated freeze-thaw cycles)
- C5-deficient human serum (Sigma)
- GVB (Sigma) supplemented with 1% (w/v) BSA and 7.5% (v/v) goat serum (optional)
- Goat anti-C3bi antibody (optional; Sigma)
- GVB (Sigma) supplemented with 5% (w/v) BSA and 7.5% (v/v) donkey serum (optional)

**BASIC
PROTOCOL 2**

**Protein
Trafficking**

15.7.9

FITC-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Labs; optional)
HEPES-buffered DMEM (e.g., Life Technologies)
20 mM phorbol 12-myristate 13-acetate (PMA) in DMSO
Methanol, prechilled to -20°C
Fluorescence mounting medium (Dako)

6-well tissue culture plates containing sterile glass coverslips (see recipe)
End-over-end rotator
Glass microscope slides
Epifluorescence microscope with bright-field capabilities and appropriate filters to visualize FITC fluorescence (see *UNITS 4.1 & 4.2* and *APPENDIX 1E*)

Additional reagents and equipment for cell culture and counting cells (*UNIT 1.1*) and microscopy (*UNITS 4.1 & 4.2*)

Plate phagocytes (day 1)

1. Grow cells of choice in DMEM with 10% FBS (*UNIT 1.1*). At a point 24 to 48 hr prior to the experiment, passage cells onto sterile glass coverslips contained in 6-well plates (prepared as described in Reagents and Solutions) so that they will reach ~75% confluence at the time of the experiment.

Opsonize sRBCs with IgG (day 2)

2. Prewarm the end-over-end rotator within an incubator to 37°C . Keep all reagents on ice until step 8.
3. Place a 200- μl aliquot of 10% sRBC suspension into a 1.5-ml microcentrifuge tube.
4. Sediment the sRBCs by microcentrifuging 5 to 30 sec at maximum speed.
5. Aspirate supernatant and resuspend cells in 1 ml ice-cold PBS.

It is best to use freshly opsonized sRBC for the phagocytosis assay. Keep all reagents on ice until step 8.

6. Wash two more times, first with ice-cold PBS and then with 1 ml of cold GVB, using the technique described in steps 4 to 5. After the last wash, resuspend the sRBCs in 200 μl GVB. Count cells using a hemocytometer (*UNIT 1.1*).

Use of GVB minimizes aggregation and spontaneous lysis of the cells.

7. Dilute 1 μl of rabbit anti-sheep IgM stock in 50 μl of GVB. Add 2 μl of the diluted IgM to the sRBC suspension.

It may be necessary to determine the optimal sub-agglutinating dilution for individual lots of IgM.

8. Incubate sRBC-rabbit IgM at 37°C for 1 hr in the prewarmed end-over-end rotator (see step 2).
9. Wash sRBCs three times with ice-cold GVB using the technique described in steps 4 to 5, then resuspend in 225 μl cold GVB. Keep on ice.
10. Add 25 μl of C5-deficient human serum (for a final concentration of 10% v/v) and incubate at 37°C for 20 min. Wash three times in GVB using the technique described in steps 4 to 5.

Under these conditions, the Fc region of the IgM pentamer activates the classical pathway of the complement cascade and deposits C3b to the IgM-coated sRBCs, where it is rapidly converted to C3bi (Newman and Mikus, 1985). While IgG-coated RBCs can also be used to opsonize particles with C3bi under comparable conditions, IgG is not an efficient activator of the complement cascade because two adjacent Fc regions are required to initiate the first step of the cascade. Furthermore, the presence of IgG could confound subsequent interpretation of phagocytosis data due to concomitant engagement of the Fc γ receptors. Use of C5-deficient serum prevents lysis of the red blood cells.

11. *Optional*: Assess opsonization efficiency using immunofluorescence.

This step is recommended for each lot of sRBC and IgM used.

- a. Take a small aliquot of the opsonized sRBC and resuspend in 200 μ l of GVB supplemented with 1% BSA and 7.5% donkey serum.
- b. Add goat anti-C3bi antibody to a final dilution of 1:100. Incubate for 20 min on end-over-end rotator at 4°C. Wash three times with GVB.
- c. Counterstain with a 1:100 dilution of FITC-conjugated donkey anti-goat IgG in GVB containing 5% bovine serum albumin and 7.5% donkey serum for 20 min.
- d. Observe under a fluorescence microscope using the appropriate filters for the fluorochrome (*APPENDIX 1E*).

A positive reaction will yield obvious, bright fluorescence.

12. Resuspend the opsonized sRBC in HEPES-buffered DMEM to yield $5\text{--}7 \times 10^8$ cells/ml

Perform phagocytosis assay (day 2)

13. Add 50 to 100 μ l of C3bi-opsonized sRBC to each well of the tissue culture plates with the cells (step 1) and allow binding to proceed for 20 min at 37°C. Use 5 to 10 C3bi-coated sRBCs per phagocytic cell for optimal assessment of phagocytosis.

C3bi binds to CR3 poorly at 4°C. At 37°C, resting cells will bind C3bi but will not initiate CR3-mediated phagocytosis.

14. To activate CR3 and initiate phagocytosis, add PMA (as 20 mM stock) to each well for a final concentration of 150 ng/ml and incubate at 37°C for the desired time period.

In resting leukocytes, CR3 is inactive, although capable of binding C3bi, and requires an additional signal for activation and initiation of phagocytosis. A number of different stimuli can activate CR3. Phorbol esters such as PMA activate CR3 by stimulation of protein kinase C (Wright et al., 1983). Other stimuli that lead to activation of CR3 include certain cytokines and chemokines, attachment to extracellular matrix proteins, and glucan binding.

15. Stop phagocytosis by placing the cells on ice. Wash with ice-cold PBS.

The duration of incubation at 37°C will depend on the stage of phagocytosis and phagosomal maturation that are of interest.

16. *Optional*: To remove the C3bi-sRBCs that have not been internalized, lyse the sRBC by incubating with 1 ml of water for 2 min.

17. Wash again with cold PBS. Keep on ice.

Fix cells

18. Aspirate PBS and add 1 ml per well of prechilled methanol. Incubate on ice for 20 min. Wash twice with PBS.

19. Mount coverslips containing cells onto slides with Dako fluorescence mounting medium. Allow to dry at room temperature for several hours prior to visualization.

It is preferable to dry the slides overnight before analysis.

Dab the coverslip on tissue wipe to remove excess fluid before mounting. This will decrease the time required for the slide to dry. To minimize air bubbles in the mounted slides, place a drop of the mounting medium on the glass slide first, then slowly ease the coverslip onto the slide. Slides can be stored for months at -20°C with little loss of fluorescence.

20. Score phagocytosis under microscope.

Only the internalized sRBC remain intact and can be readily identified by bright-field (phase or differential interference contrast) microscopy. Typically, phagocytic efficiency is scored as the number of sRBC internalized per 100 phagocytic cells counted.

Assessment of Complement-Mediated Phagocytosis Using C3bi-Opsonized Latex Beads

CR-mediated phagocytosis can also be assessed using complement-opsonized latex beads. This protocol and Alternate Protocol 3 below describe the opsonization and fluorescent labeling of beads for assessment of phagocytosis.

Coating of latex beads with C3bi is less complicated than the similar procedure using erythrocytes (see Basic Protocol 2). Furthermore, the uniform sizes of the beads allows for easy identification of phagosomes and, because lysis is not a concern, C5-deficient serum is not required. However, latex beads are significantly more expensive than sRBCs.

Additional Materials (also see Basic Protocol 2)

- 10% slurry of latex beads, ~3- μ m diameter (Bangs Laboratories cat no. PS05N or equivalent from other manufacturers)
- 1 mg/ml human IgM stock (Sigma; store in aliquots at -20°C)
- Human or mouse serum, freshly isolated, diluted 1:1 in PBS (see recipe for PBS)
- Hanks' balanced salt solution (HBSS; see recipe)
- 20 mM phorbol 12-myristate 13-acetate (PMA) in DMSO
- Goat anti-human C3bi antibody (Sigma)
- PBS (see recipe) supplemented with 1% (w/v) BSA and 7.5% (v/v) goat serum (optional)
- FITC-conjugated donkey anti-goat IgG (optional)
- 4% (v/v) paraformaldehyde in PBS (optional)
- PBS (see recipe) supplemented with 1% (w/v) BSA and 7.5% (v/v) donkey serum (optional)
- PBS (see recipe) containing 100 mM glycine (optional)
- 15-ml polystyrene tubes, sterile

Plate phagocytes (day 1)

1. Grow cells of choice in DMEM with 10% FBS (*UNIT 1.1*). At a point 24 to 48 hr prior to the experiment, passage cells onto sterile glass coverslips contained in 6-well plates (prepared as described in Reagents and Solutions) so that they will reach ~75% confluence at the time of the experiment.

Opsonize beads (day 2)

2. Place the following in a sterile 15-ml polystyrene tube:
 - 3 ml PBS
 - 60 μ l bead suspension
 - 60 μ l IgM stock solution.
3. Incubate 60 min at 37°C , rotating gently in an end-over-end rotator.
4. Sediment beads by centrifuging 10 min at $750 \times g$ (2500 rpm), 4°C , in a tabletop centrifuge. Aspirate supernatant, being careful not to remove the beads.
5. Resuspend beads in 5 ml cold PBS, centrifuge again as in step 4, and aspirate supernatant. Repeat this washing procedure a second time.
6. Resuspend beads in freshly isolated human or mouse serum diluted 1:1 in PBS. Incubate at 37° for another 20 min.

During this step, the Fc region of IgM will initiate the classical pathway of complement cascade and fix C3b to the beads where it is quickly and completely converted to C3bi (Newman and Mikus, 1985).

7. At the end of the incubation period, collect beads by microcentrifugation and wash with HBSS using the technique described in steps 4 and 5. Resuspend in 3 ml HEPES-buffered DMEM.

Perform phagocytosis assay (day 2)

8. Add 500 μ l of the C3bi-beads from step 7 to each well of the tissue culture plates with the cells (see step 1) and allow binding to proceed for 20 min at 37°C.
9. To activate CR3 and initiate phagocytosis, add PMA (as 20 mM stock) to each well for a final concentration of 150 ng/ml and incubate at 37°C for the desired time period.
10. Stop phagocytosis by placing the cells on ice. Wash with ice-cold PBS.
11. *Optional:* To identify C3bi-beads that are bound but not internalized, label the external beads with anti-C3bi antibodies on ice as follows:
 - a. Incubate each well with a 1:100 dilution of goat anti-C3bi antibody in 200 μ l PBS supplemented with 1% BSA and 7.5% donkey serum for 20 min.
 - b. Wash three times with ice-cold PBS.
 - c. Incubate with a 1:100 dilution of FITC-conjugated donkey anti-goat IgG in 200 μ l PBS with 1% BSA and 7.5% donkey serum for 20 min
 - d. Wash three times with ice-cold PBS.

Fix cells and score phagocytosis (day 2)

12. Aspirate PBS and add 1 ml of 4% paraformaldehyde in PBS per well. Incubate on ice for 20 min. Wash twice with PBS.
13. *Optional:* Add 1 ml of PBS containing 100 mM glycine to each well and incubate 15 to 30 min at 4°C to quench the free aldehyde groups. Wash three times with PBS.
14. Mount cells using fluorescent mounting medium and score phagocytosis efficiency by counting the fraction of fluorescently labeled cells using microscopy or flow cytometry.

C3bi Opsonization of Beads

It is possible to skip the IgM adsorption step and deposit C3bi onto the beads by incubating directly with fresh human or mouse serum. This requires a longer incubation period in serum to allow for the complement cascade and fixation of C3bi to the beads to proceed. It is recommended that efficiency of C3bi deposition be verified by immunofluorescence before proceeding with the phagocytosis assay.

Additional Materials (also see Alternate Protocol 2)

Human or mouse serum, freshly isolated

At step 2 of Alternate Protocol 2, place the following in a sterile 15-ml polystyrene tube instead of the items indicated in that protocol:

- 1.5 ml PBS
- 1.5 ml human or murine serum
- 60 μ l bead suspension.

Incubate 60 min at 37°C with rotation. At the end of the incubation period in step 3, continue with Alternate Protocol 2, starting from step 7.

***ALTERNATE
PROTOCOL 3***

**Protein
Trafficking**

15.7.13

ASSESSMENT OF PHAGOCYTOSIS USING FLOW CYTOMETRY

In addition to the conventional microscope-based techniques described above, phagocytosis of microorganisms and particles can be measured using flow cytometry (Bassoe and Solberg, 1984; Santos et al., 1995; Oben and Foreman, 1988; Lehmann et al., 2000). The principle of the flow cytometric technique for assessment of phagocytosis is relatively straightforward. After allowing phagocytic cells to ingest fluorescent particles (e.g., labeled microorganisms or particles), cell-associated fluorescence is quantified using flow cytometry. If the fluorescent phagocytic “prey” is evenly labeled, the amount of cell-associated fluorescence is directly proportional to the number of ingested particles, thus allowing a calculation of the phagocytic index. This technique has several advantages over the conventional techniques (Harvath and Terle, 1999), but also some limitations that are discussed below, along with several strategic considerations.

Distinguishing Particle Attachment to the Cell Surface From Internalization (“In” From “On”)

A key technical issue is the necessity to quantify only internalized particles, while excluding extracellular adherent ones. This is generally accomplished by minimizing extracellular fluorescence using impermeant quenching agents. Many fluorophores can be quenched by compounds such as trypan blue, ethidium bromide, or iodoacetate that, under optimal conditions, are able to dampen surface fluorescence by up to 95% without penetrating the plasma membrane (Steinkamp et al., 1982; Bassoe and Solberg, 1984). In addition, many fluorophores including FITC, Alexa Fluor 488, BODIPY FL, Oregon Green, tetramethylrhodamine, Texas Red, and Cascade Blue dyes can be quenched by specific antibodies. It should be emphasized that quenching is not an all-or-none phenomenon and that proper controls must be carried out to ensure that there is satisfactory separation of the internalized and adherent particle populations. It is also important to note that not all fluorophores can be quenched in this manner, and it is essential to ascertain that untested dyes can be quenched efficiently. In addition, the fluorescence of synthetic beads that are synthesized with the fluorophore incorporated within the polymer cannot be quenched in this manner.

Sensitivity To pH

It is important to consider that many fluorophores are sensitive to environmental factors—notably intracellular pH—that can affect the signal of the fluorescent probe. In the case of phagocytosis, this is particularly important because, as the phagosome matures, the luminal pH decreases, thus quenching the fluorescence of dyes such as FITC. This can result in artifactual underestimation of the phagocytic efficiency. Fluorophores such as those of the Alexa and BODIPY series, tetramethylrhodamine, and Texas Red exhibit much less quenching over a wide pH range (from pH 4 to 9), and they are much less susceptible to artifactual quenching.

On the other hand, one can take advantage of the pH sensitivity of some probes to discriminate between internalized and externally adherent particles. As described in more detail elsewhere, it is also possible to tag phagocytic targets with pH-sensitive dyes such as FITC, SNARF, SNAFL, or Oregon Green (Molecular Probes) that undergo spectral shifts or intensity changes at the acidic pH found within phagosomes. If the appropriate pH-sensitive wavelength is selected for detection, the fluorescence intensity will be proportional to phagocytosis and can be used to quantify internalized particles by flow cytometry.

Selection of Phagocytic Targets

Phagocytic targets suitable for flow cytometry include bacteria (*E. coli* and *Staphylococcus aureus*), zymosan (*Saccharomyces cerevisiae*), fungi (*Aspergillus*), and latex beads. These targets are usually opsonized with IgG or complement fragments (see Basic Protocols 1 and 2 and Alternate Protocols 1 and 2), but many present endogenous ligands such as β -glucan and other complex carbohydrates that can initiate or at least influence phagocytosis. These factors must be taken into consideration when selecting the most appropriate target and when comparing the efficiency of phagocytosis between different targets.

The protocol below delineates several different methods to assess phagocytosis by flow cytometry using bacteria, yeast, or synthetic beads.

Materials

- Zymosan A (from *S. cerevisiae*; Sigma)
- Phosphate-buffered saline (PBS; see recipe), pH 7.4
- 0.1 M sodium carbonate, pH 9.3
- 1 mg/ml solution of reactive fluorophore, e.g., fluorescein isothiocyanate (FITC) or Oregon Green 514 succinimidyl ester, in 0.1 M sodium carbonate, pH 9.3
- Bacteria of interest as phagocytic target (e.g., *E. coli* K-12 strain)
- 1 mg/ml fluorescein isothiocyanate (FITC) in PBS (see recipe), pH 8.0
- 1% (v/v) glutaraldehyde in PBS (see recipe for PBS)
- PBS (see recipe) containing 100 mM glycine
- Phagocytic cells of interest: cultured cell lines or primary isolates of peripheral blood neutrophils or monocytes (e.g., UNIT 2.2)
- PBS (see recipe) containing 0.02% EDTA
- PBS (see recipe) containing 1.25 mg/ml trypan blue
- 0.25% trypsin/EDTA or nonenzymatic cell dissociation solution (both available from Sigma)
- Quenching antibody to fluorophore (e.g., anti-FITC/Oregon Green Cat# A-889; anti-Alexa Fluor 488 Cat # A-11094 Molecular Probes)
- Bath sonicator (e.g., Branson 1200 Ultracleaner, Branson)
- Rotating shaker
- Polypropylene tubes for use with flow cytometer
- 24- or 96-well tissue culture plates (Polypropylene Cluster, Costar)
- Flow cytometer
- Additional reagents and equipment for flow cytometry (e.g., Robinson et al., 2003)

Prepare fluorophore-coupled phagocytic particles

For zymosan A

- 1a. Suspend 10 to 20 mg of dried zymosan A in 10 ml PBS. Vortex vigorously and boil for 10 min.
- 2a. Wash three times in 1 ml PBS by resuspension followed by centrifugation 1 min at $3000 \times g$ and resuspend in PBS. Sonicate in bath sonicator to disperse aggregates.
- 3a. Sediment zymosan A by centrifuging 1 min at $3000 \times g$, 4°C, remove supernatant, and resuspend pellet in 0.1 M sodium carbonate, pH 9.3, at a final concentration of 50 mg/ml.

- 4a. Initiate labeling reaction by adding 0.1 mg of fluorophore (as 1 mg/ml solution) to 1 mg of suspended zymosan particles. Allow reaction to proceed 4 hr at room temperature with constant mixing.

Reactive fluorophores (e.g., fluorescein isothiocyanate and the succinimidyl ester of Oregon Green 514) are dissolved in 0.1 M sodium carbonate at 1 mg/ml.

- 5a. Remove unbound probe by centrifugation for 1 min at $3000 \times g$, 4°C , discard the supernatant, and resuspend product in 0.5 ml of dimethylsulfoxide with 3 drops of PBS, pH 7.4. Sonicate to disperse the zymosan. Repeat centrifugation, resuspension, and sonication 2 to 3 more times while adding progressively more PBS until the supernatant is completely clear.

- 6a. Dissolve the final pellet in 1 ml PBS.

This can be frozen in aliquots or stored at 4°C in 2 mM sodium azide for several weeks (make certain to wash off the azide before using for phagocytosis assays, as azide inhibits phagocytosis).

For bacteria

- 1b. Transfer 5.0×10^8 bacteria from overnight culture to a microcentrifuge tube.
- 2b. Sediment by microcentrifuging 1 min at maximum speed and wash once by resuspension in PBS and resedimentation.
- 3b. Resuspend cells in 1 ml of 1 mg/ml FITC in PBS, pH 8.0. Incubate at room temperature on an end-over-end rotator for 25 min.
- 4b. Wash in PBS as in 2b and fix in 1 ml of 1% glutaraldehyde in PBS for 20 min.
- 5b. Quench free aldehyde groups by incubating in 0.5 ml of PBS containing 100 mM glycine for 10 min at room temperature.
- 6b. Wash as in 2b and resuspend in 500 μl sterile PBS. Keep on ice until use the same day.

Initiate phagocytosis

Using the labeled zymosan A particles or bacteria from step 6a or 6b, respectively, proceed with steps 7a to 11a if studying phagocytosis by cells in suspension (e.g., neutrophils or monocytes) or proceed with steps 7b to 1b if studying phagocytosis by adherent cells.

Phagocytosis assays for cells in suspension

- 7a. To a 1.5-ml microcentrifuge tube, add 100 μl of a suspension of 5×10^6 phagocytic cells/ml, prewarmed to 37°C . Next, mix with 100 μl of a suspension of 5×10^7 particles/ml of the labeled phagocytic target from step 6a or b.

It is crucial to ensure good contact between the phagocytes and their prey, bearing in mind that while adherent phagocytes can crawl, suspended cells cannot swim.

- 8a. To bring phagocytic cells and target particles into close proximity, cosediment the cells and beads by a rapid microcentrifugation (15 sec at 70% maximum speed or ~ 8000 rpm).

The cell-to-particle ratio should be optimized for the specific cell type, but it is usually $\sim 1:10$ (range 1:5 to 1:100).

- 9a. Incubate the cells at 37°C for the appropriate length of time. Terminate phagocytosis by adding 1 ml ice-cold PBS containing 0.02% EDTA. Keep samples on ice.

The incubation time for maximum phagocytosis should be optimized for the individual cell type and can vary from 10 to 15 min for human neutrophils to 30 to 40 min for monocytes.

- 10a. Dilute samples 1:5 with PBS containing 1.25 mg/ml trypan blue. Incubate at 4°C for at least 2 min to allow efficient quenching of fluorophore. Maintain the samples at 4°C in trypan blue-containing solution until analysis.
- 11a. Harvest cells using a micropipettor and transfer to a polypropylene tube suitable for use in the flow cytometer.

If there will be a delay before analysis, fix cells with 2% paraformaldehyde. Fluorescence will be stable for at least 24 to 48 hr in fixed cells.

For adherent cells

- 7b. Add labeled particles (from step 6 a or b; 5 to 10 per cell) to adherent phagocytes growing in wells of a 24-well tissue culture plate. Incubate for 10 to 60 min at 37°C.
- 8b. Terminate phagocytosis by adding 200 μ l per well of ice-cold PBS containing 0.02% EDTA.
- 9b. Remove the cells from the surface using 0.25% trypsin/EDTA or nonenzymatic cell dissociation solution. Pipet repeatedly yet gently to ensure dispersal into a single-cell suspension.
- 10b. If needed, filter through a mesh filter to remove clumps of cells

One risks the wrath of the flow cytometry facility if one clogs up the aspiration port of the cytometer.

- 11b. Keep samples on ice and dilute 1:5 with PBS containing 1.25 mg/ml trypan blue. Transfer to a polypropylene tube suitable for use with the flow cytometer and analyze immediately.

If there will be a delay, fix cells with 2% paraformaldehyde. Fluorescence will be stable for at least 24 to 48 hr in fixed cells.

Analyze by flow cytometry

12. Aspirate cells into the chamber of the flow cytometer and collect a minimum of 10,000 cells in the viable cell gate (also see Robinson et al., 2003).

Flow cytometers with one or several lasers are available from several commercial sources. The authors use either a BD Biosciences FACSCalibur or a Beckman-Coulter Elite.

The fluorescence measurements collected in the individual fluorescence channels are displayed on log scales. The proportion of cells that performed phagocytosis is estimated as the percentage of viable cells that have target fluorescence exceeding the background values (van Eeden et al., 1999).

When using evenly labeled fluorescent beads, the mean number of beads per phagocytic cell can be calculated by dividing the mean target fluorescence in the appropriate gate by the fluorescence of a single extracellular bead. The latter value is estimated separately using pure beads.

DETERMINATION OF PHAGOSOMAL pH

Acidification of the phagosomal lumen is apparent shortly after sealing of the phagosomal cup. As in the case of endosomes, accumulation of H⁺ is thought to result from vectorial pumping by vacuolar-type (V) ATPases (Demaurex, 2002), which are delivered to the membrane of the maturing phagosome via fusion with acidic endomembrane compartments (i.e., endosomes and lysosomes). What determines the progressive acidification is unclear, but increased density of pumps, reduced leakage of H⁺ (equivalents), and/or increasing counterion conductance have all been contemplated. All V-type ATPases are exquisitely sensitive to inhibition by macrolide antibiotics such as bafilomycin and

concanamycin (folimycin), and these drugs are routinely used to prevent or dissipate the phagosomal acidification and to validate the measurements of luminal pH. Two general approaches have been used to detect and quantify phagosomal pH, as described in Basic Protocol 4 and Alternate Protocol 4, below.

Measuring Phagosomal pH with Permeant Reagent

The simplest method for determining phagosomal pH involves the use of absorbing or fluorescent weak bases that can permeate biological membranes in their unprotonated state. Such compounds readily traverse the surface and phagosomal membranes to reach the phagosomal lumen where they become protonated because of the prevailing low pH. Because the resulting protonated species is much less permeant than its unprotonated precursor, the total concentration of the molecule in the phagosome increases beyond that of the medium and cytosol, in direct proportion to $[H^+]$. This accumulation, an indirect measure of pH, can then be detected spectroscopically either by the increased absorbance or fluorescence of the probe, or by spectral shifts that are a consequence of the formation of excimers at high fluorophore concentrations. Acridine orange, quinacrine, and LysoTracker are fluorescent weak bases that can be used to detect phagosomal acidification.

Materials

- Cells of interest (e.g., RAW264.7 macrophages; ATCC #TIB-71)
 - Na⁺-rich medium (see recipe), 37°C
 - Fluorophore-coupled phagocytic particles (see other protocols in this unit)
 - 1 mM stock of LysoTracker Red DND 99 (Molecular Probes) in DMSO
 - 6-well tissue culture plates containing sterile glass coverslips (see recipe)
 - Leiden chambers and thermostatted holder for microscope stage
 - Fluorescence imaging microscope system with rhodamine filter set
 - Light microscope with differential interference contrast (DIC) capability (*UNIT 4.1*)
- Additional reagents and equipment for light and fluorescence microscopy (*UNIT 4.1*)

Prepare cells

1. Seed cells onto sterile glass coverslips contained in 6-well plates (prepared as in Reagents and Solutions) and grow to desired level of confluence.
2. Remove coverslip from well, place in Leiden chamber and immediately cover with prewarmed (37°C) Na⁺-rich medium.

Allow cells to carry out phagocytosis and accumulate LysoTracker

3. Initiate phagocytosis by incubation with desired particles (see other protocols in this unit).
4. Wash off excess particles and allow maturation to proceed for desired time.
5. Add to the medium 50 to 100 nM LysoTracker Red DND 99 (as 1 mM stock) and allow a minimum of 10 min for complete equilibration.
6. Visualize fluorescence using a fluorescence microscope with a conventional rhodamine filter set.
7. Confirm the identity of phagosomes by visualizing the same field by differential interference contrast (DIC) microscopy.

Quantitative pH Determinations by Fluorescence Ratio Microscopy

More quantitative measurements can be made by ratio fluorimetry. This approach is based on the use of pH-sensitive fluorophores that are trapped in the phagosome along with the particle. It is best to attach the probe covalently to the particle, since the rapid remodeling of the luminal contents during maturation results in rapid clearance of soluble material that is trapped during phagosome formation. Very accurate determinations can be obtained by calculating the ratio of fluorescence at two different wavelengths that behave differentially when pH is altered. This normalization procedure corrects for overall changes in emission due to photobleaching or to alterations in focal plane, a common occurrence in the case of phagosomes, which are comparatively mobile organelles. Derivatives of fluorescein have been used extensively for this purpose.

Additional Materials (also see Basic Protocol 4)

- Fluorophore-coupled zymosan particles (see Basic Protocol 3, steps 1a to 6a)
- K⁺-rich medium, pH 7.5 and lower pHs for calibration (see recipe)
- 1 mg/ml nigericin stock in ethanol
- Ratio imaging setup (see Support Protocol 2)

Prepare cells

1. Grow cells, load in a Leiden chamber, and overlay with Na⁺-rich medium (see Basic Protocol 4, steps 1 and 2).
2. Place chamber on thermostatted holder on stage of imaging microscope.

Allow cells to phagocytose particles

3. Allow the cells to ingest fluorophore-coupled zymosan particles (see Basic Protocol 3).

Particles can be opsonized if desired by incubation with anti-zymosan antibodies (Molecular Probes), as described for red cells in Basic Protocol 1. Use a ratio of 5 to 10 particles per cell for effective phagocytosis

4. Wash off excess particles and allow maturation to proceed for desired time.
5. Acquire images, alternating the excitation between 440 nm and 490 nm and directing the light to the samples with a 510 nm dichroic mirror. Record emitted light using a 535BP25 nm filter.

Calibrate the pH

6. For calibration, replace Na⁺-rich medium with K⁺-rich medium, pH 7.5. Add 5 μg/ml nigericin (from 1 mM stock) and allow 3 to 5 min for equilibration.
7. Repeat image acquisition as in step 5.
8. Replace K⁺-rich medium, pH 7.5, with similar medium of lower pH, and nigericin as in step 6.
9. Allow equilibration and repeat image acquisition cycle
10. Repeat steps 8 to 9 for all calibration solutions.

At least four calibration points in the range of pH 5 to 7.5 are recommended.

11. Construct a calibration curve plotting fluorescence ratio versus pH. Interpolate experimental values obtained in step 5 to calculate the absolute pH from the calibration curve.

If using the Metamorph software, these steps can be expedited by using the QUICK TITRATION feature.

Calibration can be done, alternatively, by gently permeabilizing the plasma and phagosomal membranes using 0.1% Triton X-100, while ensuring that the phagocytic particle(s) of interest remain in the field of view. The K⁺-rich media of varying pH can then be introduced sequentially as above, but nigericin can be omitted. Ensure that the particle being calibrated is not displaced during solution changes.

Ratio Imaging Setup

A complete imaging system appropriate for use in quantitative pH determination via ratio imaging (see Alternate Protocol 4) consists of an excitation source, microscope with appropriate filter sets and filter-positioning devices, camera, computer, and software (Demaurex et al., 1998). Several “turn-key” complete systems are available for fluorescence imaging. On the other hand, a large variety of setups can be assembled using individual components from different manufacturers. The system that the authors have assembled in their laboratory consists of a Leica DMR IB fluorescence microscope equipped with a PL Fluotar 100×/1.3 N.A. oil-immersion objective. A Sutter wheel and controller position the excitation filters in front of a mercury lamp. Neutral-density filters are often used to minimize dye bleaching and photodynamic damage to the cells. Emitted fluorescence is captured by an Orca ER cooled charge-coupled device camera. Image acquisition is controlled by the Metafluor software (Universal Imaging Corp.) operating on a Dell computer.

The authors’ system includes a modification that makes it possible to continuously monitor the cells by bright-field (differential interference contrast) microscopy (also see *UNIT 4.1*). To this end, the sample is continuously illuminated at >620 nm by placing a red filter in front of the transmitted incandescent source. By placing an additional 660-nm dichroic mirror in the light path, the red light can be directed to a separate video camera (Dage MTI).

ASSESSMENT OF PHAGOSOMAL MATURATION

The composition of the nascent phagosome is very similar to that of the plasma membrane. However, both the limiting membrane and luminal contents of the phagosome undergo graded changes that result from progressive and finely coordinated rounds of fusion with endomembranes. These fusion events, together with the concomitant membrane budding and vesiculation steps that maintain the phagosomal area nearly constant, are collectively termed phagosomal maturation. Maturation confers upon the phagosome its microbicidal and lytic capabilities. Table 15.7.1 summarizes some of the characteristics of phagosomes at different stages of maturation.

The following protocols, outline methods to assess the occurrence of phagosomal maturation and to identify some of the intermediate stages of the process, using reagents that are readily available. It should be noted, however, that the rate and extent of maturation, and consequently the fate of the internalized particles, most likely depend on the number and type of receptors engaged. Therefore, the design and interpretation of individual experiments need to be tailored to the particular phagocytic stimulus under investigation.

Assessment of the Presence of Transferrin Receptors in Early Phagosomes

Transferrin (Tfn) receptors, which are abundant in early/recycling endosomes, are present on the phagosomal membrane at the earliest stages of maturation. The following are steps assess transferrin distribution in cells.

Materials

- Primary monocytes or macrophages (*UNIT 2.2*), or monocytic cell lines (e.g., RAW 264.7 cells, ATCC #TIB-71 or J774 cells, ATCC #HB-197)
- DMEM with 10% FBS (*APPENDIX 2B*)
- Phosphate-buffered saline (PBS; see recipe)
- Serum-free DMEM (e.g., Life Technologies)
- Fluorophore-conjugated transferrin (Tfn; Molecular Probes; store in small aliquots at -20°C)
- HEPES-buffered DMEM (e.g., Life Technologies)
- 6-well tissue culture plates containing sterile glass coverslips (see recipe)
- Fluorescence microscope with appropriate filters (*UNIT 4.2* and *APPENDIX 1E*)
- Additional reagents and equipment for phagocytosis assay using sRBCs (see Basic Protocol 1 or Alternate Protocol 1) or latex beads (see Alternate Protocol 1 or 2) and fluorescence microscopy (*UNIT 4.2*)

1. Grow cells of choice in DMEM with 10% FBS (*UNIT 1.1*). At a point 24 to 48 hr prior to the experiment, passage cells onto sterile glass coverslips contained in 6-well plates (prepared as described in Reagents and Solutions) so that they will to reach ~75% confluence at the time of the experiment.
2. Wash cells with PBS, then incubate in serum-free DMEM for 1 hr in a CO₂ incubator at 37°C.
This step will deplete the cells of endogenous transferrin.
3. While the cells are incubating, proceed with opsonization of sRBC or latex beads (see Basic Protocol 1 or Alternate Protocol 1) in preparation for the phagocytosis assay (step 6 below).

Table 15.7.1 Markers of Phagosomal Maturation

Stage	Time	pH	Interacting organelle	Markers ^a
Early phagosome	~5 min	6.0	Sorting endosomes, endoplasmic reticulum (?)	Transferrin receptor ^b , EEA1 ^b , Rab5 ^b , PI(3)P, syntaxin 13
Late phagosome	~20 min	5.0–6.0	Late endosomes, multivesicular bodies	Mannose-6-phosphate receptor ^b , LAMP1/2 ^b , Rab7 ^b , Rab9 ^b , syntaxin 7, lyso(bis)phosphatidic acid
Phagolysosome	~40 min	4.5–5.0	Lysosomes	LAMP1/2 ^b , mature cathepsin D ^b , fluid-phase markers chased for = 2 hr

^aAbbreviation: PI(3)P, phosphatidylinositol 3-phosphate.

^bAntibodies directed against these antigens are commercially available.

4. Prepare a solution of 50 µg/ml fluorophore-conjugated Tfn in HEPES-buffered DMEM. Overlay cells with this solution and incubate for 1 hr at 37°C to label the endosomes.

Molecular Probes sells human transferrin conjugated to a number of different fluorophores. One should choose the most appropriate fluorophore for one's application. Reconstitute according to manufacturer's directions and store in the dark in small aliquots at -20°C.

5. Wash off excess unbound Tfn with PBS.
6. Proceed with phagocytosis assay (see Basic Protocol 1 or Alternate Protocol 1).
7. Assess the presence of fluorescently labeled Tfn on the phagosomal membrane using fluorescence microscopy.

Tfn should be present in phagosomes at early times (5 to 15 min), but not thereafter.

Confocal microscopy is optimal for evaluation, particularly when latex beads are employed, since such beads are prone to birefringence of fluorescent light emitted outside the focal plane.

ALTERNATE PROTOCOL 5

Assessment of the Delivery of Fluid-Phase Markers to the Phagosome

The protocol detailed below takes advantage of the uptake and accumulation in lysosomes of biologically inert fluorophore-conjugated dextrans to identify phagolysosomes. For these experiments, the authors recommend the use of latex particles to avoid the conflicting fixative requirements of sRBC and the lysine-fixable dextran.

Materials

Primary monocytes or macrophages (*UNIT 2.2*), or monocytic cell lines (e.g., RAW 264.7 cells, ATCC #TIB-71 or J774 cells, ATCC #HB-197)

DMEM with 10% FBS (*APPENDIX 2B*)

Fluorophore-conjugated dextran, MW 3000, lysine fixable (Molecular Probes; store in dark in small aliquots at -20°C)

6-well tissue culture plates containing sterile glass coverslips (see recipe)

Fluorescence microscope with appropriate filters (*UNIT 4.2* and *APPENDIX 1E*)

Additional reagents and equipment for phagocytosis assay using sRBCs (see Basic Protocol 1 or Alternate Protocol 1) or latex beads (see Alternate Protocol 1 or 2) and fluorescence microscopy (*UNIT 4.2*)

1. Grow cells of choice in DMEM with 10% FBS (*UNIT 1.1*). At a point 24 to 48 hr prior to the experiment, passage cells onto sterile glass coverslips contained in 6-well plates (prepared as described in Reagents and Solutions) so that they will reach ~75% confluence at the time of the experiment.
2. Add the fluorophore-conjugated dextran at 250 µg/ml. Incubate 30 to 45 min at 37°C.

Molecular Probes sells dextran (mol. wt. 3000, lysine fixable) conjugated to a number of different fluorophores. One should choose the most appropriate fluorophore for one's application. Reconstitute according to manufacturer's directions and store in the dark in small aliquots at -20°C. The "fixable" probes have lysine residues incorporated into the dextran which allow them to be fixed by paraformaldehyde and other aldehyde fixatives.

3. Wash off excess dextran with two changes of medium.
4. Incubate for an additional 3 to 4 hr at 37°C in DMEM with 10% FBS.

5. While the cells are incubating, proceed with opsonization of sRBC or latex beads (see Basic Protocol 1 or Alternate Protocol 1) in preparation for the phagocytosis assay (step 6).
6. Proceed with phagocytosis assay (see Basic Protocol 1 or Alternate Protocol 1).
7. Assess the colocalization of fluorescently labeled dextran with the internalized latex beads by microscopy.

Dextran will be found in phagosomes only at advanced stages of maturation (>30 min).

Fresh mounts or samples fixed with 4% paraformaldehyde can be used. Confocal microscopy (UNIT 4.5) is optimal for evaluation, particularly when latex beads are employed.

Assessment of Phagosomal Maturation by Immunostaining of Endogenous Organellar Markers

As it matures, the phagosome acquires and sheds a number of different membrane and soluble proteins, characteristic of the compartments it interacts with. Antibodies directed against many of these proteins are commercially available and can be used to assess their presence in phagosomes by immunofluorescence.

It should be noted, however, that these markers are not always discretely localized to one single compartment (e.g., LAMP1 and LAMP2 are abundant in both late endosomes and lysosomes and can be present at lower density in the plasma membrane and early endosomes). Therefore, the authors recommend the use of multiple markers to validate the stage of phagosomal maturation

The following is a generic protocol that describes the immunofluorescence staining procedure following a phagocytosis assay. The species and concentrations of the primary and secondary antibodies will depend on the specific antigen under investigation. Some of the antibodies, such as those directed against some types of LAMP, are only effective in cells fixed with methanol. Please consult the specifications provided by the manufacturer for the appropriate antibody dilutions, blocking and permeabilizing conditions, and fixative to be used.

Materials

Permeabilization buffer: e.g., 0.1% (v/v) Triton X-100 in PBS or 2% (w/v) saponin in PBS (see recipe for PBS)

Blocking buffer: PBS (see recipe) containing 1% (w/v) BSA and 7.5% (v/v) of serum in which primary antibody was raised)

Primary antibody directed against the protein marker of interest (Table 15.7.1)

Fluorophore-conjugated secondary antibody

Fluorescence mounting medium (Dako)

Additional reagents and equipment for phagocytosis assay (see Basic Protocol 1 or Alternate Protocol 1) and fluorescence microscopy (UNIT 4.2)

1. Perform phagocytosis assay (see Basic Protocol 1, steps 1 to 21, or Alternate Protocol 1, steps 1 to 15).
2. Fix with methanol (see Basic Protocol 1, step 21) or paraformaldehyde (see Alternate Protocol 1, step 17). Quench with 100 mM glycine in PBS (see Alternate Protocol 1, step 17) if paraformaldehyde is used.
3. Wash fixed cells three times with PBS.

ALTERNATE PROTOCOL 6

Protein Trafficking

15.7.23

4. Permeabilize fixed cells for 20 min with the appropriate permeabilization buffer at 4°C.

Detergent-containing permeabilization buffers are required if paraformaldehyde is used to fix the cells but not if methanol is used.

5. Block for 1 hr at room temperature in blocking buffer.
6. Incubate with primary antibody diluted in blocking buffer for 1 hr at room temperature.
7. Wash three times with PBS.
8. Incubate with fluorophore-conjugated secondary antibody diluted in blocking buffer for 1 hr at room temperature.
9. Wash three times with PBS.
10. Mount in Dako fluorescence mounting medium and analyze by fluorescence microscopy.

**BASIC
PROTOCOL 6**

INHIBITION OF PHAGOCYTOSIS

Because phagocytosis is a complex phenomenon, it can be arrested at a number of steps, all of which are essential for completion of particle engulfment (Aderem and Underhill, 1999). A variety of conditions and agents have been reported to impair phagocytosis, but their detailed mode of action is not known in every instance. The simplest, most readily reversible means of preventing phagocytosis is to cool the cells. For mammalian phagocytes, temperatures below 15°C virtually eliminate particle ingestion. The precise critical step(s) affected by reducing the temperature has not been identified, and it is likely that multiple events of the phagocytic reaction are impaired simultaneously. Because it is harmless and reversible, cooling is used extensively to synchronize phagocytosis. This approach is based on the notion that binding of phagocytic receptors to their ligands is much less temperature sensitive than the consequent signaling and effector functions that lead to particle internalization.

The requirement for extensive rearrangement of the actin cytoskeleton during the uptake of large particles is universally acknowledged. For this reason, many laboratories have used cytochalasins or other antagonists of actin remodeling, such as latrunculin, to inhibit phagocytosis (also see *APPENDIX 1B*). Under these conditions, the phagocytes still associate with the target particles, but fail to extend pseudopods and do not engulf their prey. It is important to note that the effectiveness of antagonists of actin remodeling as inhibitors of phagocytosis is proportional to the size of the particle. Uptake of particles of $\leq 1 \mu\text{m}$ is only modestly affected, if at all, whereas engulfment of large targets such as apoptotic bodies is fully arrested. This divergence highlights the notion, introduced earlier, that phagocytosis is not a single phenomenon but rather a collection of phenotypically related events.

Inhibitors of phosphatidylinositol 3 kinase, such as wortmannin and compound LY294002 (*APPENDIX 1B*), also effectively impair the uptake of large particles. These drugs inactivate both class I and class III phosphatidylinositol 3 kinases at similar concentrations, and their use alone cannot distinguish the contribution of these enzyme types. However, experiments using inhibitory antibodies as well as studies of targeted gene disruption indicate that the class I, but not class III kinases are required for phagocytosis. Conversely, class III phosphatidylinositol 3 kinase is essential for normal maturation of the phagosome. Cells pretreated with wortmannin bind the target particles and initiate pseudopod extension, but the process is arrested before the particle becomes entirely trapped, resulting in the sustained formation of phagosomal cups, rich in F-actin. The critical step impaired by phosphatidylinositol 3 kinase inhibitors may be the detachment of actin from the base of the phagocytic cup, limiting the availability of actin and

associated proteins for the continued extension of the pseudopods. This may result, for instance, from sustained stimulation of Rho-family GTPases that are unable to inactivate in a timely manner. Alternatively (or additionally), phosphatidylinositol 3 kinase blockers may prevent the targeted delivery of endomembranes to the site of phagocytosis, which may be required for extension of the pseudopodia. As in the case of actin antagonists, inhibition by wortmannin and related compounds is particle size dependent. Phagocytosis of particles smaller than 2 to 3 μm can proceed to varying degrees despite inhibition of the kinase, while the uptake of larger particles is virtually eliminated.

Materials

20 to 50 μM wortmannin stock solution in DMSO *or* 1 to 2 mM cytochalasin D stock solution in ethanol (see *APPENDIX 1B* for more information on these reagents)

Additional reagents and equipment for phagocytosis assay (see Basic Protocol 1 or Alternate Protocol 1)

1. Prepare cells for phagocytosis assay (see Basic Protocol 1 or Alternate Protocol 1).
2. Prior to addition of phagocytic target particles, incubate cells in medium of choice containing either 50 to 100 nM wortmannin or 5 to 10 μM cytochalasin D. Incubate ≥ 10 min.
3. Add phagocytic particles.

Note that cytochalasin needs to be maintained in the medium during phagocytosis, as it is a reversible agent. In contrast, wortmannin is thought to alkylate and irreversibly modify phosphatidylinositol 3 kinase and, in principle, need not be present after the preincubation period is completed.

4. Mount cells and score phagocytosis (see Basic Protocol 1 or Alternate Protocol 1).

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.*

Hanks balanced salt solution (HBSS)

5.4 mM KCl
0.3 mM Na_2HPO_4
0.4 mM KH_2HPO_4
4.2 mM NaHCO_3
1.3 mM CaCl_2
0.5 mM MgCl_2
0.5 mM MgSO_4
137 mM NaCl
5.6 mM D-glucose
Adjust pH to 7.4.
Store up to 1 month at 4°C

***K*⁺-rich media**

143 mM KCl
1 mM MgCl_2
1 mM CaCl_2
5 mM glucose
Store up to 1 month at 4°C.

Titrate to desired pH between 5.0 and 7.5 using PIPES, MES, or HEPES, as appropriate.

Na⁺-rich medium

140 mM NaCl
3 mM KCl
1 mM MgCl₂
1 mM CaCl₂
5 mM glucose
20 mM HEPES, titrated to pH 7.3 at 37°C
Store up to 1 month at 4°C.

Phosphate-buffered saline (PBS)

140 mM NaCl
10 mM KCl
8 mM monobasic sodium phosphate
2 mM dibasic potassium phosphate
Check that pH is 7.4
Store up to 6 months at 4°C

Tissue culture plates, 6-well, containing sterile glass coverslips

Hold glass 25-mm round glass coverslips with 5-in. stainless steel tissue forceps and dip in a beaker containing 70% ethanol to sterilize. Gently shake off the excess ethanol and place coverslip on an angle in the well of the 6-well plate. When all six wells have been fitted with sterile coverslips, loosely place the lid over the plate and allow it to air dry in a culture hood for 10 to 15 min. Lower the lid securely onto the plate and gently tap the plate to allow the coverslips to settle to the bottom of the well. Seed cells as usual (*UNIT 1.1*).

COMMENTARY

Background Information

FcγR-mediated phagocytosis (Basic Protocol 1, Alternate Protocol 1, and Support Protocol 1)

The principal immunoglobulin (Ig) that functions as an opsonin is IgG. The constant (Fc) region of IgG binds to its cognate Fcγ receptor (FcγR). Binding of other classes of immunoglobulins, such as IgA and IgE, to their cognate Fcα and Fcε receptors can also support particle engulfment. However, phagocytosis is not the primary function of these receptors and is not discussed in this unit.

Internalization of IgG-opsonized materials is mediated by some but not all Fcγ receptors, including FcγRI, FcγRIIA, and FcγRIIIA. The cell expression and binding specificities of individual FcγR are listed in Table 15.7.2. FcγRI and FcγRIIIA are heterotrimeric proteins composed of a single α chain (which contains the Fc binding region) and two associated γ chains that contain the immunoreceptor tyrosine activation motif (ITAM). FcγRII is a monomeric

transmembrane protein containing both the Fc-binding and the signal transduction motifs. FcγRIIA, expressed exclusively on human leukocytes, contains an ITAM motif in the cytosolic region of the protein and is able to support phagocytosis.

FcγRIIB, on the other hand, has an inhibitory ITIM (immunoreceptor tyrosine inhibitory motif) in place of the ITAM motif and is a negative (inhibitory) receptor. FcγRIIB is a GPI-linked protein expressed in granulocytes. It can initiate calcium signaling and actin polymerization but it was omitted from Table 15.7.2 because its role in phagocytosis is ill defined.

Complement-mediated phagocytosis (Basic Protocol 2, Alternate Protocol 2, and Alternate Protocol 3)

Complement (C) comprises a group of proteins that are constitutively produced and secreted as inactive precursors by macrophages and hepatocytes. Complement is an important component of the innate immune response. It is not antigen-specific and can be activated

rapidly in the presence of microorganisms or foreign bodies to induce phagocytosis and cell lysis.

Activation of the complement cascade results in the cleavage of the inactive complement precursor proteins, some of which, in turn, become proteases that cleave other complement components. There are two complement cascades. The classical cascade is activated by the Fc (constant) region of antigen-bound IgG and IgM, and it is the pathway utilized in the above protocols to deposit C3bi onto erythrocytes and latex beads. The alternative complement cascade is activated by cell-surface molecules of bacteria, including the lipopolysaccharide (LPS) of gram-negative organisms, teichoic acid from gram-positive bacteria, and components from fungal and yeast cell walls.

As indicated, some fragments such as C3bi are opsonins that target coated materials for degradation. Particles that have been opsonized by complement fragments can be internalized by several complement receptors. Table 15.7.3 summarizes the different complement receptors (CR) that support phagocytosis, the receptor-binding specificities of each receptor, and the cell types where they are found. CR1 is a large monomeric glycoprotein that binds primarily C3b fragments and promotes phagocytosis and clearance of antigen-antibody complexes in conjunction with Fc receptors. CR3 and CR4 are integrin heterodimers which bind specifically to C3bi. CR3, also known as Mac1, $\alpha_M\beta_2$, or CD11b/CD18, binds to C3bi with the highest affinity and is commonly considered to be the primary receptor involved in complement-mediated phagocytosis. The binding specificities of the receptors for C3bi are as follows: CR3>CR1>CR4. Ligation of C3bi-

coated material is insufficient to initiate phagocytosis via CR3 and CR4, which, like other integrins, require a second activation (priming) stimulus such as phorbol esters (e.g., PMA), glucan binding, or adhesion of the cells to extracellular matrix proteins. For professional phagocytes such as neutrophils, cell adhesion may provide a sufficient stimulus to initiate CR3-mediated phagocytosis.

As discussed in the introduction to this unit, cell model systems using heterologous transfection of receptors have been used extensively to study phagocytosis. If using such model systems to study complement receptor-mediated phagocytosis, bear in mind that, when expressed exogenously, most integrins are in the active state. Therefore, a priming stimulus may not be necessary to elicit phagocytosis.

Flow cytometry of phagocytosis (Basic Protocol 3)

Flow cytometric analysis offers many potential advantages over microscopic assays of phagocytosis. These include: (1) the ability to assess large numbers of cells (tens of thousands), thus facilitating statistical analysis; (2) the availability of new fluorophores and multiparametric analysis that enable simultaneous measurements of structural (e.g., change in cell size or shape) and/or functional (e.g., calcium flux, oxidant production, surface adhesion molecule expression) properties of suspended cells rapidly and quantitatively (Trinkle et al, 1987); and (3) the capacity to use small volumes of whole blood. This method is particularly well suited for analysis of human samples from individuals suspected of having defects in the innate immune system, or for analysis of rodent (murine or rat) blood samples for which

Table 15.7.2 Main Types of Fc γ R

Receptor	Cell type	Binding affinity	Ligands	Function
Fc γ RI	Neutrophil, macrophage	High	IgG2a	Phagocytosis
Fc γ RIIA	Human neutrophil, macrophage	Low	IgG1, IgG2a, and IgG2b	Phagocytosis
Fc γ RIIB	Murine neutrophil, macrophage, B cell, mast cell	ND ^a	IgG1, IgG2a, and IgG2b	Inhibitory receptor
Fc γ RIIIA	Macrophage	High	IgG1, IgG2a, and IgG2b	Phagocytosis

^aAbbreviation: ND, not determined

Table 15.7.3 Complement Receptor (CR)-Mediated Phagocytosis

Receptor	Cell type	Ligands
CRI (CD35)	Monocytes, neutrophils, dendritic cells	C3b, C3bi, C4b, C4bi
CR3 (Mac1, $\alpha_M\beta_2$ CD11b/CD18)	Monocytes, neutrophils, natural killer cells, dendritic cells	C3bi
CR4 ($\alpha_V\beta_2$ integrin, CD11c/CD18)	Macrophages, neutrophils, natural killer cells, dendritic cells	C3bi
CR5aR	Macrophages, neutrophils	C5a

only small volumes are available. The method is compatible with the use of whole blood, which reduces the possibility of artifactual changes (e.g., priming or activation) caused by even the most gentle of cell purification procedures, and may be more reflective of the behavior of leukocytes *in vivo*.

Assessment of phagosomal pH (Basic Protocol 4 and Alternate Protocol 4)

The addition of labeled weak bases to assess the pH of acidic organelles is convenient and has therefore been used extensively. While rapid and convenient, this method is not exactly quantitative, because factors other than the transmembrane proton gradient contribute to the accumulation and retention of the bases. Binding of the probes to the contents of the probe can skew the determinations and limit their reversibility.

The more quantitative measurements using covalently labeled particles are far superior, though technically more demanding. The fact that fluorescence ratios are determined greatly reduces the risk of misinterpretation or miscalculation due to bleaching or defocusing. Nevertheless, the experimenter must be cautious to ensure that the appropriate background is subtracted separately from each one of the signals acquired at different wavelengths and that any changes, such as oxidation of the dye that can occur in phagosomes, affects both wavelengths to a comparable extent.

Assessment of phagosomal maturation (Basic Protocol 5, Alternate Protocol 5, and Alternate Protocol 6)

The primary purpose of internalization of opsonized particles is their ultimate destruction

by a combination of low pH, degradative enzymes, and cytotoxic mediators. To achieve this goal, the phagosome acquires proteases and other hydrolases, high concentrations of H⁺ ions, a variety of bactericidal peptides, and other factors as it matures. A detailed review of phagosome maturation has recently been published (Vieira et al, 2002).

In the maturation protocols, advantage is taken of the progressive acquisition of specific markers as the phagosome interacts with defined intracellular compartments to identify the stage of phagosomal maturation. Assessments of maturation can be complemented with measurement of phagosomal pH.

Inhibition of phagocytosis (Basic Protocol 6)

Pharmacological tools have been the source of a great deal of knowledge in the field of phagocytosis. While they are deservedly criticized for their imperfect specificity, they can be most useful when used under optimal conditions (i.e., the concentration range where their effect is most specific) and while bearing in mind their potential limitations. Molecular approaches, such as transfection of dominant negative constructs, gene ablation, or silencing using interference RNA technology are in principle more specific. However, these normally take many hours or days to develop within the cell or animal, and they can therefore generate a plethora of secondary, even developmental defects that are only distantly related to phagocytosis. Thus, pharmacology and molecular approaches are complementary. Continued improvement of the selectivity of pharmacological agonists and antagonists will surely be an

ongoing source of information about phagosome formation and maturation.

Critical Parameters and Troubleshooting

FcγR-mediated phagocytosis (Basic Protocol 1, Alternate Protocol 1, and Support Protocol 1)

Optimal adsorption of IgG onto sRBC and latex beads is critical for the success of the phagocytosis assays. The authors highly recommend that pilot experiments be performed with serial dilutions of IgG to find the concentrations required for optimal opsonization of individual lots of reagents.

Complement-mediated phagocytosis (Basic Protocol 2, Alternate Protocol 2, and Alternate Protocol 3)

For both Basic Protocol 2 and Alternate Protocol 2, the most critical parameter is the coating of the erythrocytes or latex beads with C3bi. The authors have indicated their preferred sources for some of the critical reagents in the respective protocols. It is recommended that initial experiments with serial dilutions of IgM be performed to find the optimal sub-agglutinating titers of individual lots of antibody, as well as for sRBC and latex beads from different sources.

The incubation period in C5-deficient medium is also critical. In studies of IgG- and IgM-coated erythrocytes, it has been shown that excessively long incubation periods (>20 min) lead to loss of surface-bound C3bi (Newman and Mikus, 1985).

Flow cytometry of phagocytosis (Basic Protocol 3)

The success of these experiments will depend on several factors: optimal condition of the phagocytes to be assessed, proper opsonization of phagocytic targets, and a signal-to-noise ratio that will allow clear discrimination of particles that are internalized from those that are adherent to the cell surface (“in” from “on”). For flow cytometry, this means efficient quenching of extracellular fluorescence of surface-bound particles. In studies using trypan blue to quench extracellular fluorescence, it is important not to wash the trypan blue away, or else the quenching of fluorescence will be lost.

Because of the automated nature of the procedure and the fact that the cells are never

directly visualized during the course of flow cytometry, there are several potential artifacts that need to be avoided. These include false positive scoring of particles that are adherent to the cell surface rather than internalized and enclosed within a phagosome. This may result when quenching of the fluorescence of extracellular particles is incomplete. In addition, because the technique involves the analysis of cells in suspension, it is limited to cells that are naturally suspended or to adherent cells that can be detached easily from their substrate to form single-cell suspensions. Lifting adherent neutrophils or macrophages without incurring damage is not simple, especially after the cells have performed phagocytosis. Finally, because clumps of cells are either removed by filters designed to collect debris or excluded from analysis by gating, phagocytosis by cell types that are prone to aggregate after stimulation, such as neutrophils, might be underestimated if the most responsive and likely most avidly phagocytic cells are excluded from analysis.

There are several key parameters that require attention during flow cytometric analysis:

1. *Gating.* Cells are gated on a forward versus side scatter plot to exclude debris and broken cells. Forward scatter is proportional to the size of cells and side scatter is proportional to their granularity and shape. If there is a mixed cell population (e.g., when using whole blood or buffy coat preparations), it is critical that relatively pure populations of the cells of interest be included in the gate. It is usually possible to separate neutrophils, monocytes, and lymphocytes into distinct populations based on their light scattering. However, there is some overlap between small monocytes and neutrophils, and complete separation of these two cell types requires the use of a fluorescently labeled antibody that binds to a unique surface epitope on one cell type (e.g., CD15a for neutrophils and CD14 for monocytes). A minimum of 10,000 cells are counted in the viable cell gate

2. *Coincidence.* This term refers to the simultaneous appearance through the laser beam of the cytometer of more than one cell or free target particle, and it can lead to inaccuracies in the assessment of phagocytosis. Several parameters impact on coincidence, including the flow rate of the sheath fluid, the concentration of phagocytic cells and targets in the sample, and the ratio of targets per cell (Lehmann et al., 2000).

3. *Fluorescence channels.* Each channel is designed to collect emitted light within a defined range of wavelengths. However, each fluorochrome emits light with wider emission spectra than the limits of the single channel. This gives rise to the possibility of spectral overlap, an issue that is of critical importance when two or more fluorochromes are used (e.g., FITC-labeled phagocytic particles and a phycoerythrin-labeled anti-CD15 antibody to allow identification of human neutrophils). This potential problem can be overcome by choosing fluorochromes that have minimal spectral overlap and by electronic color compensation (a discussion of this issue is beyond the scope of this unit).

Assessment of phagosomal pH (Basic Protocol 4 and Alternate Protocol 4)

In the case of partitioning probes, such as weak bases, a range of different compounds are available with varying spectral properties. For experiments where multiple fluorophores need to be used, pH-sensitive probes that absorb light in the ultraviolet, blue, or green parts of the spectrum are commercially available.

It is essential to select probes that are most pH-sensitive in the range of interest. The pK_a of the probe chosen for analysis must ideally be in the mid-range of the measurements to be performed to provide optimal linearity. In the case of macrophage phagosomes, the pH generally becomes more acidic than in neutrophils, where the oxygen radicals generated by the NADPH oxidase can scavenge protons and thereby make the lumen more alkaline.

These oxygen radicals can greatly complicate the measurements by inducing acute chemical transformation of the fluorescent probes. When the contribution of the oxidase is not important to the measurements, it is convenient, indeed advisable to inhibit the flavoprotein component of the enzyme using diphenylene iodonium. Alternatively, the contribution of peroxidases can be minimized by addition of azide, bearing in mind that this compound will block mitochondrial ATP generation.

Assessment of phagosomal maturation (Basic Protocol 5, Alternate Protocol 5, and Alternate Protocol 6)

None of the procedures outlined will, by itself, identify the stage of phagosome maturation unambiguously. Therefore, the authors suggest that two or more different markers or methods be used to confirm the precise stage

of maturation. Acquisition of defined isoforms of Rab (e.g., Rab5 versus Rab7), and differentiation between the mature and immature forms of lysosomal enzymes, such as cathepsins, can be most informative.

The second critical parameter is the choice of fluorophores used for the colocalization experiments. Care must be taken to avoid crosstalk between fluorophores when multiple determinations are performed simultaneously.

Inhibition of phagocytosis (Basic Protocol 6)

The duration of the (pre)treatment with inhibitors should be kept to the minimum required to achieve the inactivation of the desired target. Longer than necessary treatments should be avoided as they can result in secondary, indirect effects that can complicate the interpretation of the data. Extended pretreatment with wortmannin will impair vesicular traffic along the endocytic and secretory pathways and may impact negatively on the composition of the plasma membrane or on the ability of endosomes to be delivered to the surface upon activation. Similarly, prolonged exposure to cytochalasins will grossly distort cell morphology and function and can ultimately lead to detachment of the cells from the substratum. For non-adherent cells like blood neutrophils, cytochalasins have been reported to have a “priming” effect that potentiates some responses like granule secretion. The possible consequences of such priming must be borne in mind.

Anticipated Results

Fc γ R-mediated phagocytosis (Basic Protocol 1, Alternate Protocol 1, and Support Protocol 1)

Particles coated with IgG are expected to be ingested avidly by both macrophages and neutrophils and by monocytic cell lines like J774 and RAW264.7. The vast majority of the cells in any one culture will prove to be competent to internalize one or more particles over the course of 5 to 10 min.

The simplest and least ambiguous way to differentiate between extracellular adherent particles and those that have been internalized is to lyse hypotonically those on the outside. Visualization by bright-field microscopy will only detect the engulfed, intact red cells that retained their hemoglobin. Bear in mind that while fluorescently labeled red cell ghosts that remain adherent (extracellular) after hypotonic

lysis will be observable by fluorescence microscopy they will not be observable by bright-field microscopy because they have lost hemoglobin.

Complement-mediated phagocytosis (Basic Protocol 2, Alternate Protocol 2, and Alternate Protocol 3)

The efficiency of complement-mediated phagocytosis is, in the authors' experience, considerably lower than that of Fc-mediated phagocytosis. Therefore, expect only a fraction of the cells (typically 30% to 50%) to ingest one or a couple of the particles. Moreover, the kinetics of ingestion appears to be slower, perhaps because it is mediated by Rho, unlike Fc-dependent phagocytosis that involves Rac and Cdc42. The morphology of the phagocytic site also differs: complement-coated particles appear to "sink" into the cells and do not reveal the florid pseudopods seen in Fc-dependent internalization.

Flow cytometry of phagocytosis (Basic Protocol 3)

If the protocols outlined above are followed and if the phagocytic cells are healthy, the use of flow cytometry to assess phagocytosis is straightforward. For professional phagocytes such as neutrophils, phagocytosis should be very efficient, with over 70% of cells ingesting one or more phagocytic particles. By comparison, in cell lines such as COS or Chinese hamster ovary cells transfected with phagocytic receptors, phagocytosis may be much less efficient, with only 20% to 30% of cells ingesting particles.

Assessment of phagosomal pH (Basic Protocol 4 and Alternate Protocol 4)

Phagosomes formed by macrophages are expected to become acidic very rapidly, reaching steady state after 10 to 15 min at pH as low as 5.0. In human neutrophils the pH change is smaller and has been reported to be biphasic, with some authors reporting a transient alkalization due to proton consumption by superoxide. The latter can be obviated and the acidification accentuated by inhibition of the NADPH oxidase with diphenylene iodonium.

Assessment of phagosomal maturation (Basic Protocol 5, Alternate Protocol 5, and Alternate Protocol 6)

Under normal circumstances, phagosomes formed by macrophages acquire early endosomal markers almost immediately. In fact, deliv-

ery of endosomal components to the nascent (unsealed) phagosome has been detected, accounting for the increased membrane area measured by electrophysiological capacitance determinations. Late endosomal markers appear as early as 10 min after ingestion and bona-fide lysosomal contents are detected after 45 to 60 min of phagocytosis.

Neutrophils contain unique secretory organelles that are delivered to phagosomes with different kinetics. Secretory vesicles, primary, secondary, and tertiary granules are all thought to deliver their constituents to the phagosome, although the signals that mediate fusion and the kinetics of delivery vary. Bear in mind that some granulocytic cell lines, such as HL-60 cells, fail to generate and/or accumulate some of the secretory compartments, which will therefore not contribute to phagosomal maturation.

Inhibition of phagocytosis (Basic Protocol 6)

As intimated in the introductory section of this unit, phagocytosis is a generic term that describes multiple, related but not identical processes. Even in the case of a single type of receptor and ligand, the properties of the phagocytic event may vary. Thus, phagocytosis of large particles is much more sensitive to wortmannin than is the ingestion of smaller ones ($\leq 3 \mu\text{m}$). Similarly, cytochalasin effectively inhibits the uptake of particles larger than 1–2 μm , but it is not nearly as effective in preventing the uptake of smaller particles. Much less is known for phagocytosis mediated by other receptors, so the reader should be prepared to find variations in the effectiveness of drugs when testing new systems.

Time Considerations

Fc γ R-mediated phagocytosis (Basic Protocol 1, Alternate Protocol 1, and Support Protocol 1)

Growth, expansion, and plating of the cells on the appropriate substratum is the most time-consuming part of the procedure. The phagocytosis protocol proper can be performed within 1 day. Therefore, in cases where primary cells can be isolated the same day, the entire experiment can be completed in a regular working day. It is not advisable to store opsonized red cells for extended periods.

Complement-mediated phagocytosis (Basic Protocol 2, Alternate Protocol 2, and Alternate Protocol 3)

The preparation of complement-opsonized particles is more laborious than that of IgG-coated particles. Nevertheless, it can and should be performed the day of the experiment. As in the case of Fc-mediated phagocytosis experiments, the most time-consuming part of the procedure is the growth, expansion, and plating of the cells on the appropriate substratum. Bear in mind that priming with phorbol esters is required for optimal complement-mediated phagocytosis, and this increases the duration of the experiments.

Flow cytometry of phagocytosis (Basic Protocol 3)

When studying primary cells, the entire experiment will take 5 to 6 hr if the phagocytic targets are prepared ahead of time. When working with cultured cells, the entire experiment will take 2 to 3 days, with the majority of the bench work on day 2.

Assessment of phagosomal pH (Basic Protocol 4 and Alternate Protocol 4)

Qualitative assessment of phagosomal pH using fluorescent weak bases can be accomplished in under 1 hr, if the reagents are prepared in advance and the microscope is available. In contrast, the ratiometric determinations are more laborious, requiring several hours from start to finish, even when the labeled particles are prepared in advance. A sizable fraction of the time is invested in the calibrations, which last longer than the pH determinations themselves. Nevertheless, if the cells are available at the beginning of the day, multiple measurements can be accommodated in a single working day.

Assessment of phagosomal maturation (Basic Protocol 5, Alternate Protocol 5, and Alternate Protocol 6)

The delivery of fluid-phase markers to phagosomes can be assessed within hours, provided the cells and opsonized particles are prepared in advance. The marker solutions are generally stable for considerable periods and can be prepared in advance.

Assessment of maturation by immunostaining is more time-consuming, requiring long fixation, blocking, staining, and mounting steps. From start to finish, an immunolabeling experiment can be completed within 2 days.

Inhibition of phagocytosis (Basic Protocol 6)

Pretreatment with most inhibitors is generally short (under 1 hr). Therefore, assessment of their effects on phagocytosis should not take much longer than the measurements of phagocytosis themselves (see above).

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CHAPTER 16

Antibodies as Cell Biological Tools

INTRODUCTION

Monoclonal and polyclonal antibodies are powerful tools for addressing cell biological questions. These immunological reagents can be used to detect and analyze proteins and carbohydrates, characterize the subcellular distribution of proteins, and purify proteins. In addition, when taken up by cells, antibodies can be used as tags to monitor the intracellular pathways of proteins and to inhibit protein activity. These diverse applications of antibody technology have been facilitated by advances in the understanding of the molecular genetics of antibody molecules and their three-dimensional structures. The development of methods for measuring antibody binding activity and for isolating antibodies has contributed to their widespread use and extensive range of applications.

The development of monoclonal antibodies, unique and powerful reagents for detecting and measuring interactions with specific protein epitopes, has revolutionized the use of antibodies in cell biological research. These antibodies are obtained by fusing immune B cells from the spleen with tumor cells to produce hybridomas. Hybridomas each secrete a single type of antibody—the monoclonal antibody—that has precise specificity and often high affinity (*UNIT 16.1*). Because the cloned hybridoma cell line is immortal, with appropriate care it can be maintained indefinitely and the antibodies it produces can be supplied in essentially limitless quantities. Preparations containing monoclonal antibodies include hybridoma supernatants, ascites fluid from a mouse inoculated with the hybridoma, and purified monoclonal antibody. *UNIT 16.1* describes how to produce monoclonal antibodies, including basic protocols for immunization, cell fusion, and hybridoma selection. The unit also includes methods for antigen preparation, suggestions for troubleshooting to ensure production of antibodies at high titer, and approaches for increasing the specificity of the antibody.

Polyclonal antibodies (*UNIT 16.2*) take less effort to prepare than monoclonal antibodies. The process consists simply of immunizing an animal of any one of a variety of species (including goat, horse, rat, mouse, and rabbit) with purified antigen and then, after the animal develops an immune response, isolating antibodies from its serum. Because polyclonal antibodies are essentially a collection of monoclonal antibodies with different epitope specificities and affinities, they are useful for analyses of denatured forms of a protein, for immunoprecipitation, and for immunoblotting. These types of analyses are often not successful with monoclonal antibodies because the single epitope recognized by the monoclonal antibody preparation may be destroyed during protein denaturation.

The choice of animal species for immunization depends in part on the amount of antiserum required for subsequent experiments. Mice, rats, and guinea pigs yield relatively low volumes of antiserum compared to rabbits and other larger animals. For this reason, rabbits are often the animals of choice. It is often desirable to produce polyclonal antibodies in other species, however, especially when an experiment requires two distinct types of antibodies that recognize different proteins (as is often the case in indirect immunofluorescence assays; see *UNIT 4.3*). *UNIT 16.2* contains a detailed protocol for immunizing animals to produce polyclonal antibodies, with discussion of the kinetics of the antibody response, when to boost, and the use of different adjuvants; advice on how to generate the highest-titer

antiserum is also included. Using these techniques, investigators can have polyclonal antibodies in hand for use in experiments within 6 to 8 weeks.

Purified antibody preparations are crucial when accurate concentrations of antibody are needed. For example, when chemical or structural modifications of antibody are being made for antibody binding studies. *UNIT 16.3* describes how to prepare purified forms of monoclonal and polyclonal antibodies. The purification methods that are discussed include ammonium sulfate precipitation, protein A and protein G affinity chromatography, ion-exchange chromatography, and combinations of these. Rapid assays for monitoring the activity of antibody are described for ensuring that antibody activity is not lost during purification. Commercial kits for purification and fragmentation of antibodies that are available for researchers who are unable to prepare antibodies themselves are also discussed in terms of their reliability and relative cost.

UNIT 16.4 describes how to cleave antibodies into functionally distinct subunits using proteolytic enzymes such as papain. Monovalent fragments of IgG produced by papain digestion are known as Fab fragments. These fragments usually retain the binding specificity of the intact IgG molecule. They are, therefore, desirable in binding studies since the Fc portion of IgG may cause artifacts due to binding to cells that bear the Fc receptor. In addition to describing papain digestion of IgGs, the unit discusses pepsin and ficin digestion methods which produce bivalent $F(ab')_2$ fragments and allow discrimination of different IgG subclasses. Both pilot and large-scale fragmentation protocols are described.

UNIT 16.5 describes how to conjugate antibodies with fluorescent dyes, with biotin, and with enzymes. Antibodies that are directly conjugated in this manner have proven to be essential for detecting multiple structures or functions within a specimen simultaneously. The alternative method for staining multiple epitopes is by using unconjugated primary antibodies detected with labeled secondary antibodies, but this usually results in significant cross-reactivity. *UNIT 16.5* describes several simple methods for directly conjugating antibodies so that their biological activity is retained. A basic method involves derivation of the amino group(s) on the antibody with dye, biotin, or enzyme. The methods are simple to perform and have high rates of success. Alternative, more complex methods are presented; these include attaching the label at a carbohydrate site or sulfhydryl group of the antibody. The Commentary includes a detailed discussion of the types of fluorophores and enzymes and their advantages and uses for conjugation. In addition there is discussion of the critical aspects of conjugation, including the choice of conjugating species and the optimal extent of labeling to preserve antibody affinity.

A powerful new approach to elucidating the important but bewilderingly complex signal transduction pathways of cells is to use antibodies that detect specific sites on proteins only after they become phosphorylated. For example, such antibodies can recognize with high sensitivity the addition of phosphate to specific tyrosine residues in a particular signaling or structural protein—if such phosphorylation regulates activation or inhibition of functional activity, the antibodies can be used to monitor activation, e.g., in homogenates or by immunofluorescence. *UNIT 16.6* provides methods for producing either polyclonal or monoclonal antibodies against specific phosphopeptides. In order to isolate polyclonal antibodies specific only for a unique tyrosine phosphopeptide, multistep affinity chromatography is applied to achieve appropriate specificity. Similarly, monoclonal antibodies are screened in several steps to identify a clone with sufficient specificity. Although these protocols focus on sites containing phosphorylated tyrosine, similar approaches can be used for producing antibodies that recognize specific serine- and threonine-phosphorylated peptides.

Jennifer Lippincott-Schwartz

Production of Monoclonal Antibodies

UNIT 16.1

Highly specific antibodies can be obtained by fusing immune splenic B cells from the spleen with tumor cells to produce hybridomas, each of which will then secrete a single antibody. The desired antibody-producing hybridoma can be identified by a screening process. If this hybridoma is subjected to a cloning step in which clones are selected, such that all progeny are derived from a single cloned parental cell, a monoclonal antibody is obtained. Monoclonal antibodies have high specificity and can be produced in large quantities. Thus, these biological reagents have been used extensively as probes in a wide range of systems including the characterization of novel cell-surface and soluble proteins and carbohydrates, as enzyme catalysts, and for targeting in immunotherapy (see Commentary).

This unit describes the production of monoclonal antibodies beginning with basic protocols for immunization (see Basic Protocol 1) and cell fusion and selection (see Basic Protocol 2). Support protocols are provided for screening primary hybridoma supernatants for antibodies of desired specificity (see Support Protocol 1), establishment of stable hybridoma lines (see Support Protocol 2), cloning of these B cell lines by limiting dilution to obtain monoclonal lines (see Support Protocol 3), and preparation of cloning/expansion medium (thymocyte-conditioned medium; see Support Protocol 4). Figure 16.1.1 summarizes these stages and notes the protocols in this and subsequent units in which they are detailed. A major commitment of time and labor is necessary but, if successful, the monoclonal antibody may be an extremely valuable reagent that will be available in large quantities.

Submission of monoclonal antibodies to the American Type Culture Collection (ATCC) for distribution to the scientific community is encouraged. Moreover, the ATCC serves as a repository for cell lines should the line be lost in the investigator's laboratory due to unforeseen circumstances.

NOTE: All solutions and equipment coming into contact with living cells must be sterile and appropriate aseptic techniques should be used accordingly.

IMMUNIZATION TO PRODUCE MONOCLONAL ANTIBODIES

A wide variety of antigen preparations have been used successfully to produce monoclonal antibodies (see Critical Parameters discussion of antigen preparation). The following protocol provides an immunization schedule for the production of most antibodies, although several different schedules can be used. In this protocol, emulsified antigen is injected intraperitoneally into the species of choice. A booster injection is administered 10 to 14 days after the primary immunization. Three days after the booster injection, the animals' spleens are ready for cell fusion (Basic Protocol 2).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Antigen

Complete Freund's adjuvant (CFA; Sigma)

Animal: pathogen-free mouse, rat, or hamster (Armenian hamsters from Cytogen Research are recommended); see Critical Parameters for discussion of animal choice

Incomplete Freund's adjuvant (IFA; Sigma), optional

**BASIC
PROTOCOL 1**

**Antibodies As
Cell Biological
Tools**

16.1.1

Contributed by Wayne M. Yokoyama

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Supplement 3

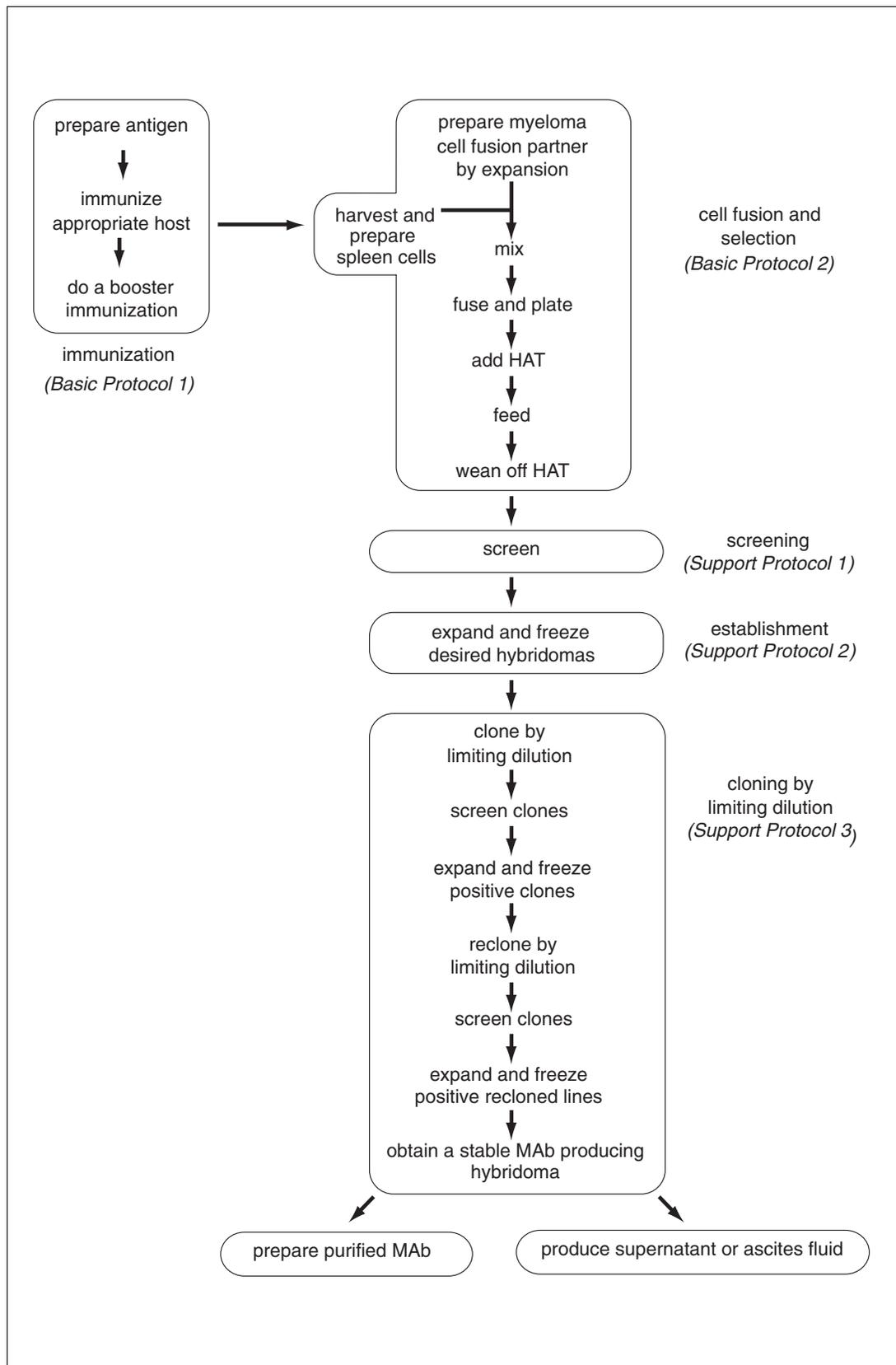


Figure 16.1.1 Stages of monoclonal antibody production, with references to the basic and support protocols in this unit that describe the steps.

- 1- to 2-ml glass syringes with Luer-Lok tips, sterile
- 3-way stopcock
- 20- and 22-G needles, sterile

CAUTION: CFA is an extremely potent inflammatory agent, and is hazardous to the investigator, particularly if introduced intradermally or into the eyes. Profound sloughing of skin or loss of sight may occur. Self-injection can cause a positive TB skin test and lead to a granulomatous reaction. Use gloves and protective eyewear when handling CFA.

1. Prepare antigen using 2×10^6 to 5×10^7 cells or 1 to 50 μg protein or peptide per animal to be immunized in normal saline.

The antigen may be in any of several different forms depending on the desired property of the MAb and the method of screening (see Critical Parameters for discussion of antigen preparation and screening assays). If cells are the immunogen, wash three times in serum-free medium before immunization. Plan the immunization of several animals (enough for several fusions) so that primed and boosted animals will be ready 3 days before fusion (see Basic Protocol 2).

To minimize the risk of introducing a pathogen into the rodent colony, screen cells for pathogens by antibody-production assay (Donovan and Brown, 1995).

2. Draw up antigen into a sterile 1- to 2-ml glass syringe with a Luer-Lok tip. Connect syringe to a 3-way stopcock.
3. Completely resuspend CFA to disperse the *Mycobacterium tuberculosis* bacilli which settle to the bottom of the container with time. Draw up a volume of CFA equal to the antigen volume in a syringe and connect to the antigen-containing syringe.
4. Emulsify antigen and CFA by discharging antigen into CFA, then discharging back and forth until a thickened mixture results. Test whether the emulsion is stable—a stable emulsion will not disperse when a drop of it is placed in water.

Figure 16.2.1 illustrates the double-syringe device.

5. Transfer all of the CFA/antigen emulsion to one syringe and remove the other syringe and stopcock. Attach a sterile 20-G needle to the syringe containing the emulsion.
6. Inject emulsion intraperitoneally into the animal using <0.2 ml per mouse, 0.5 to 1 ml per rat, or 0.2 to 0.4 ml per hamster.

Be careful not to force the syringe plunger since excessive pressure may dislodge the needle and spray the emulsion. Introduce the needle through the skin and tunnel the needle between the skin and peritoneal wall before entering the peritoneal cavity at a site distant from the dermal puncture site. Twirl needle before withdrawal to minimize leakage.

Rats are generally anesthetized whereas mice and hamsters can be manipulated with one hand and do not require anesthetic.

7. Boost animal after 10 to 14 days with approximately the same dose of antigen as in step 5. If cell fusion is planned for 3 days after boosting, immunize with antigen alone in aqueous solution, or intact cells in suspension. If a fusion is not immediately planned, boost the animal with antigen emulsified in IFA (which does not contain *Mycobacterium tuberculosis* bacilli).

Do not use CFA for the booster immunizations as this will cause intense inflammation and increased anti-TB antibody response.

If desired, antibody titers can be assayed by ELISA or immunoprecipitation (UNIT 7.2), 7 to 10 days after the primary and booster immunizations.

CELL FUSION AND SELECTION OF HYBRIDOMAS

While animals should be immunized as soon as the decision has been made to produce a monoclonal antibody and the antigen has been prepared, do not perform cell fusion until the screening assay (Support Protocol 1) has been perfected. Artifacts that may arise from conditioned media must be identified before cell fusion, because after a fusion there is only a finite amount of time available to assay for the desired monoclonal antibody.

Prior to cell fusion, the partner (myeloma) cell line is expanded and a booster injection of antigen is administered to the primed animals. On the day of fusion, the spleens are harvested. Spleen cells and partner cells are washed, harvested, and mixed. Cell fusion is performed at 37°C in the presence of polyethylene glycol (PEG). The resulting pellet is harvested and plated into tissue culture plates. After incubation with hypoxanthine, aminopterin, and thymidine (HAT) medium and feeding over ~2 weeks, the hybridomas are ready for screening (Support Protocol 1).

Materials

- SP2/0-Ag14 myeloma cell line (drug-marked, nonsecretory; ATCC #CRL 1581)
- Complete DMEM-10 and -20 media with 10 mM HEPES and 1 mM sodium pyruvate
- Primed animal; mouse, hamster, or rat (10 to 14 days after primary immunization; see Basic Protocol 1)
- Complete DMEM medium (*APPENDIX 2A*), serum-free
- 50% polyethylene glycol (PEG), sterile (see recipe)
- Ammonium chloride solution (see recipe)
- Complete DMEM-20 with 10 mM HEPES, 1 mM sodium pyruvate, and 1× HAT or 1× HT supplement (see recipe)
- 175-cm² flasks
- Fine-mesh metal screen
- 50-ml conical polypropylene centrifuge tubes
- Beckman TH-4 rotor or equivalent
- 96-well flat-bottom microtiter plates
- Additional reagents and equipment for counting cells and assessing cell viability by Trypan blue exclusion (*UNIT 1.1*)

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

Prepare myeloma cells (1 week before fusion)

1. One week before fusion, begin expansion of SP2/0-Ag14 myeloma cell line (the fusion partner cell line) in complete DMEM-10/HEPES/pyruvate (see Critical Parameters). By the day cell fusion is to be performed, the following total number of myeloma cells must be available (in multiple 175-cm² flasks containing 100 ml each), depending upon the source of the primed animal: mouse spleen, 1 × 10⁸ cells in two or three flasks; hamster spleen, 2 × 10⁸ cells in three or four flasks; and rat spleen, 5-10 × 10⁸ cells in ten flasks.

Two mouse or hamster spleens, or one rat spleen, will provide enough cells for the fusion (see step 7).

Boost primed animal (3 days before fusion)

2. Three days before fusion, boost primed animal(s) (see Basic Protocol 1, step 7).

Prepare reagents and split myeloma cells (1 day before fusion)

3. One day before fusion, prepare all reagents and media, particularly 50% PEG.
4. One day before fusion, split SP2/0-Ag14 myeloma cells (from step 1) into fresh complete DMEM-10/HEPES/pyruvate medium.

Vigorous growth of the SP2/0-Ag14 cells is generally required for good fusion.

Check myeloma cells and prewarm reagents (day of fusion)

5. Use an inverted microscope to check the SP2/0-Ag14 myeloma cells to make sure they are growing vigorously (refractile and not pyknotic), they are not contaminated (no obvious bacteria or fungi), and there are enough cells for the fusion.

It is better to postpone the fusion than to perform an ill-advised fusion, since the entire selection and screening effort will take ~3 weeks.

6. Prewarm the following in a 37°C water bath:

Three 400- and three 600-ml beakers, each containing ~100 ml H₂O
20 ml sterile complete serum-free DMEM
5 ml sterile 50% PEG solution.

Harvest spleen and prepare cells

7. Sacrifice boosted animal(s) and aseptically harvest spleen(s).

Do not use anesthetics for sacrifice. Instead, use cervical dislocation for mouse, or CO₂ asphyxiation for mouse, hamster, or rat to avoid introducing an anesthetic into the blood stream and therefore, into the cultures.

8. Transfer spleen to a sterile 100-mm-diameter petri dish filled with 10 ml sterile complete serum-free DMEM.

Perform all subsequent steps in a laminar flow hood.

9. Tease spleen into a single-cell suspension by squeezing with angled forceps or by chopping with fine-tipped dissecting scissors. Remove debris and disperse cells further by passage through a fine-mesh metal screen.
10. Transfer spleen cell suspension to a sterile 50-ml conical centrifuge tube and fill with sterile complete serum-free DMEM.

Do not use protein- or HEPES-containing medium because the PEG will precipitate proteins and HEPES can be toxic to cells during fusion.

11. Centrifuge 5 min in TH-4 rotor at 1500 rpm (500 × g), room temperature, and discard supernatant.
12. Lyse red blood cells (RBC) by resuspending pellet in 5 ml ammonium chloride solution. Let stand 5 min at room temperature.
13. Add 45 ml sterile complete serum-free DMEM, and centrifuge as in step 11.
14. Resuspend pellet in 50 ml sterile complete serum-free DMEM. Centrifuge as in step 11. Repeat DMEM addition and centrifuging once (each repeat is a wash).
15. While spleen cells are being washed, separately harvest the SP2/0-Ag14 myeloma cells (from step 5) by transferring the cells to 50-ml conical centrifuge tubes. Centrifuge as in step 11. Resuspend myeloma cells in DMEM and pool all cells into one 50-ml conical centrifuge tube. Wash myeloma cells three times as in step 14.
16. Separately resuspend the spleen and myeloma cells in 10 ml complete serum-free DMEM. Count cells and assess viability in each cell suspension using a hemacytometer and trypan blue exclusion (UNIT 1.1).

There should be nearly 100% viability of both suspensions.

17. On basis of cell counts (from step 16), calculate the amount of complete DMEM-20/HEPES/pyruvate needed to plate cells at $\sim 16.1 \times 10^6$ total cells/ml. Prewarm this amount of complete DMEM-20/HEPES/pyruvate in 37°C water bath. Prepare 96-well flat-bottom plates by labeling them sequentially: one plate is required for each 10 ml of final cell suspension.

Perform cell fusion

18. Mix SP2/0-Ag14 myeloma and spleen cells at a 1:1 ratio in a 50-ml conical centrifuge tube. Fill the tube with complete serum-free DMEM.

Other cell ratios work. Successful fusions have been performed with a ratio of myeloma/spleen cells as low as 1:20.

19. Centrifuge cell mixture 5 min at $500 \times g$, room temperature.
20. While cells are in the centrifuge, prepare three 37°C double-beaker water baths in the laminar flow hood by placing a 400-ml beaker (from step 6) containing 100 ml of 37°C water into 600-ml beaker containing 75 to 100 ml of 37°C water. Place the tubes of prewarmed 50% PEG solution and complete serum-free DMEM (from step 6) into two of the 37°C water baths in the hood.
21. Aspirate and discard supernatant from the mixed-cell pellet (from step 19).
22. Perform the cell fusion at 37°C by placing the tube containing the mixed-cell pellet in one of the double-beaker water baths in the laminar flow hood.
23. Using a 1-ml pipet, add 1 ml prewarmed 50% PEG to the mixed-cell pellet drop-by-drop over 1 min, stirring the cells with the pipet tip after each drop. Stir for an additional minute.
24. Using a clean pipet, add 1 ml prewarmed complete serum-free DMEM to the cell mixture drop-by-drop over 1 min, stirring after each drop. Repeat once with an additional 1 ml of prewarmed complete serum-free DMEM.
25. With a 10-ml pipet, add 7 ml prewarmed complete serum-free DMEM drop-by-drop over 2 to 3 min.

Macroscopic clumps of cells should be obvious at this point.

26. Centrifuge 5 min at $500 \times g$, room temperature.
27. While the cells are in the centrifuge, rewarm the beaker water baths to 37°C and place in the hood. Place prewarmed complete DMEM-20/HEPES/pyruvate (from step 17) in the beaker water bath.
28. Discard the supernatant (from step 26). Place tube in the beaker water bath.
29. With a clean 10-ml pipet, forcefully discharge 10 ml prewarmed complete DMEM-20/HEPES/pyruvate to the cell pellet.
30. Repeat step 29 until the total volume of prewarmed complete DMEM-20/HEPES (calculated in step 17) is added. If necessary, allow clumps to settle and disrupt with the pipet tip. Further warming of cell suspension is no longer required.

If the total volume exceeds 50 ml, gently aspirate and transfer to another sterile container such as a tissue culture flask.

31. Gently aspirate 10 ml of cell suspension with a 10-ml pipet. Add 2 drops (100 to 125 μ l) of suspension to each well of a 96-well flat-bottom plate (continue until entire suspension is plated). Incubate overnight in a humidified 37°C, 5% CO₂ incubator.

Vigorous pipetting of the cell suspension should be avoided at this point, as the newly formed hybrids are unstable. Moreover, the vigorous addition of cells to the wells with repeating micropipettor is not advised. Use a pipet aid and hold the 10-ml pipet at a 45° angle with the tip 1 to 2 cm above the well, bracing the pipet with a finger from the opposite hand. To avoid introducing contaminants, do not hold hands above the plate. A steady, even flow of drops from the pipet will allow the most efficient delivery of cell suspension or medium to the wells. Use a fresh pipet to withdraw additional cell suspension.

As an optional step to minimize fibroblast overgrowth, permit the fibroblasts in bulk-fused cell suspension to adhere overnight to tissue culture flasks before seeding the 96-well plates.

Many investigators select their hybridomas under bulk conditions—i.e., they incubate large numbers of cells per well in larger plates or flasks. This makes feeding easier, but allows fast-growing hybridomas to overgrow the others. Since nonproducing hybridomas tend to grow faster, especially in the hamster-mouse fusions, hybridomas are isolated initially in multiple small wells in this protocol. The primary hybridomas tend to be monoclonal. This is especially important when screening procedures are used that require differential reactivities, e.g., to different cell lines by flow cytometry analysis or to different antigen preparations. In those cases, multiple hybridomas per well will obscure the reactivity of the MAb of interest.

Monitor and feed cells

32. After one day of incubation, check wells under an inverted microscope. If seeded with the appropriate number of cells, there should be a nearly confluent monolayer of highly viable cells on the bottom and obvious clumps of cells.
33. Add 2 drops complete DMEM-20/HEPES/pyruvate/HAT to each well with a 10-ml pipet (see step 31). Place in humidified 37°C, 5% CO₂ incubator.

Use a separate pipet for each microtiter plate and keep the same covers with each plate to ensure that each plate remains a separate unit and to avoid spreading contamination. It cannot be overemphasized that it takes practice and meticulous attention to possible sources of contaminants to keep these plates sterile during the subsequent 2-to 3-week feeding and monitoring schedule.

If plates become contaminated, discarding them is advised. Alternatively, contamination in one or two wells may be treated by aspirating the contents of the contaminated well with a sterile Pasteur pipet attached to a vacuum flask, rinsing the well with 70% ethanol, and wiping with a sterile cotton swab. Wash twice with ethanol. Finally, blot the well dry with the sterile cotton swab and blot the appropriate area of the cover with a sterile cotton swab soaked in 70% ethanol. Do not open contaminated plates while other plates are in the hood.

34. On days 2, 3, 4, 5, 7, 9, and 11, aspirate half the volume of each well using a sterile, short Pasteur pipet attached to a vacuum flask, holding pipet at a 45° angle and touching tip to surface of supernatant at the point where the liquid meets the opposite wall of the well. Feed the cells by adding 2 drops complete DMEM-20/HEPES/pyruvate/HAT from a 10-ml pipet (see steps 31 and 33) to each well, and return to humidified 37°C, 5% CO₂ incubator.

Use a separate Pasteur pipet for each plate to minimize the spread of contamination. Since the frequency of successful viable hybridoma formation is $\leq 10^{-5}$, when HAT is added, profound cell death should be apparent at days 2 and 3 and the remaining viable cells should not be readily apparent until they have expanded. By day 7 to 9 for mouse-mouse fusions, day 11 for rat-mouse fusions, and day 14 for hamster-mouse fusions, clusters of hybridoma cells should become visible under the inverted microscope. If profound cell death is not apparent on days 2 and 3, check the medium containing HAT and the parental cell line by incubating an aliquot of the parental myeloma line with the medium containing HAT.

The feeding schedule is not rigid except for the first 4 days, when it is necessary to remove the toxic products of cell death. Thereafter, feedings will depend on the actual number of

cells deposited in the wells, efficiency of fusion, and appearance and growth of hybridomas. Do not allow medium to become yellow (acidic) for more than a day. Examine plates daily, even if cells are not scheduled to be fed, and feed plates if acidic wells are noted.

35. On day 14, repeat feeding protocol outlined in step 34 except use complete DMEM-20/HEPES/pyruvate/HT to feed cells. Return to 37°C, 5% CO₂ incubator.

Cells do not require more than one change of complete DMEM-20/HEPES/pyruvate/HT. After this change, the aminopterin (from prior addition of HAT medium) is apparently diluted out enough so that the cells can survive without additional HT.

36. On day 15 and subsequently, feed wells as noted using complete DMEM-20/HEPES/pyruvate without HAT or HT. The hybridomas are ready for screening when most of the wells containing growing cells demonstrate 10% to 25% confluence and when those with denser populations turn yellow within 2 days after feeding (see Support Protocol 1).

If the screening assay requires a [³H]thymidine incorporation assay, be aware that the large amount of thymidine in complete DMEM-20/HEPES/pyruvate with HAT and HT will serve as a cold-label inhibitor of [³H]thymidine incorporation. At least 3 to 4 changes of complete DMEM-20/HEPES/pyruvate without HT are required to dilute out excess thymidine.

SUPPORT PROTOCOL 1

SCREENING PRIMARY HYBRIDOMA SUPERNATANTS

The vast majority of wells will not contain the desired antibody or may contain nonproducing hybridomas. The purpose of screening is to discover which wells (<1% to 5%) contain hybridomas that secrete the antibody of desired specificity (note that the antibody is not yet monoclonal.) Screening should be performed when most of the growing wells demonstrate 10% to 25% confluence when viewed with an inverted microscope or when some of the denser wells begin to turn yellow within 2 days after feeding. This point may be reached 10 to 14 days after a mouse-mouse or rat-mouse fusion, and 14 to 21 days after a hamster-mouse fusion. Although the most dense wells can be screened first and the less dense wells when they become more dense, this duplicates the effort required and is not recommended. Thus, the wells are fed and 2 days later, aliquots of the supernatants are tested in the screening assay for the presence of the desired antibody.

Additional Materials (also see Basic Protocol 2)

Growing hybridomas (Basic Protocol 2)

Additional reagents and equipment for ELISA (APPENDIX 3) and indirect immunofluorescence (UNIT 4.3)

1. Estimate the number of wells with growing hybridomas using an inverted microscope. Determine whether it will be more efficient to screen all wells or only the wells that contain hybridomas.
2. Allow hybridomas in the viable wells to grow in a humidified 37°C, 5% CO₂ incubator without feeding for ≥2 days.

This is usually enough time to build a saturating titer of antibody in the culture supernatant.

3. Remove 100 μl from each well to be tested and use in a screening assay, such as an ELISA or indirect immunofluorescence (see Critical Parameters).

A micropipettor with disposable sterile tips is convenient. Use a new pipet tip with each well. If all wells are screened, a multichannel pipet is convenient for transfer to another 96-well assay plate.

For each sample, keep track of the plate number and well by its row letter and column number. This is frequently the origin of the MAb's name.

4. After harvesting one entire plate, feed wells with fresh complete DMEM-20/HEPES before harvesting the next plate (Basic Protocol 2, step 33).

ESTABLISHMENT OF HYBRIDOMA LINES

Once candidate hybridomas are identified (see Support Protocol 1), they are expanded and fed, then the cells are both frozen and cloned by limiting dilution (see Support Protocol 3). This additional work ensures that viable antibody-producing hybridomas are available after the screening process. Unfortunately, this must be done for all candidate lines before the monoclonal antibody specificity is fully characterized. To limit the amount of extra work involved, select the twenty best candidate wells. All twenty should be frozen and limiting-dilution plates set up while the supernatants are checked.

Additional Materials (also see Basic Protocol 2)

Growing hybridomas (Basic Protocol 2)

Cloning/expansion medium (Support Protocol 4)

24-well microtiter plates

Additional reagents and equipment for cryopreservation of cells (*UNIT 1.1*)

1. When the growing hybridoma is 25% to 50% confluent in the 96-well plate (master well), expand the candidate hybridoma to a well in a 24-well plate by resuspending the cells in the master well with a sterile pipet (a micropipettor set at 100 μ l is convenient). Transfer the entire contents of the master well to a well in the 24-well plate.

Sufficient numbers of cells will remain to serve as backup to the expanded cells.

If a small number of cells are transferred, they may not expand. However, if there are fibroblasts in the master well, the hybridoma cells should be transferred as soon as possible to avoid fibroblast overgrowth in the well (if necessary, transfer to another 96-well plate).

When transferring cells from a well with fibroblasts, be careful not to scrape the bottom of the well (which will loosen the fibroblasts).

2. Feed cells in the master well with 3 drops complete DMEM-20/HEPES/pyruvate from a 1-ml pipet (Basic Protocol 2, step 33). Incubate cells in humidified 37°C, 5% CO₂ incubator.
3. Use a fresh pipet and feed cells in 24-well plate with 1 to 1.5 ml cloning/expansion medium. Incubate 2 to 3 days in humidified 37°C, 5% CO₂ incubator.

Remember that there are only two wells in the world that contain this hybridoma and that each is a backup to the other in case of contamination or other undesirable circumstance. Therefore, once established, each is treated as an individual entity and fed with medium from different bottles and different pipets.

4. When cells in the 24-well plate are 25% to 50% confluent (2 to 3 days), they are ready to be used for cloning by limiting dilution (see Support Protocol 3).

Repeat steps 1 to 3 as necessary when cells are 25% to 50% confluent in master well.

5. After taking cells for limiting-dilution cloning, transfer remainder of cells in the 24-well plate to a 4-ml sterile capped tube and feed the cells in the 24-well plate with complete DMEM-20/HEPES/pyruvate.
6. Centrifuge the cells in the 4-ml tube 5 min at 500 \times g, room temperature. Keep supernatant for further characterization of the antibody and as a control, and freeze cell pellet (*UNIT 1.1*).

The supernatant usually contains sufficient antibody that could be reassayed in the original screening test and/or in any confirmatory test. The cell line is not established until stable

clones can be identified, frozen, and successfully thawed. If the limiting-dilution plate does not yield an antibody-producing line, the original cells from the 24-well plate can be thawed, seeded back into a 24-well plate, grown overnight, used to seed another series of limiting-dilution plates, and refrozen for safekeeping.

CLONING BY LIMITING DILUTION

Monoclonal antibodies are secreted by the progeny of a single cell that can produce only a single antibody (assuming a nonsecretory fusion-partner line). Cloning is required to ensure that the problems of polyspecificity are avoided and the risk of overgrowth by nonproducing cells is minimized. Although cloning can also be performed by the soft-agar technique, clones derived by this technique must be adapted to liquid culture before the supernatants can be tested (Coffino et al., 1972). Since cloning by limiting dilution allows direct testing of the supernatants, this method is much more advantageous (hence, the soft-agar technique will not be discussed here).

Additional Materials (also see Basic Protocol 2)

Candidate hybridoma line (Support Protocol 2)
Cloning/expansion medium (Support Protocol 4)

1. Resuspend the candidate hybridoma line (Support Protocol 2, step 4) in their wells and count and assess viability of a small aliquot (50 μ l) of cells using a hemacytometer and Trypan blue (*UNIT 1.1*).
2. Prepare 10 ml of cells at 50 viable cells/ml and 10 ml at 5 viable cells/ml in cloning/expansion medium.

The degree of dilution is usually very large and thus serial dilution may be needed.

3. Seed a 96-well plate with the cell suspensions at 200 μ l/well. Incubate 7 to 10 days in a humidified 37°C, 5% CO₂ incubator.

There will be enough to seed half of the wells in the plate with each dilution at final concentrations of 10 cells/well and 1 cell/well.

4. Determine which dilution was optimal for monoclonal growth by determining the number of wells that show growing hybridomas.

Usually hybridoma growth is obvious by macroscopic visualization of the well bottoms. A microplate reading mirror (Flow Laboratories or Dynatech) is useful to avoid the necessity of holding the plate above one's head.

Poisson statistics indicate that if <22% of the wells have growing cells (the proportion expected if the cells were seeded at 0.3 per well and had a cloning efficiency of 100%), then 88% of these wells have only one clone. However, primary hybridomas generally have a poor "plating efficiency" and thus, more cells need to be seeded in each well to derive a reasonable number of growing clones. Use the wells seeded with 1 cell/well if there are "growing" wells and, if necessary, the wells seeded with 10 cells/well. The frequency of antibody-producing clones is dependent on the time after the initial testing that the cloning plates were set up and the MAb species. Generally, the longer the wait before plating the primary hybridomas and the more phylogenetically distant from the mouse the source of spleen cells, the less frequent the positive clones.

5. Inspect wells for monoclonality with an inverted microscope before feeding cells, looking for tight single clusters of cells as evidence of monoclonal growth. Polyclonal growth is evidenced by more than one cluster of cells; if possible, do not use these wells.
6. Use the screening assay that was used in the initial identification of the master well (see Critical Parameters) to test the monoclonal wells for desired antibody activity on day 7 to 14. Use an aliquot of the original hybridoma supernatant (Support Protocol 2, step 6) as a positive control.

Mouse-mouse hybridomas could be checked as early as 7 days after plating, whereas hamster-mouse hybridomas may require up to 14 days for growth. If any of the wells begin to turn yellow, they should be tested. Since the wells were not fed, any well with growing cells should have readily detectable antibody if the cells produce the desired MAb. Wait 2 days before testing if the wells were fed.

7. When the desired clone is identified, expand and freeze the well as for the primary hybridoma (Support Protocol 2).
8. Reclone one of the positive hybridoma clones as in steps 1 to 3; seed two new 96-well plates at 0.3 cells/well (60 viable cells in 40 ml cloning medium).
9. Repeat steps 4 to 6.
10. Expand and freeze the recloned hybridoma (Support Protocol 2).
11. Wean the recloned cells to complete DMEM-10/HEPES/pyruvate by splitting the cells 1:2 every day (for 3 days) with complete DMEM-10/HEPES/pyruvate. At this point, the desired hybridoma should be stable as a cell line.

Freeze multiple vials on different days with different aliquots of freezing medium (UNIT 1.1). Thaw representative vials and check cells for growth and supernatant for MAb activity in appropriate assay. The hybridoma can then be used for ascites production and for large-scale production of hybridoma supernatants. The isotype of the MAb can now be determined.

Even recloned hybridomas have an unstable phenotype, especially some hamster-mouse hybridomas, which may require additional recloning. Prolonged cultures in vitro may result in loss of MAb production. To minimize this problem, frozen aliquots of cells known to produce the MAb are necessary and should be verified as sources of viable cells.

An occasional cloned hybridoma will not tolerate complete DMEM-10/HEPES/pyruvate and will require higher concentrations of FBS. It may be necessary to wean cells first to DMEM-15 before weaning to DMEM-10. Mycoplasma contamination should be considered (see UNIT 1.5).

PREPARATION OF CLONING/EXPANSION MEDIUM

Many investigators add feeder cells (i.e., peritoneal washout cells, splenocytes, or thymocytes) to produce conditioned media that seem to enhance hybridoma growth and cloning. The direct addition of irradiated, freshly isolated cells to wells, however, sometimes results in contamination. Therefore, cell-free, sterile-filtered supernatants of all suspensions are recommended to enhance hybridoma cloning efficiencies.

The following procedure describes the preparation of a cell-free thymocyte-conditioned medium from mice. After sacrificing several mice and removing each thymus, a single cell suspension is prepared and grown for several days. The supernatant is harvested, filter sterilized, and stored at -20°C .

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

1. Sacrifice 5 or 6 mice, avoiding use of anesthetics (see Support Protocol 2, step 7, annotation).

Mice (e.g., BALB/c) should be 4 to 6 weeks old. Obtain pathogen-free mice from a reliable supplier that screens for mycoplasma contamination.

2. Aseptically remove the thymus. Tease thymus into a single-cell suspension (Basic Protocol 2, steps 8 to 11). Resuspend cells in 20 ml of complete DMEM-20/HEPES/pyruvate.
3. Add 10 ml thymus cells to a 75-cm² flask. Add complete DMEM-20/HEPES/pyruvate

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medium to final amount of ~20 ml medium per thymus (maximum 60 ml cell suspension/flask). Incubate flask 4 to 5 days in upright position in a humidified 37°C, 5% CO₂ incubator.

4. Transfer suspension to a sterile 50-ml conical centrifuge tube. Centrifuge suspension 5 min at 1000 × *g*, room temperature, and harvest supernatant.
5. Filter sterilize supernatant through 0.45-μm filter. Freeze at -20°C in 10-ml aliquots.
6. Thaw and use at 10% to 20% final concentration in desired medium.

An alternative to feeder cells and thymocyte-conditioned medium is the use of a source of IL-6 (plasmacytoma growth factor), such as liposaccharide-stimulated P388D₁ supernatant.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Ammonium chloride solution

0.02 M Tris·Cl, pH 7.2

0.14 M NH₄Cl

Store up to 6 months at 4°C

Complete DMEM-20/HEPES/pyruvate/HAT (or HT)

To complete DMEM-20 medium (APPENDIX 2A) containing 10 mM HEPES and 1 mM pyruvate, add 100× HAT (hypoxanthine/aminopterin/thymidine) or 100× HT supplement (APPENDIX 2B) to 1× final. Store at 4°C for up to 1 month.

100× HAT and 100× HT supplement are available commercially (e.g., Sigma).

50% polyethylene glycol (PEG)

Autoclave 10 g PEG 4000 (Merck or ATCC) in a Wheaton glass bottle and cool. Before it solidifies (at ~55°C), add 10 ml complete serum-free DMEM. This makes enough for ~20 fusions. The solution may be kept at room temperature for several months; it will become alkaline but this does not affect its performance.

Be sure not to use protein-containing medium because PEG precipitates proteins.

COMMENTARY

Background Information

Since the original description of a technique to produce monoclonal antibodies (MAbs) of defined specificity (Köhler and Milstein, 1975), MAbs have proven to be powerful tools to analyze a myriad of biological systems. As a testimony to the broad utility of this technique, the original authors noted above received the Nobel Prize for medicine in 1984. Furthermore, a search of Medline (computerized medical literature data) for 1975 to 1999 shows that more than 90,000 citations referred to MAb.

To produce MAb, a suitable host is immunized with an antigen and antibody-secreting B cells are immortalized by the fusion of the host immune B cells with a nonsecretory, drug-marked myeloma cell line. Since the unfused

normal B cells cannot survive long in an in vitro culture, they derive immortality by fusion to a partner tumor cell line. The tumor line is resistant to the purine analogue 6-thioguanine because of deficiency of hypoxanthine-guanine phosphoribosyl transferase (HGPRT). This deficiency results in lethal sensitivity to aminopterin, which blocks de novo synthesis of purines. The normal B cell is not sensitive to aminopterin when hypoxanthine and thymidine are supplied, since it possesses the salvage pathways utilizing HGPRT which are necessary for survival. Thus, only hybridomas (normal B cells fused to tumor cells) will survive selection in HAT. If a hybridoma that produces the antibody of interest is identified and subjected to a cloning procedure that results in monoclonality (i.e., derivation of progeny cells

from a single cell), a MAb of desired specificity is produced.

Although monoclonal antibodies are powerful tools, it cannot be overemphasized that a major commitment is necessary to identify a MAb of interest. The work is tedious and labor intensive. Moreover, in some cases, specific polyclonal antisera, which are generally easier to produce, may be as suitable as—and in certain instances superior to—MAb. For example, specific antisera against defined synthetic peptides that will immunoprecipitate cell-surface antigens of interest can be made. Such antisera frequently will be useful in immunoblots whereas MAb, unless directly screened for such a purpose, may not recognize determinants on denatured antigens. Nevertheless, if the desired MAb is produced, this reagent has a wide variety of uses, particularly for the characterization of novel molecules, and as a specific antagonist or agonist of ligand-receptor interactions. Moreover, large quantities of the MAb can be readily purified.

There is a large body of literature on MAb production. More specific elaboration of the methods described here are discussed by Goding (1996) and Harlow and Lane (1999). Furthermore, many reports describing refinements of the basic technique are frequently published in the *Journal of Immunological Methods*. With general basic skills in animal handling, tissue culture, and screening assays, the novice should be able to produce MAb using the protocols in this unit. The major obstacle, as with any screening effort, is identification of the MAb of desired specificity. Because the technique produces many MAb-secreting hybridomas, the temptation exists to keep all or selected hybridomas even though they are not producing the MAb of initial interest. A large number of hybridomas secreting antibodies to unique antigens may be isolated, but the extra effort required is considerable. Nevertheless, it is a strategy that may prove useful while the attempt to identify the desired MAb proceeds. Many antibodies described in the literature are such by-products.

Critical Parameters

There are several major factors, other than technical, that should be considered before any MAb production project is begun.

While several fusion partners are available, the SP2/0-Ag14 myeloma is a good general-purpose cell line. The best sources of the cell line are ATCC or a laboratory actively producing hybridomas. Some sublines do not fuse

well, perhaps due to some genetic variation or to undetected contaminants such as mycoplasma.

The SP2/0-Ag14 cells should grow in suspension with minimal adherence to the tissue culture flask. The cells should not be overgrown and the medium should not become acidic (yellow). Healthy cells will be refractile and none of the cells should be pyknotic when viewed with an inverted microscope. In general, the cells should be diluted (split) into fresh medium ($\geq 1:5$ if the cells will be used on the following day) when the cells in an undisturbed flask form a monolayer on the bottom of the flask ($1-2 \times 10^6$ cells/ml). If the cells do not appear healthy even when grown at lower densities, mycoplasma contamination should be suspected.

Freeze multiple large aliquots of cells (*UNIT 1.1*) so that only a few days of expansion after thawing will be necessary for future fusions. If planned correctly, a primed animal can be boosted, cells thawed on the same day, and a fusion performed 3 days later.

Choice of animal for immunization

The protocol on immunization (Basic Protocol 1) outlines the production of MAb by *in vivo* immunization and concentrates on the use of rodents for immunization. Specialized work may require the use of splenic injections, *in vitro* immunizations, or human monoclonals; these techniques are much more difficult to implement successfully and are not recommended for the novice. Other protocols designed to eliminate unwanted MAb against particularly immunogenic epitopes, involve the use of cytotoxic agents immediately after the initial immunization with the unwanted antigens and should be considered only after some experience has been gained (Sharpe et al., 1985).

Four animal species (mouse, rat, hamster, and rabbit) can be used for MAb production. The spleen cells from a given species must be able to produce stable MAb-producing hybridomas with a rodent myeloma cell line. Although several rat and mouse tumor cell partners exist and all have been successfully used, the SP2/0-Ag14 mouse cell line from ATCC is recommended because it is a drug-marked, nonsecretory myeloma that does not constitutively produce either light or heavy chains. Hybridomas derived from fusion to SP2/0-Ag14 will not make chimeric MAb. This cell line also forms stable hybridomas with mouse, rat, and hamster B cells. Rabbit MAb have been described (Raybould and Takahashi, 1988) but little collective experience has accumulated.

It is technically more difficult to produce stable MAb-producing hybridomas from animals that are phylogenetically distant from the mouse. First attempts at producing MAb should use either the mouse or rat. Of the two, the mouse is the best choice for most xenogeneic antigens, such as human antigens, because many more antibodies have been produced in the mouse. Isotype-matched MAbs to defined antigens are available commercially, and can be used as controls in the assays of interest. Moreover, the mouse is easier to handle, anti-Ig reagents specific for each mouse Ig isotype are more commonly available, and generally mouse MAb are easier than rat MAb to purify. If a mouse is to be immunized, the best choice is a BALB/c mouse, because the hybridomas resulting from fusion to SP2/0-Ag14 will be entirely of BALB/c origin and thus should grow in a BALB/c host for ascites fluid production. Moreover, BALB/c spleens are generally larger than spleens from other mouse strains.

In contrast, immunization of mice would not be appropriate for most mouse antigens unless an allotypic difference is known. Fortunately, the rat is a reasonable choice for many mouse antigens because rat MAb will frequently recognize framework determinants on mouse proteins. Most of the commonly available rat strains can be used, although this laboratory usually employs Lewis rats. If rat MAb are produced, the mouse anti-rat κ MAb, MAR 18.5 (ATCC) is useful, since MAR 18.5 recognizes rat κ light chains (present in 90% of rat MAbs) and binds protein A, and thus is easy to purify. Moreover, the hybridoma produces high titers of MAb in culture supernatants, making MAR 18.5 useful in detection and purification of rat MAbs.

Although there are mouse MAb that recognize many major, functionally important human cell-surface and soluble antigens, it has proven difficult, despite considerable effort, to produce rat MAb to several of the homologous mouse antigens. It is possible that these mouse molecules are not antigenic in rat. Some of these mouse molecules may be more antigenic in hamsters, presumably due to the greater phylogenetic distance involved (Schreiber et al., 1985). However, hamster MAb are more difficult to produce because of fibroblast overgrowth and instability of hybridomas. Hamsters are therefore not ideal choices for the novice until experience with several fusions is acquired. The Armenian hamster strain is the best suited for hybridoma production because the hybridomas are more stable and fibroblast

overgrowth is less than with other available hamster strains. Unfortunately some Armenian hamster MAb are nonreactive with standard, commercially available anti-hamster immunoglobulin antibodies. However, many hamster MAb are reactive with protein A and/or with the mouse anti-rat κ MAb, RG7/7.6, which cross-reacts with hamster κ chains (Sanchez-Madrid et al., 1983). Finally, there are few serologic reagents available for hamster pathogens and thus it is wise to quarantine hamsters—and the hybridomas derived from them—in case there are occult infections that could be spread to other rodents (Donovan and Brown, 1995).

The sex of the host animal does not appear to be important. It is usually advisable to immunize young adult animals because the immunization schedule may be prolonged. Fusions of spleens from older hamsters (>6 months old) tend to have more fibroblasts.

Antigen preparation and immunization

Many types of antigen preparations have been used successfully including whole cells, partially purified lymphokines and cytokines, solubilized cell membranes and protein bands isolated from SDS-polyacrylamide gels (*UNIT 6.1*). The nature of the antigen preparation to be used is dependent on several factors, particularly the ease in preparing the antigen, the screening assay, and the desired specificity and property of the MAb.

Although it is desirable to immunize with a purified antigen to increase the frequency of hybridomas secreting the desired antibodies, in contrast to polyclonal antisera production, this is not a major requirement.

It is important to note that MAb are quite specific. It is possible to immunize with impure antigens or with multiple antigens, and with a highly specific screening assay, pick the MAb that identifies a specific antigen. In many instances, this is exactly the protocol used. For example, animals are usually immunized with whole cells to derive MAb that recognize a specific cell-surface antigen. The major advantage to whole-cell immunization is that the proteins will be in their native conformation and thus, the MAb produced can recognize these antigens in their native form. The major disadvantage to whole-cell immunization is the production of MAb to many other antigens, particularly those that have been previously produced (since they tend to be the most commonly made); immunization of rats with whole mouse T cell suspensions will produce $\geq 25\%$ anti-Thy-1 MAb (W. M. Y., unpub. observ.).

Nevertheless, collective experience with whole-cell immunizations suggest that although the antigen preparation may be impure, it is important to consider the nature of the antigen relative to its native form.

Because protein purification frequently denatures molecules and synthetic peptides usually do not achieve native conformations, immunization with gel-purified proteins and synthetic peptides generally has resulted in the production of MAb that recognize the antigen in its denatured form. While such MAb may be useful for immunoprecipitation and immunoblot studies, often they are not useful for flow cytometry analysis of cell-surface antigens or functional assays that require binding of the antigen in its native conformation.

There are many immunization protocols used to produce MAb. The major requirements appear to be a primary immunization with an adjuvant and fusion 3 to 4 days after the boost. However, there are notable exceptions. For example, a single primary immunization and fusion 4 to 5 days later have been successfully used to produce MAb against cell-surface antigens (Logdberg et al., 1985). It is generally agreed that a successful fusion requires the presence of activated B cells.

Most injections can be given intraperitoneally with good results. Many investigators prefer to boost intravenously with antigen without adjuvant. While this can be done via tail vein in the mouse, this vein is inaccessible in the rat or Armenian hamster and intravenous injections are generally not used in these animals. *Be aware that intravenous injections may occasionally result in fatal systemic reactions.*

Many investigators screen their immunized animals for serum antibody titers before a final boost and fusion. This depends on the purity of the antigen and an assay that is not influenced by serum. However, many MAb have been successfully produced against cell-surface antigens without screening the sera before fusion even if whole cells are used to immunize.

Screening assays

The cell-fusion protocol outlined in this unit should yield hybridomas in ~50% of the wells. The purpose of the screening assay (Support Protocol 1) is to exclude all hybridomas that are unlikely to produce the MAb of interest and yet include all likely candidates. In that regard, a reliable assay that will detect a few false positives but no false negatives will help decrease the number of hybridomas from several hundred to ~20. Depending on the difficulty of

the assay and the availability of the antigen, all wells can be screened for reactivity or—especially if most wells do not contain hybridomas—the wells can be screened first for visual evidence of growing hybridomas. Visual screening can be problematic since it requires inspection with an inverted microscope and a significant amount of bookkeeping to record which wells contain hybridomas (and which do not). More troublesome is the possibility of identifying a candidate hybridoma only to clone and save the wrong master well due to a bookkeeping error.

Screening assays should be perfected before the fusion is done. The assay should be rapid, with results available within 1 to 2 days, reliable, sensitive, and simple to perform in large numbers (hundreds to thousands of wells). In the protocol described here, the assay must be performed with 100- μ l volumes. Alternatively, the supernatants from several wells could be pooled to screen fewer samples. One clever screening approach is to pool 50 μ l from each well in each horizontal row and then separately pool 50 μ l from wells in each vertical column of a single plate. The well containing the desired hybridoma can be pinpointed by the positive row and column. Because each supernatant is tested twice, the percentage of false positives is diminished. This technique should not be used for low-sensitivity assays as the supernatants are diluted significantly. Many different screening assays and techniques have been described. ELISA (APPENDIX 3) and radioimmunoassay (RIA; APPENDIX 3) have been popular because of the above considerations.

Because MAb are exquisitely specific, those which recognize the same antigen may bind to slightly different epitopes and thus have different functional properties. For example, if a MAb to a cell-surface antigen that inhibits ligand-receptor interaction is required, flow cytometry analysis may not be the best choice for the initial screening assay because a MAb that binds well by flow cytometry analysis may not be functionally active and vice versa. However, if the desired property of the MAb could be improved by a change of the heavy chain, it is possible to isolate isotype-switch variants that may improve the usefulness of a particular MAb. Such studies have been performed with isotype-switch variants of anti-CD3 MAb (van Lier et al., 1987). Nevertheless, the ideal screening assay should identify MAb with the desired property, not just the desired specificity.

Troubleshooting

The production of MAb is a prolonged procedure. Success requires the optimization of several steps. The antigen preparation and immunization protocol must be adequate; this can be checked by serum titers after immunization. If no hybridomas are produced, then the immunization protocol may not have produced activated B cells, an unlikely situation if an adjuvant was used. Alternatively, hybridoma formation and growth may be inadequate, particularly if the cells (spleen or fusion partner) were contaminated with mycoplasma or if the lot of fetal bovine serum (FBS) does not support hybridoma growth. Obtain the fusion partner from a reliable source, either a lab that is successfully producing hybridomas or from ATCC. Freeze aliquots of these cells as soon as possible. Obtain animals from specific pathogen-free suppliers and cage them in uncontaminated rooms. The FBS lot can be tested by assaying the cloning efficiency of stable hybridoma lines and using a lot that supports high-efficiency cloning.

If viable hybridomas are produced at the anticipated level but the desired MAb is not found, assay the master wells for other antibodies that should react with the immunogen. For example, if whole cells were used as immunogens and the original assay was a functional one (e.g., inhibition of proliferation), several wells should be screened for antibodies that recognize cell-surface antigens by flow cytometry; under these conditions, 25% to 50% of growing wells will produce antibodies that recognize cell-surface antigens. This result would prove that MAb were produced but that the desired specificity was not found. Unfortunately, this is a typical result and the source of the major commitment in time and labor that MAb production requires. The solution to the problem is to perform more fusions and screening. It is useful to consider alternatives to the immunization, choice of animals, and screening procedure.

During the cloning procedures, there are several problems that may be encountered. If none of the cloned hybridomas secrete the desired MAb, either the hybridoma was not producing the MAb initially or the clones have lost the ability to produce the MAb. An aliquot of supernatant from an expanded master well can be tested in confirmatory assays. If none of the clones produce the MAb of interest, the frozen master-well cells should be carefully thawed, reseeded in multiple cloning plates, and then refrozen. Beware that mycoplasma contamina-

tion will affect cloning efficiency adversely.

Anticipated Results

With the cell-fusion protocol outlined in this unit (Basic Protocol 2), hybridoma growth should be apparent on the days mentioned and $\geq 50\%$ of the master wells will contain hybridomas. Depending on the purity of the antigen used and the immunization protocol, $\leq 1\%$ to 5% of the wells will contain the desired hybridoma. If the screening assay demonstrates many positive wells, it is likely that these are false positives.

Cloning by limiting dilution of the primary hybridomas (Support Protocol 3) should yield ≥ 10 to 50 "growing" wells/plate when seeded as described. The second cloning under more stringent conditions should yield ≥ 10 wells when seeded at 0.3 cells/well. Remember that the cloning efficiency of the primary hybridomas is relatively poor. If no viable clones are derived, seed additional wells at higher density and/or use a source of IL-6 (plasmacytoma growth factor; Nordan and Potter, 1986; Bazin and Lemieux, 1989).

Time Considerations

The initial immunization to the final cloning of the desired MAb requires ≥ 2 to 3 months if the MAb is produced and identified in the first fusion. Usually, several fusions are required and thus, a fusion every 3 weeks may be necessary. During much of this time, especially during the 3 weeks after fusion, daily or every-other-day tasks are required. An entire day's work is required when the actual cell fusion and screening assays are performed. Feedings require 5 to 10 min/plate.

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Key References

Goding, 1996. See above.

An in-depth discussion of MAb production.

Köhler and Milstein, 1975. See above.

The first description of MAb, for which the authors were awarded the Nobel Prize.

Oi, V.T. and Herzenberg, L.A. 1980. Immunoglobulin-producing hybrid cell lines. In *Selected Methods in Cellular Immunology* (B.B. Mishell and S.M. Shiigi, eds.) pp. 351-372. W.H. Freeman, New York.

This reference is the basis for this protocol and for the development of many MAb in the literature.

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Production of Polyclonal Antisera

Production of good antisera depends in large part upon the quality, purity, and amount of available antigen as well as on the specificity and sensitivity of the assay. For protein antigens, if possible, the material should be biochemically homogeneous and, depending on the intended use, should be in either a native or denatured conformation. Be aware that minor contaminants are often (unfortunately) more antigenic than the immunogen of interest, and antisera resulting from immunization may have more activity against the contaminants than against the protein of interest. Antisera to be used for screening bacterial expression cDNA libraries or for immunoblots are best made against denatured protein, whereas those to be used for screening cDNAs expressed in eukaryotic transfection systems or for immunoprecipitation of native-cell-synthesized structures might best be made against native protein.

Although the advances offered by the development of monoclonal antibody techniques have revolutionized the specificity, uniformity, and quantity of antibodies, there remain many circumstances in which polyclonal antibodies are more desirable than monoclonal antibodies. Production of polyclonal antisera takes less time and effort than production of monoclonal antibodies, requires relatively simple and readily available equipment, and produces reagents that can be used for immunoprecipitation, immunoblotting, and enzyme-linked immunosorbent assays (ELISAs; *APPENDIX 3*). The protocols describe the production of polyclonal antisera specific for protein antigens in rabbits, rats, mice, and hamsters using complete Freund's adjuvant (CFA; see Basic Protocol) or other adjuvants (see Alternate Protocol). In addition, the unit presents a method for preparing serum from blood (see Support Protocol). Polyclonal anti-peptide antisera can be produced by substituting carrier-conjugated peptides for the purified protein antigens.

Choice of animal for the production of antibodies depends upon the amount of antiserum desired, the evolutionary distance between the species from which the protein of interest has been derived and the species of the animal to be immunized, and prior experience with the immunogens. Rabbits are the usual animal of choice because they are genetically divergent from the human and mouse sources of the proteins most often studied. Rabbits, depending on their size, provide as much as 50 ml of serum from each bleed without significant harmful effects. For smaller-scale experiments, or for those that rely on precisely defined antibody specificities, inbred mouse strains may be the system of choice. Because mice are smaller, the volume of antigen suspension used for immunization is significantly less and the amount of serum that can be obtained from a single bleed does not exceed 0.5 ml. Rats and hamsters may be used when larger amounts of serum are needed, or when the greater evolutionary distance is advantageous. With repeated bleeding, as much as 5 ml of serum can be obtained from these species. Additional discussion of the choice of species for the production of monoclonal antibodies is given in *UNIT 16.1*.

The choice of adjuvant for in vivo animal use has become problematic in recent years. Freund's adjuvant has been reliably and widely used for over fifty years (Freund et al., 1937). However, there is a degree of distress and discomfort to the animal associated with its use which requires that the responsible investigator explore alternatives (McWilliam and Niemi, 1988). Most studies that have compared available alternatives to Freund's adjuvant have indicated that Freund's adjuvant gives antibody titers that are far superior to the commonly used commercially available adjuvants (Johnston et al., 1991; Deeb et al., 1992; Smith et al., 1992). However, in some instances TiterMax (Bennett et al., 1992) and Ribi Adjuvant Systems (Mallon et al., 1991) have performed as well as Freund's adjuvant. The use of these adjuvant systems as alternatives to Freund's adjuvant is described in the Alternate Protocol.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

IMMUNIZATION TO PRODUCE POLYCLONAL ANTIBODIES USING FREUND'S ADJUVANT

In the presence of adjuvant, the protein antigen is injected intramuscularly, intradermally, or subcutaneously into an animal of the chosen species. Booster immunizations are started 4 to 8 weeks after the priming immunization and continued at 2- to 3-week intervals. Prior to the priming immunization, following the primary and each booster immunization, the animal is bled and serum prepared from whole blood (see Support Protocol).

Factors important in preparing specific high-titer antisera, procedures for modifying protein antigens to enhance their immunogenicity, and choice of host animal are discussed in the Commentary. Protocols for immunization prior to production of monoclonal antibodies (*UNIT 16.1*) should be reviewed for these purposes.

Materials

- Rabbit, rat, mouse, or hamster of appropriate strain
- Complete Freund's adjuvant (CFA; Sigma)
- 1 to 2 mg/ml purified protein antigen in PBS (*APPENDIX 2A*)
- Incomplete Freund's adjuvant (IFA; Sigma)
- 50-ml disposable polypropylene centrifuge tubes
- 3-ml glass syringes with 19-, 21-, and 22-G needles
- Double-ended locking hub connector (Luer-Lok, Becton Dickinson) *or* plastic 3-way stopcock

CAUTION: CFA is an extremely potent inflammatory agent, particularly if introduced intradermally or into the eyes and may cause profound sloughing of skin or loss of sight. Self-injection can cause a positive TB test and lead to a granulomatous reaction. Use gloves and protective eyewear when handling CFA.

1. Bleed the animal prior to immunization and collect blood sample in a 50-ml centrifuge tube. Prepare serum from blood and assay and store (see Support Protocol).

This preimmune bleed is critical as a control to ensure that the antibody activity detected in later bleeds is due to the immunization.

2. Shake CFA to disperse insoluble *Mycobacterium tuberculosis* bacilli. Add 2 ml CFA to 2 ml of 0.25 to 0.5 mg/ml purified protein antigen in PBS at 4°C.

These volumes produce immunogen sufficient to immunize 4 rabbits or up to 80 mice. Do not use Tris-based buffers for generating the emulsion.

An effective and simple method for preparing purified protein antigen is by preparative SDS-PAGE (UNIT 6.1). If a standard-size 1.5-mm slab gel is used with a large-toothed comb, as much as 2 mg of a homogeneous protein can be loaded across the entire gel. Following electrophoresis, an edge can be sliced off with a razor blade, fixed and stained, and used to identify the region containing the protein band. The gel slice containing the protein may then be directly added to several milliliters of PBS (APPENDIX 2A) and emulsified as described below with an equal volume of CFA. The acrylamide serves as an additional component for the protein depot provided by the adjuvant.

3. Draw up the CFA/antigen mixture into a 3-ml glass syringe with a 19-G needle. Remove needle, expel as much air as possible, and attach syringe to the double-ended locking hub connector or the plastic 3-way stopcock (see Fig. 16.2.1). Attach an empty 3-ml glass syringe at the other end and force the mixture back and forth from

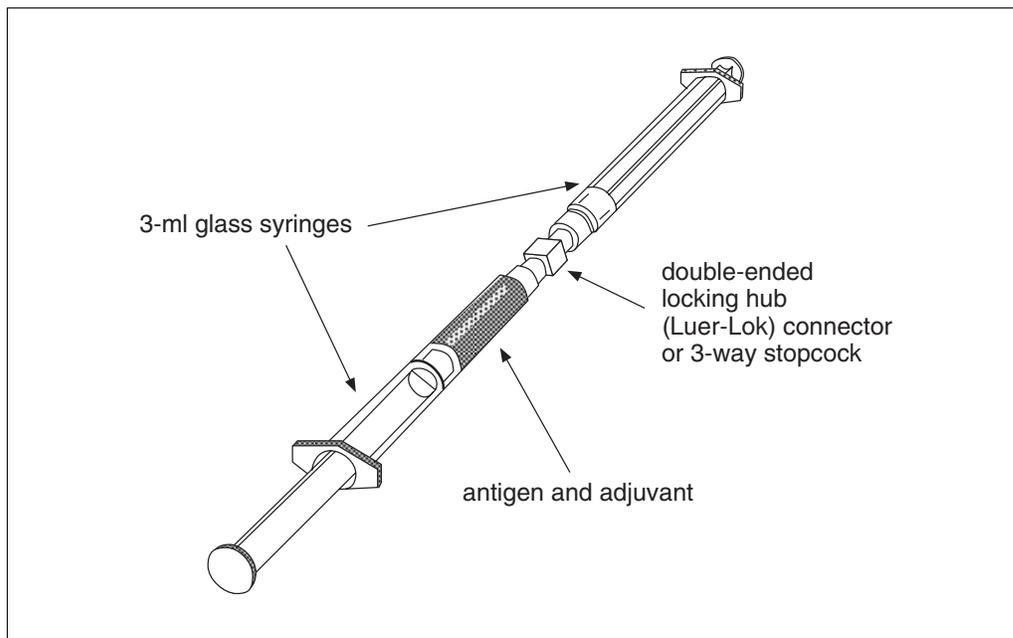


Figure 16.2.1 Double-syringe device for preparation of antigen-adjuvant emulsions.

one syringe to the other repeatedly. When the mixture is homogeneous and white, disconnect the connector or stopcock, attach a 21-G needle, and test whether the emulsion is stable by extruding a small drop onto the surface of 50 ml cold water in a 100-ml beaker. A good oil-in-water emulsion should hold together as a droplet on the surface of the water. If the drop disperses, mix the antigen using the hub-connected syringes until it forms an emulsion.

Heat will be generated by this procedure. Chill on a bed of ice from time to time to keep the mixture as close to 4°C as possible.

4. Transfer all of the adjuvant-antigen emulsion to one syringe and remove the connector or stopcock. Attach a 22-G needle to the syringe and remove air bubbles.
5. Restrain the animal and inject the adjuvant/antigen emulsion into multiple intramuscular (i.m.), intradermal (i.d.), or subcutaneous (s.c.) sites.

For intradermal immunizations, use no more than 50 μ l per site. For subcutaneous immunizations, use 100 μ l per site. For intracellular inoculation of rabbits, use 300 μ l in three sites in one leg muscle or 500 μ l in each lumbar muscle.

Discard the unused immunogen. For extremely valuable antigens, the emulsion may be stored at 4°C for several weeks and reemulsified before use. However, denaturation of protein antigens may take place under these conditions. For immunization of small rodents (e.g., mice), it is often better to carry out injections intraperitoneally (i.p.).

6. Bleed the animal 10 to 14 days following the priming immunization and collect blood sample. Prepare serum from blood (see Support Protocol).
7. Prepare antigen for booster immunizations, following steps 2 to 4. When CFA is the primary adjuvant, use IFA as the adjuvant for all subsequent immunizations.
8. Administer the first booster immunization 4 to 8 weeks after the priming immunization, bleed the animal 10 to 14 days later, and collect blood sample. Prepare serum from blood (see Support Protocol).

Some investigators will administer the first booster immunization as early as 2 weeks after the primary immunization.

- Administer further booster immunizations at 2- to 3-week intervals. Bleed animal 10 to 14 days after each boost and collect blood sample. Prepare serum from blood (see Support Protocol).

Repeated intradermal immunization should be avoided as it can cause skin ulceration. Following primary intradermal or subcutaneous immunization, it is preferable to boost with intramuscular injections for the rabbit. Some investigators prefer primary intramuscular injections with boosters at other sites.

**ALTERNATE
PROTOCOL**

**IMMUNIZATION TO PRODUCE POLYCLONAL ANTISERUM USING
OTHER ADJUVANTS**

For highly immunogenic antigens the use of Freund's adjuvant can certainly be avoided. For other immunogens it may be necessary to test a number of adjuvant systems. The use of two commercially available adjuvants is described in this protocol.

Additional Materials (also see Basic Protocol)

TiterMax #R-1 (CytRx; store <24 months at 4°C) or Ribi Adjuvant System (RAS; Ribi ImmunoChem; store at 2° to 8°C and do not freeze)
1-ml plastic syringes

- Using TiterMax:* Emulsify aqueous antigen with TiterMax adjuvant (see Basic Protocol; follow steps 1 through 5, except use 0.5 ml antigen and 0.5-ml vial TiterMax in step 2 and plastic syringe in step 3).

TiterMax contains microparticulate silica coated with block copolymer CRL-8941, sorbitan mono-oleate, and squalene.

Although glass syringes are recommended for Freund's adjuvant emulsions, all-plastic syringes should be used with TiterMax.

Each reconstituted 0.5-ml vial will immunize 20 mice, 10 rabbits, or 1 goat. Unused antigen/adjuvant emulsion can be stored at 4°C, -20°C, or -70°C for as long as the antigen is stable. It may be necessary to re-emulsify before using.

- Using Ribi Adjuvant System:* Warm the vial of RAS 5 to 10 min at 40° to 45°C. Add 2 ml antigen in PBS directly through the rubber stopper using a syringe with a 21-G needle. Vigorously vortex the vial 3 min at room temperature with the cap seal in place. The final volume of adjuvant/antigen is 2 ml containing 2% squalene oil.

Each vial of RAS contains 0.5 mg each of monophosphoryl Lipid A (MPL), synthetic trehalose dicorynomycolate (TDM), and cell wall skeleton (CWS) in 44 µl squalene and Tween 80.

Each reconstituted vial will immunize 10 mice or 2 rabbits or goats. Unused adjuvant/antigen emulsion can be stored several months at 4°C. However, if the entire vial will not be used initially, it is better to reconstitute to 1.0 ml with saline alone, store at 4°C, and mix aliquots 1:1 with antigen in saline as needed.

- Transfer the antigen emulsion to a 1-ml syringe, attach a 22-G needle to the syringe, and remove air bubbles.
- Restrain the animal and inject the adjuvant/antigen emulsion.

Rabbits should receive 40 µl TiterMax/antigen emulsion i.m. in each thigh. High antibody titers have been obtained with 30 to 50 µg of antigen per rabbit. Rabbits should be immunized with 1.0 ml RAS containing 50 to 250 µg of antigen in multiple sites: 0.05 ml i.d. at six sites, 0.3 ml i.m. in each thigh and 0.1 ml s.c. in the neck.

- Bleed the animal and prepare antigen for booster immunization (see Basic Protocol, steps 6 and 7).

Serum antibody responses have been reported to be slower for both RAS and TiterMax than for Freund's adjuvant (Smith et al., 1992).

5. Administer booster immunizations at 4, 8, and 12 weeks. Bleed the animal 10 to 14 days after each booster immunization. Prepare serum from blood (see Support Protocol) and cease immunization when high antigen-specific titers have been achieved.

Boosting with TiterMax may not be necessary for all antigens. If a second immunization is necessary, use soluble antigen in place of antigen/adjuvant at 4 weeks. If titers are still low after 10 to 14 days, a booster dose of antigen/TiterMax adjuvant can be given immediately. Increasing the amount of antigen may also help.

Ribi ImmunoChem strongly recommends that booster injections of RAS adjuvant/antigen be repeated no more frequently than every 4 weeks.

PREPARATION OF SERUM FROM BLOOD

Each blood sample is allowed to stand 4 hr at room temperature and overnight at 4°C until a clot forms. After removal of the clot and debris, the serum is assayed and stored at -20°C.

Additional Materials (also see Basic Protocol)

Blood samples (see Basic Protocol)
Sorvall H-1000B rotor or equivalent

Additional reagents and equipment for immunoblotting (UNIT 6.2), immunoprecipitation (UNIT 7.2), ELISA (APPENDIX 3), or double-immunodiffusion assay in agar (APPENDIX 3)

1. Allow blood to stand in the 50-ml centrifuge tube 4 hr at room temperature to allow clot to form, then place overnight at 4°C to allow clot to retract.
2. Gently loosen the clot from the sides of the tube with a wooden applicator stick (do not break up the clot), then remove the clot from tube with the applicator.

If a clot has not formed, initiate clotting by placing a wooden applicator stick into the tube containing the collected blood, then begin again at step 1.

3. Transfer serum to a 50-ml centrifuge tube. Pellet any remaining blood cells and debris by centrifuging 10 min in H-1000B rotor at 4000 rpm (2700 × g), 4°C, and save supernatant.
4. Assay antibody titer by the appropriate method: immunoprecipitation, immunoblotting, ELISA, or double-immunodiffusion assay in agar.
5. Store serum in aliquots in screw-top tubes at -20°C.

Some sera lose activity on repeated freezing/thawing. Sera can be stored for up to 6 months at 4°C. If storing sera at 4°C for long periods of time, 0.02% sodium azide should be added to prevent bacterial growth.

COMMENTARY

Background Information

The kinetics of development of a specific antibody response upon immunization of a rabbit with antigen are illustrated in Figure 16.2.2. After the primary immunization, naive B cells are stimulated to differentiate into antibody-secreting plasma cells. For most soluble protein antigens, specific antibody begins to appear in

the serum 5 to 7 days after the animal is injected. The antibody concentration (titer) continues to rise and peaks around day 12, after which it decreases. Similar kinetics are observed with mice, rats, hamsters, and rabbits.

In addition to differentiating into antibody-forming cells, the antigen-stimulated B cells proliferate to form a large population of mem-

SUPPORT PROTOCOL

Antibodies As
Cell Biological
Tools

16.2.5

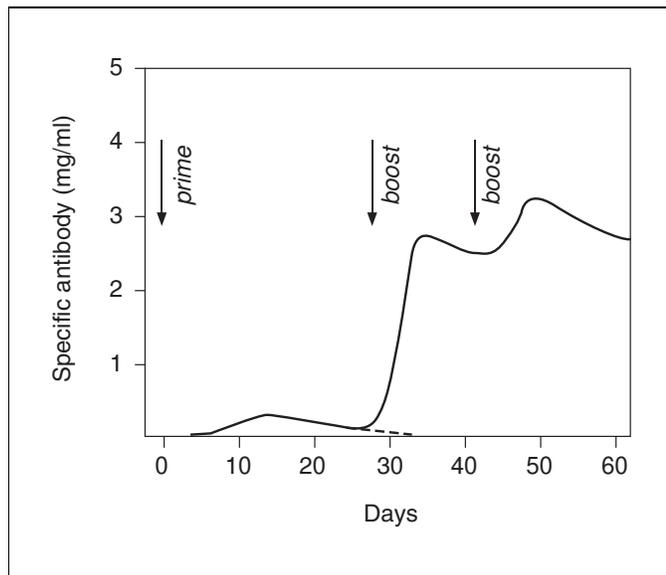


Figure 16.2.2 Kinetics of development of the specific antibody response. Arrows indicate when priming and boosting immunizations were administered. Actual amounts of specific antibody produced will vary considerably depending on immunogenicity of the protein.

ory B cells, which quickly become activated after the booster injection is administered. Thus, the lag period before the appearance of the specific antibody is much shorter after a booster injection than that observed for the initial immunization. In addition, a significantly higher titer of specific antibody is achieved and sustained for a longer period of time. The peak of antibody production occurs 7 to 14 days after boosting. As a consequence of the existence of the memory B cells, less antigen is required to stimulate a strong secondary response. Memory B cells are long-lived; therefore, a specific antibody response can be elicited as much as 6 months to a year after the last booster. Finally, the average affinity and degree of specificity of the antibody population for the antigen increase with repeated immunizations (Klinman and Press, 1975).

Adjuvants greatly enhance the specific antibody titer, as they allow the antigen to be released slowly, thus ensuring the continual presence of antigen to stimulate the immune system. Freund's adjuvant has been used extensively in the preparation of antigen because it induces a high, long-lasting antibody titer that is often still measurable 25 weeks or more after boosting. The presence of mycobacteria in complete Freund's adjuvant (CFA) activates the T cell population, providing necessary lymphokines for B cell stimulation and maturation. CFA may cause granuloma and subsequent necrotic abscesses, so it should be used only for primary immunization. Incomplete Freund's adjuvant (IFA) is adequate for booster injections. In the past decade, adjuvant research has concentrated on the production of effective ad-

juvants that minimize animal trauma. Wherever possible, to reduce animal discomfort, less noxious adjuvants should be used (see Alternate Protocol). In addition some countries are restricting the use of CFA in laboratory animals. Published comparisons of commercial adjuvants with the basic CFA/IFA protocol vary widely in their conclusions. In most cases, however, CFA/IFA produces higher titers of higher-affinity antibodies in a shorter time period (Johnston et al., 1991; Deeb et al., 1992; Smith et al., 1992). Conflicting results (Mallon et al., 1991; Bennett et al., 1992) may reflect differences in the immunogenicity of the antigens used.

Critical Parameters

New Zealand red or white rabbits are generally the best source of specific antisera because 30 to 50 ml of whole blood can be obtained at each bleed, depending on their body weight. The life span of a rabbit is 5 to 6 years, so a continual source of specific antiserum can be provided over a period of time by one rabbit after booster injections. In this regard, the recommended times between booster injections are not critical; the animal may be rested for several months between subsequent boosters, after the primary and secondary booster injections. Blood collection, however, must take place 10 to 14 days after each booster to ensure a high titer.

Preimmune serum from the same animal is the preferred negative control. If additional control serum is required, either immune serum from animals immunized with totally unrelated antigens or pooled serum from naive animals will be adequate. Occasionally, spurious anti-

body activities from nonimmunized animals may mimic the activity of the immune serum.

Antibody specificity may vary widely between individual animals with respect to the dominant antigenic epitopes recognized on a given protein antigen. Therefore, antiserum from a single animal should be used throughout a study. If more than one animal must be used for particular antisera, the antisera should be pooled. Large-scale production of antisera can be carried out in goats, sheep, and horses with appropriate veterinary guidance. If serum is taken from inbred animal strains, the variability in antibody specificity, as observed in outbred rabbits, is less of a problem.

The most important factor in producing a highly specific polyclonal antiserum is the purity of the antigen preparation used for immunization. The immune system is very sensitive to the presence of foreign proteins. Any contaminating proteins in the antigen preparation can potentially induce a strong immune response when injected in the presence of adjuvant. When antisera are employed in sensitive techniques such as immunoblotting or the screening of cDNA or genomic libraries, significant antibody titers to protein contaminants can be a major problem. Thus, the antigen preparation should contain no significant contaminating proteins. Ideally, there should be no visible contaminating bands when 10 to 20 μg are analyzed on an SDS-polyacrylamide gel stained with Coomassie brilliant blue (UNITS 6.1).

If the antiserum is to be used in functional assays, extra care must be taken to ensure that the immunizing antigen is in its native form, because antibodies directed to denatured forms of the protein antigen will interact weakly, if at all, with the antigen in its native conformation. On the other hand, antibodies used in immunoblots, immunoprecipitation of primary in vitro translation products, and immunoscreening of $\lambda\text{gt}11$ expression libraries may be most effective if generated against a denatured protein with reduced and carboxymethylated disulfide bonds.

Troubleshooting

Inability to attain high-titer antiserum after several booster injections may be due to a variety of factors as described below.

Use of inappropriate adjuvant. Some experimentation may be necessary to optimize the antigen/adjuvant ratio for different antigens. If the alternate protocol still fails to produce a good antibody titer after three immunizations, switch to the Basic Protocol.

Inadequate antigen emulsification. If the emulsion fails the drop-on-water test described in the basic protocol (step 3), repeat the emulsification process. Be sure to use phosphate-buffered saline. Avoid plastic syringes and Tris-based buffers with CFA and IFA.

The antigen is a poor immunogen. In general, the immunogenicity of a protein is related to the degree to which it differs from “self” proteins (Benjamin et al., 1984). Large bacterial or viral proteins such as hemagglutinin or bacterial-coat proteins are highly immunogenic, whereas proteins from mammalian sources, such as polypeptide hormones or cell-surface receptors, may be poorly immunogenic due to tolerance. Protein antigens can be made more immunogenic in two ways. First, they can be chemically linked to a carrier protein that is known to be a good immunogen. Common carrier proteins include keyhole limpet hemocyanin (KLH), fowl immunoglobulin, and bovine serum albumin (BSA). Coupling peptides to carrier proteins is described in Maloy et al. (1991); the same protocols can be used to couple the protein antigen of interest to the desired carrier. Second, the immunogenicity of an antigen may be enhanced by its polymerization into large aggregates via a cross-linking agent such as glutaraldehyde. The protocol in Maloy et al. (1991) for the coupling of peptide antigens to a carrier protein with glutaraldehyde can also be used to polymerize any protein antigen. With both the coupling and polymerization procedures, any insoluble antigen complexes formed should be removed prior to immunization by centrifuging 10 min at $15,000 \times g$, 4°C .

Host animal's immune system may be compromised by bacterial or viral infection. Refer to Donovan and Brown (1995) for discussion of the consequences of poor animal husbandry. Utilize animals from reliable, pathogen-free sources and maintain them in appropriate infection-free facilities.

Only a few animals have been immunized. Because of the vagaries of immune-response genes in outbred animals such as rabbits, some antigens may not induce a good antibody response in a significant proportion of randomly selected animals. Thus, it is best to immunize several different animals and to screen the sera for the best responder. Obviously, this is less of a problem in homozygous inbred strains, but with a new antigen it is wise to test several strains for their antibody response.

An insufficient amount of antigen was used. Although recommended concentrations of antigen for rabbits are 0.25 to 0.5 mg/ml injected

into multiple sites, for a total of 1 to 2 ml in the same animal, good results can be obtained with $\frac{1}{10}$ to $\frac{1}{20}$ of the concentration in the same volume. It is always tempting to use less of a precious antigen, but often too low a dose leads to too low a response.

Anticipated Results

For large or nonevolutionarily related proteins, a titer of 5 to 10 mg/ml of serum can be expected after repeated boosts (hyperimmunization). When immunizing with small or highly conserved protein species, a titer of 1 to 2 mg/ml of specific antibodies is more likely. Antibody titers and affinity for the antigen will be low after primary immunization and the first booster immunization, but both titer and affinity will increase with subsequent immunizations.

Time Considerations

Preparation of the immunogen and immunization will take up to 3 hr on each occasion. Collection of antisera will take 1 to 2 hr, depending on the number and species of animals.

Collection of antiserum after the primary immunization will be at 10 to 14 days. This will be a low-titer, low-affinity serum. The first booster normally is given 4 to 8 weeks after the primary immunization but can be given as early as 2 weeks after the primary if time is critical. Ideally, there should be at least 19 days between the primary and the secondary bleeds. A second booster is given at 6 weeks with a bleed on day 52 to 59. This will usually be the first high-titer bleed. If a titer of <1 mg/ml of specific antibody is obtained, subsequent boosting immunization will be necessary.

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A comprehensive methods book with many modern techniques.

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Purification of Immunoglobulin G

For many applications, both monoclonal and polyclonal antibodies may be used in impure form: monoclonal antibodies may be used either as ascites fluid or as tissue culture supernatant, and polyclonal antibodies may be used as antiserum. Such unpurified antibodies are perfectly suitable for use in indirect flow cytometry assays, for most ELISAs, or for cytotoxicity studies if the concentration of antibody is not important. Purified antibodies must be used, however, when accurate concentrations are required, when chemical modifications such as labeling with fluorescent and radioactive probes for binding studies are required, or when structural modifications such as removal of the Fc portion to produce bivalent $F(ab')_2$ or monovalent Fab fragments are required. IgM can be modified and the single bivalent subunit (IgMs) of the pentamer produced as a low-molecular-weight alternative to intact IgM. Bivalent $F(ab')_2\mu$ fragments of IgM are more difficult to produce but are sometimes required. Detailed methods for production of these fragments are provided in *UNIT 16.4*.

Choice of procedure for antibody purification depends on the intended use of the antibodies and on the available resources. This unit presents purification methods that can be tailored to the laboratory's resources. If a highly purified product is required, an assay for antibody activity and a reliable means of assessing the degree of protein contamination are essential. The method of choice for determining purity is SDS-PAGE (*UNIT 6.1*). The antibody assay must be rapid and accurate to monitor activity throughout the purification. Some preferred assays are: (1) labeling of cell-surface antigens with the antibody, followed by incubation with a fluorescent anti-Ig of the correct specificity and microscopic (*UNIT 4.3*) or flow cytometry analysis of the sample; (2) ELISA; (3) complement-mediated lysis of the appropriate cells; (4) immunodiffusion; (5) radioimmunoassay (RIA; Cooper and Paterson, 1993); and (6) inhibition of purified fluorescent or radiolabeled antibody binding to its appropriate ligand. Assays will generally not provide a measure of contaminating proteins that remain in the preparation at different stages of the purification. These can be detected by SDS-PAGE.

Several companies produce kits for the purification and fragmentation of antibodies derived from all common animal species. These kits are primarily designed for IgG and IgM classes of antibody, but there is also a kit for purification of egg yolk-derived IgY. The kits mentioned here are the most reliable in the opinion of the authors, but they may not be the least expensive option, so classical methods of purification and fragmentation are described in these units.

IgG can be purified by ammonium sulfate precipitation followed by size-exclusion (SE) chromatography (see Basic Protocol 1). This is the least expensive option available for purification of antibodies. Protein A- and protein G-affinity chromatography (see Basic Protocol 2 and Alternate Protocol 1) are the fastest methods for purifying antibodies, but they are not effective for all subclasses of rat antibody. Affinity chromatography using anti-rat antibody can be used to purify rat antibodies (see Alternate Protocol 2). Ion-exchange (IEX) chromatography (see Basic Protocol 3) can also be used to purify intact monoclonal and polyclonal antibodies and antibody fragments. All these methods give a product with a high degree of purity. Ammonium sulfate precipitation followed by IEX chromatography can be applied to any type of antibody. However, affinity chromatography, either as described in Basic Protocol 2 and Alternate Protocol 1 or carried out using a commercially produced kit, is much more efficient and less tiresome to carry out. There are many commercial kits available for purifying IgG (see Table 16.3.1). The T-Gel purification kit (thiophilic gel; Pierce) produces results similar to those of ammonium

Table 16.3.1 Commercial Kits for Purifying Antibody

Kit	Supplier ^a	Features
HiTrap protein A and protein G columns ^b	Amersham Pharmacia Biotech, Sigma	Ready-packed columns in a variety of sizes for use with syringe loading or a peristaltic pump
Immunopure (A) and Immunopure (G) IgG ^c	Pierce	Protein A and protein G purification
Immunopure mouse IgG ₁ ^c	Pierce	Mild elution buffer kit for purification of mouse IgG ₁ on protein A
Immunopure and Immunopure <i>Plus</i> (A/G) IgG	Pierce	Binds all IgGs that bind to both protein A and protein G; contains sufficient gel to purify 6 or 16 mg, respectively
MAb Trap GII ^b	Amersham Pharmacia Biotech	A complete protein G kit with buffers, column, and syringe
Protein A and Protein G Superose columns ^b	Amersham Pharmacia Biotech	Ready-made, reusable columns for use with the Pharmacia FPLC system; good for purifying large amounts of antibodies
<i>Antibodies of other types/classes or general kits</i>		
T-Gel ^c	Pierce	One-step purification by adsorption to a thiophilic gel; the gel has broad specificity for immunoglobulins of any type or subclass derived from various animal species; used to purify antibodies from serum, ascites fluid, or culture supernatant
HiTrap IgY ^b	Amersham Pharmacia Biotech	Concentrates IgY antibodies from egg yolk by centrifugation in a liquid linear polymer
HiTrap IgM Purification Column	Amersham Pharmacia Biotech	Prepacked column containing thiophilic adsorption medium coupled to Sepharose High Performance. Interacts with IgM in hydrophilic-hydrophobic interaction. Kit of buffers provided.

^aSee SUPPLIERS APPENDIX.

^bThese kits may require specialized equipment or preparation of additional reagents.

^cThese kits come complete or reagents may be purchased separately.

sulfate precipitation because it has a broad specificity toward immunoglobulins derived from various animal species, irrespective of the type or subclass. The E-Z-SEP system (Pharmacia Biotech) consists of a group of kits with applications for all types of antibodies—e.g., ascites IgG, ascites IgM, bioreactor medium IgM, serum-free tissue culture IgG, serum polyclonal antibodies.

BASIC PROTOCOL 1

AMMONIUM SULFATE PRECIPITATION AND SIZE-EXCLUSION CHROMATOGRAPHY

Ammonium sulfate precipitation coupled with size-exclusion (SE) chromatography is a method for purifying proteins of all types and may be the procedure of choice for purifying certain antibodies. Ammonium sulfate precipitation can be used to purify all subclasses of mouse antibodies as well as antibodies of other species. This method can be used to purify IgM, IgG, and IgA of all species. Although more time-consuming than affinity chromatography (see Basic Protocol 2), it has a wider range of applications.

After removing cell debris from ascites fluid or a monoclonal antibody supernatant, ammonium sulfate is added to precipitate the proteins. The precipitate is recovered by centrifugation and dissolved in PBS or borate buffer, then dialyzed and concentrated. It is purified by SE chromatography and the pure IgG is analyzed.

Purification of IgG

16.3.2

Materials

Ascites fluid or MAb supernatant
PBS (see recipe)
Saturated ammonium sulfate (SAS; *APPENDIX 2A*)
Borate-buffered saline (optional; see recipe)
Sephacryl S-200 Superfine (Amersham Pharmacia Biotech)
PBS containing 0.02% sodium azide (optional)

Glass wool (Polysciences)
Sorvall centrifuge and SS-34 rotor (or equivalent)
26 × 900–mm column (Amersham Pharmacia Biotech)

Additional reagents and equipment for protein dialysis (*APPENDIX 3C*), column chromatography (*APPENDIX 3*), concentrating proteins (*APPENDIX 3C*), and reducing and nonreducing PAGE (*UNIT 6.1 & APPENDIX 3*)

- 1a. *For ascites:* Remove lipid from (5 to 12 ml) ascites fluid by placing enough glass wool into a funnel to cover the opening, pouring ascites through, rinsing glass wool with PBS, and squeezing glass wool gently with gloved fingers to obtain all the sample. Centrifuge filtered ascites 30 min at $20,000 \times g$ (13,000 rpm in SS-34 rotor) either 4°C or room temperature. Decant and save the supernatant and discard membranous material and cell debris remaining in the pellet.

Wear gloves when handling glass wool.

- 1b. *For MAb supernatant:* Centrifuge (~100 ml) MAb supernatant 30 min at $20,000 \times g$, either 4°C or room temperature. Decant and save the supernatant.
2. Add SAS slowly with stirring to the ascites or tissue culture supernatant to 45% (v/v). Leave 1 to 2 hr or overnight at 4°C to ensure precipitation of all the protein.
3. Centrifuge 1 hr at $20,000 \times g$, either 4°C or room temperature, and save precipitate to use in step 4. Save the supernatant to check for antibody activity.
4. Dissolve precipitate in a minimum volume of PBS or borate buffer (10 to 20 ml is usually suitable).
5. Place the dissolved precipitate in dialysis tubing. Dialyze against ≥ 20 vol PBS or borate buffer for 24 to 48 hr total at 4°C. Change the dialysis buffer four to six times during dialysis.

Generally, accurate molecular weight cutoffs are not required and it is not necessary to boil the tubing; merely soak for a few minutes in distilled water to soften.

The solution in the dialysis tubing will turn from a yellowish liquid to a cloudy or clear solution.

6. Concentrate the protein solution to ≤ 5 ml (*APPENDIX 3C*).
7. Prepare a 26 × 900–mm Sephacryl S-200 Superfine column and load the concentrated protein solution onto it. Elute protein with PBS, PBS containing 0.02% sodium azide, or borate buffer, and collect 100 fractions (1% of the column volume).

Monitor the protein fractions with a UV spectrophotometer at 280 nm. Alternatively, a precalibrated column can be used.

8. Check the purity of the fractions with A_{280} values >0.5 on nonreducing and reducing 10% polyacrylamide gels (*APPENDIX 3 & UNIT 6.1*).

In nonreducing PAGE, a band at ~150 kDa indicates IgG; in reducing PAGE, two bands at ~55 kDa (heavy chain) and ~25 kDa (light chain) indicate IgG. IgG_{2b} has asymmetric

Table 16.3.2 Extinction Coefficients and Molecular Size of Immunoglobulins and Their Fragments

Molecule	A_{280} (1 mg/ml solution)	Molecular weight (kDa)	
		Nonreduced	Reduced ^a
IgG	1.43	150	50, 25
IgM	1.18	900	78, 25
IgM subset	1.18	180	78, 25
Fab	1.53	50	25 ^b
F(ab') ₂	1.48	100-110	25 ^b
F(ab') ₂ μ	1.38	135	44, 25
F(ab')μ	1.38	65	44, 25

^aNumbers to the left of the comma represent molecular weights of the heavy chain of immunoglobulins, and numbers to the right are weights of the light chain of immunoglobulins.

^bMay appear as a doublet on SDS-PAGE.

glycosylation of the heavy chains and will therefore appear as a doublet on the gel (Table 16.3.2). If the fractions are not sufficiently concentrated for detection by SDS-PAGE, concentrate as described in APPENDIX 3C).

9. Assess IgG concentration spectrophotometrically at A_{280} (Table 16.3.2). Pool the eluates containing pure IgG.

Antibodies purified by ammonium sulfate precipitation followed by SE chromatography are of sufficient purity for any manipulation. They may be used for fragmentation after dialysis to the desired buffer (APPENDIX 3C), for ELISA, or for labeling with fluorescein isothiocyanate (FITC) or biotinylation.

10. Adjust IgG concentration to 0.1 to 30 mg/ml in borate buffer or PBS with 0.02% (w/v) sodium azide and store at 4°C up to several years. For back-up frozen stocks, freeze IgG at -70°C.

Thawing and freezing once from -70°C is usually fine, although as a general rule this should be avoided. Do not store antibodies at -20°C for >1 month. Do not repeatedly freeze/thaw from -20°C.

BASIC PROTOCOL 2

AFFINITY CHROMATOGRAPHY USING PROTEIN A-SEPHAROSE

This protocol describes the purification of antibody using protein A-Sepharose affinity chromatography. Protein A can be used to isolate monoclonal and polyclonal IgG from ascites, serum, and tissue culture and bioreactor supernatants. Protein A purification is recommended for human (except IgG3; mouse IgG₁ may bind only weakly), rabbit, guinea pig, and pig antibodies (see Table 7.2.1). The protocol requires addition of the antibody to a protein A-Sepharose column at pH 8.0, followed by elution at a lower pH. The antibody is then dialyzed back against PBS.

Materials

Ascites fluid or MAb supernatant
PBS, pH 8.0 and pH 7.3 (see recipe)
1 M NaOH
Protein A-Sepharose CL-4B, hydrated (Amersham Pharmacia Biotech or Sigma)
0.1 M citric acid at pH appropriate for subclass of antibody (see step 5)
Borate-buffered saline (optional; see recipe)
3 M potassium thiocyanate, filtered

continued

Sorvall centrifuge and SS-34 rotor (or equivalent)
0.45- μ m filter
1.5 \times 10-cm column
HiTrap protein A column (Amersham Pharmacia Biotech or Sigma; optional)
Additional reagents and equipment for dialysis (*APPENDIX 3C*) and column chromatography (*APPENDIX 3*)

For ascites fluid

- 1a. Clarify ascites fluid and remove lipids (see Basic Protocol 1, step 1a).
- 2a. Dilute ascites fluid 10-fold with PBS, pH 8.0.

For MAb supernatant

- 1b. Centrifuge MAb supernatant at 20,000 \times g (13,000 rpm in SS-34 rotor), 4°C, and filter through a 0.45- μ m filter.
- 2b. Adjust MAb supernatant to pH 8.0 by dialysis against PBS, pH 8.0, or by adding 1 M NaOH.

It is important to have the protein sample at pH 8.0 if IgG1 is to be purified. Other subclasses should bind to protein A at pH 7.4.

3. Prepare protein A-Sepharose column and attach to fraction collector. Equilibrate column with PBS, pH 8.0, at either 4°C or room temperature. Layer antibody solution onto resin bed.

Volumes of 1 ml to several liters can be loaded onto a protein A-Sepharose column. Use a peristaltic pump or gravity to assist in loading large volumes.

There is a limit to the binding capacity for immunoglobulin (~5 mg mouse IgG, and 8 mg human IgG, per milliliter resin). The expected concentrations can be assessed from the yields (see Anticipated Results). The antibody activity of the unbound fraction can be tested to check for overloading. Use a flow cytometry assay or ELISA for assessing activity of the eluate.

The HiTrap protein A column is ready-to-use. Alternatively, a column can be prepared in a 10-ml syringe. Add glass wool to the syringe before adding hydrated protein A-Sepharose CL-4B.

4. Wash column with several volumes PBS, pH 8.0.

Eluate should have an A_{280} at baseline before proceeding to the next step.

5. Elute with 0.1 M citric acid at suitable pH (bring to appropriate pH with 1 M NaOH): for mouse IgG₁ use pH 6.5, for IgG_{2a} use pH 4.5, and for IgG_{2b} and IgG₃ use pH 3.0.

It is thought that standing in low pH may damage the antibody; therefore, 50 μ l of 2 M Tris base buffer (Boehringer-Mannheim)/ml of eluate may be placed in the fraction collector tubes prior to elution. Reverse elution is undertaken by reversing the leads of the column so that the pump is pushing buffer up the column in the opposite direction to that in which the column was loaded. Samples that do not occupy the entire capacity of the column can be eluted at a higher concentration by this method. Reverse elution can concentrate the antibody if the protein A-Sepharose column is underloaded.

6. Pool protein-containing fractions, place eluates in dialysis tubing, and dialyze eluates against 1 liter PBS, pH 7.3, with or without 0.02% (w/v) sodium azide, at 4°C. Change the dialysis buffer twice. Store samples in PBS or borate-buffered saline at 4°C.

If desired, check purity by SDS-PAGE (UNIT 6.1).

7. Clean column with 1 column volume of filtered 3 M potassium thiocyanate before reequilibrating it in PBS, pH 7.3. Store at 4°C.

Wash the column with several volumes of PBS, pH 7.3, to remove residual potassium thiocyanate, which absorbs at 280 nm and could interfere in later purifications.

Antibodies purified by protein A–Sepharose affinity chromatography will be of similar purity to those obtained by ammonium sulfate precipitation and can be used for the same purposes (see Basic Protocol 1).

ALTERNATE PROTOCOL 1

AFFINITY CHROMATOGRAPHY USING PROTEIN G–SEPHAROSE

Affinity chromatography using protein G–Sepharose is useful for purifying antibody from serum, ascites fluid, tissue culture supernatant, and bioreactor supernatant. Protein G (Akerstrom and Bjorck, 1986) has a binding profile opposite to that of protein A with respect to pH: antibodies bind better at a low pH and badly at high pH. However, some antibodies (mouse IgG₁, and rabbit and human antibodies) do remain bound to protein G at high pH (8 to 10), so it is best to bind the antibody at pH 5 and elute at pH 2.8. This method is useful for mouse IgG₁, rat (most subclasses bind weakly although IgG_{2b} may not), monkey, rabbit, cow, goat, horse, and sheep antibodies (see Table 7.2.1). As with protein A purification, there is the possibility of some loss of antibody binding ability due to low-pH elution.

Additional Materials (also see Basic Protocol 2)

0.1 M sodium acetate, pH 5.0

0.1 M glycine·HCl, pH 2.8

HiTrap protein G column (Amersham Pharmacia Biotech or Sigma)

1. Prepare ascites fluid or MAb supernatant (see Basic Protocol 2, step 1a or 1b).
2. Dilute with 0.1 M sodium acetate, pH 5.0.

Dilute >2-fold for tissue culture supernatant or 10-fold for ascites fluid and bioreactor supernatant.

3. Equilibrate HiTrap protein G column in 0.1 M sodium acetate, pH 5.0. Layer antibody solution onto protein G gel.

Protein G has a higher capacity for IgG than protein A: ~10 mg protein/ml of gel with some species variation.

4. Wash column with several column volumes of 0.1 M sodium acetate, pH 5.0.
5. Elute bound antibody with 0.1 M glycine·HCl, pH 2.8.

To minimize exposure to low pH, add 50 µl Tris-based buffer (Boehringer-Mannheim) per milliliter of eluate to each tube of the fraction collector..

6. Pool protein-containing fractions and dialyze (APPENDIX 3C).
7. Recycle the column by washing it with 0.1 M glycine·HCl, pH 2.8, then reequilibrating it to pH 5.0 with 0.1 M sodium acetate, pH 5.0.

AFFINITY CHROMATOGRAPHY USING ANTI-RAT κ CHAIN MONOCLONAL ANTIBODY COUPLED TO SEPHAROSE

ALTERNATE
PROTOCOL 2

Occasionally, a monoclonal antibody (particularly one derived from the rat) cannot be purified by either protein A- or protein G-Sepharose chromatography, either because the antibody fails to bind the protein A or protein G, or because the elution conditions are too harsh for the retention of activity. In such a case, a column consisting of an anti-rat-Ig light-chain monoclonal antibody coupled to Sepharose can be used to bind the rat monoclonal antibody from a tissue culture supernatant or from ascites fluid. A series of buffers of decreasing pH can be used to assess the mildest conditions for elution of the rat antibody from the anti-Ig column. This protocol describes the production of such a column and the conditions for binding and elution from it.

Additional Materials (also see Basic Protocol 2)

Mouse anti-rat κ MAb: MAR 18.5 (ATCC TIB 216) purified using protein A-Sepharose (see Basic Protocol 2)

CNBr-Sepharose CL-4B (Amersham Pharmacia Biotech)

Binding buffer: 0.05 M Tris·Cl/0.15 M NaCl/0.02% (w/v) NaN₃, pH 8.6

Crude rat antibody solution to be purified (MAb supernatant or ascites fluid)

pH 7.0 elution buffer: 0.05 M sodium phosphate/0.15 M NaCl/0.02% (w/v) NaN₃, pH 7.0

pH 5.5 elution buffer: 0.05 M sodium citrate/0.15 M NaCl/0.02% (w/v) NaN₃, pH 5.5

pH 4.3 elution buffer: 0.5 M sodium acetate/0.15 M NaCl/0.02% (w/v) NaN₃, pH 4.3

pH 2.3 elution buffer: 0.5 M glycine/0.15 M NaCl/0.02% (w/v) NaN₃, pH 2.3

Additional reagents and equipment for preparation of antibody-Sepharose (APPENDIX 3)

Prepare column

1. Covalently couple ≥ 10 mg purified MAR 18.5 antibody to the CNBr-Sepharose 4B.

Coupling ratio should be ~10 mg MAR 18.5/ml wet gel. Such a column will have a capacity to bind ~1 mg of protein.

2. Prepare the column and wash extensively with the binding buffer at 4°C or room temperature.

Purify antibody

3. Clarify ascites fluid or MAb supernatant (see Basic Protocol 2).
4. Load the column with ~10 ml ascites or ~100 ml MAb supernatant of crude rat antibody solution.
5. Wash the column extensively with 10 to 15 column volumes of binding buffer. Monitor the A_{280} to be certain the absorbance returns to baseline. Set up a fraction collector to collect all fractions from steps 6 to 9.
6. Elute with 5 column volumes of pH 7.0 elution buffer, watching the UV monitor.

For most antibodies, this does not elute the bound antibody and serves as a preliminary wash step. Be sure A_{280} has returned to baseline before beginning the next step.

7. Elute with 5 column volumes of pH 5.5 elution buffer, watching the UV monitor.

Some antibodies will elute under these mild conditions. Be sure A_{280} has returned to baseline before beginning the next step.

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Biological Tools

16.3.7

8. Elute with 5 column volumes of pH 4.3 elution buffer, watching the UV monitor.

Most antibodies will elute under these conditions. Be sure A_{280} has returned to baseline before beginning the next step.

9. Elute with 5 column volumes of pH 2.3 elution buffer, watching the UV monitor.

All antibodies will elute at pH 2.3. This also serves as a final wash step. Be sure A_{280} has returned to baseline before beginning the next step.

10. Equilibrate column with binding buffer by washing with ≥ 10 column volumes. Store column wrapped in Parafilm at 4°C.

11. Identify eluted protein peaks (including the unbound initial fractions), pool, assay for antibody activity, and concentrate if necessary (see Basic Protocol 1, step 9).

DE52 ION-EXCHANGE CHROMATOGRAPHY WITH TRIS-Cl

DE52 ion-exchange (IEX) chromatography can be used to purify antibodies from a tissue culture supernatant, ascites fluid, and serum or ammonium sulfate precipitates derived from any of these antibody-containing fluids. The protocol may also be used as a second step following purification by size exclusion (SE) chromatography (see Basic Protocol 1 and Alternate Protocols 1 and 2). The major contaminant protein in all these preparations is albumin, which binds DE52 tightly under conditions of low-to-moderate ionic strength. Antibody either fails to bind to DE52, in which case it elutes in the void volume as the column is loaded, or it binds loosely, and can be eluted with a gentle salt or pH gradient. Fractions eluted are assayed first by A_{280} and then by either ELISA (APPENDIX 3) or SDS-PAGE (UNIT 6.1).

Caution should be exercised in that different monoclonal antibodies as well as antibody fractions from different immunized animals' sera elute from DE52 under different conditions. In this protocol, antibody in 0.01 M Tris·Cl at pH 8.6 is passed over the DE52 column and bound antibody is eluted with a gradient in the same buffer approaching 0.5 M NaCl.

Materials

DE52 powder (Whatman)

0.01 M Tris·Cl, pH 8.6 (APPENDIX 2A)

0.5 M NaCl/0.01 M Tris·Cl, pH 8.6 (see recipe)

Antibody sample (ascites fluid, tissue culture supernatant, immune serum, or ammonium sulfate precipitate)

1.5 × 50-cm column

Additional reagents and equipment for column chromatography (APPENDIX 3), dialysis (APPENDIX 3C), and ELISA (APPENDIX 3) or SDS-PAGE (UNIT 6.1)

1. Swell DE52 powder in 0.01 M Tris·Cl, pH 8.6, and remove fine particles.

It is very important to adjust the pH prior to pouring the DE52 gel into the column.

2. Pour a 1.5 × 50-cm DE52 column and equilibrate with 0.01 M Tris·Cl, pH 8.6.

The size of the column is dependent upon the total amount of protein in the crude antibody sample. The capacity of DE52 is ≥ 100 mg total protein/ml hydrated gel, so a column of 1 to 5 ml is commonly sufficient.

3. Place antibody sample in dialysis tubing. Dialyze twice against ≥ 20 times the sample volume of 0.01 M Tris·Cl, pH 8.6, overnight at 4°C.

4. Load the dialyzed antibody sample onto the column.
5. Elute column with 0.01 M Tris-Cl, pH 8.6, until all of the protein that does not bind to DE52 at this pH comes through the column (detected by monitoring A_{280} with a UV monitor). Collect the first ten fractions (~12 ml each).

Some antibodies elute at this stage (i.e., they do not bind DE52 in 0.01 M Tris-Cl, pH 8.6). They will thus be free of the major contaminant, albumin, which remains on the column. However, the great majority of antibodies will adsorb to the column under these conditions.

6. Elute the remaining material using a 200- to 250-ml linear gradient of 0.01 M Tris-Cl, pH 8.6, to an equal volume of 0.5 M NaCl/0.01 M Tris-Cl, pH 8.6. Collect 2-ml fractions.
7. Monitor column fractionation as follows: (1) A_{280} , to measure protein concentration; (2) conductivity, as an indication of the progress of the gradient as the concentration changes; (3) activity of the antibody by ELISA (APPENDIX 3); and (4) protein structure by SDS-PAGE (UNIT 6.1).

A typical example of the elution profile of a mouse MAb is shown in Figure 16.3.1. The first peak that comes off as the ionic strength is increased is the IgG. This is confirmed by SDS-PAGE or ELISA.

IEX chromatography is also useful for separating Fab fragments. Following size-exclusion chromatography of the digestion mixture to remove intact IgG (APPENDIX 3), the 50-kDa fraction is dialyzed (APPENDIX 3C) exhaustively against 0.01 M Tris-Cl, pH 8.6, and separated as described in this protocol using DE52. Elution profiles can vary greatly

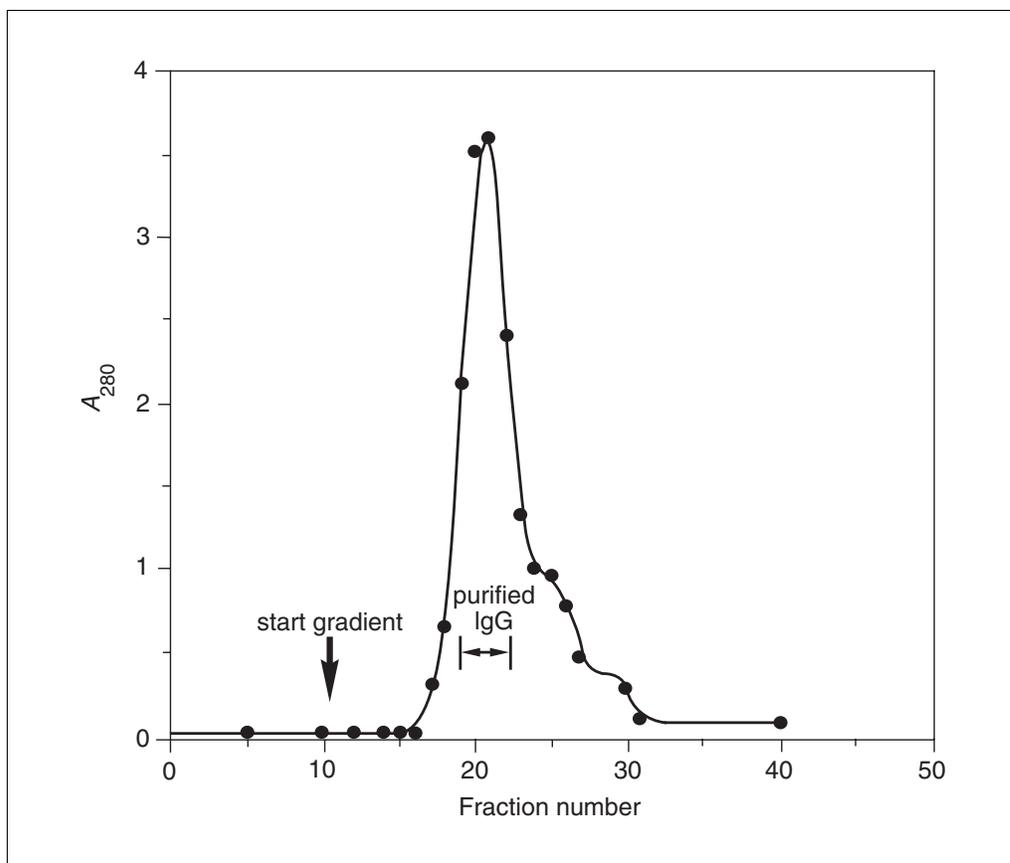


Figure 16.3.1 IEX chromatography elution profile from a DE52 column (1.5 × 50 cm). The IgG fractions from an ACA-34 column were dialyzed against 0.01 M Tris-Cl, pH 8.6, and applied to the column. Ten fractions (12 ml) were collected using the starting buffer, then a linear gradient of 0 to 0.5 M NaCl in Tris-Cl, pH 8.6, was started. Fractions 19 to 22 contained pure IgG as determined by SDS-PAGE (UNIT 6.1).

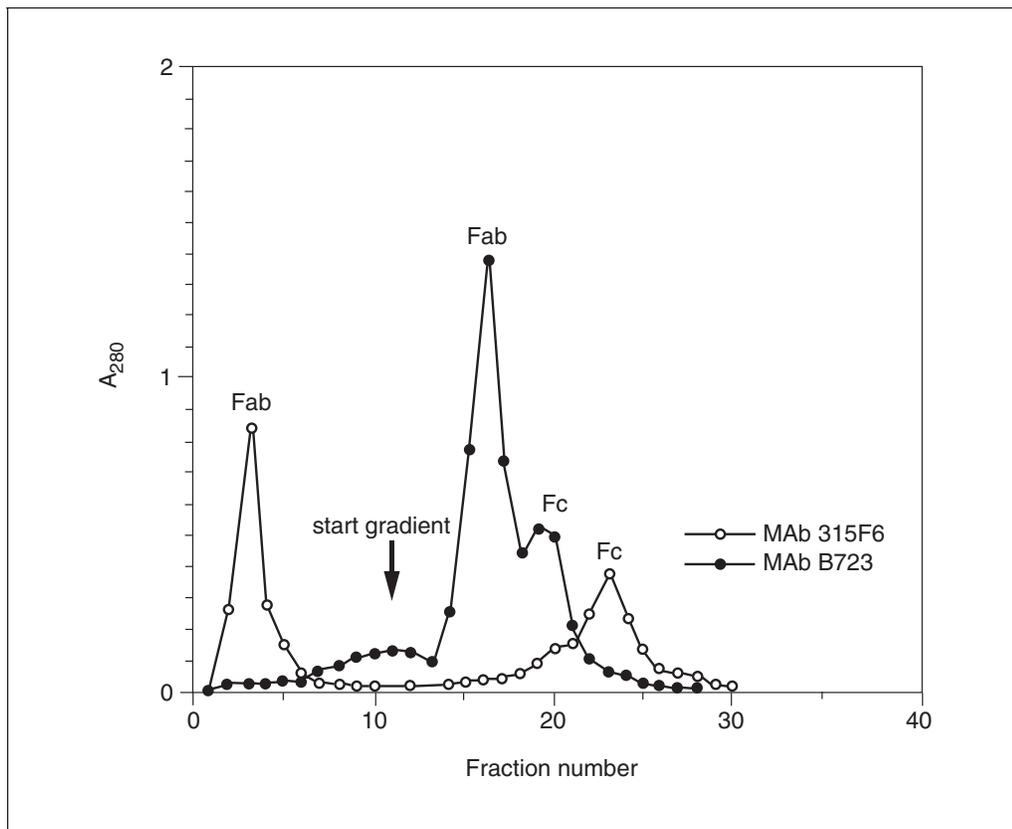


Figure 16.3.2 IEX chromatography elution profile of Fab from two different antibodies using a DE52 column (1.5 × 50 cm). Elution conditions and gradient were the same as for the example in Figure 16.3.1. For MAb 315F6 (open circles), the Fab fragment eluted in the starting buffer and the Fc portion much later in the gradient. Fab fragments from MAb B72.3 (closed circles) eluted at the beginning of the gradient.

between antibodies as shown in Figure 16.3.2. For one of the antibodies (open circles), the Fab elutes at 0.01 M Tris·Cl, pH 8.6. In contrast, the Fab from the other antibody (closed circles) elutes 40 ml after the salt gradient is started. These examples reinforce the need to monitor the location of the protein during the purification process.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2D; for suppliers, see SUPPLIERS APPENDIX.

Borate-buffered saline

0.015 M sodium borate
 0.15 M NaCl
 Adjust to pH 8.5 with 1 M NaOH and filter sterilize
 Store indefinitely at room temperature

0.5 M NaCl/0.01 M Tris·Cl, pH 8.6

1.21 g Tris base
 29 g NaCl
 800 ml H₂O
 Adjust pH to 8.6 with HCl and add H₂O to 1 liter

Phosphate-buffered saline (PBS)

0.23 g NaH₂PO₄ (anhydrous, 1.9 mM final)
 1.15 g Na₂HPO₄ (anhydrous, 8.1 mM final)

9 g NaCl (154 mM final)
Add H₂O to 900 ml
Adjust desired pH using 1 M NaOH or 1 M HCl
Add H₂O to 1 liter
Store indefinitely at room temperature

COMMENTARY

Background Information

Before purifying antibody from any preparation, consideration should be given to the potential use of the final product. If the antibody is to be used in an assay with internal controls or to saturate cell-surface antigen, then impure ascites fluid or a dialyzed ammonium sulfate precipitate will usually suffice. At the other extreme, biochemical modifications such as conjugation to drugs or certain fluorochromes (e.g., phycobiliproteins) may require an extremely pure product. In this case ion-exchange (IEX) chromatography (see Basic Protocol 3) should be used in addition to ammonium sulfate precipitation and affinity chromatography. For uses such as conjugation to fluorescein isothiocyanate (FITC), biotin or radioisotopes, antibody that has been purified by protein A or G is perfectly adequate.

Protein A is a cell wall component produced by several strains of *Staphylococcus aureus*; it is a single polypeptide chain with a molecular weight of 42,000 Da. Protein A binds specifically to the Fc region of immunoglobulin molecules, especially IgG for which it has four high-affinity binding sites. It is heat stable and retains its native conformation even after exposure to denaturing reagents such as 3 M thiocyanate, or in acidic conditions. Thus antibody can be stripped from protein A and the reagent used again for purification. Not all IgG molecules bind to protein A; rat IgGs bind particularly weakly, and many mouse IgG₁ subclass antibodies will not bind. Some of these antibodies can be affinity purified with the similar reagent, protein G.

Protein G is a bacterial cell wall protein isolated from group G streptococci. Protein G also binds IgG molecules through their Fc portions. There are two binding sites for IgG in native protein G; it also has binding sites for Fab regions of antibody, albumin, and cell membranes. Sigma sells a recombinant form of protein G which has been truncated so the sites for Fc binding remain, but the Fab-, albumin-, and membrane-binding sites have been removed. Protein G is particularly useful for purification of human IgG3 and rat IgG_{2b}; al-

though protein G can be used for other rat IgG molecules, the binding is frequently weak, especially in the case of IgG₁. Protein G is usually better than protein A for purification of mouse IgG₁.

Rat antibody can be problematic to purify, and an anti-rat affinity column (Alternate Protocol 2) may be required. The authors have had little success with protein G purification for rat monoclonal antibodies.

Elution profiles for IEX chromatography are different for each antibody or antibody fragment. The activity of eluted antibodies should be monitored using an ELISA (APPENDIX 3) in addition to the absorbance (A_{280}) profile.

Table 16.3.1 describes kits available for antibody purification. On first inspection, these may not seem to be the least expensive option, but it is important to remember that the reliability of kits and their excellent performance in terms of yield usually make them quite economic in the long term. It is a false economy to spend many hours of staff time obtaining a decreased percentage yield for the sake of saving a few dollars at the outset.

Critical Parameters

Because activity of the purified product is of prime importance, this should be evaluated for the impure antibody before purification is started and throughout the steps of purification. Some investigators report that the acid conditions required to elute from protein A can damage the antibody. Care should be taken to avoid excessively long periods of incubation at low pH. Elution from protein A- or protein G-Sepharose can also be accomplished using 3 M potassium thiocyanate, high ionic strength, or high pH. For particular antibodies these conditions may be more gentle, but in the authors' experience the best results are obtained with the methods described here.

Ammonium sulfate precipitation is a more gentle procedure than protein A purification, but it is more time-consuming. It can sometimes be difficult to remove samples from protein G. Use lower pH and increased salt concentration if problems are encountered.

Protein A columns can be used at flow rates of ~1 ml/min, while SE columns should be limited to ≤0.5 ml/min. Optimal results for SE are obtained using samples containing 5 to 100 mg of antibody. Protein A should be loaded within the limits given.

The antibody-binding capacity of protein A is variable between species. For mouse IgG, 1 ml of hydrated protein A will bind ~5 mg of antibody; for human IgG, 1 ml of hydrated protein A will bind ~8 mg of antibody.

For IEX (DE52) chromatography, the pH is very important and buffers should be checked on a pH meter immediately before starting. All column fractions should be retained until SDS-PAGE and antibody activity assays have been performed. In some cases, antibody may elute from the IEX column in the same fractions as serum albumin, in which case SE chromatography should be used.

DE52 has an enormous capacity for binding protein, as estimated by the manufacturer at 130 mg/ml bed volume. Thus, dilute antibody preparations can be concentrated effectively by this method.

Anticipated Results

Different batches of ascites fluid and monoclonal antibody supernatant can vary widely in the amount of antibody they contain. Generally, 1 ml of ascites should yield 1 to 4 mg of purified antibody and 1 ml of MAb supernatant should yield 0.5 to 50 µg of purified product. If cells are grown in automated culture systems that recycle the medium (bioreactors), yields equivalent to those of ascites fluid can be obtained. The lowest yields are usually from ammonium sulfate precipitation and the highest yields are usually from IEX or affinity methods.

Yields significantly lower than these should be a warning that the antibody-producing hybridomas are being overgrown by nonproducers. This can be remedied by returning to an earlier freeze of cells, or by recloning (and rescreening) the hybridoma.

Time Considerations

Procedures such as dialysis and running columns can be lengthy, but large amounts of antibody can be purified in 2 to 3 days. FPLC (fast peptide, protein, and polynucleotide liquid chromatography) systems are expensive, but

can reduce the time taken to obtain purified antibody.

Quickest results can be obtained when culture supernatant or ascites is purified by one of the affinity methods described here (Basic Protocol 2): pure antibody can be obtained in <1 day. The ammonium sulfate, size-exclusion, or ion-exchange methods will take longer.

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Contributed by Sarah M. Andrew
Chester College
Chester, United Kingdom

Julie A. Titus
National Cancer Institute
Bethesda, Maryland

Fragmentation of Immunoglobulin G

To study the distinct regions of an immunoglobulin, it is useful to cleave them with proteolytic enzymes into various fragments (see Fig. 16.4.1 for a schematic of the structure of antibody molecules and their proteolytic fragments). The Fc portion of the antibody molecule is responsible for certain biological effects of antibodies, such as binding to Fc receptors on cells, mediating antibody-dependent cellular cytotoxicity, and complement fixation. It is also thought to be the more immunogenic portion of the molecule in vivo. For experiments concerning effects mediated by specific binding of antibody to cells in vitro or localization of antibodies to certain organs in vivo, it may be necessary to eliminate the nonspecific effects of Fc-receptor binding. The affinity of antibody for an antigen on the surface of viable cells can only be measured accurately using monovalent antigen-binding fragments (Fab).

Classically, fragmentation to the monovalent Fab fragment is carried out using papain digestion (see Basic Protocols 1 and 2), and the bivalent $F(ab')_2$ fragment is obtained by pepsin digestion (see Basic Protocols 3 and 4). Although papain digestion produces Fab fragments from IgG of all subclasses from all species, pepsin is not so universally useful. Alternative methods of fragmentation to $F(ab')_2$ include use of papain that is preactivated with cysteine (see Alternate Protocol 1) and use of the enzyme ficin (see Alternate Protocol 2). These alternate methods are particularly useful for mouse IgG₁ antibodies. When using pepsin and papain for the first time on an antibody, pilot experiments are performed to determine the correct reaction conditions; with preactivated papain and ficin these pilot experiments are unnecessary.

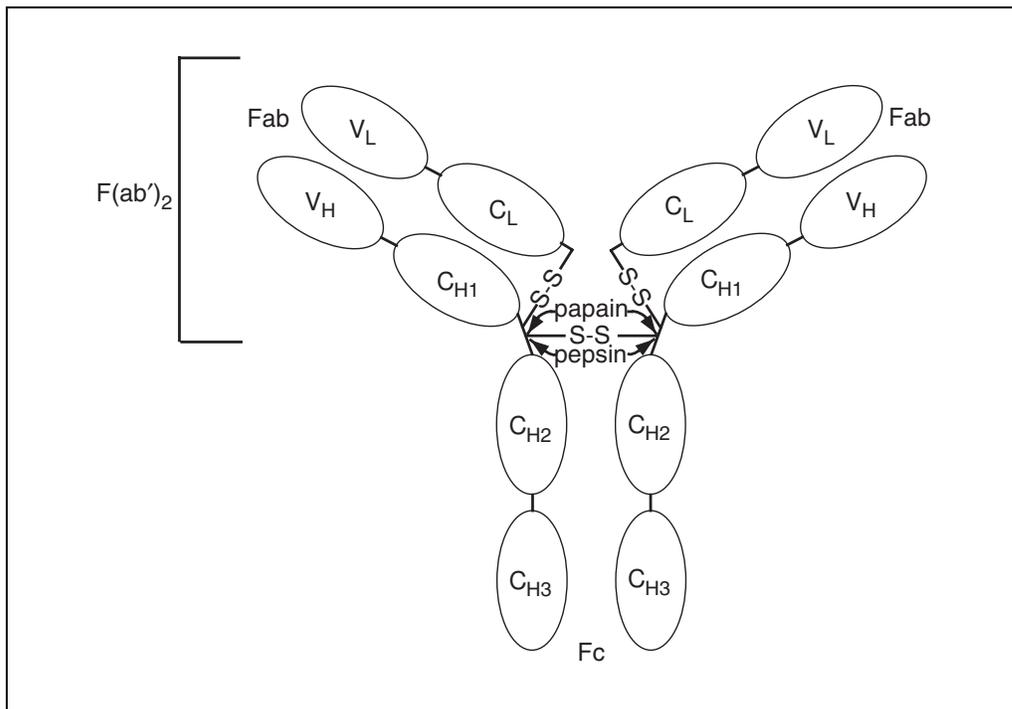


Figure 16.4.1 Proteolytic digestion fragments of immunoglobulins. Structural domains of the variable and constant regions of the light chain (V_L, C_L) and heavy chain (V_H, C_H1, C_H2, C_H3), as well as interchain disulfide bonds (S-S), are indicated. Monovalent fragments of IgG produced by papain digestion are known as Fab, while bivalent papain fragments are $F(ab)_2$. $F(ab')_2$ refers to the bivalent fragments of IgG produced by pepsin digestion; those derived from IgM are called $F(ab')_2\mu$. Bivalent subunits of IgM with both Fab and Fc are known as IgMs (subunit), and fragments of IgG containing a single antibody-binding site and an intact Fc portion are Fab/c.

PILOT FRAGMENTATION OF IgG TO Fab USING PAPAINE

Papain digestion of IgG produces Fab fragments. This protocol is designed to determine conditions for large-scale fragmentation, and can be used for IgG from mouse (all subclasses), rat, human, goat, sheep, horse, chicken, guinea pig, and cow. Once the method for a particular antibody is established, it will be reproducible for each fragmentation.

Purified monoclonal antibody is digested with papain in the presence of the reducing agent cysteine. To determine the optimal conditions, both the concentration of papain and the time of digestion are varied. After terminating the digestions, the fragments are dialyzed and analyzed by SDS-PAGE. By assessing the gel results, optimal conditions are chosen. These conditions are used in the large-scale fragmentation of IgG to Fab (see Basic Protocol 2).

Materials

2 mg/ml purified IgG (UNIT 16.3) in PBS (UNIT 16.3)

Papain (2× crystallized suspension; Sigma)

Digestion buffer (see recipe)

0.3 M iodoacetamide (prepare fresh from crystalline material; Sigma) in PBS

PBS (UNIT 16.3)

Additional reagents and equipment for nonreducing gel electrophoresis (UNIT 6.5) and dialysis of proteins (APPENDIX 3C)

1. Pipet 100 μ l of 2 mg/ml purified IgG in PBS into each of 24 numbered microcentrifuge tubes.
2. Prepare two papain solutions in 5 ml digestion buffer—one at 0.1 mg/ml papain and the other at 0.02 mg/ml.

Because papain is a suspension, mix well without vortexing before pipetting.

3. Add 100 μ l of 0.1 mg/ml papain to eight of the IgG tubes. Add 100 μ l of 0.02 mg/ml papain to a second group of eight IgG tubes. Finally, add 100 μ l digestion buffer without papain to the remaining eight tubes. Close the lids on all of the tubes.

Thus, reactions have been set up with enzyme/antibody (E/A) ratios of 1:20 and 1:100, and control tubes with no enzyme.

4. Prepare a digestion time curve by incubating tubes in a 37°C water bath and removing them at different times in groups of three—one tube each of E/A at 1:20 and 1:100, and one control tube. Remove the first group after 1 hr, and the subsequent groups at 2, 4, 6, 12, 18, 24, and 48 hr. As each group of three tubes is removed from the water bath, terminate the reaction by adding 20 μ l of 0.3 M iodoacetamide in PBS and then vortexing.

The final concentration of iodoacetamide (0.03 M) irreversibly inactivates papain.

5. Dialyze the reaction mixture (APPENDIX 3C).

Dialysis can be performed using a homemade microdialysis chamber or commercial microdialysis cassettes (e.g., Slide-A-Lyzer Dialysis Cassettes, Pierce).

6. Analyze digestion products by loading an 80- μ l aliquot of the dialyzed mixture from each tube onto a 10% nonreducing polyacrylamide gel (UNIT 6.5).

Antibodies that have been digested with papain in the presence of cysteine to produce Fab fragments show a major protein band on a nonreducing polyacrylamide gel at a molecular weight of ~50 kDa, and a second band at 27 kDa which is the remaining Fc portion. There are usually a couple of smaller bands at 24 and 15 kDa which are other unimportant products of digestion. Obviously, undigested antibody will appear as a band at 150 kDa. Frequently, effects of cysteine alone are seen in antibody which has not been treated with papain, causing a protein band on the gel at 120 kDa. This 120-kDa band is also seen in

papain, causing a protein band on the gel at 120 kDa. This 120-kDa band is also seen in the digestion mix at short incubation times (up to 4 hr). The digestion is complete when no protein bands of 150 kDa or 120 kDa are seen in the gel lanes containing the digestion mixture. The time of complete digestion will vary between antibodies.

The effect of incubation with cysteine alone will be seen in the control lanes, probably because cysteine causes some unfolding of the antibody molecule, thus altering its electrophoretic mobility. Fab fragments have a molecular weight of 50 kDa (see Table 16.3.2). At early time points some intact antibody is expected on the gel, with the reaction moving towards completion as incubation times increase.

The amounts of antibody used in this method are suitable for analysis by SDS-PAGE using standard 150 × 1.5-mm gels. If a minigel apparatus is available (e.g., the Phast system from Pharmacia), modify the amounts accordingly and use less antibody.

7. Choose the optimal conditions to obtain Fab from the IgG by assessing the gel results and use these conditions for Basic Protocol 2.

LARGE-SCALE FRAGMENTATION OF IgG TO Fab USING PAPAINE

Exact conditions for optimum yield of Fab from each antibody should be assessed from the pilot experiment (see Basic Protocol 1); those conditions are then scaled up in this procedure. The procedure is similar to Basic Protocol 1 where papain is used to digest the antibody and the digested antibody is dialyzed. The dialyzed antibody fragment is then purified by protein A–Sepharose affinity chromatography (UNIT 16.3) and can be further purified by size-exclusion chromatography (APPENDIX 3). The purity of the product is assessed by nonreducing gel electrophoresis (UNIT 6.5).

Materials

Papain (2× recrystallized suspension; Sigma)

Digestion buffer (see recipe)

≥1 mg/ml IgG (≥5 mg total; UNIT 16.3) in PBS

Iodoacetamide crystals

PBS, pH 8.0 (UNIT 16.3)

Protein A–Sepharose CL-4B

Sephacryl S-200 Superfine (Pharmacia Biotech)

5 × 100-mm column (Bio-Rad)

26 × 900-mm column (Pharmacia Biotech)

Additional reagents and equipment for protein dialysis (APPENDIX 3C), column chromatography (UNIT 16.3 & APPENDIX 3), concentrating protein solutions (APPENDIX 3C), and nonreducing gel electrophoresis (UNIT 6.5)

1. Dissolve the required amount of papain in a volume of digestion buffer equal to the volume of antibody solution to be digested; use optimum amounts of enzyme and antibody as well as incubation times from the results of the pilot fragmentation in Basic Protocol 1.

Digestion buffer is made at twice the required final strength so that when added to the antibody solution in equal volume, the mixture will be at the correct molarity with respect to EDTA and cysteine. Because papain is a suspension, mix well without vortexing before pipetting.

2. Add the papain buffer solution to the ≥1 mg/ml IgG in PBS, mix, and incubate for the required time (determined from Basic Protocol 1) in a 37°C water bath.
3. Stop the reaction by adding crystalline iodoacetamide to 0.03 M final. Mix carefully to ensure that the iodoacetamide dissolves completely.

BASIC PROTOCOL 2

4. Transfer to dialysis tubing and dialyze the mixture against 2 liters PBS, pH 8.0, for 12 to 20 hr at 4°C.

More detailed procedures for large-volume dialysis, and selection and preparation of dialysis membranes can be found in APPENDIX 3C.

5. Prepare a 5 × 100–mm protein A–Sepharose CL-4B column (UNIT 16.3 & APPENDIX 3) and load dialyzed mixture onto it. Collect unbound fraction containing the Fab fragment and enzyme. If necessary, wash column with PBS to completely recover Fab fragments.

The remaining intact antibody will bind to the column.

6. Concentrate the mixture containing the Fab fragments to ≤5 ml (APPENDIX 3C).
7. Prepare a 26 × 900–mm Sephacryl S-200 Superfine size-exclusion column (APPENDIX 3) and load concentrated mixture onto it. Collect fractions corresponding to a molecular weight of 50 kDa.

Fractions can be collected and their molecular weight assessed by SDS-PAGE or a precalibrated size-exclusion column.

8. Check the purity of the final product (1 to 80 μl) on a 10% nonreducing polyacrylamide gel (UNIT 6.5).
9. Assess the concentration of the Fab fragments at A_{280} (Table 16.3.2). Store fragments in borate buffer (UNIT 16.3) at 4°C or in PBS containing 0.02% NaN_3 at –70°C.

BASIC PROTOCOL 3

PILOT FRAGMENTATION OF IgG TO F(ab')₂ USING PEPSIN

Pepsin fragmentation of IgG to produce F(ab')₂ is similar to the pilot fragmentation with papain (see Basic Protocol 1) except that the pH and time of digestion are varied. The optimum pH for pepsin digestion is ~2; however, this low pH may damage the antibody, so a pH of ~4 is the minimum recommended. Two different pH values are tested in this pilot experiment, as some antibodies will fragment to Fab or smaller fragments at lower pH values. Because pepsin treatment can be slightly harsher than papain, an enzyme/antibody (E/A) ratio of no higher than 1:20 is recommended, as it is possible that pepsin could damage the binding site of the antibody molecule.

NOTE: F(ab')₂ fragments cannot be obtained from IgG_{2b}. Treatment of IgG_{2b} with pepsin will result in fragments of about the correct molecular weight (120 kDa), but these will be Fab/c fragments (consisting of one Fab fragment and the Fc portion; Fig. 16.4.1).

Materials

3 mg/ml purified IgG (UNIT 16.3)
Acetate buffer, pH 4.0 and 4.5 (see recipe)
0.1 mg/ml pepsin (Sigma) in pH 4.0 and pH 4.5 acetate buffers
2 M Tris base
PBS (UNIT 16.3)

Additional reagents and equipment for protein dialysis (APPENDIX 3C) and nonreducing gel electrophoresis (UNIT 6.5)

1. Dialyze 2 ml of 3 mg/ml purified IgG against 200 ml acetate buffer, pH 4.0, and another 2 ml of 3 mg/ml purified IgG against 200 ml acetate buffer, pH 4.5, both for 4 hr at 4°C.
2. Determine the concentrations of both dialyzed IgG solutions by A_{280} readings using the acetate buffer of corresponding pH as the blank (Table 16.3.2).

3. Adjust each dialyzed antibody solution to a concentration of 2 mg/ml in the acetate buffer of corresponding pH.
4. Pipet 100 μ l of the 2 mg/ml IgG, pH 4.0, into each of 16 numbered microcentrifuge tubes. Into another 16 numbered microcentrifuge tubes, pipet 100 μ l of the 2 mg/ml IgG, pH 4.5.

Thus, there are two groups of 16 tubes—one group at pH 4.0 and the other at pH 4.5.

5. Add 100 μ l of 0.1 mg/ml pepsin in acetate buffer, pH 4.0, to eight tubes in the IgG, pH 4.0 group. To the other eight tubes in this group, add 100 μ l of acetate buffer, pH 4.0. Add 100 μ l of 0.1 mg/ml pepsin in acetate buffer, pH 4.5, to eight tubes in the IgG, pH 4.5 group. To the other eight tubes in this group, add 100 μ l of acetate buffer, pH 4.5.

Thus, there are two groups of 16 tubes with pepsin at an E/A ratio of 1:20 at two different pH values, and control tubes without enzyme.

6. Incubate the 32 tubes in a 37°C water bath, removing one tube from each group of eight tubes after 1, 2, 4, 6, 12, 24, and 48 hr. As each tube is removed, stop the reaction by adding 40 μ l of 2 M Tris base.
7. Transfer to dialysis tubing and prepare microdialysis chamber (*APPENDIX 3C*). Dialyze the samples against 1 liter PBS for 4 hr at 4°C.
8. Analyze 80- μ l aliquots of the fragmentation products on a 10% nonreducing polyacrylamide gel (*UNIT 6.5*).

F(ab')₂ has a molecular weight of 110 kDa. Bands of this molecular weight should be seen in the fractions treated with pepsin. Lower-molecular-weight bands (~50 kDa) are probably the Fab' fragment of the antibody, a further degradation product from pepsin. Amounts of F(ab')₂ and Fab' produced will vary among antibodies, depending on the fragmentation conditions and individual susceptibilities.

9. Choose the best conditions to obtain maximum yield of F(ab')₂ by assessing the gel results and use these conditions in the following basic protocol.

LARGE-SCALE FRAGMENTATION OF IgG TO F(ab')₂ USING PEPSIN

As with the papain large-scale protocol, optimal fragmentation conditions should be assessed for each antibody from the pilot fragmentation (Basic Protocol 3). Purification is by protein A–Sepharose affinity chromatography (Basic Protocol 2). It is important to remember that IgG_{2b} generally cannot be fragmented to F(ab')₂.

Materials

- ≥1 mg/ml purified IgG (*UNIT 16.3*)
- Acetate buffer at appropriate pH (see Basic Protocol 3 and recipe)
- 0.1 mg/ml pepsin in acetate buffer at appropriate pH (see Basic Protocol 3)
- 2 M Tris base
- PBS, pH 8.0 (*UNIT 16.3*)
- Protein A–Sepharose CL-4B
- Sephacryl S-200 Superfine (Pharmacia Biotech)
- 5 × 100–mm column (Bio-Rad)
- 26 × 900–mm column (Pharmacia Biotech)

Additional reagents and equipment for protein dialysis (*APPENDIX 3C*), concentrating protein solutions (*APPENDIX 3C*), column chromatography (*UNIT 16.3 & APPENDIX 3*), and reducing and nonreducing gel electrophoresis (*UNIT 6.5*)

BASIC PROTOCOL 4

**Antibodies as Cell
Biological Tools**

16.4.5

Digest IgG

1. Dialyze ≥ 1 mg/ml IgG against acetate buffer at the appropriate pH.
2. Determine the concentration at A_{280} .
3. Add 0.1 mg/ml pepsin dissolved in acetate buffer at appropriate pH to give an E/A ratio of 1:20 (see Basic Protocol 3, step 5). Incubate the reaction mixture for the required time (see Basic Protocol 3, step 6) in a 37°C water bath.
4. Stop the reaction by adding ~ 50 μ l of 2 M Tris base/ml reaction. Check with pH paper that pH = 8.0 and add more Tris base if necessary.
5. Transfer the mixture to dialysis tubing and dialyze against 1 liter PBS, pH 8.0, at 4°C (APPENDIX 3C).

Purify $F(ab')_2$

6. Prepare a 5 \times 100–mm protein A–Sepharose CL-4B column (UNIT 16.3 & APPENDIX 3) and load dialyzed mixture onto it. Collect the unbound fraction.

At this stage, the $F(ab')_2$ fragment can be reduced to Fab' if desired. Add cysteine to a concentration of 0.01 M, mix well and incubate 2 hr at 37°C. Cysteine should reduce and alkylate the inter-heavy chain disulfide bonds. If further alkylation is thought necessary, make the mixture 0.15 M with respect to iodoacetamide. Purify the Fab' using the steps below.

7. Concentrate the mixture to ≤ 5 ml.
8. Prepare a 26 \times 900–mm Sephacryl S-200 Superfine size-exclusion column (APPENDIX 3) and load concentrated mixture onto it. Collect fractions corresponding to a molecular weight of 110 kDa.

Measure the protein eluting from the column with a UV monitor or spectrophotometer. Assess their contents by SDS-PAGE or use of a precalibrated column.

9. Check the purity of the final product (1 to 20 μ l) on a 10% SDS–polyacrylamide gel under reducing and nonreducing conditions (UNIT 6.5).

$F(ab')_2$ will give a single band at 110 kDa on a nonreducing gel and a single band or a doublet at 25 kDa on a reducing gel.

10. Assess the concentration of the $F(ab')_2$ fragments at A_{280} (Table 16.3.2). Store fragments in borate buffer (UNIT 16.3) at 4°C or in PBS containing 0.02% NaN_3 at -70°C .

ALTERNATE PROTOCOL 1

FRAGMENTATION OF IgG USING PREACTIVATED PAPAINE

Mouse IgG₁ has shown significant resistance to pepsin digestion. The antibody is missing a leucine at residue 234; this restricts the hinge region and causes pepsin resistance. This method can be used to obtain $F(ab)_2$ fragments from IgG₁. Additionally, Fab fragments can be obtained from mouse IgG_{2a} and IgG_{2b} by this cleavage. It is a gentler procedure and can give a more stable fragmentation and a high yield of fragments. The procedure is time-consuming but incubation periods are usually less critical. The conditions used here are typical of large-scale fragmentation and purification. It is not necessary to carry out a pilot fragmentation experiment to assess conditions when using this procedure, although the type of fragment obtained varies between subclasses.

Fragmentation of IgG

16.4.6

Additional Materials (also see Basic Protocol 4)

10 mg IgG (UNIT 16.3) in 2 to 5 ml PBS
Acetate/EDTA buffer (see recipe)
2 mg/ml papain in acetate/EDTA buffer
0.05 M cysteine (free base, crystalline; Sigma)
Iodoacetamide crystals

PD-10 column (Pharmacia Biotech)
26 × 900-mm column

Additional reagents and equipment for protein dialysis and concentration (APPENDIX 3C), column chromatography (UNIT 16.3 & APPENDIX 3), and SDS-PAGE (UNIT 6.1)

Prepare papain

1. Dialyze 10 mg IgG in 2 to 5 ml PBS in acetate/EDTA buffer (APPENDIX 3C).
2. Determine the concentration at A_{280} with the acetate/EDTA buffer as a blank.
3. Incubate 2 mg/ml papain and 0.05 M cysteine for 30 min in a 37°C water bath.

Papain requires a free sulfhydryl group for its catalytic activity. In the native crystalline form, this group is blocked and the enzyme exhibits extremely low proteolytic activity. Cysteine activates the group and activation is optimal in the presence of a heavy-metal binding agent such as EDTA.

4. Equilibrate a PD-10 column with 20 ml of acetate/EDTA buffer (APPENDIX 3).
5. Apply papain/cysteine (from step 3) to the PD-10 column. Collect ten 1-ml fractions, eluting with acetate/EDTA buffer.

This process removes the cysteine from preactivated papain. Use preactivated papain within 2 hr of preparation and keep on ice until use.

6. Assay the fractions at A_{280} and pool the two or three fractions containing papain. Calculate the preactivated papain concentration using the following formula:

$$A_{280}/2.5 = \text{mg preactivated papain/ml.}$$

Digest IgG

7. Add 0.5 mg preactivated papain to dialyzed, 10-mg IgG solution (from step 2) and vortex. Incubate 6 to 12 hr in a 37°C water bath.
8. Stop reaction by adding crystalline iodoacetamide to a final concentration of 0.03 M.
9. Dialyze against 1 liter PBS, pH 8.0, at 4°C for 6 to 12 hr (APPENDIX 3).

Purify fragments

10. Prepare a protein A-Sepharose column equilibrated in PBS, pH 8.0 (UNIT 16.3), and apply reaction mixture. Collect unbound 2-ml protein fractions and pool those represented by the first peak.

The antibody fragments will elute in the first protein peak. Assess this using a UV monitor or spectrophotometer.

11. Concentrate the unbound fractions to ≤ 5 ml (APPENDIX 3C).
12. Prepare a size-exclusion (SE) column (APPENDIX 3) and load the sample. Collect 100 fractions (1% of the column volume). Assess the fractions containing the appropriate molecular weight by SDS-PAGE (UNIT 6.1) or on a precalibrated column. Store in borate buffer (UNIT 16.3) at 4°C or in PBS containing 0.02% NaN_3 at -70°C .

Monitor the fractions with a UV spectrophotometer at A_{280} nm. The fragments obtained from this procedure are Fab from IgG_{2a} and IgG_{2b} , and F(ab)_2 from IgG_1 .

FRAGMENTATION OF MOUSE IgG₁ TO F(ab')₂ USING FICIN

IgG₁ can also be fragmented to F(ab')₂ by treatment with ficin in the presence of cysteine. Ficin is a thiol protease isolated from fig latex.

Additional Materials (also see *Basic Protocol 4*)

10 mg mouse monoclonal IgG₁ (UNIT 16.3) in 2 to 5 ml PBS
50 mM Tris/2 mM EDTA, pH 7
Ficin solution: 1 mg/ml ficin (Sigma) in 50 mM Tris/2 mM EDTA, pH 7
Cysteine
100 mM *N*-ethylmaleimide (Sigma)
PBS (UNIT 16.3)

Additional reagents and equipment for protein dialysis (APPENDIX 3C) and column chromatography (UNIT 16.3 & APPENDIX 3)

1. Dialyze 10 mg mouse monoclonal IgG₁ in PBS against 1 liter of 50 mM Tris/2 mM EDTA, pH 7, for 6 hr at 4°C.
2. Determine the concentration of the antibody by reading the A₂₈₀ with the dialysis buffer as a blank.
3. Add ficin solution to give a 1:30 enzyme/immunoglobulin ratio.
4. Add solid cysteine to the mixture to give a final cysteine concentration of 1 mM.
Cysteine is required to activate the enzyme.
5. Incubate 4 to 5 hr in a 37°C water bath with gentle shaking.
The incubation time is not critical.
6. Stop reaction by adding 1/10 vol of 100 mM *N*-ethylmaleimide.
7. Dialyze the mixture against PBS, then purify the fragments by protein A–Sepharose and size-exclusion (SE) chromatography (see Alternate Protocol 1, steps 10 to 12 and UNIT 16.3 & APPENDIX 3).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Acetate buffer, pH 4.0 and 4.5

Bring 0.2 M sodium acetate to pH 4.0 and 4.5 (as required) with glacial acetic acid.

Acetate/EDTA buffer, pH 5.5

Bring 0.1 M sodium acetate to pH 5.5 with glacial acetic acid and add EDTA to 0.003 M.

Digestion buffer

PBS (UNIT 16.3) containing:
0.02 M EDTA (disodium salt)
0.02 M cysteine
Prepare fresh; store <10 hr on ice

COMMENTARY

Background Information

Immunoglobulins can be cleaved by proteolytic enzymes into functionally distinct subunits (Fig. 16.4.1). Generally, fragments that bind antigens are of interest. Fab and F(ab')₂ fragments contain the antibody-binding portion of the molecule; the Fc fragment is generally only of interest if studying the crystal structure of antibodies. The Fab or F(ab')₂ portion of the antibody may be required in certain experiments. For instance, in mixed lymphocyte response (MLR) and cytolytic T lymphocyte (CTL) assays, the Fc portion may cause artifacts because it binds to cells bearing the Fc receptor. It has also been suggested that the Fc portion of the antibody may be more immunogenic *in vivo* (Hellstrom and Hellstrom, 1985). The Fc portion of IgG_{2a} and IgG₃ can invoke antibody-dependent cell-mediated cytotoxicity responses *in vivo* and *in vitro*.

The monoclonal antibodies routinely used for *in vitro* and *in vivo* experiments are from the mouse, and the protocols of this unit are designed for use with mouse antibodies. Fragmentation of antibodies from other species can be evaluated and optimized using the pilot protocol provided. Fragmentation with papain is similar for many species.

Subclass-dependent susceptibility of mouse antibodies to proteolytic cleavage has been widely reported (Parham, 1986). The order of sensitivity to proteolytic enzymes has been found to be IgG_{2b}>IgG₃>IgG_{2a}>IgG₁. Not all antibodies, however, fall into this ranking; IgG_{2a} can be extremely sensitive to the action of papain in the presence of cysteine. If such sensitivities are found, then the method in which cysteine is removed from the enzyme following preactivation is recommended (see Alternate Protocol 1; Parham et al., 1982).

It must be stressed that all monoclonal antibodies are different and, for this reason, standard conditions for fragmentation are not given for papain and pepsin digestion. A pilot experiment should always be performed to assess optimal conditions before carrying out a large-scale fragmentation. The pilot experiment is very similar to the large-scale procedure. Microdialysis (Overall, 1987) is recommended so that the conditions from small- to large-scale are identical.

IgG can be fragmented to yield monovalent Fab fragments using the proteolytic enzyme papain in the presence of the reducing agent cysteine. IgG subclasses (e.g., IgG₁ and IgG₂)

have different susceptibilities to papain digestion. IgG₁, IgG_{2a}, and IgG₃ can be digested to bivalent F(ab')₂ fragments using pepsin. Although IgG₁ may be sensitive to pepsin degradation, a more stable digestion is achieved using papain in the absence of cysteine. Ficin is also useful for digestion of IgG₁ to F(ab')₂ (Mariani et al., 1991). It is a thiol protease purified from fig latex. It requires activation with cysteine; the concentration of cysteine determines the extent of digestion. Generally, IgG_{2b} cannot be fragmented to F(ab')₂. Prior to fragmentation, a pilot experiment should be undertaken to determine individual conditions for fragmentation of each antibody. Separation of fragments can be carried out very efficiently by ion-exchange (IEX) chromatography on DE52 (UNIT 16.3 & APPENDIX 3); other methods for separation have been described in the protocols.

Pepsin cleavage of mouse IgG subclasses results in two sites of proteolytic sensitivity, one on either side of the inter-heavy chain disulfide bonds as suggested by Parham (1983). Primary cleavage may occur at a site on the COOH-terminal side of the bridges, giving F(ab')₂, followed by secondary cleavage on the NH₂-terminal side, giving Fab'. In the case of IgG_{2b}, the relative sensitivities of these two sites is reversed. Because the heavy chains of IgG_{2b} are asymmetrically glycosylated and the products of pepsin digestion of this subclass are Fab and Fab/c, F(ab')₂ has a molecular weight close to that of Fab/c. F(ab')₂, however, has rarely been reported to be obtained from IgG_{2b}, and therefore is not a feasible product of this subclass.

Pierce produces several kits for production of Fab and F(ab')₂ fragments from mouse (and other species) antibodies. These kits have been used with great success by the authors. Such kits are strongly recommended to those with the funds to purchase them. Each kit comes complete with all buffers and instructions.

Critical Parameters

Because each monoclonal IgG is different, if one antibody of a particular subclass is cleaved under a certain set of conditions, it cannot be expected that all other antibodies of that subclass will behave in the same manner. A pilot experiment should always be carried out when a new antibody is to be fragmented. It is probably better to choose a reaction time that will leave some antibody unfragmented rather than risk damaging the antibody-binding site by excessive exposure to proteolytic en-

zymes. Intact antibody can be easily removed from the fragmentation mixture by passage through a protein A–Sepharose column. For those antibodies that do not bind well to protein A, protein G–Sepharose (UNIT 16.3) or ion-exchange chromatography (APPENDIX 3) are reasonable alternatives.

Troubleshooting

Optimum reaction conditions may vary after scaling up from the pilot fragmentation. Occasionally the incubation times appear shorter in the small-scale procedure, so it may be necessary to increase the incubation time a little when scaling up.

Another possible difficulty is that the fragmentation does not proceed at all. Probably, this is related to the enzyme. Ascertain that there are no enzyme inhibitors, such as azide, present in the antibody solution (UNIT 16.3) before ordering new enzyme.

Anticipated Results

Yields are frequently subclass-dependent. A 100% yield of Fab fragment would be twice the number of moles of intact antibody in the starting mixture, and a 100% yield of F(ab')₂ would be equal to the number of moles of antibody in the starting mixture. Typically, yields of 45% to 55% are achieved, although some investigators report yields as high as 70% to 80%.

Time Considerations

Dialysis is usually carried out overnight, although 4 hr is sufficient for samples up to 100 ml. Running a protein A–Sepharose column takes ~4 hr, and running a size-exclusion column takes overnight.

Dialysis and running of columns are time-consuming procedures. The total time required to obtain fragments from a new antibody—

beginning with the pilot experiment to the end of the large-scale production—is 4 to 5 days for Fab fragments and 6 to 8 days for F(ab')₂ fragments.

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Key Reference

Parham, 1983. See above.

A lucid and comprehensive guide to the production of fragments from mouse monoclonal antibodies.

Contributed by Sarah M. Andrew
Chester College
Chester, United Kingdom

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Antibody conjugates are extremely useful reagents for probing many biologically and chemically important molecules *in vitro* or *in vivo*. This unit presents some basic protocols for conjugating antibodies with fluorescent dyes, with biotin (see Basic Protocol 1), and with enzymes (see Basic Protocol 2). These protocols have been selected because they are relatively easy to perform and have high rates of success. The basic approach for conjugating antibodies is to derivatize amino groups on the antibody with the dye or biotin derivative of choice. Conjugation at amine sites is simple to perform, and, with a fluorescent dye, it generally yields brightly fluorescent conjugates. The conjugates retain high levels of biological activity, notwithstanding the fact that the labels react with random amines on the antibody molecule, and they may even react near or at the antigen-binding site. Labeling of the amines is the most widely used commercial method to tag antibodies. In this unit, suggestions are presented for variations on these basic protocols that are useful when quantities of available antibody are limited. Methods for estimating antibody concentration are also included (see Support Protocol).

Alternate protocols for attaching label at the carbohydrate site of the Fc portion or at the sulfhydryl groups of the hinge region, which join the two heavy chains of the antibody molecule (Fig. 16.5.1), are also possible (see Alternate Protocol 1). These alternate protocols, which entail more steps, are more complex and require more meticulous optimization. Furthermore, they are difficult to perform when <1 mg of antibody is available. Among these alternate protocols, a general procedure is described for conjugating antibodies (see Alternate Protocol 2) or enzyme (see Alternate Protocol 3) at the carbohydrate site. Although this alternate protocol is presented for antibody-enzyme conjugates only, the basic principles can easily be adapted for conjugation of antibodies with fluorophores or biotin, as detailed in the Background section. Background Information also includes references for labeling antibodies at the hinge region.

CONJUGATING ANTIBODIES TO FLUOROPHORES OR BIOTIN

The procedural steps for antibody-fluorophore conjugation and for antibody-biotin conjugation are quite similar. These steps consist of mixing the dye or the biotin-derived reagent at an appropriate molar ratio and pH with the antibody solution and, after incubation, purifying the conjugate from the reaction mixture by size-exclusion chromatography (UNIT 5.5) or dialysis (APPENDIX 3C). The antibody-dye or antibody-biotin conjugate can then be analyzed to determine the degree of labeling, *i.e.*, the number of moles of dye or biotin per mole of antibody. See Critical Parameters for helpful hints.

Materials

- Antibody to label
- 1.0 M sodium bicarbonate (see recipe)
- Phosphate-buffered saline (PBS; see recipe)
- Probe (fluorophore or biotin)
- Anhydrous dimethylformamide (DMF) or dimethylsulfoxide (DMSO; see recipe)
- 1.5 M hydroxylamine, pH 8.0 (see recipe)
- Size-exclusion chromatography matrix: MATREX Cellufine GH25 (Amicon/Millipore), BioGel P30 (BioRad Laboratories) or equivalent matrix
- Toyopearl HW-40F (TosoHaas)
- Reaction buffer, pH 7.5 (see recipe)
- Appropriate TLC solvent (see Table 16.5.1, optional)

continued

BASIC PROTOCOL 1

Antibodies as Cell
Biological Tools

16.5.1

Contributed by Rosaria P. Haugland

Current Protocols in Cell Biology (2000) 16.5.1-16.5.22

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Supplement 6

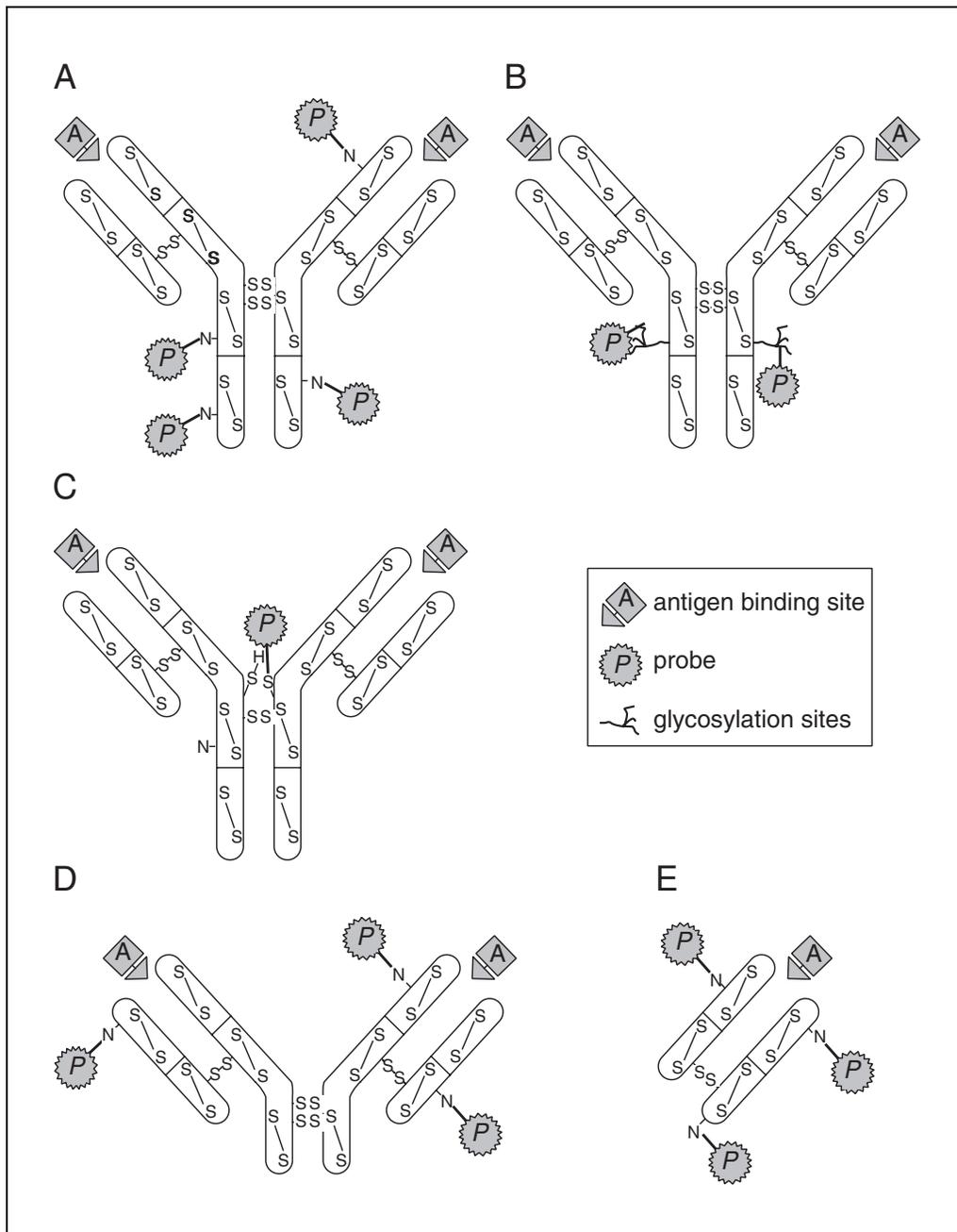


Figure 16.5.1 Schematic representation of possible modes of conjugation of antibodies or their fragments to probes. An antibody or Fab fragment is shown conjugated to one or more probe molecules. The probe symbol indicates a fluorophore, biotin, or an enzyme. **(A)** An antibody conjugated to a probe at several amine groups; **(B)** antibody conjugated at carbohydrate groups in the Fc regions; **(C)** antibody conjugated at thiol group in hinge region; **(D)** Fab fragment conjugated at amine groups; **(E)** $F(ab')_2$ fragment conjugated at amine groups. The molecular weight of intact IgG molecules (with all four subunits) is 140 to 160 kDa; where heavy chains are 50 to 75 kDa, and light chains are each ~25 kDa (A-C). Papain digestion of intact IgG yields Fab fragments of ~50 kDa, consisting of one light chain and a slightly larger fragment of the heavy chain, still attached by a disulfide bond (E). Cleavage of intact IgG antibodies with pepsin yields bivalent $F(ab')_2$ fragments of ~105 kDa (D); and subsequent reduction of the disulfides in the hinge region of $F(ab')_2$ yields two Fab' fragments (not shown), each ~53 kDa.

Table 16.5.1 TLC Solvents and Suggested Incubation Molar Ratios of Probe to Antibody for Several Commonly Used Fluorophores^a

Fluorophore	MR (Ab at 1-3 mg/ml)	MR (Ab at 4-10 mg/ml)	MW (Da)	Probe (mg/ml)	TLC solvent
Alexa Fluor 350	15-25	10-15	410	10	H ₂ O:AcCN :: 20:80
Alexa Fluor 430	15-20	8-10	623	10	H ₂ O:AcCN :: 20:80
Alexa Fluor 488	20-25	15-20	643	10	H ₂ O:AcCN :: 20:80
Alexa Fluor 532	14-18	10-12	724	10	H ₂ O:AcCN :: 20:80
Alexa Fluor 546	12-15	8-10	1079	10	H ₂ O:AcCN :: 20:80
Alexa Fluor 568	25-30	10-20	792	10	H ₂ O:AcCN :: 20:80
Alexa Fluor 594	20-30	10-20	819	10	H ₂ O:AcCN :: 20:80
BODIPY FL	15-30	10-15	641	10	C:M:A :: 70:25:5
Cascade Blue	15-20	10-15	607	10	D:H ₂ O:P:NH ₄ :: 50:16:19:15
Cy3 Bis SE	15-28	5-15	944	10	C:M:A :: 70:25:5
Cy5 Mono SE	10-20	5-10	792	10	C:M:A :: 70:25:5
Eosin	50-70	40-60	705	10	C:M:A :: 70:25:5
FITC	40-60	30-60	389	10	C:M:A :: 70:25:5
Fluorescein-EX	20-25	15-20	590	10	C:M:A :: 70:25:5
Marina Blue	8-12	6-10	367	10	C:M:A :: 70:25:5
Oregon Green 488	15-20	10-15	509	10	C:M:A :: 70:25:5
Oregon Green 514	15-20	10-15	609	10	C:M:A :: 70:25:5
Rhodamine Red-X	8-10	5-8	771	5	C:M :: 85:15
Rhodol Green	10-15	5-10	472	10	C:M:A :: 70:25:5
Tetramethyl-rhodamine (TMR)	15-20	8-10	528	10	C:M:A :: 70:25:5
Texas Red-X	5-8	5-8	817	5	C:M:A :: 70:25:5
Biotin					
Biotin-XX	20-25	10-20	568	10	

^aMR = Incubation molar ratio of fluorophore to antibody, when antibody is at 1 to 3 mg/ml (column 2) or at 4-10 mg/ml (column 3); SE = succinimidyl ester. Solvents: C = chloroform; M = methanol; A = acetic acid; D = dioxane; P = isopropyl alcohol; NH₄ = ammonia; AcCN = acetonitrile.

10 mM 4'-hydroxyazobenzene-2-carboxylic acid (HABA) in 10 mM NaOH (for biotinylation)
 Assay buffer: 50 mM sodium phosphate/150 mM NaCl, pH 6.0 (for biotinylation)
 0.5 mg/ml avidin in assay buffer (for biotinylation)
 0.25 mM biotin in assay buffer (for biotinylation)
 Thin layer chromatography (TLC) silica gel/aluminum plates (EM Science; optional)
 TLC chamber (optional)
 Dialysis tubing
 Chromatography column of appropriate size (see step 7)
 Ultrafrel centrifugal filter devices, Biomax-50K or-100K (Amicon/Millipore; optional)
 Additional reagents and equipment for size exclusion chromatography (*UNIT 5.5*) and dialysis (*APPENDIX 3C*)

Prepare the antibody for conjugation

1. Dissolve the antibody, if lyophilized, at 5 to 10 mg/ml or as concentrated as possible, in 0.1 M sodium bicarbonate (obtained by 10-fold dilution of a 1 M stock). If conjugating with isothiocyanates or sulfonyl chlorides of fluorophores, adjust the pH

of the bicarbonate solution to 9.0. If the antibody to be conjugated is already in solution in 10 to 20 mM phosphate, 0.15 M NaCl (PBS) to obtain the appropriate pH by simply adding 1/10 of the volume of 1 M bicarbonate, with pH unmodified, or pH 9.0 as needed. If the antibody is in a buffer containing Tris, glycine or other amines, dialyze the antibody against PBS or 0.1 M bicarbonate to “exhaustion,” because the probes will react with any available free amine.

There is no need to modify the pH when reacting with succinimidyl esters.

Prepare probe solution

2. Calculate the volume of 10 mg/ml solution of probe (fluorophore or biotin) required to react with the antibody to be labeled, as follows:

$$\text{ml of 10 mg/ml probe} = \{[(\text{mg of Ab}) / (10 \text{ mg/ml of probe})] / (\text{MW of Ab})\} \times (\text{MR} \times \text{MW of probe})$$

where Ab = antibody; MW = molecular weight; and MR = incubation molar ratio of probe to antibody.

For example, for labeling 5 mg of antibody with a 1:10 incubation molar ratio of carboxytetramethylrhodamine succinimidyl ester (TAMRA, SE; MW 430.5):

$$\text{volume of TAMRA, SE at 10 mg/ml} = [(5/10)/(145,000)] \times (10 \times 430.5) = 0.0148 \text{ ml.}$$

3. Immediately prior to starting the reaction, prepare a 10 mg/ml solution of the probe by weighing an amount of probe appropriate for the precision of the balance, for example, 3 mg, and dissolve it in 0.3 ml of DMF or DMSO.

Sulfonyl chlorides, such as Texas Red sulfonyl chloride and Lissamine rhodamine B sulfonyl chloride, should be dissolved in DMF, because they react with DMSO.

4. Briefly, vortex or sonicate the reactive probe until it dissolves completely.

In general, reactive probes are very sensitive to hydrolysis; thus, solutions of any reactive probe should not be stored. However, in some cases, the loss of reactivity during storage at -20°C may be within an acceptable range.

Label antibody

5. While stirring, slowly add the probe solution to the antibody solution, in the amounts determined in step 2. Mix thoroughly.
6. Incubate the reaction mixture 60 to 90 min at room temperature with gentle stirring.
7. (Optional) Stop the reaction by adding 1/10 vol of 1.5 M hydroxylamine hydrochloride that has been adjusted to pH 8.0 with NaOH and incubating an additional 20 to 30 min.

Treatment with hydroxylamine is potentially useful for removing the dye or biotin from unstable conjugates with hydroxyl-containing amino acids (Wong, 1991).

Purify conjugated antibody

8. Purify the antibody from unreacted dye or biotin by size-exclusion chromatography (UNIT 5.5) using PBS or reaction buffer.

The size of the column should be selected based on the amount and concentration of the antibody. A 10×400 -mm column is adequate for 5 to 10 mg of antibody at 5 to 10 mg/ml, while a 5×200 - to 250 -mm column can be used for 1 to 5 mg of antibody at ≥ 2 or more mg/ml. For dyes that are relatively hydrophobic, such as rhodamines or Texas Red, it is very useful to layer a small amount of ToyoPearl (a 2- to 3-cm layer) over the matrix used in the column, to help retain the unreacted dye.

If the antibody concentration is <2 mg/ml, purify the conjugate from excess probe by dialysis (APPENDIX 3C) to “exhaustion,” i.e., until no free probe is detectable by TLC analysis (see step 9). Although it is more time consuming, dialysis is a very easy method to purify small, diluted amounts of antibody conjugates, avoiding the further dilution inevitable with size-exclusion chromatography performed with free-standing columns.

Alternatively, the solution of the antibody conjugate can be concentrated using a centrifugal spin filter device, with a mwco of 30 to 50 kDa. For amounts of antibody <1 mg, at the concentration of ≥ 1 mg/ml, separation from unreacted probe can be achieved by centrifuging the antibody, in volumes of ≤ 0.2 ml, on disposable spin columns, following the manufacturer’s directions. Spin columns do not cause appreciable dilution of the sample, and they offer a rapid method to obtain small amounts of purified conjugates. To avoid denaturation, dilute solutions of antibody conjugates (<1 mg/ml) should be stabilized by adding bovine serum albumin (BSA) or gelatin to a final concentration of 1 mg/ml.

9. Identify the fractions from the fluorophore-antibody column chromatography.

For dye conjugates, the antibody conjugate is the first colored fraction to elute from the column, while the unreacted fluorophore is retarded. A clear band should be visible between the conjugate and the unreacted probe. Depending on the type of fluorophore and the type of size-exclusion matrix used, the unreacted dye could appear as a single band or multiple bands, because the succinimidyl ester might be only partially hydrolyzed and/or the dye might consist of isomers. Neither the band of unreacted biotin on the top of the column nor the biotin-antibody conjugate in the eluate from a spin column can be seen directly. Consequently, the biotin conjugate eluting from the column must be identified by measuring the A_{280} .

Analyze conjugates for purity

10. For fluorescent antibodies only: Analyze the purity of the eluted antibody conjugate by TLC. Spot 1 to 2 μ l of sample on a small strip ($\sim 1.5 \times \sim 10$ cm) of a silica gel thin layer chromatography sheet and run it in a small, saturated chamber in the appropriate solvent (see Table 16.5.1), in parallel with the dye as a control. Remove the strip when the solvent front reaches $\sim 3/4$ of the strip height, and let it dry.

The antibody-conjugate will remain at the origin, while the contaminating unreacted dye will migrate towards the solvent front. The spots can be seen when excited by the long wavelength light of a hand-held UV lamp. If unreacted dye is present, the conjugate can be further purified by dialysis or by spinning it on a new spin column, according to the conjugate volume. This method is not applicable to antibody-biotin conjugates.

Determine the degree of antibody labeling by the probe

For antibody-fluorophore conjugates

- 11a. Measure the absorbance of the conjugate at the wavelength at which the dye absorbance is maximal (A_{Dye}) and at 280 nm (A_{280}). Then calculate the dye-to-antibody ratio using the following equation:

$$(A_{Dye}/\epsilon_{Dye}^M) \div [(A_{280} - A_{Dye} \times CF)/\epsilon_{A280}^M] = (\text{mol of dye/mol of antibody})$$

where A_{Dye} = dye absorbance at peak wavelength; $\epsilon_{A280}^M = 203,000 \text{ cm}^{-1}\text{M}^{-1}$, the approximate molar extinction coefficient of the antibody (IgG) at 280 nm; ϵ_{Dye}^M = molar extinction coefficient of the dye at the same wavelength as A_{Dye} . CF is a correction factor equal to $A_{280}(\text{for dye})/A_{Dye}$.

Note that, while this formulation applies to fluorophore conjugates with intact IgG molecules, it can approximately apply as well to conjugates with antibody fragments, Fab, Fab’, and $F(ab')_2$ (see Fig. 16.5.1), when used with the ϵ_{A280}^M appropriate for the antibody fragment. In this case, the ϵ_{A280}^M of the antibody fragment can be calculated from the measured absorbance of antibody solution at 1.0 mg/ml.

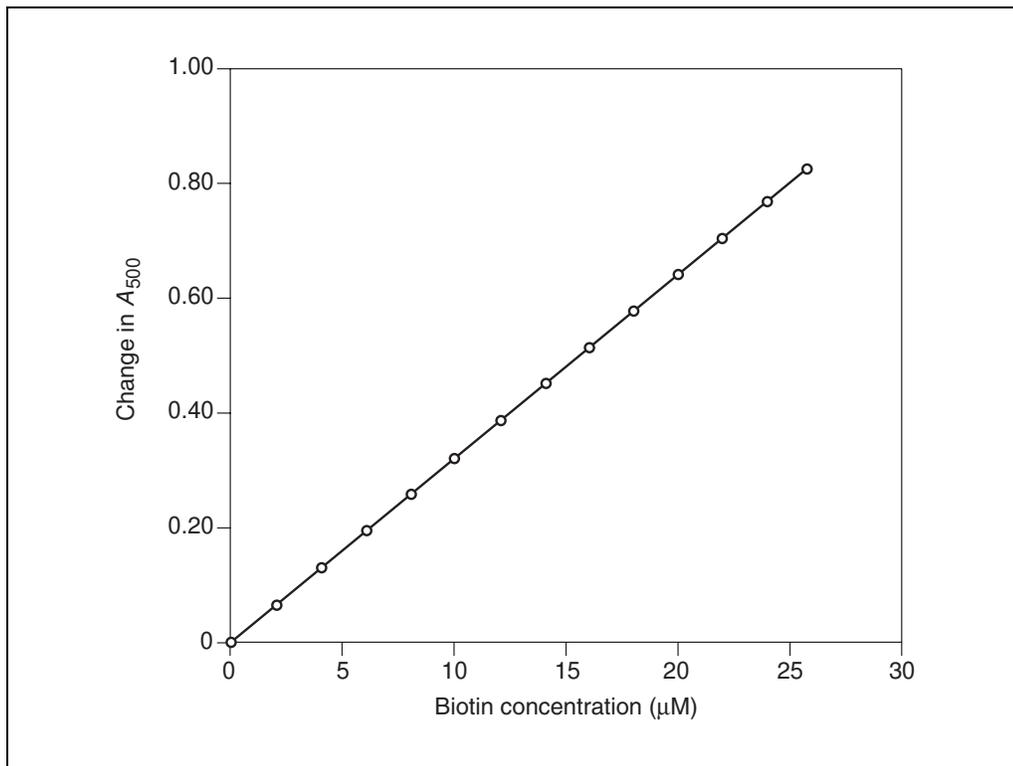


Figure 16.5.2 Standard curve of biotin concentration versus ΔA_{500} . The standard curve for biotin assay with avidin-HABA reagent relates measured difference in absorption at 500 nm to biotin concentration.

For antibody-biotin conjugates

The normal degree of biotinylation of antibodies can be determined by taking advantage of the high affinity of biotin for avidin. The dye, HABA, interacts with avidin, yielding a complex with an absorption maximum at 500 nm. Because of its higher affinity, biotin will displace HABA, decreasing the absorbance at 500 nm (A_{500}) by an amount proportional to the amount of biotin present in the assay. This relation can be quantified in a standard curve.

- 11b. Prepare the standard curve by adding 0.25 ml of HABA to 10 ml of the 0.5 mg/ml avidin solution. Incubate 10 min at room temperature.
- 12b. Distribute 0.9 ml of the avidin/HABA solution into each of seven tubes. Add 0.1 ml buffer, pH 6.0, to one tube (reference) and 0.005 to 0.1 ml biotin solution to the other six tubes. Bring the final volume to 1.0 ml (when necessary) with assay buffer, mix well and incubate 10 min at room temperature.
- 13b. Measure the A_{500} for each concentration point, using assay buffer as a blank, and subtract it from the value obtained from the reference absorption.
- 14b. Plot a standard curve of the nanomoles of biotin versus the decrease in A_{500} .

An example of such a standard curve is shown in Figure 16.5.2.

- 15b. To measure the degree of biotinylation of the sample, add an aliquot of biotinylated antibody of known concentration to 0.9 ml avidin-HABA complex. For example, add 0.05 to 0.1 ml of biotinylated antibody at 1 mg/ml to 0.9 ml of avidin-HABA mixture. Bring the volume to 1.0 ml with assay buffer (when necessary), incubate for 10 min, and measure the decrease in A_{500} .

- 16b. Using the standard curve, determine the nanomoles of biotin corresponding to the observed change in A_{500} .

The ratio between nanomoles of biotin used to displace HABA and nanomoles of antibody represents the degree of biotinylation. For example, the calculation for an IgG conjugate is:

$$(15 \text{ nmol biotin} \times 145,000 \text{ g/mol}) \div (4.35 \text{ mg/ml antibody} \times 0.1 \text{ ml}) = 5 \text{ mol of biotin per mol of antibody}$$

where 145,000 is the MW of the IgG antibody, and 0.1 ml is the volume of 1 mg/ml of biotinylated antibody sample.

CONJUGATION OF FLUOROPHORES OR BIOTIN WITH F(ab')₂ OR Fab ANTIBODY FRAGMENTS

**ALTERNATE
PROTOCOL 1**

As described in *UNIT 16.4* of this series, antibody fragments that retain antibody binding activity can be generated by selective enzymatic digestion of intact antibodies. Conjugates made with antibody fragments may offer enhanced signals because their smaller size can permit penetration and reaction with the more recessed antigen sites or more extensive binding to closely located binding sites (see Critical Parameters for helpful notes).

Alternative conjugation procedure for fluorophores or biotin. In cases where conjugation with fluorophores or biotin at lysines appears to interfere with antibody binding, labeling can be performed with biotin hydrazides or fluorophore hydrazides at the carbohydrate prosthetic group, located in the Fc portion of the molecule (see Fig. 16.5.1), as described in the protocol for conjugation of antibodies with enzymes (see Basic Protocol 2). The hydrazide of the chosen fluorophore or biotin should be used in place of the enzyme, and purification of the conjugate performed as described (see Basic Protocol 2). As for the succinimidyl ester forms, biotin hydrazides are available with one (X) or two (XX) aminohexanoic acid spacers.

METHODS TO ESTIMATE ANTIBODY CONCENTRATION

**SUPPORT
PROTOCOL**

The antibody concentration can be estimated by three different methods:

Correct the A_{280} of the conjugate for the A_{280} due to the dye, using the correction factor of the specific dye given in Table 16.5.2 and the value, $\epsilon_{A_{280}}^M = 203,000 \text{ cm}^{-1} \text{ M}^{-1}$, for the IgG antibody. For example, conjugation of a goat anti-guinea pig IgG antibody with Alexa Fluor 488 succinimidyl ester yielded the following data, after 1:10 dilution: $A_{488} = 0.533$; $A_{280} = 0.314$; $\epsilon_{\text{Dye}}^M = 71,000 \text{ cm}^{-1} \text{ M}^{-1}$ (Table 16.5.2). In this case, the degree of labeling is

$$0.533/71,000 \text{ cm}^{-1} \text{ M}^{-1} \div [(0.314 - 0.533 \times 0.11)/203,000 \text{ cm}^{-1} \text{ M}^{-1}] = 5.96 \text{ moles of dye per mole of antibody}$$

where 0.11 is the CF for the Alexa Fluor 488 dye (from Table 16.5.2).

Estimate the protein concentration from the initial amount, assuming a loss of 15% to 20%, or more if some precipitation occurs during purification.

Use a protein assay method (*APPENDIX 3B*) to measure the concentration of the conjugate. This can be problematic, however, because most protein assays suffer from interference by fluorophores.

**Antibodies as Cell
Biological Tools**

16.5.7

Table 16.5.2 Optical Properties of Commonly Used Fluorophores for Antibody Labeling

Dye	Abs/Em ^a	ϵ^{Mb}	CF ^c	Notes
Alexa Fluor 350	347 nm/442 nm	19,000	0.19	Blue-fluorescent labeling dye with higher fluorescence per conjugated dye than AMCA.
AMCA-X	350 nm/448 nm	18,000	0.15	Widely used blue-fluorescent labeling dye. Compact structure
Marina Blue	365 nm/460 nm	20,000	0.20	Blue-fluorescent dye. Compact structure. pH-insensitive at pH >6.
Cascade Blue	400 nm 420 nm	28,000	0.65	Resistant to quenching upon protein conjugation. Exhibits better spectral separation from FITC than does AMCA. Water soluble.
Cascade Yellow	402 nm/545 nm	20,000	0.61	Large Stokes shift. Useful for multicolor analysis in combination with blue-fluorescent dyes.
Pacific Blue	405 nm/455 nm	30,000	0.20	Bright blue fluorescence emission. Longer-wavelength alternative to AMCA and Marina Blue dye.
Lucifer yellow	428 nm/536 nm	11,900	0.30	Water soluble. Available as Lucifer yellow iodoacetamide.
Alexa Fluor 430	431 nm/541 nm	16,000	0.28	Large Stokes shift. One of relatively few dyes that absorb between 400 and 450 nm and have appreciable fluorescence beyond 500 nm.
Fluorescein	494 nm/518 nm	68,000	0.30	Widely used green-fluorescent labeling dye (FITC). Absorption overlaps the 488-nm spectral line of the argon-ion laser. Prone to photobleaching. pH-sensitive from pH 5 to 8.
Alexa Fluor 488	495 nm/519 nm	71,000	0.11	Best fluorescein substitute for immunofluorescence and other labeling applications. Exceptionally photostable and pH-insensitive from pH 4 to 10. Produces brighter conjugates than FITC. Ideal for 488-nm excitation.
Oregon Green 488	496 nm/524 nm	70,000	0.12	Fluorescein substitute. pH-insensitive at pH >6.
BODIPY FL	505 nm/513 nm	68,000	0.04	Insensitive to solvent polarity and pH from pH 4 to 8. Narrow spectral bandwidth. Neutral dye. Succinimidyl ester with a cysteic acid spacer is the preferred reactive form for protein conjugation.
Oregon Green 514	511 nm/530 nm	70,000	0.19	Very photostable. pH-insensitive at pH >6. Absorption matched for 514-nm excitation.

*continued***BASIC
PROTOCOL 2****CONJUGATION OF ANTIBODIES WITH ENZYMES**

Conjugation of antibodies with enzymes involves crosslinking of the two proteins in successive procedural steps. The method involves (1) derivatization of the antibody with a bifunctional reagent that introduces reactive maleimide groups into the molecule and (2) derivatization of the enzyme with a thiol group that can form a stable thioether bond with the maleimide group on the antibody. This approach can be tailored to obtain either heteroconjugates consisting of one molecule of antibody and one molecule of enzyme or conjugates consisting of one antibody labeled with multiple enzyme molecules. The most commonly used enzymes are horseradish peroxidase (HRPO), alkaline phosphatase (APase), β -galactosidase (β Gase), and glucose oxidase (GO).

**Antibody
Conjugates for
Cell Biology****16.5.8**

Table 16.5.2 Optical Properties of Commonly Used Fluorophores for Antibody Labeling, continued

Dye	Abs/Em ^a	ϵ^{Mb}	CF ^c	Notes
Eosin	524 nm/544 nm	90,000	0.25	Useful photooxidizer of diaminobenzidine (DAB). Phosphorescent (lifetime ~1 msec.) Useful for phosphorescence anisotropy measurements. Efficient FRET ^d acceptor from fluorescein, BODIPY FL dye, dansyl and coumarin.
Alexa Fluor 532	531 nm/554 nm	81,000	0.09	Bright and photostable dye with spectra intermediate between those of fluorescein and tetramethylrhodamine. Water soluble. Excellent protein-labeling dye.
Tetramethyl-rhodamine (TMR)	555 nm/580 nm	65,000	0.30	Widely used orange-fluorescent labeling dye (TRITC). pH-insensitive. Very photostable. May form nonfluorescent aggregates when attached to proteins.
Cy3	553 nm/570 nm	150,000	0.08	Orange fluorescing, bright and water soluble cyanine dye.
Alexa Fluor 546	556 nm/573 nm	104,000	0.12	Very bright and photostable tetramethylrhodamine (TAMRA or TRITC) and Cy3 dye substitute. Ideal for 546-nm excitation. Water soluble.
Rhodamine Red-X	570 nm/590 nm	120,000	0.17	Rhodamine Red-X succinimidyl ester generally yields higher fluorescence per attached dye than Lissamine rhodamine B sulfonyl chloride and is more stable in water.
Alexa Fluor 568	578 nm/603 nm	91,300	0.46	Bright and photostable Lissamine rhodamine B substitute. Ideal for 568-nm laser excitation. Excellent protein-labeling dye. Water soluble.
Alexa Fluor 594	590 nm/617 nm	73,000	0.56	Bright and photostable Texas Red dye substitute. Ideal for 594-nm laser excitation. Excellent protein-labeling dye. Water soluble.
Texas Red	595 nm/615 nm	80,000	0.20	Good spectral separation from fluorescein. Texas Red-X succinimidyl ester typically yields higher fluorescence per attached dye than Texas Red sulfonyl chloride and is more stable in water.
Cy5	650 nm/667 nm	250,000	0.05	Excitable by 632-nm line of the HeNe laser.
LaserPro	807 nm/812 nm	229,000	0.12	Relatively low quantum yield. Emission is not visible to the eye.

^aAbs/Em are the absorbance and emission maxima of the dye.

^b ϵ^M is the molar extinction coefficient of the dye in $\text{cm}^{-1}\text{M}^{-1}$.

^cCF is the percentage correction factor determined by the following formula: $\text{CF} = A_{280} / \text{dye}/A_{\text{max}}$ for dye.

^dFRET, fluorescence resonance energy transfer.

It should be emphasized that β Gase contains a large number of free thiols (8 to 12 or more, according to the different preparations of the enzyme) and need not be modified; rather, it can be reacted directly in its native form with the maleimide derivatives of antibodies.

Some protein-to-protein crosslinking kits, adaptable to the conjugation of antibodies with enzymes, are commercially available (Molecular Probes, Pierce, Prozyme).

Materials

HRPO, APase, GO, β Gase (Boehringer Mannheim, Sigma)
Reaction buffer, pH 7.5 (see recipe)
TEA buffer (see recipe; for APase only)
Succinimidyl 3-(2-pyridyldithio)propionate (SPDP, MW 312)
Anhydrous dimethylsulfoxide (DMSO) or dimethylformamide (DMF)
BIO-GEL P-30 (Bio-Rad) or Cellufine GH-25 (Amicon/Millipore)
Dithiothreitol (DTT)
Antibody to label
Succinimidyl *trans*-4-(*N*-maleimidylmethyl)cyclohexane-1-carboxylate (SMCC, MW 334)
Tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP, MW 287; Molecular Probes, Pierce Chemical)
50 mM *N*-ethylmaleimide (MW 125.13)
Sephacryl S-200 for HRPO conjugates (Amersham Pharmacia Biotech or Sigma Chemical)
BIO-GEL A-0.5m for APase conjugates (Bio-Rad Laboratories)
BIO-GEL A-1.5m for β Gase conjugates (Bio-Rad Laboratories)
Chromatography column of appropriate size (see step 4)
Ultrafrel centrifugal filter devices, Biomax-50K or-100K (Amicon/Millipore)
Additional reagents and equipment for dialysis (*APPENDIX 3C*) and size exclusion chromatography (*UNIT 5.5*)

Prepare the thiolated enzyme

1. Dissolve or dialyze HRPO (or GO) in reaction buffer at a concentration of 10 mg/ml. Dialyze APase in TEA buffer.
2. Weigh 3 mg of SPDP, and dissolve in 0.3 ml DMF to obtain a 10 mg/ml solution. Prepare this solution fresh immediately before using.
- 3a. *HRPO*: To 10 mg of HRPO (0.25 μ mol) in 1 ml reaction buffer, add, while stirring, 40 μ l of SPDP solution, to obtain a molar ratio of reagent to HRPO of 5. Incubate 1 hr at room temperature.
- 3b. *APase*: To 10 mg of APase (0.07 μ mol) in 1 ml of pH 7.6 TEA buffer, add 10 μ l of the SPDP solution and incubate 1 hr at room temperature.
- 3c. *GO*: To 10 mg of GO (0.063 μ mol) in 1 ml reaction buffer, add 10 μ l of the SPDP solution and incubate 1 hr at room temperature.

APase and GO have many more amines available for thiolation compared to HRPO, which has only 4 to 6 amines (Welinder, 1979). Thus the molar ratio of reagent to enzyme is ~5, notwithstanding the disparity in molecular weight between the two proteins.

4. Purify the thiolated enzyme by molecular exclusion column chromatography, using BIO-GEL P-30 or Cellufine GH-25, in reaction buffer for HRPO or TEA buffer, pH 7.6, for APase.

A column bed size of ~10 \times 400-mm is required for 10 to 20 mg enzyme.

Determine the degree of thiolation

5. Prepare a 100 mM solution of DTT by dissolving 7.7 mg of DTT in 0.5 ml of distilled water.
6. Transfer 1 ml of 0.5 to 1 mg/ml enzyme solution to a spectrophotometer cuvet. Record the A_{280} and A_{343} of the solution.

7. Add 50 μl of DTT solution. Mix well, incubate 3 to 5 min, and record the A_{280} and A_{343} again.
8. Using an extinction coefficient of $8080 \text{ cm}^{-1}\text{M}^{-1}$ at 343 nm (Carlsson et al., 1978), calculate the amount of pyridine-2-thione liberated during the reduction.

The amount of this product is equivalent to the number of thiols introduced in the protein.

9. To determine the enzyme concentration, correct the A_{280} of the enzyme solution for the absorbance contributed by 2-pyridyldithio propionate at this wavelength, according to the following equation:

$$A_{280} \text{ of enzyme} = A_{280} \text{ of conjugate} - (\Delta A_{343} \times 5100 \text{ cm}^{-1}\text{M}^{-1} / 8080 \text{ cm}^{-1}\text{M}^{-1})$$

where $\Delta A_{343} \times 5100 \text{ cm}^{-1}\text{M}^{-1} / 8080 \text{ cm}^{-1}\text{M}^{-1}$ reflects the amount of pyridine-2-thione generated during the reaction.

For HRPO, the enzyme concentration can be determined at 403 nm using the extinction coefficient shown in Table 16.5.2, with no correction. The number of thiols per mole of enzyme can be calculated using the following equation:

$$\text{moles of -SH groups per mole of enzyme} = (\Delta A_{343} / 8080 \text{ cm}^{-1}\text{M}^{-1}) \div \{ [A_{280} - (\Delta A_{343} \times 5100 \text{ cm}^{-1}\text{M}^{-1} / 8080 \text{ cm}^{-1}\text{M}^{-1})] / \epsilon_{\text{enzyme}}^M \}$$

or, for HRPO:

$$(\Delta A_{343} / 8080 \text{ cm}^{-1}\text{M}^{-1}) \div (A_{403} / \epsilon_{\text{enzyme}}^M).$$

10. From the preceding equations, determine the average number of moles of antibody that can be reacted with each mole of enzyme.

To obtain a 1:1 enzyme-antibody conjugate, the enzyme should be derivatized with 1.2 to 1.5 pyridyldithiol residues per mole. Thiolated enzymes remain stable for a few weeks when stored refrigerated in presence of 2 mM azide or, for HRPO, 0.01% (w/v) thimerosal, because azide inhibits the activity of this enzyme.

Prepare the antibody-maleimide derivative

11. Dissolve or dialyze the antibody in reaction buffer.

The antibody should be as concentrated as possible to increase both the yield and the efficiency of the conjugation reaction. Ideally, the concentration should be 5 to 10 mg/ml; however, modifications of the labeling procedures can accommodate lower concentrations, especially when labeling small amounts of antibody (0.1 to 1.0 mg). Thus, the antibody concentration may be 2 to 10 mg/ml.

12. Weigh 3 to 5 mg of SMCC and immediately before use dissolve it in DMSO or DMF to obtain a 10 mg/ml solution. Vortex or sonicate to assure complete dissolution.
13. While stirring, add 15 μl of SMCC solution to each 5 mg of antibody to obtain a molar ratio of reagent to antibody of ~ 15 .

The amount of SMCC should be tailored to the concentration of the antibody. For example: 5 mg of antibody at the concentration of 2 mg/ml would require 15 to 17 μl of the above SMCC solution, while antibody at 10 mg/ml would yield a better conjugate when using a molar ratio of SMCC of 10.

14. Incubate 1 hr at room temperature with gentle stirring.
15. Dialyze 24 hr in the cold with four changes of 1 liter or more reaction buffer.

Deprotect the enzyme thiol groups

NOTE: It is essential that this procedure be carried out simultaneously to the labeling of the antibody with maleimide groups, because both the deprotected thiols of the enzyme and the maleimide groups of the antibody are unstable in water and are best used within a day from their preparation.

Deprotect with DTT

- 16a. Add 8.0 mg of DTT to each milliliter of the enzyme solution. Incubate 15 to 20 min at room temperature.
- 17a. Dialyze the thiolated enzyme 24 hr in the cold against 2 liters reaction buffer, with at least four changes of buffer to eliminate as much of the DTT as possible.

Deprotect with TCEP

- 16b. Calculate the volume of 1 mg/ml TCEP stock solution needed to obtain an incubation molar ratio of 5 moles of TCEP per mole of enzyme

$$\mu\text{l of TCEP} = (\text{mg of enzyme}) / (1 \text{ mg/ml TCEP}) \times 286.7 / (\text{MW of enzyme}) \times 5 \times 1000$$

where 286.7 is the MW of TCEP, 5 is the incubation molar ratio, and 1000 is a unit conversion factor. Weigh out 3 to 5 mg of TCEP powder, and dissolve it in 3 to 5 ml reaction buffer to make a 1 mg/ml solution of TCEP.

Alternatively, TCEP can be used in place of DTT to deprotect sulfhydryl groups (Getz et al., 1999). TCEP does not react with maleimides. Thus, one need not eliminate excess TCEP from the reaction mixture, avoiding the need to dialyze to exhaustion. TCEP is very reactive and should be used at an incubation molar ratio of 5 TCEP per mole of enzyme.

- 17b. Add the appropriate amount of TCEP stock solution to the thiolated enzyme solution, and mix well. Incubate the mixture 10 to 15 min at room temperature.

Synthesize the antibody-enzyme conjugate

- 18a. *For HRPO, APase, and GO:* Transfer the antibody and the HRPO or APase from the dialysis tubes into two separate test tubes. Slowly drip the antibody solution into the enzyme solution with stirring. Incubate 1 hr at room temperature and overnight at 4°C.
- 18b. *For β Gase:* For each 5 mg of antibody dissolve 15 mg of enzyme in 1.5 ml reaction buffer and proceed as in step 18a.
19. Stop the reaction for HRPO, GO, and APase conjugates with the addition of *N*-ethylmaleimide (NEM) at a final concentration of 50 μ M, by diluting the NEM solution 1:1000 in the conjugate reaction mixture. Incubate 30 min at room temperature.

The conjugate is now ready to be purified by column chromatography, immediately or after overnight storage at 4°C.

The reaction of β Gase cannot be stopped because of the large number of -SH groups of this enzyme.

Purify the antibody-enzyme conjugate

20. Concentrate the antibody-enzyme conjugate in a centrifugal filter device at the speed recommended by the manufacturer.

21. Pack appropriate size columns (e.g., 1 × 60-cm for 10 mg of protein in the conjugate reaction mixture) with the appropriate matrix.

For purification of HRPO-antibody conjugate use Sephacryl S-200 in reaction buffer. For GO and APase-antibody conjugates use Bio-Gel A-0.5m in 3 M NaCl/30 mM TEA/1 mM MgCl₂, pH 7.6; for BGase Bio-Gel A-1.5m in reaction buffer, with the addition of 3 mM MgCl₂ and 2 mM 2-mercaptoethanol.

22. Load the conjugate onto the column, elute with the buffer used to pack the column, collecting 1-ml fractions.

The first peak to elute, measurable by absorbance at 280 nm, or 403 nm for HRPO conjugates, corresponds to the conjugate. However, each fraction should be checked for antibody binding and enzymatic activity in an ELISA-type (APPENDIX 3) or other appropriate assay.

An alternative method to purify antibody-enzyme conjugates from the unreacted enzyme relies on the affinity of the Fc portion of the antibody for nickel chelates. An affinity column based on this principle is available from Pierce Chemical. The Fc region complexes with nickel on the column while the unconjugated enzyme passes through. The bound antibody-enzyme conjugate can be eluted by lowering the pH, by increasing the salt concentration, or by simply adding EDTA to the elution buffer. A detailed procedure for using this affinity column accompanies the product. This column cannot be used for F(ab')₂ or Fab fragments, as they are devoid of the Fc region (Hermanson, 1996a).

CONJUGATION AT THE CARBOHYDRATE SITE OF THE ANTIBODY

The sugar constituents of glycoproteins contain vicinal hydroxyl groups that can be oxidized to dialdehydes by treatment with periodic acid or its salts. The dialdehydes formed easily react with amines, including the hydrazide form of fluorophores, the ε-amino group of lysines, and the amino terminus of proteins. The carbohydrate-containing portion of an antibody molecule is located in the Fc region; thus, the periodate oxidation method of conjugating antibodies allows labeling at a specific location, remote from the antigen binding site.

CAUTION: Some monoclonal antibodies are not glycosylated and cannot, therefore, be conjugated by this procedure.

Materials

Antibody to label in solution (see Basic Protocol 1, step 1)
0.1 M acetate buffer, pH 6.0
20 mM sodium metaperiodate in acetate buffer, pH 6.0, ice cold
Reaction buffer, pH 7.5 (see recipe)
100 mM aqueous sodium cyanoborohydride, freshly prepared
HRPO, APase, βGase, GO
Column chromatography matrices (see Basic Protocol 2)

NOTE: Protect the sample from light during the entire procedure.

1. Dialyze the antibody at 2 to 10 mg/ml in 0.1 M acetate buffer, pH 6.0, at 4°C.
2. Keeping the dialyzed antibody on ice, add an equal volume of ice-cold metaperiodate solution. Incubate 2 hr in the dark at 4°C.
3. Purify the oxidized antibody: If the antibody is dilute (<1 mg/ml), dialyze it against the same buffer, protecting it from light. If the antibody is concentrated, purify it on a desalting column (Bio-Gel P-30 or equivalent) in free-standing or spin-column form (Basic Protocol 1, step 8) to remove the iodate that is produced during the oxidation.

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4. Prepare a solution of the enzyme of choice in reaction buffer in the amount needed to obtain an incubation molar ratio of enzyme to antibody of 2 to 10, according to the type of conjugate desired.

For instance, if a 1:1 ratio of probe to antibody is desired, use an incubation molar ratio of 2; otherwise, the higher the desired enzyme-antibody ratio, the higher will be the necessary incubation molar ratio.

5. Slowly add the antibody to the enzyme solution with stirring and incubate the mixture for 2 hr at room temperature with gentle stirring.
6. (Optional) To stabilize the imine bond into a substituted hydrazide, add sodium cyanoborohydride to a final concentration of 10 mM, adding 1/10 vol of a 100 mM stock solution of sodium cyanoborohydride in distilled water. Incubate for 2 hr at 4°C.

Some researchers consider the imine bond between the sugars on the antibody and the amines on the enzymes to be relatively unstable.

7. Purify the conjugate by any of the chromatography methods (see Basic Protocol 2, step 21).

ALTERNATE PROTOCOL 3

CONJUGATION AT THE CARBOHYDRATE SITE OF THE ENZYME

Since HRPO and GO are glycoproteins, Alternate Protocol 2 can be modified by oxidizing the sugar on the enzyme and, following removal of excess reagent, adding it to the native antibody solution. The steps, molar ratios, and times suggested in Alternate Protocol 2 can be applied to this “reversed” version of the procedure. GO is a flavoenzyme consisting of two 80-kDa subunits that are rich in carbohydrates and joined by a disulphide bond (O'Malley and Weaver, 1972). The conjugation method of choice for GO involves oxidizing the enzyme with periodate at its carbohydrate site. The antibody can then be conjugated to GO through one or more of its amino groups (Rodwell et al., 1986; Husain and Bieniarz, 1994; Hage et al., 1997).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Hydroxylamine, 1.5 M, pH 8.0

4.2 g hydroxylamine, hydrochloride
25 ml H₂O
Adjust the pH to 8.0 with 5 M NaOH
Add H₂O to 40 ml
Prepare fresh

Phosphate-buffered saline (PBS)

1.1 g K₂HPO₄
0.43 g KH₂PO₄
9 g NaCl
900 ml H₂O
Adjust the pH to 7.2 or 7.4, if necessary, with 1 M sodium bicarbonate (see recipe)
Add H₂O to 1 liter
Store 1 week at 4°C

The final concentrations are 0.01 M potassium phosphate, 0.15 M NaCl, pH 7.2.

Reaction buffer

11.5 g Na₂HPO₄
2.6 g NaH₂PO₄
5.8 g NaCl
900 ml H₂O
Adjust the pH to 7.5 with 5 M NaOH
Add H₂O to 1 liter
Store 2 weeks at 4°C

For labeling IgM, adjust the pH to 7.2.

Sodium bicarbonate, 1.0 M

8.3 g NaHCO₃
100 ml H₂O
Store 2 weeks at 4°C

The resulting pH is generally 8.3 to 8.5

This bicarbonate solution is used to raise the pH of PBS.

TEA buffer

For each liter of buffer, weigh 175 g of NaCl and dissolve it in 750 ml H₂O. Add 4.08 ml of TEA (98%, ρ = 1.12 g/ml). Prepare a stock solution of 0.5 M MgCl₂ and 0.05 M ZnCl₂ by weighing 10.16 g of MgCl₂ and 0.68 g of ZnCl₂ and dissolving them in distilled water to obtain a final volume of 100 ml. Add 2.0 ml of this stock to the NaCl-TEA solution. Adjust the pH to 7.6 with 3 M HCl, and bring the volume to 1.0 liter with H₂O. Store at 4°C. For prolonged storage (>1 week), add azide to 2 mM final concentration.

This buffer is used for APase only.

Final concentrations are 3.0 M NaCl, 30 mM TEA, 1.0 mM MgCl₂, 1.0 mM ZnCl₂, pH 7.6.

COMMENTARY

Background Information

Antibodies

The use of antibodies as probes has become widespread in the biosciences. In immunocytochemical applications, the use of labeled secondary antibodies is limited by the number of targets to be detected by different antibodies. This is due to the fact that secondary antibodies, even when purified by immunoadsorption against related species, nonetheless exhibit significant residual cross-reactivity when more than two antibodies are used to stain multiple features of the same sample. Thus, to detect multiple structures or functions simultaneously, direct labeling of primary antibodies with fluorophores, biotin, or enzymes is essential. Biotinylation of antibodies allows detection with fluorescently labeled avidin (or analogous molecules), generally without interference from the secondary antibody or antibodies required in the experimental procedure. Investigators new to the field might refer for general information on the theory of fluorescence and applications of fluorescent probes to the in-

troductory to the topic by Johnson (1996; see also *UNIT 4.2*) and for avidin-biotin technology to the review by Wilchek and Bayer (1990).

For procedures employing fluorescent labeling of primary antibodies, it is important to select probes with the highest quantum yield, because signals from these antibodies lack the amplification obtained with the secondary antibodies. Signal amplification can be achieved by conjugating antibodies with enzymes that use fluorogenic or colorimetric substrates. These substrates should be of the precipitating type for histological preparations or soluble for ELISA and other microplate assays.

Very useful antibody labeling kits, containing detailed instructions, premeasured reactive fluorophores or biotin are now available from a few commercial sources (Amersham Pharmacia Biotech, Molecular Probes, Pierce). These kits generally allow conjugations of 0.2 to 10 mg of an antibody. They are ideal for researchers who need to perform labeling procedures only occasionally or do not have the necessary equipment (e.g., separation columns and solvent for the la-

being reagent) readily available. They also represent a good starting step for researchers who want to familiarize themselves with the labeling procedure before acquiring larger amounts of the materials and reagents needed for the conjugation.

Fluorophores

The most commonly used amine-labeling reagents belong to three classes of acylating compounds:

1. *Isothiocyanates*. The isothiocyanate derivatives of fluorophores are relatively stable in aqueous solution. They react with amines at pH 9 to 9.5 to yield thiourea bonds. In this class, fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) are the most widely used.

2. *Sulfonylhalides*. The most commonly used probes in this category are the sulfonyl chloride of Texas Red and that of Lissamine rhodamine B. This reactive form of fluorophores is extremely labile in water. Consequently, in conjugation with proteins the reaction rate is strongly counteracted by the hydrolysis rate of the dye, making reproducibility of the reaction difficult. Also sulfonyl chlorides require relatively basic conditions, pH 9.0 to 9.5, to react with amines.

3. *Succinimidyl esters*. Reagents of this form react more selectively with aliphatic amines than the isothiocyanates by forming stable carboxamide bonds at pH 7.5 to 8.5 (Haugland, 1996a). Most fluorophores, including fluorescein, rhodamine, Cy3, Cy5, and the new Alexa Fluor dyes, are available as succinimidyl esters (Haugland, 1999). These are highly preferred because of their greater selectivity for amino groups (Wong, 1991), relatively slow hydrolysis rate (Wong, 1991), and high reactivity at lower pH (Hermanson, 1996b), closer to physiological pH. Because of their still high reactivity at pH 7.2, succinimidyl esters can be used efficiently to label IgM antibodies, which tend to denature when the pH is above 7.4.

The author strongly recommends using fluorescein, tetramethylrhodamine, and Texas Red in their succinimidyl ester forms. In the past, fluorescein and tetramethylrhodamine were available only as isothiocyanates, broadly known in their abbreviated forms as FITC and TRITC, respectively. Texas Red was available only as its sulfonyl chloride, which has an extremely rapid rate of hydrolysis in buffer. The new, succinimidyl ester forms of these dyes are preferable, because they will generally yield more reproducible and stable antibody conjugates (Banks and Paquette, 1995; Lefevre et al., 1996).

In determining the degree of antibody labeling in fluorophore conjugates, one should note that the values, both of the extinction coefficients and of the correction factors described in Table 16.5.2, are only approximate. These values are not corrected for the change in extinction coefficients that may occur when the dyes are conjugated to proteins. This change in extinction coefficient can vary from protein to protein, and it can vary with the number of dyes reacted with the protein, due to interference between dye molecules (Ravdin and Axelrod, 1977). Thus, determination of the degree of labeling is relative and approximate. Nonetheless, it is invaluable when comparing different preparations of the same antibody conjugate and for determining the relative optimal number of moles of fluorophores per mole of antibody for particular biological applications.

Phycobiliproteins

This unit does not include protocols for conjugation of antibodies with phycobiliproteins—large, highly fluorescent pigments that are isolated from the photosynthetic apparatus of algae. The conjugation procedure of phycobiliprotein to antibodies is very similar to Basic Protocol 2. Molecular Probes offers a protocol for conjugation with phycobiliproteins, and details are published (Haugland, 1996b). A kit that greatly facilitates phycobiliprotein conjugation is available from Prozyme, although it does not lend itself to optimizing the procedure. The Protein-to-Protein Crosslinking Kit from Molecular Probes could be used for this purpose. However, obtaining the phycobiliprotein of interest, R-phycoerythrin (R-PE) or allophycocyanin (APC), already derivatized with 1 to 2 pyridyldisulfide groups per molecule is strongly recommended. This is more economical than preparing and optimizing the activated protein.

Other fluorophores

Fluorophores with enhanced water solubility and fluorescence output have been developed by introducing sulfonic groups in the rings of their molecule. This procedure also confers negative charges that help to decrease interaction between fluorophores and to minimize the loss of probe fluorescence that generally occurs upon conjugation to biological molecules. The excitation wavelengths for these sulfonated fluorophores span the entire spectrum from excitation in the UV range to the 633-nm excitation of the HeNe laser. They include the Alexa Fluor dyes, prepared by sulfonating coumarins or rhodamines (Panchuk-Voloshina et al., 1999), Cy dyes, prepared by sulfonating carbocyanines (Mujumdar et al.,

1989, 1993, 1996), and Cascade Blue, which is a pyrenetrisulfonic acid derivative (Whitaker et al., 1991). Each of these dyes generally offers enhanced signal-to-noise ratios. Many of these dyes form conjugates that tolerate a higher number of moles of dyes per mole of antibody in comparison with the non-sulfonated ones, while avoiding quenching of the fluorescence or precipitation of the antibody (Panchuk-Voloshina et al., 1999). In rare cases the negative charges on these dyes may cause nonspecific interaction with positive charges of the cells under investigation. In these cases, which unfortunately can be determined only experimentally, the nonsulfonated form of the dye (e.g., TAMRA, instead of Alexa Fluor 546, or Texas Red-X, instead of Alexa Fluor 594) should be used. Fluorescein itself carries two negative charges, and in cases of background problems the neutral BODIPY FL can be tried as an alternative.

Biotin

Biotin is a small, ubiquitous molecule that acts as a coenzyme of carboxylases. Due to its strong affinity for avidin and avidin-like molecules ($K_d = 1.3 \times 10^{-15}$ M at pH 5.0), it is broadly used as a label that can be probed with fluorescent or enzyme-linked avidin or streptavidin. Avidin (MW = 66 kDa) and streptavidin (MW = 60 kDa), both tetrameric proteins with ~33% homology in their amino acids composition, can each bind four molecules of biotin (Green, 1975).

Iminobiotin, a modified biotin with lower affinity for avidin, is occasionally used in place of biotin to prepare conjugates that can be separated by affinity chromatography. The succinimidyl ester of iminobiotin can be used to effect labeling of a tag only transiently because the iminobiotin-avidin complex dissociates at low pH (Hoffman et al., 1980; Orr, 1981). Affinity purification of biotinylated conjugates is also possible with monomeric avidin, which has a lower affinity for biotin than the tetrameric form and is now commercially available (Pierce Chemical). Nitroavidin, another form of monovalent avidin with a lower affinity for biotin, can be prepared by selectively nitrating three of the tyrosines, each located at the active site of avidin or streptavidin subunits, leaving just one site available for biotin binding (Morag et al., 1996). Another commercially available form of reactive biotin is the DNP biocytin succinimidyl ester, which carries the colorimetric properties of dinitrophenyl (DNP) and the avidin-binding properties of biotin (Briggs and Panfili, 1991; Haugland, 1996c). With this reagent, direct spectrophotometric

measurement of the number of moles of biotin per mole of antibody can be achieved by using the molar extinction coefficient of DNP ($15,000 \text{ cm}^{-1}\text{M}^{-1}$ at 364 nm). This reagent (MW = 862 Da) is larger than most common fluorophores and the reactive biotins described above. Thus, more reagent is necessary to obtain an efficient incubation molar ratio of reagent to antibody during the reaction, because larger molecules react more easily with surface groups and have difficulty reaching internal lysines. However, the presence of the DNP group on the conjugate offers a second tag on the target and the conjugate can be further probed with an anti-DNP antibody (Haugland, 1996c).

Enzymes

Antibody-enzyme conjugates are probably the most widely prepared antibody-based reagents because they are broadly used in diagnostic clinical tests. HRPO conjugates represent ~70% to 80% of the antibody-enzyme conjugates in common use, while APase makes up for most of the remainder; β Gase and GO represent ~2%, and they are used primarily in research. The major applications of enzyme-conjugated antibodies lie in immunocytochemical studies of cells or tissue slices and in enzyme-linked immunosorbent assays (ELISA) for quantifying and detecting a wide variety of analytes. The activity of the conjugated enzyme causes the accumulation of measurable enzyme product in the test system, offering amplification of the signal and high assay sensitivity.

It is important to consider the experimental application for which the antibodies are to be conjugated with the enzymes. For cytological or histological studies, conjugates comprised of one enzyme and one antibody molecule are preferable, because, in general, larger conjugates are accompanied by higher background signals. The MW of IgG is ~150 kDa, and IgM is often >850 kDa. Enzymes can also be rather large (e.g., glucose oxidase (GO, MW = 160 kDa), alkaline phosphatase (APase, MW = 140 kDa), β -galactosidase (β Gase, MW = 540 kDa)). Consequently, the mass of the antibody-enzyme conjugate can be considerable, especially when multiple enzyme molecules are bound to one antibody molecule. In contrast, for ELISA-based tests, in which only the total signal is essential, larger enzyme-antibody aggregates often increase the sensitivity of the assay.

The simplest method (and one of the oldest) to prepare enzyme-antibody conjugates involves mixing the two conjugate components and adding a "condensation" reagent, generally glutaralde-

hyde, to crosslink the two proteins at random amines. This method presents problems, however. Although conditions can be determined for obtaining an optimal yield of the enzyme-antibody conjugate, nonetheless, unwanted homopolymers of enzyme or of antibody are invariably obtained. Furthermore, this method is impractical for obtaining antibodies labeled with only one enzyme molecule.

Another commonly used method relies on the reaction of a maleimide-labeled enzyme (such as with SMCC, as described in Basic Protocol 2 for derivatizing the antibody) with an antibody or F(ab')₂ fragment. The maleimide groups interact with sulfhydryl groups in the hinge region after the disulfide bonds have been reduced by 2-mercaptoethylamine, in the presence of EDTA. Optimizing this procedure involves minimizing the disruption of other disulfide bridges within the antibody molecule and at the same time reducing a sufficient fraction of the hinge disulfides to give an optimal yield of conjugate. 2-Mercaptoethylamine is chosen for this reduction because it is more controllable than dithiothreitol. Details for this method can be found in Hermanson (1996c).

The basic approach described in this unit for preparing antibody-enzyme conjugates is based on forming thioether bonds between a maleimide group on the antibody and a free thiol on the enzyme. These two reactive groups are obtained by derivatizing the conjugate components with the heterobifunctional crosslinking reagents, SMCC and SPDP. This approach is "controllable" and easier to optimize than are other methods. Similar methods that involve reaction of the maleimide crosslinking reagent to the enzyme and conjugation of the sulfhydryl crosslinking reagent to the antibody have been also reported. This "reverse" method, however, is recommended only when *S*-acetylthioglycolic acid *N*-hydroxysuccinimide ester (SATA) is used as the thiolating reagent, because iminothiolane yields very unstable free thiol groups, and SPDP entails reduction with DTT or TCEP, which might disrupt the disulfide groups of the antibody. Furthermore, because deprotection of the thiol in SATA requires only exposure to hydroxylamine (Duncan et al., 1983), the antibody molecule is less likely to be altered. However, one should also consider that assaying for the free thiols generated with either iminothiolane or SATA requires a relatively large amount of antibody. In contrast, the release of pyridyl-2-thione from SPDP reduction permits one to promptly determine the number of free thiols obtained per molecule of enzyme by measuring absorption (Carlsson et al., 1978), yielding the precise number of reactive sites. This

offers two advantages: (1) the enzyme can be derivatized with only 1 to 2 sulfhydryl groups in order to obtain mostly heteroconjugates of one enzyme per antibody molecule, and (2) the activity of the enzyme can be disrupted to a lesser degree when only 1 or 2 amines are derivatized.

The protocols presented in this unit for linking enzymes to antibodies can be tailored to the antibody-specific application by modulating the number of reactive crosslinking groups with which the antibody or the enzyme is modified. When free thiols are available, as with β Gase, no chemical modification of the enzyme is necessary. Enzymes derivatized with only 1 to 1.5 thiol and antibodies carrying only a few maleimide groups generally yield heteroconjugates, most of which have one enzyme and one antibody. By introducing a larger number of thiols on the enzyme or a larger number of maleimide groups on the antibody, it is possible to obtain small aggregates consisting of one or more antibody molecule conjugated to two or more enzyme molecules.

Critical Parameters

Preparation of the antibody for conjugation

Before being conjugated to a probe, antibodies should be as free as possible of other proteins. Association with extraneous proteins can arise from inadequate purification of the antibody or from stabilizers added to diluted antibody preparation such as bovine serum albumin (BSA) or gelatin. The proper buffer and pH for the antibody conjugation can be obtained by dialysis or by adding 1 M bicarbonate when the antibody is dissolved in PBS. When antibodies are in PBS solution, the pH can be easily changed by adding 1/10 volume of 1 M sodium bicarbonate at pH 8.3 or 9.0, depending on which probe is used. This obviates the need for dialysis, accompanied by losses of material during manipulation of the antibody solution. One should note, however, that the buffer in which the antibody is dissolved should contain no primary amines (e.g., TRIS, glycine, or traces of ammonium sulfate), because they will react with the amine-reactive probe.

Solubility, storage, and stability

Most dyes, biotin, and cross-linking reagents are poorly soluble in buffers, and they require solubilization in an organic solvent such as dimethylformamide (DMF) or dimethylsulfoxide (DMSO). Some of the most recently introduced dyes that exhibit greater fluorescence output and photostability (Cy and Alexa Fluor series) have

improved or good solubility in water, because of the sulfonic groups introduced in the ring of their molecule (Panchuk-Voloshina et al., 1999). Some labeling reagents are available as sulfosuccinimidyl esters, with a sulfonic acid as part of the reactive group, which increases water solubility but may decrease the overall conjugation efficiency. However, in the author's experience, dissolving the labeling reagent as a concentrated solution in DMF or DMSO (10 to 20 mg/ml) and then adding it to the antibody solution in amounts such that the solvent does not exceed 10% of the reaction mixture, does not generally decrease the antibody binding activity. The presence of the solvent, in fact, helps to maintain the labeling reagent in solution and favors the efficiency of the conjugation reaction.

All labeling reagents should be stored desiccated at -20°C . Antibody conjugates of fluorophores, biotin, or enzymes can be stored frozen in small aliquots, avoiding repeated freezing and thawing. The defrosted aliquots should be stable at 4°C for ≥ 1 week. Labeled antibodies generally maintain the storage requirements of their native form. However, introduction of dyes or biotin to the molecule, with concomitant loss of the positive charges of the tagged lysines or addition of enzyme molecules, may favor aggregation. Therefore, it is good practice to centrifuge antibodies and particularly antibody conjugates briefly in a microcentrifuge before use. Antibodies at concentrations < 1 mg/ml are often unstable and should be stored in presence of 1 to 10 mg/ml BSA to avoid denaturation.

Appropriate degree of labeling

The appropriate number of labels per antibody molecule depends on the probe. In general, the number of labels that a protein tolerates before losing its biological activity is proportional to its molecular weight. In the author's experience, a 10- to 20-kDa protein preserves its activity when only one probe molecule per protein molecule is conjugated to the ϵ -amino group of a lysine. Thus 4 to 8 moles of probe per mole of an IgG antibody are possible in the case of relatively small or hydrophilic molecules (such as fluorescein, biotin, or sulfonated dyes) or 2 to 4 moles, in the case of hydrophobic probes, such as rhodamines. Excessive labeling may present problems: the antibody conjugate might aggregate or precipitate, lose binding activity to the protein, exhibit low fluorescence output due to quenching, or exhibit high background fluorescence in biological applications. The latter phenomenon can arise from a loss of specificity due to structural changes in

the antibody caused by the high number of fluorophores substituting the positive charged amines. The Fab fragment (Fig. 16.5.1E), tolerates two or three fluorescein or Alexa Fluor 488 molecules well, but only one Texas Red or rhodamine. $\text{F}(\text{ab}')_2$ fragments (Fig. 16.5.1D) can be labeled with three to four fluorescein molecules or one to two Texas Red molecules. Consequently, when using Fab fragments, one should use smaller molar ratios of reactive probe to antibody than those recommended in Table 16.5.1 for native antibodies. Precipitated antibodies generally cannot be recovered.

Choosing the appropriate fluorophore

The choice of fluorophore for a particular application should be based on the following considerations. When the fluorescence microscope is the detection instrument, for single-color experiments the choice should be dictated by the spectral properties of the dye relative to the excitation source, the brightness of the probe, and its photostability. Photostability is less essential in flow cytometry experiments. For multiple-color experiments, minimal spectral overlap among the chosen fluorophores is essential. For samples with pronounced autofluorescence, longer wavelength emission dyes are better than the green fluorescence-emitting ones because the autofluorescence background will be lower. Table 16.5.2 describes the optical properties of the most commonly used fluorophores for the labeling of antibodies (also see *APPENDIX 1E*).

Choosing the appropriate incubation molar ratio of fluorophore or biotin to antibody

The factors that determine the number of moles of probe per mole of antibody (degree of labeling) that will be present in the purified conjugate are:

- (1) Reactivity of the dye. This varies from dye to dye; it is pH and temperature dependent, and it must be taken into consideration when planning the reaction molar ratio of reagent to protein to use.
- (2) Concentration of the antibody. As in any chemical reaction, the reagent concentrations determine the rate and efficiency of the reaction and will generate higher yields of conjugate at higher concentrations.
- (3) Structure of the antibody. Although the Fc portion of the antibody molecule is relatively preserved, the amino acid composition of the remaining regions is quite varied and contains lysine residues in different numbers and positions. Consequently, the reactivity of each antibody is different. When possible, three or more molar incubation ratios of dye, biotin, or crosslinking reagent to antibody should be tried

Table 16.5.3 Performance of Commercially Available Anti-Photobleaching Reagents^{a,b}

Dye	Reagent						
	PBS	Slow-Fade	SlowFade Light	ProLong	Vecta-Shield	Cyto-seal	Others ^{b,c}
Alexa Fluor 350	---	+++	---	+++	---	---	***
Alexa Fluor 430	---	---	+++	+++	---	---	***
Alexa Fluor 488	---	+++	---	+++	+++	---	***
Alexa Fluor 532	---	---	---	+++	+++	---	***
Alexa Fluor 546	---	---	---	---	---	---	***
Alexa Fluor 568	---	---	---	+++	+++	---	***
Alexa Fluor 594	---	+++	---	+++	---	---	***
BODIPY FL	---	+++	---	---	---	+++	nt
BODIPY TR-X	---	---	nt	+++	nt	---	nt
Carboxytetramethylrhodamine	---	+++	---	+++	+++	---	nt
Cascade Blue	---	+++	+++	+++	---	+++	***
Cy3	---	+++	---	+++	+++	---	nt
Cy5	---	+++	---	+++	+++	---	nt
Fluorescein	---	+++	---	+++	+++	---	nt
Oregon Green 488	---	---	nt	+++	nt	+++	nt
Oregon Green 514	---	---	nt	+++	---	+++	nt
Rhodamine Red-X	---	---	nt	+++	nt	---	nt
Rhodol Green	---	+++	nt	+++	nt	+++	nt
Texas Red	---	---	nt	+++	+++	---	nt
Texas Red-X	---	---	nt	+++	nt	---	nt

^aFading was measured for 90 sec.^bAbbreviations: nt: not tested; PBS: phosphate-buffered saline.^cNotes: In addition to testing anti-photobleaching reagents listed in the table, the authors tested the performance of the following anti-photobleaching reagents when used with the dyes in the Alexa Fluor series: Crystal Mount, Gel Mount, Immu-Mount, Fluor-Save, Clarion, Aqua-Poly Mount, Mowiol. Aqua-Poly Mount and Mowiol worked well with Alexa Fluor 546.

to obtain a degree of labeling of the probe-antibody conjugate that will work best in the specific application. Table 16.5.1 gives guidelines of suggested incubation molar ratios of probe to antibody for different concentrations of antibody. The guidelines are very general and individual antibodies may require customized variations. As previously mentioned, it is desirable to try three different degrees of labeling or, if that is not possible, aim for a moderate degree of labeling of three to four fluorophore moles per mole of antibody. Also note that it is possible to label an antibody for the second time, but it is not possible to reduce the moles of dye per antibody following a conjugation because they are covalently attached.

Choosing the appropriate biotin-binding protein

Avidin is a highly positively charged glycoprotein, rich in mannose and glucosamine. Thus, it may interact nonspecifically with negatively charged molecules within the cell or with sugar receptors of the cell membrane. For this reason,

a chemically modified avidin, deglycosylated, and with a lower isoelectric point has been developed. It is commercially available in both unconjugated and conjugated forms under the trademark, NeutrAvidin (Haugland, 1999). Streptavidin is not glycosylated, and it generally yields less nonspecific binding than avidin. However, streptavidin contains the amino acid sequence Arg-Gly-Asp (RYD) that mimics the binding peptide of fibronectin (RGD), a universally recognized domain of the extracellular matrix. Thus, although it is rare, nonspecific interaction is possible also with streptavidin (Alon et al., 1990), in which cases, avidin or its NeutrAvidin form may be preferable.

Choosing the appropriate biotinylation reagent

As described for conjugations with fluorophores, the most broadly used reactive biotin for conjugation with antibodies is the succinimidyl ester form. Three types of succinimidyl esters are available: either biotin derivatized directly at the carboxyl with the reactive group or with a 7- or

14-atom spacer between the carboxyl and the reactive group (called biotin-X and biotin-XX, respectively). The addition of a spacer favors the efficiency of formation of the complex between avidin and the biotinylated antibody, possibly by facilitating the interaction of the biotin group with the hydrophobic crevice of the avidin binding site (Gretch et al., 1987; Hnatowich et al., 1987). Biotin-X and biotin-XX can be used interchangeably, although in this laboratory the authors have found that biotin-XX gives a somewhat higher signal in ELISA (Haugland and You, 1998). Sulfosuccinimidyl esters of biotin, which are highly water soluble are also commercially available. However, this form is generally less reactive and requires a higher molar incubation ratio of reagent to antibody.

Fluorescence intensity in histological preparations

For detection by microscopy, either conventional or confocal, the resistance of the fluorophores to photobleaching is a critical concern. It is very frustrating to see an image disappear as soon as it is in focus. There is considerable variation in the performance of anti-photobleaching products with different fluorophores. The author has tried many of the commercially available anti-photobleaching reagents in cellular preparations stained with the most commonly used fluorophores. The results from this study are reported in Table 16.5.3.

Anticipated Results

The protocols in this unit are established and have a high probability of success. The exact degree of labeling depends on the antibody to be labeled and the application for which it is used. The reactions may be scaled up or down.

Time Considerations

Fluorophore or biotin conjugation and purification of the conjugated antibody will take ~3 hr. Checking the degree of labeling will take 30 to 60 min. Longer times (~2 days) are required when working with very dilute antibodies that require dialysis for purification. Conjugation with enzymes and purification of the conjugated antibody will take ~5 days.

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Hermanson, G.T. 1996. *Bioconjugate Techniques*. Academic Press, San Diego.

These two citations are excellent reference sources covering concepts behind all the procedures discussed in this unit.

Haugland, 1996b, 1999. See above. (The 7th edition of this Handbook (1999) is available free on CD-ROM from Molecular Probes and at www.probes.com).

Contains excellent background material for all procedures, as well as chemical structures, spectra, and other product information for many of the probes discussed in this article.

Internet Resources

<http://www.probes.com>

This Molecular Probes site is a searchable, complete handbook and catalog of fluorescent probes, protein conjugates, and other probes for research. It contains downloadable spectra, structures, images, product information sheets, material safety data sheets, full scientific references, and link to contact the manufacturer.

<http://www.piercenet.com>

This Pierce Chemical Company site is also a searchable handbook and catalog with technical tips and selection guides, literature, application notes, and link to contact the manufacturer.

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Production of Antibodies That Recognize Specific Tyrosine-Phosphorylated Peptides

It is possible to produce anti-phosphopeptide antibodies (i.e., antibodies recognizing phosphorylated peptides) that recognize a protein only in its phosphorylated state, and that do not cross-react with either the cognate unphosphorylated protein or other phosphoproteins. Because the state of phosphorylation is often indicative of a protein's functional state or activity, such antibodies provide a convenient probe of the functional state of a protein. Thus, unlike conventional antibodies, anti-phosphopeptide antibodies provide information regarding not only the abundance of a protein but also its activity. Unlike general anti-phosphoamino acid (e.g., anti-phosphotyrosine) antibodies, which have broad reactivity, anti-phosphopeptide antibodies may have unique specificity toward the cognate proteins. Such reagents not only facilitate conventional *in vitro* analysis of phosphoproteins, but also allow heretofore impossible applications—e.g., differential isolation of species of a particular protein that have been phosphorylated at individual phosphorylation sites, as well as analysis of the functional state of a protein *in situ* by immunohistochemical techniques.

Basic Protocol 1 describes the production of polyclonal anti-phosphopeptide antibodies and Basic Protocol 2 describes the production of monoclonal anti-phosphopeptide antibodies. Both of these procedures are based upon immunizing animals with oligopeptides that have been synthesized containing phosphorylated tyrosine; hence, a knowledge of the sequence and phosphorylation site of the protein of interest is a prerequisite for either protocol. Such an immunization will generate an immune response with at least four components: (1) anti-carrier protein reactivity, (2) general anti-phosphotyrosine reactivity, (3) phosphorylation-independent anti-peptide reactivity, and (4) phosphorylation-dependent anti-peptide reactivity. For the production of polyclonal antibodies (see Basic Protocol 1), a multiple-step affinity chromatographic purification with several negative-selection steps is carried out to produce a final antibody preparation having the desired reactivity. For production of monoclonal antibodies (see Basic Protocol 2), ELISAs are performed to screen candidate hybridoma supernatants against the cognate phosphopeptide, as well as against related phosphorylated and nonphosphorylated peptides, until one is found with the desired reactivity and specificity. Although monoclonal antibodies have a number of advantages, production of polyclonal antibodies is likely to be more predictable, as in the authors' experience a monoclonal hybridoma clone of stringent specificity may occur with very low frequency (see Critical Parameters). Production of monoclonal antibodies is also generally more time-consuming and expensive. The relative merits of monoclonal and polyclonal antibodies are discussed in the Chapter 16 introduction and in general antibody guides (see Key References). Support Protocols are provided for the coupling of peptides (see Support Protocol 2) and phosphotyrosine (see Support Protocol 3) to the affinity matrix (Affi-Gel 10); BSA-agarose affinity matrix is commercially available.

This unit describes production of antibodies against tyrosine-phosphorylated peptides, with which the authors have the most expertise, but the principles discussed here also apply to peptides phosphorylated on serine and threonine (see Key References).

Strategic Planning

The first step of the planning phase is design and synthesis of the required peptides (see Support Protocol 1 and Background Information). The authors employ a peptide containing fifteen amino acids with the phosphorylation site Tyr in the center (residue 8). This peptide consists of fourteen amino acids from the actual sequence of the protein plus an N-terminal Lys to enhance coupling of the peptide to a carrier protein (BSA is used as a carrier in this protocol). The peptide, coupled to BSA, is then used to immunize rabbits. The cognate phosphopeptide is also immobilized on an affinity-chromatography column support (see Support Protocol 2); this affinity matrix will ultimately be used to purify the antibody from the rabbit serum. In addition, a series of negative-selection affinity-chromatography columns are used to adsorb antibodies from the serum that cross-react with epitopes other than the cognate phosphopeptide. These columns are composed of matrix coupled to various synthetic peptides—principally the cognate nonphosphorylated peptide (to remove phosphorylation-independent antibodies) and phosphotyrosine itself (to remove indiscriminant anti-phosphotyrosine reactivity; see Support Protocol 3 for preparation of phosphotyrosine affinity columns). Additional negative-selection steps may be used if needed, depending upon the particular target chosen, to remove antibodies that cross-react with phosphorylation sites of other proteins having known homology to the target site. This cross-reactivity occurs particularly when the target protein is a member of a family of homologous proteins—e.g., a subgroup of related receptor tyrosine protein kinases. If such cross-reactivity is anticipated, it is possible to synthesize not only the cognate phosphorylated and nonphosphorylated peptides, but also phosphopeptides based upon the related homologous sequences, to be used for negative selection. The authors also pass the serum over a column consisting of immobilized carrier protein (commercially available BSA-agarose) to remove the majority of the antibodies generated against the much larger carrier protein. Thus, a typical purification scheme might consist of the following:

Negative-selection affinity-purification columns

- BSA (carrier)
- phosphotyrosine
- cognate nonphosphopeptide
- homologous phosphopeptides (if desired)

Positive-selection affinity-purification column

- cognate phosphopeptide.

The second step in planning is to raise and test antisera in rabbits. To prepare the phosphopeptide-carrier conjugate for immunization of rabbits, the authors couple a 30-fold molar excess of phosphopeptide (14.4 mg dissolved in water and neutralized with 1 N NaOH) to BSA (20 mg dissolved in 0.4 M sodium phosphate buffer, pH 7.5), using glutaraldehyde as the cross-linking reagent as described in Doolittle (1986). The reaction is allowed to proceed 30 min at room temperature; the development of a yellow color in the coupling solution is an indication that the glutaraldehyde has reacted. Conjugation can be confirmed by performing an anti-phosphotyrosine immunoblot on a small test aliquot of the conjugate (although a smear should be expected on the blot, resulting from variation in the amount of peptide coupled per BSA molecule as well as from possible multimers of BSA). Guidelines for immunization of rabbits are provided in *UNIT 16.2*, and many institutions have core facilities for the immunization of rabbits and production of antisera. The authors immunize with 1 mg immunogen (the peptide-BSA conjugate

prepared above, dissolved in PBS) in 1 ml of a 50% emulsion with complete Freund's adjuvant. This is administered subcutaneously at multiple sites, followed by four boosts at 2-week intervals using the same quantity of immunogen in incomplete Freund's adjuvant. An adequate immune response can be seen as early as week 6.

The specificity of the final antibody is demonstrated most simply by immunoblot analysis (*UNIT 6.2*) using a panel of relevant phosphorylated and nonphosphorylated proteins. Electrophoresis is carried out with purified cognate protein in both its phosphorylated and unphosphorylated states, as well as with any proteins toward which there could conceivably be cross-reactivity. These are most easily isolated by immunoprecipitation using conventional antibodies. The blot is probed with the purified anti-phosphopeptide antibody to demonstrate appropriate reactivity. This immunoblot assay may also be used after each chromatographic step to monitor the success of the purification.

An assay also must be devised for estimating the titer of the immune response in the sera of the immunized rabbits. Convenient screening assays for this purpose include tests for the ability of crude sera to immunoprecipitate the target phosphoprotein from a cell lysate known to contain it, as well as immunoblotting of purified phosphorylated target protein with the crude serum.

The relationship among the above procedures is illustrated by the flow chart in Figure 16.6.1.

Purification Method

The purification of polyclonal antibodies consists of multiple affinity-chromatography steps for the negative and positive selection of antibodies of the appropriate reactivity (see Fig. 16.6.1). All of the following chromatographic steps may be carried out at room temperature, with the solutions applied to the columns by gravity.

Materials

- BSA-agarose affinity matrix (Sigma) packed (see Support Protocol 2) in a 10-ml bed volume column
- Phosphotyrosine affinity matrix column (10-ml bed volume; see Support Protocol 3)
- Crude serum from rabbit immunized with phosphopeptide-BSA conjugate (refer to Strategic Planning, above)
- PBS/azide: PBS (*APPENDIX 2A*) containing 0.02% (w/v) sodium azide (store indefinitely at 4°C or room temperature)
- Cognate nonphosphopeptide affinity matrix column (3-ml bed volume; see Support Protocols 1 and 2 and Strategic Planning)
- 3 M NaSCN
- Homologous phosphopeptide affinity matrix columns (optional; 3-ml bed volume; see Support Protocols 1 and 2 and Strategic Planning)
- Positive-selection phosphopeptide affinity matrix column (3-ml bed volume; see Support Protocols 1 and 2 and Strategic Planning)
- 3.5 M and 4.5 M MgCl₂ (optional; see Critical Parameters)
- Spectrophotometer (optional)
- Dialysis tubing (MWCO 12,000 to 14,000; 10 mm width, 6.4 mm diameter; e.g., Spectra/Por 4 from Spectrum)
- Additional reagents and equipment for dialysis (*APPENDIX 3C*), protein quantitation (*APPENDIX 3B*), and analysis of antibodies by ELISA (*APPENDIX 3A*) or immunoblotting (*UNIT 6.2*)

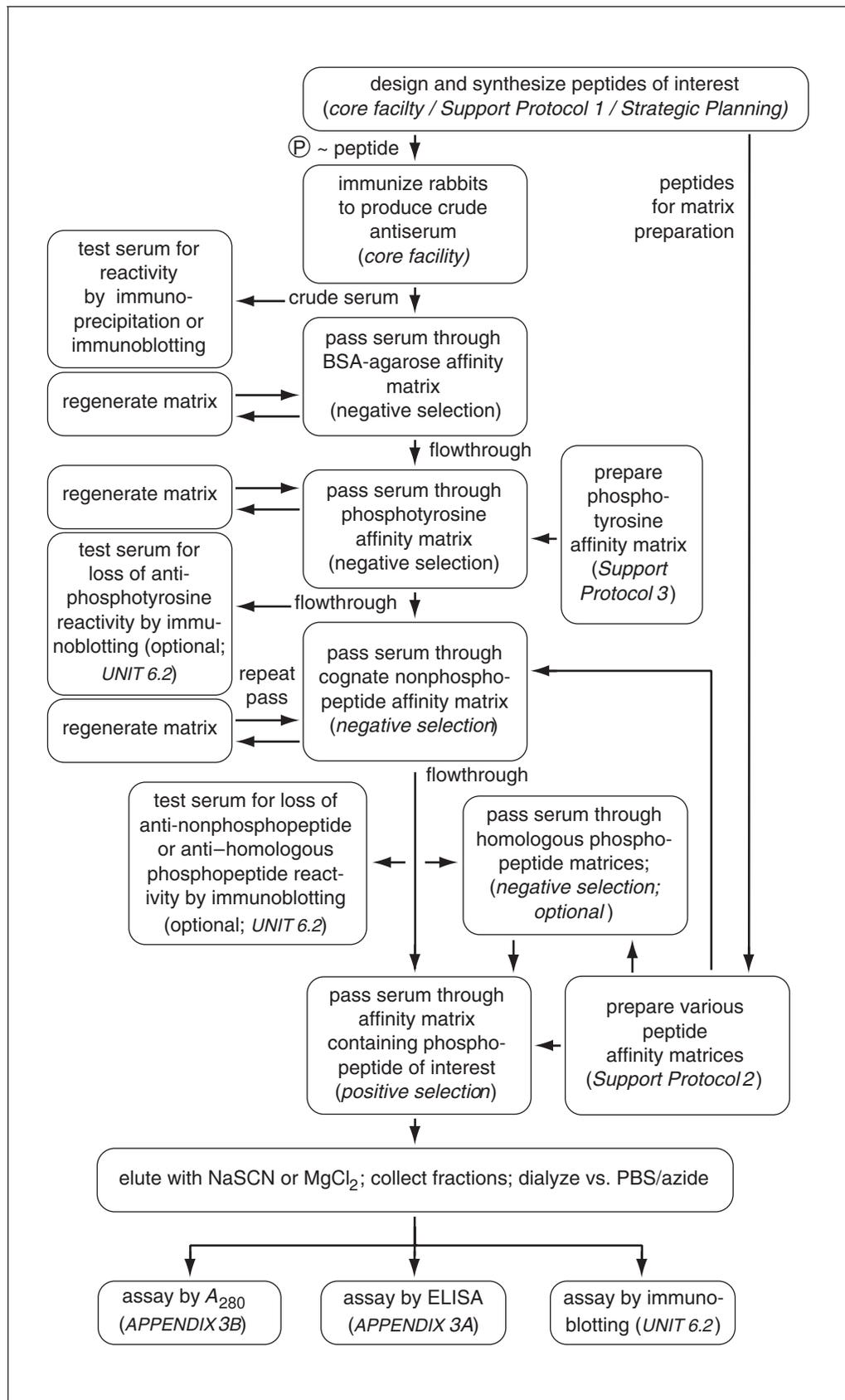


Figure 16.6.1 Flow chart for production of polyclonal anti-phosphopeptide antibodies.

Deplete reactivity of serum toward phosphotyrosine and carrier protein

1. Connect the washed BSA-agarose and phosphotyrosine affinity matrix columns in series.
2. Pass ~15 ml crude serum through the two columns, allowing it to flow through by gravity. Wash with PBS/azide until all of the yellow color of the serum has passed through the columns (or monitor the A_{280} of the column effluent spectrophotometrically until baseline absorbance is reached), then wash with an additional 5 to 10 ml PBS/azide, collecting all washings in the flowthrough fraction, which will contain the antibody of interest.

Aliquots of the crude serum as well as this first flowthrough may be saved for subsequent analysis and comparison of individual purification fractions. The serum may be analyzed at this time for elimination of anti-phosphotyrosine reactivity by performing immunoblotting (UNIT 6.2) of samples containing a variety of phosphotyrosyl proteins; alternatively, it is possible to save an aliquot for analysis and proceed to the next step (see Troubleshooting).

Expect that with each passage through a column, the volume of the serum will increase, which will prolong the amount of time needed for subsequent columns. Note that if flowthrough is monitored by the passage of the yellow color of the serum, this will become more difficult as the serum becomes more dilute.

After the serum passes through, regenerate the columns by washing with 10 bed volumes of 3 M NaSCN followed by 10 bed volumes of PBS/azide, and store at 4°C in PBS/azide for future use.

Deplete reactivity of serum toward cognate nonphosphopeptide

3. Pass the flowthrough serum through the cognate nonphosphopeptide affinity matrix column by gravity as many times as necessary to deplete cross-reactivity, regenerating the column between passes by washing with 10 bed volumes of 3 M NaSCN followed by 10 bed volumes PBS/azide.

A 15-ml starting serum sample may have to be passed over this column several times to quantitatively deplete cross-reactivity (see Critical Parameters). The serum may be analyzed after each or several passes, or an aliquot can be saved for analysis at a later time.

4. If cross-reactivity with homologous phosphoprotein(s) is anticipated, pass the flowthrough serum through the homologous phosphopeptide affinity matrix column(s) using the methodology described in step 3.

Refer to Strategic Planning, above, for discussion of cross-reactivity with homologous phosphoproteins.

Purify antibodies by positive-selection affinity chromatography and dialysis

5. Hydrate and wash ~25-cm strips of dialysis tubing in advance for collection of fractions from the positive-selection affinity purification. Secure one end of each length of tubing with a dialysis clamp and check for leaks (APPENDIX 3C). Also prepare 6 liters PBS/azide and cool to 4°C in advance to use as the dialysis solution.

To minimize the time that the antibodies are exposed to the elution solution, fractions (~3 ml each) from the positive-selection affinity column will be collected directly into preprepared dialysis tubing.

6. Pass the flowthrough serum from the previous chromatography step through the positive-selection phosphopeptide affinity column three times, without washing between passes.

Three passes are performed to maximize the interaction of the antibody with the affinity matrix.

7. Collect the flowthrough serum from the final pass, then wash the column with 5 to 20 ml PBS/azide (depending upon the precolumn volume and column bed volume) and combine the washings with the flowthrough serum. Wash with an additional 20 ml PBS/azide, and collect the washings separately as the “wash” fraction.
8. Elute with chaotropic agent of choice: either 20 ml of 3 M NaSCN, or 10 ml of 3.5 M MgCl₂ followed by 10 ml of 4.5 M MgCl₂. Upon starting the elution, immediately begin collecting ~3-ml fractions directly into the dialysis bags prepared in step 5, securing the proximal end of the tubing with dialysis clamps and dropping the bag immediately into the PBS/azide dialysis solution. Collect at least six fractions.

Alternatively, ~3-ml fractions can be collected in tubes and the liquid immediately placed into dialysis tubing upon completion of each fraction. Most of the antibody will elute in the first three fractions from a column of 2- to 3-ml bed volume. Regenerate the column as in step 3.

See Critical Parameters for a discussion of the relative merits of NaSCN versus MgCl₂ as eluants.

9. Dialyze all fractions collected in step 8 exhaustively against PBS/azide at 4°C (APPENDIX 3C).

Analyze and store purified antibody

10. Determine protein concentration of dialyzed fractions by measuring absorbance at 280 nm or by a colorimetric protein assay (APPENDIX 3B) and calculate yield.

The authors have generally recovered ≥1 mg of purified antibody from a 15-ml serum sample.

11. Store the antibody in aliquots at –70°C; store working aliquot at 4°C.
12. Assay the reactivity and cross-reactivity of the final samples, as well as any aliquots saved from previous steps, using ELISA (APPENDIX 3A) or immunoblotting (UNIT 6.2).

BASIC PROTOCOL 2

PRODUCTION OF MONOCLONAL ANTI-PHOSHOPEPTIDE ANTIBODIES

Strategic Planning

As in the production of polyclonal anti-phosphopeptide antibodies (see Basic Protocol 1), the first step of the planning phase for anti-phosphopeptide monoclonal antibodies is the design and synthesis of the required peptides (see Support Protocol 1 and Background Information). Here too, the authors employ a peptide containing fifteen amino acids with the phosphorylation site Tyr in the center (residue 8), consisting of fourteen amino acids from the actual sequence of the protein plus an N-terminal Lys to enhance coupling to a carrier protein. A different carrier protein must be employed for immunization than will be used as substrate for the ELISA screen, to avoid detection of anti-carrier antibodies in the ELISA (see Purification Method, below). The authors use keyhole limpet hemocyanin (KLH) as the carrier for immunization and BSA as the carrier for the ELISA substrate. The cognate phosphopeptide can also be immobilized to an affinity-chromatographic column support (see Support Protocol 2) and used ultimately to affinity-purify the antibody produced by the desired hybridoma clone. A number of additional peptides must be produced for use in ELISA screening of candidate hybridoma supernatants, to screen both for the presence of the desired reactivity and the absence of undesired cross-reactivity. For ELISA screening, the peptides are coupled to a carrier protein different from the one used for immunization. Bovine serum albumin (BSA) is used for screening, because many of the clones are likely to react against the carrier used in the immunization of the

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mice (KLH) and because unconjugated peptides have not been found to function well as ELISA substrates (Doolittle, 1986). In addition, because antibodies may be generated against the cross-linking reagent, the method used for coupling the phosphopeptide to the BSA to produce the substrate for ELISAs should be different from that used to link peptides to the KLH carrier for immunization (Doolittle, 1986; Czernik et al., 1991).

The second step of planning is to raise antibodies in mice. General guidelines for the immunization of mice and the production of monoclonal antibodies are described in *UNIT 16.1*, and many institutions have core facilities that will perform that task. The authors have immunized BALB/c mice by intraperitoneal injection with 1 mg/ml of immunogen (dissolved in PBS) in a 50% emulsion with complete Freund's adjuvant on day 1 and boosted intraperitoneally on days 15 and 37 with immunogen in incomplete Freund's adjuvant. Test bleeds from day 47 were analyzed by ELISA. On day 57 the mouse with the best titer was boosted intravenously, and its spleen was harvested on day 60. Freshly harvested spleen cells were prepared for cell fusion to generate hybridoma lines, which were subsequently screened by ELISA (*APPENDIX 3A*). The purpose of the ELISA screening is to identify clones that react with the cognate phosphopeptide and to eliminate clones that cross-react with other epitopes in addition to the cognate phosphopeptide. Epitopes screened by ELISA generally consist of the cognate nonphosphorylated peptide (to eliminate clones with phosphorylation-independent reactivities) and at least one unrelated phosphopeptide (to eliminate clones with indiscriminant anti-phosphotyrosine reactivity). In addition, depending upon the particular target chosen, it may be necessary to screen for reactivity with phosphopeptides from proteins of known homology. This occurs particularly when the target protein is a member of a family of homologous proteins—e.g., the subgroups of related receptor tyrosine protein kinases. If such cross-reactivity is anticipated, it is essential to synthesize not only the cognate phosphorylated and nonphosphorylated peptides and at least one unrelated phosphopeptide, but also phosphopeptides based upon the related homologous sequences. Thus, a typical ELISA screening scheme might consist of the following steps.

1. Screen for clones that exhibit reactivity against the cognate phosphopeptide. These clones, which will likely be <10% of all the clones, are potential candidates for the desired clones.
2. Screen for clones with reactivity against the cognate nonphosphopeptide. These clones, which are phosphorylation state-independent, are then eliminated.
3. Screen for clones with reactivity against at least one unrelated phosphopeptide. These clones, which are likely to represent indiscriminant anti-phosphotyrosine reactivity, are also eliminated.
4. Screen for and eliminate clones with reactivity against potential cross-reacting homologous peptides, if any are anticipated.

In the ELISAs performed in the authors' laboratory, 96-well polyvinyl chloride assay plates are generally used and bound monoclonal antibody is detected using biotinylated horse anti-mouse secondary antibody followed by horseradish peroxidase (HRP)-conjugated Avidin D (Vector Labs) and 4-aminoantipyrine (Sigma)/H₂O₂ as the substrate. This gives a deep red color in positive wells and absence of color in negative wells, allowing easy visual scoring without the need for quantitative measurements. The clones that pass all the screening steps are candidates for desirable hybridoma cell lines. These are expanded, frozen down as a pool, subcloned by limiting dilution, then rescreened against cognate phosphopeptide to ensure continued production of antibody through the early passages. Those continuing to produce are expanded further and a larger quantity of either culture supernatant or ascites fluid is screened in further immunoassays. Reactivity is

demonstrated most simply by immunoblot analysis using a panel of relevant phosphorylated and nonphosphorylated proteins. Electrophoresis is carried out with purified (generally by immunoprecipitation using conventional antibodies; *UNIT 7.2*) cognate protein in both its phosphorylated and unphosphorylated states, as well as with any proteins toward which there could conceivably be cross-reactivity. The blot is probed with the monoclonal antibody preparation to demonstrate appropriate reactivity. The antibody preparation can ultimately be purified on a cognate phosphopeptide affinity column.

The relationship among the above procedures is illustrated by the flow chart in Figure 16.6.2.

Purification Method

This protocol presents procedures for isolating hybridoma clones of the desired reactivity, starting from the point at which candidate hybridoma clones have been seeded in 96-well culture plates (see Fig. 16.6.2). Candidate hybridoma supernatants are screened in ELISA assays against a series of peptides (as described in Strategic Planning). Identified clones are expanded, subcloned by limiting dilution, and further purified.

Materials

Candidate hybridoma cell lines from fusion (refer to Strategic Planning and *UNIT 16.1*)

HT medium; prepare as for HAT medium (*UNIT 1.2*) except omit aminopterin

Screening diluent (see recipe)

BSA-conjugated cognate phosphopeptide (for use as ELISA antigen; refer to Strategic Planning and Support Protocol 1)

Preimmune serum from mouse used to produce hybridoma line (negative control)

Immune serum from mouse used to produce hybridoma line (positive control)

BSA-conjugated cognate nonphosphopeptide (for use as ELISA antigen; refer to Strategic Planning and Support Protocol 1)

BSA-conjugated noncognate phosphotyrosyl peptide (for use as ELISA antigen; refer to Strategic Planning and Support Protocol 1)

BSA-conjugated homologous phosphopeptides (optional, for use as ELISA antigens; refer to Strategic Planning and Support Protocol 1)

96-well polystyrene tissue culture plates

96-grid note sheets

Additional reagents and equipment for cloning by limiting dilution, ELISA, and freezing and recovery of hybridoma cell lines (*APPENDIX 3A*)

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

Identify and sample conditioned media from candidate hybridomas

1. Seed hybridoma cells from fusion at a low density (aiming for one cell per three wells) in multiple 96-well polystyrene tissue culture plates using HT medium. Examine at ~2 weeks and identify all single-colony hybridoma culture wells as potential candidates by recording the number of colonies per well on a 96-grid note sheet. Mark the underside of each well containing one colony for easy identification.

The supernatant fluid from wells possessing a single colony will be screened for reactivity.

2. Using sterile technique, remove an aliquot of supernatant (e.g., 100 μ l from a 200- μ l culture) from each potential candidate well and transfer to a separate 96-well polystyrene plate (the "screen plate"). Record the original plate number and well position and the corresponding plate number and well position for the screen plate (e.g., on a grid sheet representing the screen plate, record the original plate number

and well position from which the supernatant in each well was transferred). Replace the aliquot removed from each original well with 100 μ l 37°C fresh HT medium.

A brightly colored dot sticker the size of one well, attached to the tissue culture hood surface, will help to keep the plate in register, as the plate may be moved one well at a time along the sticker. If the supernatants are not to be tested immediately, store the screen plates at 4°C, wrapped in plastic wrap.

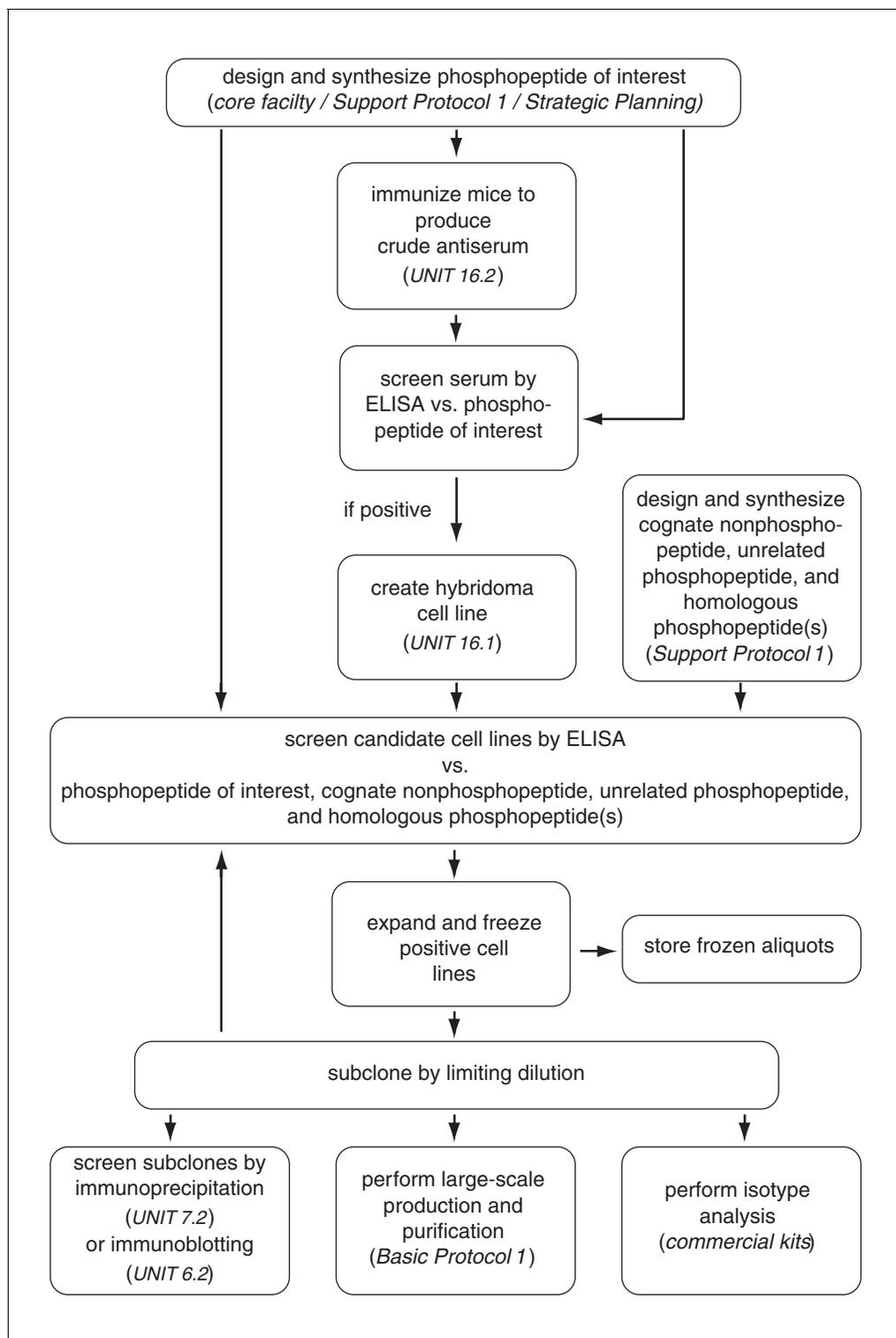


Figure 16.6.2 Flow chart for production of monoclonal anti-phosphopeptide antibodies.

3. To each supernatant in the screen plates, add 150 μ l screening diluent

The components of the screening diluent prevent microbial contamination; this reagent also serves to expand the volume should multiple rounds of screening be required.

Screen candidate hybridomas by ELISA

4. Screen an aliquot (e.g., 50 μ l) of each candidate hybridoma supernatant from the screen plate in an ELISA using the BSA-conjugated cognate phosphopeptide as antigen. Record the screen-plate number and well position and corresponding to original culture plate number and well position for each positive sample. Also assay preimmune serum from the mouse used for fusion (negative control) and immune serum from the same mouse (positive control), each at a 1:100 dilution in a 1:1 mixture of HT medium and screening diluent. Include an ELISA with screening diluent only (additional negative control).

It is important to use a different carrier protein in the ELISAs (BSA) than that used for the immunizations (KLH), to avoid detecting reactivity against the carrier (refer to Strategic Planning, above).

5. Screen an aliquot of each positive sample from step 4 in an ELISA against the BSA-conjugated cognate nonphosphopeptide to eliminate clones with phosphorylation-independent reactivity, against the unrelated BSA-conjugated phosphotyrosylpeptide to eliminate clones with indiscriminant phosphotyrosine reactivity, and, if cross-reactivity with homologous phosphoprotein(s) is anticipated, against phosphopeptide-BSA conjugates corresponding to any potentially cross-reacting homologous phosphoproteins.

These screens may be done sequentially or, if the number of candidate hybridomas has diminished sufficiently, simultaneously. Isolation of clones with phosphorylation-independent cognate peptide specificity may also be useful.

6. Expand clones that satisfy all screening requirements and freeze aliquots as backup; use remainder for subcloning by limiting dilution.
7. Screen culture supernatants from subclones as in steps 4 and 5 to check for continued production and specificity of the antibody.

Keep representative independent subclones from each original parental colony, as it cannot be predicted whether each parental monoclonal antibody will recognize the cognate holophosphoprotein or function in all types of immunoassays desired in the future. Candidate subclones should then also be expanded and frozen. It is a good idea to continue to check for production of the appropriate antibody after several rounds of serial passaging and after freezing, thawing, and reexpansion.

8. To characterize the subclones further and identify the most useful clones, assay the culture supernatants for reactivity with cognate holophosphoprotein in individual immunoassays (e.g., immunoprecipitations, as described in *UNIT 7.2*, or immunoblot analyses, as described in *UNIT 6.2*).

*Antibody from the desired clones can be produced on a large scale by harvesting large volumes of culture supernatant and performing affinity purification (see *Basic Protocol 1*), or by producing ascites fluid, which can be purified by ammonium sulfate precipitation followed by affinity purification. Isotype analysis can be performed by using commercially available kits (Pierce or Sigma).*

SYNTHESIS OF PEPTIDES

Many institutions have core facilities for synthesis of peptides using automated solid-phase peptide synthesizers. For phosphotyrosine-containing peptides, the authors originally used standard Merrifield solid-phase procedures with *t*-butyloxycarbonyl (Boc) amino acids and Boc-*O*-(dibenzylphosphono)-L-tyrosine as the phosphorylated residue (Barany and Merrifield, 1979). For cleavage of phosphopeptide from the resin, 1 g phosphopeptide resin was stirred 10 min at 0°C with 2 ml dimethyl sulfide; then an ice-cold mixture of 4 ml trifluoromethanesulfonic acid (TFMSA) and 10 ml trifluoroacetic acid was added slowly with stirring. The resulting reaction mixture was stirred under a nitrogen atmosphere 4 hr at 0°C and the cleaved phosphopeptide precipitated with methyl *t*-butyl ether at -30°C. The precipitate was dissolved in 40% acetic acid and lyophilized several times from water. This original method suffers from low yields. Currently, most phosphotyrosyl-containing peptides are synthesized with 9-fluorenylmethyloxycarbonyl (Fmoc) phosphotyrosine. The lack of a side chain protecting group on the phosphotyrosine (Kitas et al., 1994) allows standard Fmoc synthesis and cleavage procedures to be employed (Chang and Meienhofer, 1978). However, a higher molar excess of activated Fmoc amino acids than that used in standard procedures may be required for efficient coupling of amino acids after unprotected phosphotyrosine has been added. In some circumstances, a double coupling procedure may be employed to add the few amino acids following phosphotyrosine to the growing peptide chain. This alternative requires the sequential, repeated addition of the next particular amino acid to be added to the peptide. A support protocol for the coupling of peptides to an affinity matrix is provided (see Support Protocol 2).

COUPLING OF PEPTIDES TO AFFI-GEL 10 AFFINITY MATRIX

This protocol describes coupling of phosphopeptides or nonphosphopeptides to Affi-Gel 10 affinity matrix for use in affinity column chromatographic purification of antibodies. Anhydrous coupling, detailed here, is the most efficient coupling method for peptides. The steps here result in production of 3 ml final bed volume of affinity resin; 3 mmol of peptide is coupled per milliliter Affi-Gel 10. According to the manufacturer's specifications, Affi-Gel 10 resin contains ~15 μmol of active ester per milliliter of gel, and the gel has a capacity for 35 mg of protein or 15 to 20 μmol of a low-molecular-weight ligand per milliliter of gel. Note that these capacities are dependent upon the molecular weight and nature of the protein. After coupling, the matrix may be poured into columns of the appropriate size.

Materials

- Synthetic oligopeptide for coupling (see Support Protocol 1)
- Dimethyl sulfoxide (DMSO)
- N*-methylmorpholine (99% purity; Acros Organics)
- Affi-Gel 10 (Bio-Rad) or equivalent activated support matrix
- Ethanolamine
- 0.1 M ethanolamine-HCl, pH 8.0
- High-salt/high-pH solution: 0.5 M NaCl/0.4% (w/v) sodium bicarbonate
- High-salt/low-pH solution: 0.5 M NaCl/100 mM sodium acetate, pH 4.2
- PBS/azide: PBS (APPENDIX 2A) containing 0.02% (w/v) sodium azide
- 0.5 M NaCl
- 3 M NaSCN
- Polypropylene screw-cap centrifuge tubes (do not use polystyrene tubes with DMSO)
- End-over-end rotator
- IEC Clinical centrifuge (or equivalent)
- Vacuum aspirator
- Glass chromatography columns, ≥ 5 -ml capacity

SUPPORT PROTOCOL 1

SUPPORT PROTOCOL 2

Prepare peptide solution

1. Dissolve oligopeptide in DMSO at a volume ~0.5 to 4 times the desired bed volume.

For a 3-ml bed volume, dissolve a total of 9 μmol peptide, which for an average 15-mer peptide of mol. wt. ~1700 would be 15.3 mg.

2. Neutralize the peptide solution by titration as follows: add peptide synthesis-grade *N*-methylmorpholine (full-strength or diluted to 1:1 to 1:9 in DMSO) in 1 to 2 μl increments, remove a 2- to 3- μl aliquot after each addition, dilute this with 50 μl water, then spot it onto a strip of pH paper. Repeat this process until a pH in the range of 7 to 8 is reached.

*Titrate carefully, as it is very easy to overshoot the desired pH. Typically 7 to 8 μl of *N*-methylmorpholine is required per 3 μmol peptide, although the precise amount will depend greatly upon the particular amino acid sequence. The aliquots must be mixed with water because pH is a measure of hydrogen ion concentration, and hence the pH of a nonaqueous solution cannot be measured.*

Add peptide to Affi-Gel 10

3. Mix contents of the bottle of Affi-Gel 10 matrix well to resuspend the resin, then transfer the required amount of resin to a screw-cap polypropylene centrifuge tube and wash three times in DMSO, each time by centrifuging 5 min at $700 \times g$ (2000 rpm in an IEC Clinical centrifuge, setting 5), room temperature, aspirating the supernatant by vacuum, resuspending the resin in 5 vol DMSO, then centrifuging again at $700 \times g$.

Be sure to take at least twice as much Affi-Gel 10 as ultimately will be needed, as the volume will shrink. The resin is supplied as a 50% slurry; therefore, it is necessary to take at least four times the required bed volume. Avoid centrifugation at higher than recommended speed as this may result in collapse of the resin.

4. Aspirate the excess DMSO from the resin and add the peptide solution prepared in step 2. Incubate overnight at room temperature with end-over-end rotation.

Quench and wash the resin

5. Add 2 μl pure ethanolamine per milliliter resin and incubate 2 hr at room temperature with end-over-end rotation.

Ethanolamine is added directly to the coupling reaction to quench unreacted ester groups.

6. Wash twice in DMSO as in step 3.
7. Wash twice in 0.1 M ethanolamine-HCl, pH 8.0, as in step 3 (performing the first wash on ice as a great deal of heat will be generated). Remove the 0.1 M ethanolamine-HCl from the second wash and replace with fresh, incubate tube overnight at 4°C with end-over-end rotation, then centrifuge at low speed and aspirate the supernatant.
8. Wash three times with high-salt/high-pH solution using the technique described in step 3.
9. Wash three times with high-salt/low-pH solution using the technique described in step 3.
10. Wash three times with PBS/azide using the technique described in step 3 and store washed resin at 4°C until ready to pack in column.
11. Mix matrix into a slurry with 0.5 M NaCl and pour into glass chromatography columns.

Commercially available glass chromatography columns can be used; alternatively, 5-ml plastic syringes with 1-cc plugs of silanized glass wool (UNIT 5.6) in the bottom may function as columns.

12. Wash each column with 10 column volumes 3 M NaSCN followed by 10 column volumes PBS/azide.

COUPLING OF PHOSPHOTYROSINE TO AFFI-GEL 10 AFFINITY MATRIX

**SUPPORT
PROTOCOL 3**

This protocol describes coupling of phosphotyrosine to Affi-Gel 10 affinity matrix for use in affinity column chromatographic purification of antibodies. Phosphotyrosine is insoluble under the anhydrous conditions used for coupling peptides (see Support Protocol 2); therefore, coupling is done under aqueous conditions. The steps here result in production of 10 ml final bed volume of affinity resin, as for peptides, 3 μmol phosphotyrosine is coupled per milliliter of Affi-Gel 10. According to the manufacturer's specifications, Affi-Gel 10 resin contains $\sim 15 \mu\text{mol}$ active ester per milliliter gel, and the gel has a capacity for 35 mg of protein per milliliter gel or 15 to 20 μmol of a low-molecular-weight ligand per milliliter gel. Note that these capacities may vary depending upon the molecular weight and nature of the protein. Phosphotyrosine-agarose affinity matrix is now also available commercially from Sigma.

Additional materials (also see Support Protocol 2)

Phosphotyrosine
0.4% (w/v) sodium bicarbonate
1 M NaOH (optional)

Fritted-glass funnel and vacuum aspirator
Glass chromatography column, ≥ 14 -ml capacity

Additional reagents and equipment for coupling peptides to Affi-Gel 10 affinity matrix (see Support Protocol 2)

Prepare phosphotyrosine solution

1. Dissolve phosphotyrosine in 0.4% sodium bicarbonate at a volume ~ 0.5 to 4 times the desired bed volume.

For a 10-ml bed volume, dissolve a total of 30 μmol phosphotyrosine. Based on its molecular weight of 261, this amounts to 7.8 mg, although it is acceptable (and may be easier) to use an excess, as phosphotyrosine is relatively inexpensive.

2. Check that the pH is in the range of 7 to 8 using pH paper. If adjustment is necessary, neutralize the solution by titration—i.e., adding small amounts of 1 M NaOH, removing a 2- to 3- μl aliquot after each addition, then spotting the aliquots onto a strip of pH paper. Repeat this process until a pH in the range of 7 to 8 is reached.

Add phosphotyrosine to Affi-Gel 10

3. Mix contents of the bottle of Affi-Gel 10 matrix well to resuspend resin, then transfer the required amount of resin to a fritted-glass funnel attached to a vacuum aspirator. Wash three times, each time pouring cold distilled water over the resin in the funnel and aspirating, then perform one final wash in the same manner using ice-cold 0.4% sodium bicarbonate.

Be sure to take at least twice as much Affi-Gel 10 as ultimately will be needed, as the volume will shrink. The resin is supplied as a 50% slurry; therefore, it is necessary to take at least four times the required bed volume. Do not allow >20 min to elapse from the time the resin is removed from the bottle to the time it is mixed with the phosphotyrosine solution.

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4. Remove most of the excess liquid from the gel by vacuum aspiration without letting it dry completely, transfer it to a screw-cap centrifuge tube, and add the phosphotyrosine solution from step 2.
5. Incubate overnight at 4°C with end-over-end rotation.

Quench and wash resin

6. Add 2 µl pure ethanolamine per milliliter resin and incubate 2 hr at room temperature with end-over-end rotation.

Ethanolamine is added directly to the coupling reaction to quench unreacted ester groups.

7. Wash resin twice, each time by centrifuging at low speed (see Support Protocol 2, step 3), aspirating the supernatant, resuspending the resin in 5 vol of 0.4% sodium bicarbonate, then centrifuging again at low speed.
8. Wash resin twice with 0.1 M ethanolamine-HCl, pH 8.0, as in step 7. Remove the 0.1 M ethanolamine from the second wash and replace with fresh. Incubate tube overnight at 4°C with end-over-end rotation, then centrifuge at low speed and aspirate the supernatant.
9. Wash, store, and pack resin in columns (see Support Protocol 2, steps 8 to 12).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Screening diluent

- 6.18 g boric acid (100 mM final)
- 9.54 g sodium borate (47 mM final)
- 4.38 g NaCl (75 mM final)
- 10 g bovine serum albumin (BSA; 1% w/v final)
- 1 g sodium azide (1% w/v final)
- H₂O to 1 liter

The pH of the solution will be 8.4 to 8.5.

COMMENTARY

Background Information

Conventional antibodies have proven to be invaluable tools in numerous techniques for the biochemical analysis of proteins. In the past antibodies with specificity toward the phosphorylated forms of proteins were produced serendipitously. Using techniques such as those described in this unit, such antibodies may now be produced by design. Whereas phosphorylated holoproteins might in general be poor candidates for immunogens in the production of such antibodies as a result of their susceptibility to dephosphorylation, it has been suggested that short phosphopeptides are relatively resistant to phosphatases (Czernik et al., 1991), thereby providing a better chance of success.

A dramatic advance in the analysis of protein tyrosine phosphorylation and the regula-

tion of signal transduction pathways by such phosphorylation occurred with the production of polyclonal and monoclonal anti-phosphotyrosine antibodies (Frackelton et al., 1983; Ross et al., 1981; reviewed in Stern, 1991). These antibodies proved capable of recognizing phosphorylated tyrosine residues in the context of virtually any flanking peptide sequence, and were shown to be exquisitely specific in the requirement for a phosphorylated tyrosine yet at the same time remarkably promiscuous in their acceptance of any peptide sequence. Techniques using polyclonal and monoclonal anti-phosphotyrosine antibodies have supplanted the standard methodology of metabolically labeling proteins with ³²P_i, which was cumbersome, time-consuming, hazardous, and of relatively lower sensitivity.

Antibodies that recognize a specific tyrosyl-phosphorylated peptide as described in this unit represent a marriage of anti-phosphotyrosine technology with the stringent sequence-dependent specificity of conventional antibodies (Roussel, 1990; Bangalore et al., 1992; Epstein et al., 1992; DiGiovanna and Stern, 1995). Conceptually, they may be thought of either as anti-phosphotyrosine antibodies that have strict sequence specificity, or as conventional antibodies possessing the additional specificity of phosphorylation dependence.

Anti-phosphotyrosine antibodies have been most useful in the analysis of tyrosine phosphorylation of proteins in a technique using a combination of immunoprecipitation and immunoblotting (known as the "IP-western" method). In this procedure, a protein typically is immunoprecipitated either with conventional anti-protein antibody or with anti-phosphotyrosine antibody, then immunoblotted with whichever of these two antibodies was not used for the immunoprecipitation. Because anti-phosphopeptide antibodies recognize the cognate protein, but only when it is phosphorylated, they are capable of performing both functions in a single step—i.e., either an immunoprecipitation alone or an immunoblot alone is sufficient to supply the desired information.

The most useful functions for such antibodies are likely to be those that in fact are not possible using traditional reagents. For example, using anti-phosphopeptide antibodies, it is possible to identify and isolate distinct phosphorylated species of a phosphoprotein containing multiple phosphorylation sites. Thus, phosphorylation at each site can be examined independently, and a preparative separation of individual phosphospecies of a holoprotein can be achieved. Another unique application is analysis of the abundance and phosphorylation state of individual proteins *in situ* in preparations of cells or tissues. Because phosphorylation is a major mode of regulation of protein function, the phosphorylation state is often an indicator of the functional status of a protein. The mere identification of a protein in a cell or tissue specimen gives no indication of its functional status. The ability of anti-phosphopeptide antibodies to demonstrate the phosphorylation state, and by extrapolation the functional state, of a single protein with high specificity places these reagents in a unique class. Hence, identity and functional status may be probed simultaneously using one simple assay. In tissue sections, one may probe to determine

whether a particular protein is present, and, if present, in what functional state it is found. The authors have employed this strategy in immunohistochemical staining of formalin-fixed, paraffin-embedded human breast tumors using antibody to the phosphorylated form of the receptor tyrosine kinase Neu (DiGiovanna and Stern, 1995), and were able to demonstrate that Neu is phosphorylated, and therefore functionally active, in only a subset of the tumors that overexpress this protein.

Anti-phosphopeptide antibodies possess unique properties that render them capable of performing functions not possible with conventional reagents. From the technical considerations regarding their production, it would follow that antibodies with specificity for the non-phosphorylated state should also be achievable, and the production of such antibodies has also been reported (Nairn et al., 1982; Roussel, 1990; Czernik et al., 1991; Epstein, 1995; Tzartos et al., 1995; Kawakatsu et al., 1996).

Critical Parameters

In the production of polyclonal anti-phosphopeptide antibodies, a major challenge is the depletion of cross-reactivity. The first consideration in achieving this is the anticipation of potential cross-reacting proteins in the planning phase of the procedure. Cross-reactivity is most likely to occur when the target protein is a member of a family of closely related homologous proteins—e.g., of the subfamilies of tyrosine protein kinases. The authors have produced a monoclonal anti-phosphopeptide antibody with specificity for the Tyr-1248 autophosphorylation site of Neu (DiGiovanna and Stern, 1995). A homologous site exists in the epidermal growth factor receptor (EGFR). The antibody produced in the authors' laboratory does not cross-react with the homologous EGFR site in spite of having identical residues in seven of the 14 amino acids of the peptide sequence (five of which are N-terminal to the phosphotyrosine, one C-terminal, and the phosphotyrosine itself). Thus, specificity is achievable even with at least up to 50% identity. Nevertheless, such a close relationship among the phosphorylation sites of different proteins highlights the importance of considering all known related proteins.

The second consideration in depleting cross-reactivity is the quantity of cross-reactive antibodies in a given aliquot to be purified relative to the capacity of the peptide columns used for the negative selection. Because of the expense of the peptides, the authors have gen-

erally prepared small columns (with 2- to 3-ml bed volumes) and passed the sera over the columns multiple times, empirically determining the number of passes necessary to quantitatively deplete cross-reacting material. In these columns, 3 μmol of ligand is coupled per milliliter of Affi-Gel 10. The manufacturer states that the resin contains ~ 15 μmol active ester per milliliter gel, and that the gel has a capacity for 35 mg of protein or 15 to 20 μmol of a low-molecular-weight ligand per milliliter gel. The BSA and phosphotyrosine columns are relatively inexpensive to produce; thus, a single large column of each is practical and sufficient.

Because the authors elute the antibodies from the positive-selection affinity column in strongly chaotropic solutions that are potentially deleterious to the stability of the antibody (3 M NaSCN or 3.5 M followed by 4.5 M MgCl_2), care is taken to collect the fractions in preprepared dialysis tubing so that they may be immediately placed into the PBS dialysate, thus minimizing the time that the antibodies are exposed to the eluting solutions. Although MgCl_2 is thought to be “gentler,” the authors have found that gravity-driven flow rates from phosphopeptide columns eluted with this salt quickly become extremely slow, possibly because of precipitation of the salt in the column or an interaction of the Mg^{2+} ion with the phosphate groups. Thus use of NaSCN is preferred, and this salt appears to permit recovery of comparable activity.

In the production of monoclonal anti-phosphotyrosyl peptide antibody, which the authors have carried out once, a major technical hurdle was the low frequency with which clones of the desired specificity were produced (DiGiovanna and Stern, 1995). In that work, in which antibody was produced to the phosphorylated form of the receptor tyrosine kinase Neu, >1200 hybridomas (obtained from a single fusion) were screened to obtain a single clone that satisfied all requirements. The authors found 68 candidate clones that recognized the cognate phosphopeptide, of which only 20 were unreactive toward the cognate nonphosphopeptide. Of those, seven cross-reacted with an unrelated phosphopeptide (i.e., exhibited indiscriminant phosphotyrosine activity), and of the remainder three cross-reacted with the homologous EGFR. The remaining ten were subcloned by limiting dilution, after which only five continued to produce antibody. Of these five, only one reliably detected the phosphorylated holoprotein in immunoblots and immunoprecipitations. Thus, the “hit rate” for an antibody that

satisfied all requirements was <1 in 1000 clones. As this is the only monoclonal anti-phosphotyrosyl peptide antibody produced by the authors to date, it is impossible to be sure whether this low frequency will be a general phenomenon. It is crucial to devise a convenient and reliable ELISA assay for such extensive screening. Other assays may require extensive optimization. For example, the monoclonal antibody produced in the above project performed poorly in immunoblotting when any detection system other than a very sensitive chemiluminescence-based assay was used. This was possibly due to a low affinity of the antibody for the cognate protein. In addition, extensive optimization, particularly aimed toward enhancing the strength of the signal, was required for use of the antibody in immunohistochemistry. Finally, it is important to use different carrier proteins for the immunizing conjugate and the ELISA conjugate, so that antibodies to the carrier used for the immunization will not give positive reactions in the ELISA. Some authorities also advocate using different cross-linking agents, as antibodies against the cross-linker itself could theoretically be generated as well (Doolittle, 1986; Czernik et al., 1991).

Troubleshooting

In the production of polyclonal anti-phosphopeptide antibodies, the most common unfavorable outcomes are persistence of cross-reactivity, loss of specific reactivity, and poor yield. Although it may be tempting, in the interest of saving time, to perform the entire purification and postpone all analyses until the end (a reasonable course of action once a scheme is known to work), it may be prudent, especially with a new protocol, to analyze the serum at each step. For example, the authors have produced antibody to phosphorylated EGFR (M. DiGiovanna, M.A. Lerman, and D. Stern, unpub. observ.). The crude antiserum cross-reacted with the related receptors Neu and HER-4, which had been anticipated. The purification was monitored by probing, after each column, an immunoblot consisting of four lanes containing lysates from cells overexpressing (1) phosphorylated EGFR, (2) nonphosphorylated EGFR, (3) phosphorylated Neu, and (4) phosphorylated HER-4. In following the depletion of cross-reactivity, such stepwise analysis demonstrates whether undesired cross-reactivity has been eliminated or whether further passes through a particular negative-selection column are necessary.

If loss of specific reactivity is also a problem, stepwise analyses can also demonstrate where the loss is occurring. Loss of specific reactivity can occur for three major reasons. The first possible reason is that the antibody is not stable over the course of the purification, which may be the case if the procedure is very prolonged. If this is suspected, the pace of the purification may be hastened or the procedure may be carried out at 4°C instead of room temperature. With prolonged purifications, it is especially important to include sodium azide in the PBS used to dilute the serum, to prevent microbial growth. Activity may also be lost nonspecifically as a result of the multiple manipulations in a protocol requiring multiple columns and/or multiple passes through each column. In this case, fraction analysis may indicate which columns are absolutely essential, and also indicate the minimum necessary number of passes through each column. Also, creating larger columns with greater capacity should reduce the number of times the serum will need to be passed over each to deplete cross-reactivity. Finally, specific activity may be lost as a result of the presence in one of the negative-selection columns of a cross-reacting peptide that is so similar to the peptide of interest that essentially any antibody that recognizes the cognate phosphopeptide will recognize this phosphopeptide as well. In this last theoretical scenario, ultimate unique specificity may not be achievable.

The columns used in the purifications can be regenerated and used repeatedly if stored at 4°C in sodium azide-containing buffer to prevent microbial contamination and consequent damage to the matrix. A theoretical consideration is that with repeated use, phosphatases in serum may eventually cleave enough phosphate groups from the peptide moieties on the column to render it ineffective as a phosphopeptide column. Thus, it may be prudent to periodically prepare fresh columns. An alternative would be to add phosphatase inhibitors to the serum prior to purification. Similarly, proteases in serum may eventually cleave peptides from the resin, and this may be prevented by adding protease inhibitors to the serum.

As discussed in Critical Parameters, the main technical hurdle in the production of monoclonal antibodies appears to be the large number of clones that it may be necessary to screen to find one with the desired reactivity. Here, time and persistence are the primary defenses. Another obstacle may be the failure of hybridoma cells to continue to produce antibody upon subcloning, which is likely due to

the continuing genetic instability of hybridomas at early passage. If the parental hybridoma colony has been expanded and frozen prior to subcloning, this will serve as a potential source for additional subclones, although it is possible that these additional daughter clones may also be nonproducing. The use of the final monoclonal antibody in a variety of immunoassays is more likely to require potentially extensive optimization of protocols and higher concentrations (because of lower affinities) than the use of polyclonal antibodies.

Anticipated Results

In the production of polyclonal antibodies, the authors have obtained yields of ~1 mg of purified anti-phosphopeptide antibody from a single 15-ml serum sample (M. Digiovanna, M.A. Lerman, and D. Stern, unpub. observ.). These antibodies have been used in the immunoprecipitation of phosphoproteins from both denaturing and nondenaturing solutions. They have also been used in immunoblotting, in immunofluorescence on fixed cultured cells, and in immunohistochemistry on formalin-fixed, paraffin-embedded tissue sections. Specificity can be demonstrated when such assays are inhibited by cognate phosphopeptide but not by cognate nonphosphopeptide or unrelated phosphopeptides.

Through the production of monoclonal antibodies a permanent supply of a uniform reagent is ensured. Many milligrams can be obtained from the ascites produced by a single mouse. The authors have used such an antibody in all of the same assays as described above for polyclonal antibodies. More extensive optimization of protocols is likely to be required with monoclonal antibodies, and these may be expected to have a lower affinity than typical polyclonal antibodies. Isotype analysis can easily be performed by use of commercially available kits (Pierce or Sigma).

Time Considerations

For both protocols, the principal time-consuming step is the production of an immune response in the immunized animals. For the production of polyclonal antibodies, the authors have obtained high titers of reactive sera as early as 6 to 8 weeks in animals that have been boosted every 2 weeks. Later bleeds with continued boosts have yielded even higher titers. Once adequate bleeds have been obtained and the necessary affinity columns prepared, purification can generally be carried out within 1 week, with the exact length of time depending

upon the number of columns and the necessity of analyzing the product after each step.

In the production of monoclonal antibodies, the authors have harvested splenocytes at ~2 months from the initial immunization, with several boosts in between. The screening of hybridoma supernatants can take 1 to 2 weeks, and their expansion and subcloning several weeks more.

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CHAPTER 17

Macromolecular Interactions in Cells

INTRODUCTION

The capacity of proteins to perform different functions within cells is essentially conferred by information provided within the proteins themselves. That is, the function of the protein is determined by the one-dimensional sequence of amino acids encoded within the mRNA from which the protein is translated. However, in order for proteins to accomplish the complex tasks required for cell viability, this information must direct not only the three-dimensional folding and post-translational processing of the protein itself, but also the interaction of the protein with other molecules of the cell. These interactions may include binding to small ligands, enzyme substrates, nucleic acids, or other proteins. It is therefore not surprising that elucidating the interactions of proteins with other cellular components has become a major task in transforming molecular biological knowledge of protein sequences into full understanding of protein functions within cells.

Many traditional methods for detecting protein-protein interactions have relied upon biochemical co-purification of tightly associated protein complexes. In addition to conventional column chromatography, rapid affinity methods for co-purification have been developed (*UNIT 17.5*). These methods typically rely upon purification using engineered expression constructs producing proteins with tags that allow retention on an affinity matrix or upon purification by immunoprecipitation (*UNIT 7.2*). After isolation of the protein of interest through these methods, associated proteins can be detected by protein staining (*UNIT 6.6*) of the purified fraction. Alternatively, the interaction of known proteins can be assayed through Western blotting (*UNIT 6.2*), and novel interactions can be documented through mass spectrometry and bioinformatics means (*UNIT 17.5*).

Alternative methods to identify protein-protein interactions through modified ligand blotting protocols have also been developed (*UNIT 17.2*). In such far-western blotting methods, a protein of interest is immobilized on a membrane support and probed with another non-antibody protein. This method has been particularly useful in examining the interaction between proteins within complex mixtures and in examining proteins whose insolubility or biochemical behavior make them ill-suited for traditional approaches.

A number of methods have been developed for broad-based genetic screening to find novel interactions. The two-hybrid system or interaction trap screening (*UNIT 17.3*) allows evaluation of peptides encoded within large cDNA libraries for interactions with a protein of interest. Interactions between the protein of interest and peptides encoded within the library result in transcriptional activation that can be assayed using simple selection criteria. Moreover, these protocols can also be adapted to test specific association between two proteins for which there is a prior reason to expect an interaction, again by measuring the extent of transcriptional activation. Smaller epitopes involved in protein-protein interactions can be found using phage-display combinatorial peptide libraries (*UNIT 17.4*). In this method, peptides are fused to the capsid proteins of the filamentous M13 bacteriophage, such that they remain exposed after the assembly of viable phage particles. Peptides are selected by affinity of the phage particles to immobilized proteins of interest. Through multiple rounds of affinity selection, it is possible to isolate phage expressing fused peptides that are characteristic of optimal ligand preferences or protein interaction

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modules. Peptides found through this method either can be utilized directly for study of the protein of interest, or they can be used to search databases in order to identify potential protein-protein interaction partners.

Once interaction partners have been identified, a number of biophysical methods offer the capacity for detailed characterization of binding affinities and kinetics. One attractive method that has become increasingly valuable during the past decade is the use of optical biosensors (*UNIT 17.6*). Optical sensors provide precise measurement of the refractive index near the surface of a sensor. When a protein of interest is immobilized on the sensor, its interactions with partners in the solution above can thus be monitored. Analysis of steady-state interactions using biosensors can provide equilibrium binding constants over a wide range of affinities. If set up appropriately, optical biosensors can also provide accurate real-time measurements of association, from which chemical on- and off-rates can be deduced.

Precise information about the thermodynamics of a broad variety of macromolecular interactions can be obtained through isothermal titration calorimetry (ITC; *UNIT 17.8*). Changes in macromolecular interactions, for instance the binding of two proteins, are accompanied by the release (exothermic reaction) or absorption (endothermic reaction) of heat. The heat thus produced or consumed is monitored in ITC using a heat-flux calorimeter, which measures the amount of power required to maintain a constant temperature difference between the reaction cell and a reference cell. This measurement can be used to calculate the Gibbs energy, enthalpy, entropy, and heat capacity of the reaction under study.

The obvious limitation of the methods discussed above is that they assay protein interactions outside of the normal context of intact cells. The absence of a cellular context frequently causes a loss of spatial or temporal resolution regarding how macromolecular interactions occur *in vivo*. Chromatin immunoprecipitation (*UNIT 17.7*) is frequently used to monitor the association between proteins and DNA within intact cells. In this method, native interactions are preserved by formaldehyde crosslinking of protein-DNA and protein-protein complexes. After the DNA is fragmented into shorter pieces, complexes are precipitated with antibodies against proteins of interest, resulting in co-precipitation of the DNA sequences that are directly or indirectly associated with individual proteins. The cross-links can then be chemically reversed, allowing analysis of the DNA sequences by direct methods such as PCR. Recently, methods have also been developed to assay protein-protein interactions directly within cells through the use of fluorescence resonance energy transfer (FRET; *UNIT 17.1*). FRET is a process through which a donor fluorophore transfers energy to an acceptor fluorophore through dipole-dipole coupling. Protein-protein interactions can be detected through this photophysical process by covalently coupling donor and acceptor fluorophores with appropriate excitation and emission spectra to two proteins of interest. Due to the sixth-order distance dependence of the FRET process, efficient FRET occurs only when the fluorophores are in immediate proximity. FRET can be measured effectively by donor quenching through acceptor photobleaching. These powerful new techniques will complement more traditional biochemical methods and extend the study of interactions between molecules in their true biological context.

In an era of rapidly expanding databases of nucleic acid and protein sequences, a true appreciation of how macromolecules function within cells will require not only data about the expression of these molecules, but also knowledge about their localization and relationship to other cellular components. Thus, techniques for determining how macromolecules recognize and interact with each other will provide a key element of cell biological studies, essential for opening the door between genomic information and a deeper understanding of biology.

Mary Dasso

Imaging Protein-Protein Interactions by Fluorescence Resonance Energy Transfer (FRET) Microscopy

Specific protein-protein interactions, whether induced by covalent protein modifications or not, are generally considered to mediate cellular signaling and function. Detection of these processes has long been restricted to bulk biochemical methods such as immunoprecipitation (UNIT 7.2) and immunoblotting (UNIT 6.2). These approaches have proven invaluable, e.g., in uncovering the major signal transduction pathways by delineating the hierarchy of protein-protein interactions and kinase-substrate relationships in the different phosphorylation cascades. However, immunoblotting techniques lack spatial information and the interactions that are detected depend on the stability of the complex during the experimental conditions that exist outside the cell in homogenates. Detection of proteins using immunofluorescence provides spatial information on the micrometer scale, and it is therefore not possible to infer protein-protein interactions.

Limited information on the phosphorylation status of proteins can be obtained by the application of phospho-specific antibodies against specific phosphorylated residues in a given protein (see UNIT 14.2). The use of these antibodies is, however, restricted by availability and specificity. Only a small number of antibodies against phosphoproteins exist and phosphotyrosine residues have proven to produce the most specific antibodies thus far, leaving a large number of modifications undetectable by microscopy.

This unit describes the preparation and execution of a typical fluorescence resonance energy transfer (FRET) experiment. The following procedures will specifically refer to the use of a GFP-tagged protein as donor and Cy3-labeled antibodies as acceptor. As an example, covalent modification by tyrosine phosphorylation of the epidermal growth factor receptor (EGFR) is determined by FRET between C-terminal GFP-tagged EGFR and Cy3-labeled anti-phosphotyrosine antibodies (Wouters and Bastiaens, 1999). FRET is measured by release of donor quenching through acceptor photobleaching. An advantage of this method is the convenient setup; it does not require specialized equipment but can be performed using common microscopy equipment, preferably a confocal laser scanning microscope (Bastiaens and Jovin, 1996; Bastiaens et al., 1996; Wouters et al., 1998). The method is quantitative, and by simple relationships, a FRET efficiency is obtained. The sample preparation described in this unit is identical for other FRET microscopy techniques or other donor-acceptor pairs. As an illustration, the result of a more advanced FRET measurement using fluorescence lifetime imaging microscopy (FLIM), which is based on the change in GFP fluorescence lifetime (see Background Information), is also included (Bastiaens and Squire, 1999; Ng et al., 1999; Wouters and Bastiaens, 1999).

This unit describes FRET microscopy based on release of quenched donor fluorescence after acceptor photobleaching (see Basic Protocol), microinjection of reagents into the nucleus or cytosol (see Support Protocol 1), and labeling antibodies with Cy3 (see Support Protocol 2).

FRET MICROSCOPY OF FIXED CELLS

A number of quantitative FRET microscopy techniques are available at this time. A technique based on the release of quenched donor fluorescence after acceptor photobleaching will be described in this unit. This technique requires a pixel-by-pixel reference image to be created by photobleaching of the acceptor through continuous illumination at the absorption maximum of the acceptor. The time scale of the pho-

BASIC PROTOCOL

Molecular Interactions in Cells

17.1.1

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photobleaching process is on the order of minutes. In a lifetime-based FRET approach, the photobleaching step is used at the end of a time-lapse sequence of measurements to introduce an intracellular reference in live cells. In the donor intensity approach (unquenching), described here in detail, the time scale of photobleaching restricts the technique to fixed cells but has the advantages of being relatively simple and quantitative.

Materials

Cells of interest
Plasmid for GFP-tagged protein
Transfection reagent (e.g., Fugene 5 from Boehringer Mannheim, Lipofectin from Life Technologies, Effectene from Qiagen, or Superfect from Qiagen)
Serum-free medium
Low-background fluorescence CO₂-independent medium (Life Technologies, or see recipe)
Phosphate-buffered saline (PBS; see recipe), pH 7.4
4% (w/v) formaldehyde fixative solution (see recipe)
Quench solution: 50 mM Tris·Cl (pH 8.0)/100 mM NaCl (*APPENDIX 2A*)
0.1% (v/v) Triton X-100 in PBS
Antibody (e.g., PY72 monoclonal anti-phosphotyrosine antibody) labeled with Cy3 (see Support Protocol 2)
1% (w/v) bovine serum albumin (BSA, fraction V) in PBS
Mowiol mounting medium (see recipe)
6- and 12-well tissue culture plates
Coverslips
Microscope slides
Confocal laser scanning microscope (e.g., Zeiss LSM 510), equipped with argon (488 nm) and He/Ne (543 nm) lasers selected by the HFT 488/543 double dichroic filter, GFP fluorescence selected by the NFT 545 dichroic and BP 505-530 emission filter, and Cy3 fluorescence selected by the LP560 emission filter
Imaging software package (e.g., NIH-image or IPLab Spectrum from Scanalytics)
Additional reagents and equipment for transfection of mammalian cells (*APPENDIX 3A*)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

Prepare cells

1. Seed the cells onto culture dishes containing coverslips.

For live-cell FRET experiments, seed the cells onto glass-bottom MatTek culture dishes.

The cells should be adherent and grow in a monolayer.

2. Transfect cells with the plasmid for GFP-tagged protein of interest using calcium phosphate precipitation, lipofection, or the activated dendrimer reagent, Superfect.

For cells that are difficult to transfect, due to resistance to transfection (e.g., primary cell lines), or due to cytotoxicity of the transfection reagent, the DNA for the GFP-tagged protein can be introduced by nuclear microinjection (see Support Protocol 1). The latter method is also particularly useful when the expression levels of the protein of interest have to be controlled accurately.

3. Grow the cells, typically 1 to 2 days post transfection, until expression levels of the GFP-tagged protein are high enough to detect with fluorescence microscopy. When needed, starve the cells overnight with serum-free medium to make them semi-quiet.

For cells that undergo apoptosis upon serum deprivation, lower the serum concentration in the medium to 0.5%.

4. On the day of the experiment, transfer the coverslips to a 12-well tissue culture plate.

Subject the cells to the desired experimental conditions (e.g., growth factor/hormone stimulation, or incubation with drugs or inhibitors).

Fix cells

5. Wash the cells with ice-cold PBS, aspirate, and add 1 ml 4% formaldehyde fixative solution. Allow cells to fix at room temperature for 10 min.

The common alternative fixation protocol, where cells are incubated in -20°C methanol for 5 min, is not advised. Strictly speaking, this treatment does not fix the tissue, but precipitates the cellular proteins. Protein-protein interactions are therefore likely to be affected.

6. Quench excess fixative with quench solution by briefly washing the cells, then changing to fresh quench solution and incubating 5 min at room temperature.

In a first short wash, the excess fixative is removed. In a second 5-min incubation, the remaining aldehyde groups are allowed to react with the primary amino group on the Tris molecule. Alternative, equally effective, quench solutions are 0.1 M hydroxylamine or 0.1 M glycine.

7. Permeabilize cellular membranes by incubating 5 min with 0.1% Triton X-100 to allow penetration of acceptor-labeled molecules into fixed cells.

In this treatment, a compromise is made between optimal morphology and permeabilization. A milder detergent treatment with 0.1% saponin in PBS or a harsher permeabilization with -20°C methanol for 5 min can be considered if morphology or permeabilization, respectively, is compromised.

8. Wash the cells with PBS to remove the permeabilization solution.

Add antibody

9. Dilute Cy3-labeled antibodies appropriately in 1% BSA/PBS. Incubate the coverslips with appropriately diluted Cy3-labeled antibodies for 1 hr, at room temperature.

Typical concentrations are 0.1 to 10 $\mu\text{g/ml}$, but it is advised that a titration series be performed to determine the antibody concentration where epitope binding saturates.

To minimize the amount of antibody needed, press a sheet of Parafilm to the bench with some water; place 25- μl drops of antibody solution on the Parafilm, place the coverslips cells-down on these drops, and incubate 1 hr. To facilitate handling of the coverslips at the end of the 1-hr incubation, place a pipet to the edge of a coverslip and add ~ 100 μl PBS underneath it. This will lift the coverslip so that it can be easily picked up with jeweler's forceps.

10. Transfer the coverslips to 6-well tissue culture plates and wash four times, each time with 3 ml PBS, to remove excess antibody.
11. Blot off excess PBS with tissue and mount the coverslips on slides with ~ 10 μl Mowiol mounting solution. Allow Mowiol to harden overnight at 4°C before imaging.

If necessary, cells can be imaged after a short drying period of 1 hr if the coverslip is attached to the slide by painting the edge of the coverslip with molten agarose or rubber cement at four points. Do not use nail polish since this has been shown to quench GFP fluorescence.

Examine cells and calculate results

12. View the specimen on a confocal microscope using a 63× or 100× oil-immersion objective. Acquire an image in the GFP channel (excitation, 488 nm; emission, NFT 545, BP505-530). Either take an image of the entire field of vision or select a region of interest containing the cell that is to be imaged.

GFP fluorescence is selected by the NFT 545 dichroic and BP 505-530 emission filter.

Do not use the full dynamic range of the detector, since unquenching will result in added fluorescence. Depending on the make of the confocal microscope, make sure that a second image can be made at exactly the same location. Do not adjust the settings (i.e., pinhole size, contrast, brightness, laser power, or averaging) for the GFP channel.

This acquisition provides the FRET-quenched donor image (F_{DA}), since the acceptor is present throughout the cell, causing FRET with the GFP donor.

Minimize GFP photobleaching at this point by limiting the illumination.

13. Change to the Cy3 channel (excitation, 543 nm; emission, LP560) and take an image of the cell, minimizing Cy3 photobleaching. Select a portion of the cell in which the FRET efficiency is to be determined.

Cy3 fluorescence is selected by the LP560 emission filter.

This region is where the acceptor will be photobleached, thereby revealing the unquenched donor intensities enabling FRET calculation.

Consequently, any portion of the cell where the acceptor is not photobleached serves as control [i.e., $1 - F_{DA}/F_{DA} = 0$]. This area in the same cell will provide an essential control to judge the effects of photobleaching GFP, lateral movement, or focal mismatch between the two consecutive donor images.

14. Photobleach the selected acceptor region by repeated scanning with the 543-nm He/Ne laser line at full power. Follow the progress of photobleaching by monitoring the intensities of the respective images (emission: NFT 545, BP505-530). Continue until there is no more discernible Cy3 intensity.

Depending on the staining intensity and the area that is bleached, this will typically take 1 to 20 min.

When selecting the photobleaching region on the Zeiss LSM 510 confocal microscope, crop the acceptor image to this region (and note the zoom settings to return to the original image after bleaching) instead of selecting a region of interest (ROI). In the latter option, the entire area will be scanned but the laser will only switch on in the ROI. Consequently, the duty cycle is low and bleaching will take considerably longer.

15. Return to the GFP channel and make the second acquisition, using identical settings and location of the prebleached image.

This provides the F_D reference in the region where the acceptor was photobleached.

16. To calculate the FRET efficiency in the bleached area, use an appropriate imaging software package. Subtract the prebleach donor image from the post-bleach donor image (i.e., $F_D - F_{DA}$). Divide this image by the postbleach donor image: $(F_D - F_{DA})/F_D$; this is identical to $1 - (F_{DA}/F_D) = E$, the image of FRET efficiencies.

A software package capable of performing the abovementioned image processing is NIH Image. This package is freely available from <http://rsb.info.nih.gov/nih-image/> and ver-

sions are available for Apple Macintosh, Windows (Scion image) and even a Java version is available that runs on any platform (Image J). A more versatile commercial package is IPLab Spectrum.

Image arithmetic for calculating the FRET efficiency should be performed on a region of interest obtained by thresholding the images in order to prevent the amplification of background noise. Therefore, thresholds should be chosen in such a way that the background is omitted in the calculation. Accuracy can be improved by subtracting the average background intensity from the images before calculating the FRET efficiency.

NUCLEAR AND CYTOSOLIC MICROINJECTION

The following is a generic protocol for introduction of DNA or labeled proteins into a cell. DNA is microinjected directly into the nucleus to achieve controlled and high expression of the protein of interest. Labeled proteins are injected into the cytosolic region next to the nucleus since the cell is at its highest here, facilitating injection.

Materials

Cells of interest, cultured in MatTek glass-bottom 35-mm dishes (MatTek)
DNA (e.g., human EGFR cDNA in the Clontech pEGFP-N1 expression vector;
APPENDIX 3A)
HPLC-grade water
Millex-GV4 0.22- μ m filtration unit
GELoader tips (Eppendorf)
Needles for microinjection (e.g., Femtotip from Eppendorf)
Microinjector (e.g., Eppendorf model 5244)
Micromanipulator (e.g., Eppendorf model 5170)
Inverted microscope with 10 \times and 40 \times air objectives
Additional reagents and equipment for preparation of DNA (*APPENDIX 3A*)

1. Prepare DNA, to be microinjected, of the highest possible quality.

In the authors' experience, double cesiumchloride-banded DNA and DNA purified by Qiagen ion-exchange resin (Qiaprep midi/maxiprep columns) perform equally well.

Cy3-labeled protein (e.g., antibody against protein of interest; see Support Protocol 2) may also be microinjected.

- 2a. *For optimal expression a few hours after microinjection:* Dilute DNA in HPLC-grade water to 100 μ g/ml.
- 2b. *For expression overnight:* Dilute DNA in HPLC-grade water to 1 μ g/ml.
3. Clear the DNA solution to prevent blocking of the glass needle during microinjection as follows. Place a 0.22 μ m Millex filtration unit in a 0.5-ml microcentrifuge tube and place the entire unit in a 1.5-ml microcentrifugation tube to enable centrifugation. Filter 10 μ l of the DNA solution by microcentrifuging 1 min at maximum speed, room temperature.

Since these membranes have low-protein-binding characteristics, they can also be used for clearing Cy3-labeled proteins (see Support Protocol 2).

The recovery of Cy3-labeled antibodies when cleared this way is also generally very high. Alternatively, microcentrifuge the Cy3-labeled protein for 20 min at maximum speed. Sacrifice a small amount of solution to prevent disturbing the pellet of aggregated protein.

SUPPORT PROTOCOL 1

4. Load 2 μ l of DNA solution (or Cy3-protein solution) using GELloader tips into the capillary glass needle of the microinjector.

Commercially available needles (Femtotip from Eppendorf) can be used. These needles fit directly into the needle holder of the Eppendorf microinjection device. Carefully remove the pipet tip that protects the needle. In the authors' experience, this is most easily performed by holding the needle pointing downwards and loosening the tip by rotation until it falls to the ground. If access is available to a needle-pulling device, make sure that the diameter of the needle opening is $\sim 0.25 \mu\text{m}$. The diameter of the needle opening can be estimated using a simple syringe-operated micropipet bubble meter (Clark Electronic Instruments) by measuring the air pressure required to expel air bubbles from the pipet into a liquid (Mittman et al., 1987).

5. On an inverted microscope, microinject the DNA solution (or Cy3-protein solution) into the nucleus (or perinuclear cytosol) of cells grown in MatTek culture dishes.

Typical settings are: 0.3 sec, 150 to 400 hPa injection pressure with 20-hPa back-pressure to prevent medium from entering the needle.

The injection pressure may be varied according to the needle opening and cell type.

No major movement of the nucleus (or cell organelles) should be observed. A visual indication for excessive pressure is the separation of the nucleus from the surrounding cellular material (i.e., light ring around the nucleus) and leakage into the cytosol, visible by movement of the cellular organelles.

Restrict the microinjection procedure to a maximum of 10 min. In the authors' laboratory, microinjection is performed at room temperature in normal CO_2 -dependent medium. After 10 min, the medium starts to acidify significantly (i.e., purple medium). CO_2 -independent (or HEPES-buffered) media can be used for longer periods.

SUPPORT PROTOCOL 2

PROTEIN LABELING WITH Cy3

Proteins are labeled on unprotonated free amino groups (i.e., α -amino terminus or ϵ -amino groups on lysine side chains) by the succinimide esters of the fluorescent sulfoindocyanine (Cy) dyes (also see *UNIT 16.5*). Alkaline labeling conditions ensure deprotonation of amino groups. Cy-dyes are water soluble and have high extinction coefficients, making them particularly useful for sensitive detection of proteins with minimal disturbance of protein function. The following protocol describes the labeling of antibodies with Cy3, a suitable donor for GFP in a FRET experiment, and also explains how to remove stabilizing compounds (e.g., gelatin, BSA), which are often added to prolong shelf life, as these contain amino groups that would compete with the labeling reaction.

Materials

- Antibody (PY72 monoclonal anti-phosphotyrosine antibody)
- 1 M Tris-Cl, pH 8.0 (*APPENDIX 2A*)
- 10 mM and 100 mM Bicine/NaOH, pH 8.0
- 100 ml citric acid/NaOH, pH 2.8
- 1 M Bicine/NaOH, pH 9.0
- 1 M NaCl (*APPENDIX 2A*)
- Labeling buffer: 100 mM Bicine/NaOH (pH 8.0)/100 mM NaCl
- Cy3.29-OSu monofunctional sulfoindocyanine succinimide ester (Amersham Pharmacia Biotech)
- Dimethylformamide (DMF) dried by addition of 10 to 20 mesh 3- \AA pore diameter molecular sieve dehydrate (Fluka)
- 1-ml Protein G HiTrap columns (Amersham Pharmacia Biotech)
- Centricon YM30 concentrators (Amicon)

Biogel P6DG Econopac prepacked size-exclusion columns (5.5×1.5 -cm, ~10 ml; Bio-Rad)

1-ml and 10-ml syringes with HPLC Luer-Lok fitted tubing

Additional reagents and equipment for spectrophotometric protein determination
(APPENDIX 3B)

Prepare antibody solution

1. Resuspend antibody to 1 mg/ml in PBS.

Excess stabilizing agents containing free amino groups (e.g., BSA and gelatin) in commercially available antibody preparations compete for the labeling reagent and have to be removed by Protein A or Protein G affinity chromatography (UNIT 16.3). If the protein solution to be labeled is free of additional amino groups, proceed directly to step 10. A number of suppliers of antibodies, e.g., Transduction Laboratories and New England Biolabs, can provide their products free of these compounds. Request that the antibodies be provided at 1 mg/ml concentration in PBS.

2. Prepare a syringe-operated 1-ml protein G HiTrap column. Equilibrate column with 10 ml PBS at a maximum flow of 4 ml/min.

All fluid handling is performed manually using appropriately sized syringes. These columns are easy to use and result in minimal loss of protein.

A number of subclasses of IgG molecules do not bind to protein A. When using protein G or A chromatography, make sure that the antibodies are compatible (see Table 7.2.1).

3. Add 0.1 vol 1 M Tris-Cl, pH 8.0, to the antibody solution.

Commercial antibody solution is typically 0.5 ml at 0.1 mg/ml IgG.

4. Load antibody solution onto the column and wash column with 10 ml of 100 mM Bicine/NaOH, pH 8.0. Collect run-through.

5. Wash column with 10 ml of 10 mM Bicine/NaOH, pH 8.0. Collect run-through.

6. Elute column with 5 ml of 100 mM citric acid/NaOH, pH 2.8, and collect 0.5-ml fractions (i.e., 8 drops) in 1.5-ml microcentrifuge tubes containing 100 μ l of 1 M Bicine/NaOH, pH 9.0, to neutralize the pH. Mix immediately and store on ice.

7. Determine the A_{280} of the eluted fractions using a spectrophotometer (APPENDIX 3B) and pool the fractions that contain protein.

Under the given conditions, the protein will typically elute in the first four fractions.

8. Add 0.1 vol of 1 M NaCl. Concentrate the solution in a YM30 Centricon to ~200 μ l by centrifuging at $5000 \times g$, 4°C.

9. Redilute to 2 ml with labeling buffer and repeat the concentration and redilution steps (steps 8 and 9).

10. Concentrate to ~50 to 100 μ l as in step 8 and collect the concentrated protein solution.

When labeling proteins from solutions containing relatively high concentrations (i.e., millimolar) of compounds that contain free amino groups (e.g., Tris, glycine, glutathione), repeat the concentration-redilution cycle more often. Each cycle dilutes the compound ~10 fold. Allow a maximum of 10% of contaminating primary amino groups, compared to the protein to be labeled.

For example, the protein concentration in a 0.5 mg/ml antibody solution is 3.3 μ M, assuming a molecular mass of 150 kDa. If this solution contains 50 mM Tris buffer (a 1.5×10^4 fold excess of free amino groups), a 1.5×10^5 -fold dilution, corresponding to 5.2 concentration cycles, is needed to reach the 10% contamination level.

When labeling proteins other than antibodies, a Bicine concentration of 50 mM is recommended in the labeling buffer. Additional compounds that are needed to maintain the function of the protein to be labeled should be included. At this point, no compounds containing free amino groups should be added. High concentrations of reducing agents are also known to inhibit the labeling reaction. It is recommended that these be included in the chromatography step following labeling to prevent these compounds from interfering with the labeling reaction. Choose the cut-off value of the Centricon carefully to ensure retention of the protein of interest.

Prepare dye

11. Reconstitute Cy3.29–OSu in 20 μ l dry DMF to give a \sim 10 mM Cy3 solution. Determine the exact concentration by measuring the absorption of a 10^4 -fold diluted solution in PBS. From the ϵ_{550} of $150 \text{ mM}^{-1}\text{cm}^{-1}$, calculate the concentration.

Cy3.29–OSu is supplied as a desiccated pellet in microcentrifuge tubes.

DMF is dried by addition of hygroscopic beads to the container.

The ϵ_{650} of Cy5 is $250 \text{ mM}^{-1}\text{cm}^{-1}$.

Perform labeling reaction

12. Determine the protein concentration of the antibody (or protein) solution to be labeled based on A_{280} reading (*APPENDIX 3B*).

Antibody concentration at $A_{280} = 1.0$ is typically 1 mg/ml. At low protein concentrations (i.e., $<0.1 \text{ mg/ml}$), or for smaller proteins (where the $A_{280} = 1.0 = 1 \text{ mg/ml}$ protein relation does not hold true), the protein concentration can be determined using the Bio-Rad Coomassie-based protein determination assay.

Antibodies are known to give a lower apparent concentration when BSA is used as a standard in this assay. Multiply the concentration found by a factor of two or use an IgG standard.

13. Slowly add a 10- to 20-fold molar excess of dye to the protein solution while simultaneously stirring the solution with the pipet tip.

The added volume of Cy3/DMF should not exceed 10% of the total volume, to prevent protein denaturation by DMF.

14. Incubate 30 min at room temperature, shaking the tube gently every 10 min.

Remove unconjugated dye

15. Remove the unconjugated dye by size-exclusion chromatography on a Bio-Rad P6DG Econopac (6-kDa exclusion size) column. Equilibrate the column with 30 ml PBS (or a buffer that is specifically formulated for the protein to be labeled).

16. Load the labeling reaction mixture onto the column and wash with a small amount (i.e., \sim 200 μ l) of PBS (or equilibration buffer). Discard the first 2.5 ml (void volume \sim 3.3 ml).

17. Collect the colored protein fraction (or 2 ml of eluate if the staining is too weak to be visible) in a YM30 Centricon concentrator and concentrate to approximately the volume of the labeling reaction.

Determine labeling ratio

18. Estimate the labeling ratio by either of the following formulas:

$$A_{554} \times M/[A_{280} - (0.05 \times A_{554}) \times 150] \text{ for Cy3}$$

$$A_{650} \times M/[A_{280} - (0.05 \times A_{650}) \times 250] \text{ for Cy5}$$

where A_x is the absorption at wavelength x and M is the molecular weight of the protein in kDa.

The extinction coefficients (in units of $\text{mM}^{-1}\text{cm}^{-1}$) 150 and 250 are for Cy3 and Cy5, respectively. These formulas assume the $A_{280} = 1.0 = 1 \text{ mg/ml}$ relationship as found for larger proteins (i.e., $>50 \text{ kDa}$) and correct for the 5% absorption of Cy3 at 280 nm. For smaller proteins or lower protein concentrations (i.e., $<0.1 \text{ mg/ml}$) that cannot be reliably estimated from the A_{280} , determine the protein concentration by the Bio-Rad protein assay and estimate the labeling ratio from:

$$A_{554} \times M/(P \times 150) \text{ for Cy3}$$

$$A_{650} \times M/(P \times 250) \text{ for Cy5}$$

where P is the protein concentration in mg/ml as determined by Bio-Rad assay.

As previously mentioned, IgG concentration obtained with BSA as standard has to be multiplied by 2.

Using mass spectrometry, the authors have observed that ~50% of the Cy3 molecules in the preparation provided by Amersham are reactive. The molar excess is thus 7.5-fold for a 15-fold excess and, the typical labeling ratio of 2 to 3 that is reached under these conditions is therefore reasonably efficient.

19. Verify covalent labeling of the antibody by SDS-PAGE of the labeled product (UNIT 6.1). Use transillumination with a UV source (302 nm) to visualize labeled protein.

Ensure that there is no fluorescence at the running front of the gel; this indicates contamination with unconjugated dye. Where applicable, verify the specific activity of the protein and compare to the specific activity before labeling to ensure that labeling did not interfere with the biological function of the protein.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Formaldehyde fixative, 4% (w/v)

Dissolve 4 g of paraformaldehyde (Sigma) in 50 ml water. Add 1 ml 1 M NaOH solution and stir gently on a heating block ($>60^\circ\text{C}$) until the paraformaldehyde is dissolved. Add 10 ml of 10 \times PBS (see recipe) and allow to cool to room temperature. Adjust the pH to 7.4 using 1 M HCl (~1 ml). Adjust to 100 ml with water and filter through a Millipore 0.45- μM filter using a syringe to remove traces of undissolved paraformaldehyde. Store up to several months at -20°C .

Low-background-fluorescence CO_2 -independent medium

Adjust the formulation of the standard medium by omitting the pH indicator phenol red, the antibiotics penicillin and streptomycin, folic acid, and riboflavin. Before use, supplement the medium with 50 mM HEPES/NaOH, pH 7.4. Store 1 to 2 months at 4°C .

Mowiol mounting medium

Mix 6 ml glycerol, 2.4 g Mowiol 4-88 (Calbiochem), and 6 ml water. Shake for 2 hr. Add 12 ml of 200 mM Tris \cdot Cl, pH 8.5 (APPENDIX 2A), and incubate at 50°C with occasional mixing until the Mowiol dissolves (i.e., ~3 hr). Filter through 0.45- μM Millipore filtration unit and store in aliquots up to several weeks at 4°C or up to several months at -20°C .

Phosphate-buffered saline, 10×

68 g NaCl
18.8 g Na₂HPO₄
2 g KH₂PO₄
H₂O to 1 liter

COMMENTARY

Background Information

Fluorescence resonance energy transfer (FRET) is a photophysical process that can be exploited to obtain information on protein-protein interactions and protein modification in addition to location (Clegg, 1996). Its usefulness lies in the ability to sense the presence of acceptor fluorophores in the extreme vicinity of a donor fluorophore with a maximum separation distance that is in the order of magnitude of single protein molecules. FRET is a radiationless process whereby an excited donor fluorophore transfers energy to an acceptor by dipole-dipole coupling (Förster, 1948). Due to the 6th-order distance dependence of the FRET efficiency, two discrete states of the fluorophores can be discriminated: exhibition of efficient FRET when donor and acceptor are in close proximity or no occurrence of FRET due to distance.

The transfer of energy (FRET) from an excited donor fluorophore to a nearby acceptor fluorophore has a number of consequences with respect to the fluorescent properties of both fluorophores and this can be exploited to measure the efficiency of this process.

In the methods presented in this unit, FRET is determined by measuring the donor fluorescence emission exclusively (Bastiaens and Squire, 1999). The acceptor fluorophore is excluded from the measurement by the choice of optical filters. As a consequence, a large excess of acceptor can be used and, when using antibodies, specificity can be sacrificed to gain higher occupancy.

In photophysical terms, FRET provides an extra channel of nonradiative decay by which the excited state of the donor fluorophore is depopulated. This results in a reduced quantum yield (Q), the ratio of emitted photons over absorbed photons. The reduction in quantum yield can be determined in two ways: (1) by the decrease in steady-state fluorescence emission; or (2) the decrease in fluorescence lifetime (τ), which characterizes the duration of the excited state of the fluorophore. The measurement of the change in quantum yield by steady-state emission has to be calibrated due to its depend-

ence on concentration and light path. In contrast, the fluorescence lifetime is proportional to Q and is independent of concentration and light path. Fluorescence lifetimes are measured by fluorescence lifetime imaging microscopy (FLIM; Lakowicz and Berndt, 1991; Gadella et al., 1993; Gadella and Jovin, 1995) and can be performed sufficiently fast to enable real-time live cell experiments. FLIM requires a specialized microscopy setup enabling high-frequency modulation of the excitation light and the gain on the detector. This technique can be combined with acceptor photobleaching to provide an internal lifetime reference, e.g., at the end of a live-cell time-lapse sequence. A detailed description of a frequency domain FLIM set-up is given by Squire and Bastiaens (1999). This type of imaging system is gradually being made commercially available by companies such as LaVision or Lambert Instruments (see *SUPPLIERS APPENDIX*).

Calibration in the intensity-based FRET method is achieved by photobleaching the acceptor to provide the unquenched donor (Bastiaens et al., 1996; Bastiaens and Jovin, 1998; Wouters et al., 1998). In this method, an exposure of the donor fluorescence emission is made (F_{DA} , fluorescence emission of donor in presence of the acceptor). Removal of the acceptor fluorophore from the sample by photobleaching enables the acquisition of an unquenched donor emission image when the exposure is taken with settings identical to those used for the original image (F_D , fluorescence emission of the donor in absence of the acceptor). This essentially recovers that part of the fluorescence intensity that is lost to FRET. A pixel-by-pixel FRET efficiency map is obtained by simple image arithmetic: $E = 1 - (F_{DA}/F_D)$. The specificity of the acceptor photobleaching techniques lies in the steep edge at the long-wavelength (i.e., red edge) of the absorption spectrum of the donor, enabling exclusive photobleaching of the acceptor. Essential to the success of this technique is that the photoproduct of the acceptor does not exhibit residual absorption and does not fluoresce at donor emission wavelengths. Cy3 meets both

criteria to act as a proper acceptor for GFP; the same is the case for Cy5 acting as acceptor for Cy3. Since photobleaching of the acceptor occurs on a minute time scale, this type of FRET determination is restricted to fixed cells.

FRET microscopy can be used to detect protein-protein interactions in single cells as if one were performing an immunoprecipitation experiment. There is, however, no need to isolate the complex or remove it from its physiological environment prior to investigation, and the experiment can be performed in living cells. A number of approaches can be followed: (1) the donor molecule can be purified, labeled with Cy3, and introduced into cells by microinjection (Bastiaens and Jovin, 1996); (2) the donor molecule can be detected by a Cy3-labeled antibody or Fab fragment (provided the antibody is highly specific) on fixed cells (Ng et al., 1999); (3) the donor protein can be fused to one of the mutants of the intrinsically fluorescent green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* (Tsien, 1998), genetically encoded, and expressed; (4) acceptor proteins (mostly antibodies or Fab fragments) can be labeled with Cy5 (for Cy3 donors), or labeled with Cy3 (for GFP donors) and introduced by microinjection or incubation in the case of antibodies; and (5) the recently discovered and commercial available red fluorescent protein from the *Anthozoa* sp. (Matz et al., 1999) can be employed. The latter opens up a wide array of possibilities since an ideal acceptor for GFP can be coexpressed in the same cell, obviating the need for exogenous labeling and microinjection.

Alternative methods for measuring FRET on the basis of fluorescence intensities are available. In these methods FRET is not determined from the donor fluorescence exclusively, but also from the sensitized emission of the acceptor, excited by receiving energy from the donor. These approaches fall into two categories (1) those based on changes in donor/acceptor emission ratio (Adams et al., 1991; Miyawaki et al., 1997); and (2) those based on acceptor sensitized emission (Day, 1998; Gordon et al., 1998; Mahajan et al., 1998). These approaches are attractively simple to perform but may suffer from a number of drawbacks that cannot always be corrected for.

In the ratiometric approach, the decrease in donor emission and concomitant increase in acceptor emission are imaged by calculating the ratio of these intensities at each pixel. Since this is a relative measure, a change in FRET can only be observed in live cells where the ratio

changes over time. A decrease in the donor/acceptor emission ratio can be taken as an indication for the occurrence of FRET. However, this ratio is also dependent on the local concentrations of the donor and acceptor molecules measured in each pixel, which can complicate the interpretation by differential translocation of the biomolecules conjugated to donor and/or acceptor fluorophores. This problem does not occur when the donor and acceptor fluorophores are present on the same molecule (e.g., the “chameleon” Ca^{2+} biosensors; Miyawaki et al., 1997), since the relative concentrations are identical at each location in the cell.

Measuring FRET from the sensitized emission intensity alone (by using a “FRET filter set”) is the most widely used method. Emission from the acceptor would be an exclusive readout for FRET when contamination with donor fluorescence (i.e., donor bleed-through) and direct excitation of the acceptor did not complicate the approach. Correction for these effects by extensive control measurements, some of which have to be performed in different samples where the acceptor is absent, is described in Gordon et al. (1998). Here, both donor-quenching and acceptor-emission information are used to derive the sensitized emission contribution, which is proportional to the FRET efficiency. However, sensitized emission is also proportional to the concentration of the acceptor. Therefore, relative populations of associated molecules cannot be determined. Molecules that are not participating in FRET are not detected. In contrast to donor-based measurements, no estimation can be made of the bound/unbound fractions from the sensitized emission alone. Since excitation spectra generally exhibit an extensive tail at the lower wavelength (blue-edge) the acceptor can easily be excited at the wavelength used for excitation of the donor. Direct excitation will be especially problematic when the recently discovered red fluorescent protein from the *Anthozoa* sp. is used as an acceptor, since its absorption spectrum shows multiple absorption peaks at the excitation optima for the green and yellow fluorescent protein.

Critical Parameters and Troubleshooting

FRET measurements are prone to false negative results. While the finding of FRET by unquenched fluorescence is highly unlikely to be caused by artifactual processes or nonspecific proximity of donor and acceptor, the absence of FRET does not provide proof for the

absence of an interaction for a number of reasons.

The presence of a GFP moiety on the protein of interest might interfere with its function or targeting, thus affecting the interaction with the target protein. Cloning vectors for the construction of GFP fusion proteins routinely contain a multiple cloning site that has been optimized for a maximum number of restriction sites to facilitate cloning. As a consequence, the random amino acid residues that they translate into can adopt an unfortunate secondary structure that hinders the proper behavior of the fused protein. In the case of the EGFR-GFP, the amino acid residues encoded by the cloning site were replaced by a flexible six-glycine linker between the GFP moiety and the EGFR to prevent the GFP from affecting EGFR function (Wouters and Bastiaens, 1999). Another problem might arise when the separation distance between the GFP moiety on the donor protein and the Cy3 groups on the antibody is too large (i.e., >10 nm) for energy to be transferred efficiently. This might be overcome by using antibodies raised against a different epitope, or the entire protein, thus increasing the chance of a favorable orientation of the antibody to the donor fluorophore.

The labeling ratio of the antibodies is a common reason for failure to detect FRET. The labeling ratio should exceed 1 to prevent unlabeled antibody from competing with the labeled antibodies. A higher labeling ratio is beneficial for FRET detection since the R_0 distance (i.e., characteristic value for a given donor-acceptor pair, the distance at which 50% of the excited state energy is transferred by FRET) is effectively increased by a larger number of acceptor molecules per donor molecule. In the authors' experience, labeling ratios up to 5 do not significantly affect the specificity of the antibody. However, this should be verified by using the Cy3-labeled antibody in an immunofluorescence experiment and judging the staining pattern as compared to unlabeled antibody. Remember that absolute specificity is not a requirement in the FRET assay, because only the donor photophysical properties are used. However, in extreme cases, the highly labeled antibody could become nonspecific to a point where significant amounts bind to the donor.

It is essential that the donor images before and after acceptor photobleaching be taken under identical conditions. Any change in parameters that influence the collection efficiency of donor fluorescence (i.e., brightness, contrast, laser power, and averaging) will influence the

calculation of the FRET efficiency. This will affect the entire image, and the control region in the E map will no longer be distributed around 0 but will be uniformly shifted. This also occurs with donor photobleaching. When the laser is used at high intensity, or when too many scans are made for averaging, a substantial amount of the GFP becomes photobleached. This will lead to a shift to negative E values in the control region. Furthermore, it is essential to verify that the sample has not moved and that the same focal plane is imaged in both donor images. Movement causes structures in the control region in the E map with positive values at the leading edge and negative values at trailing edges. Movement is most often caused by temperature differences between the slide and the objective but can also be caused by drift in the slide holder. To correct for translation of the image after acceptor photobleaching, the maximum in the correlation function between pre- and post-bleaching images gives the shift between the two images (Bastiaens and Jovin, 1998). The correlation function of the two images is obtained by Fourier transformation on both images, followed by multiplication of the conjugate of the Fourier transform of one image by the Fourier transform of the other, and performing an inverse Fourier transform on the resulting image. Another source of structured contrast in the control region in the E map can be caused by focal mismatch between the two images, originating from the apparent appearance (positive E) or disappearance (negative E) of structures. This problem can be prevented by observing the z position of the stage between exposures. A larger pinhole can reduce these problems by decreasing the depth of focus but at the cost of z resolution. When using pre- and post- z sections rather than single-focus plane images, the maximum in a three-dimensional correlation function can be used for registration of the two data sets and a correct three-dimensional E map can be obtained (Bastiaens et al., 1996).

Anticipated Results

After photobleaching of the acceptor, an increase in donor emission intensity can be observed when substantial FRET is present. Image arithmetic should produce FRET efficiencies that are closely distributed around zero in the portion of the cell that was not photobleached. Deviations from this condition indicate donor bleaching, lateral movement, or focal mismatch. With the occurrence of FRET, a separate efficiency distribution is expected in

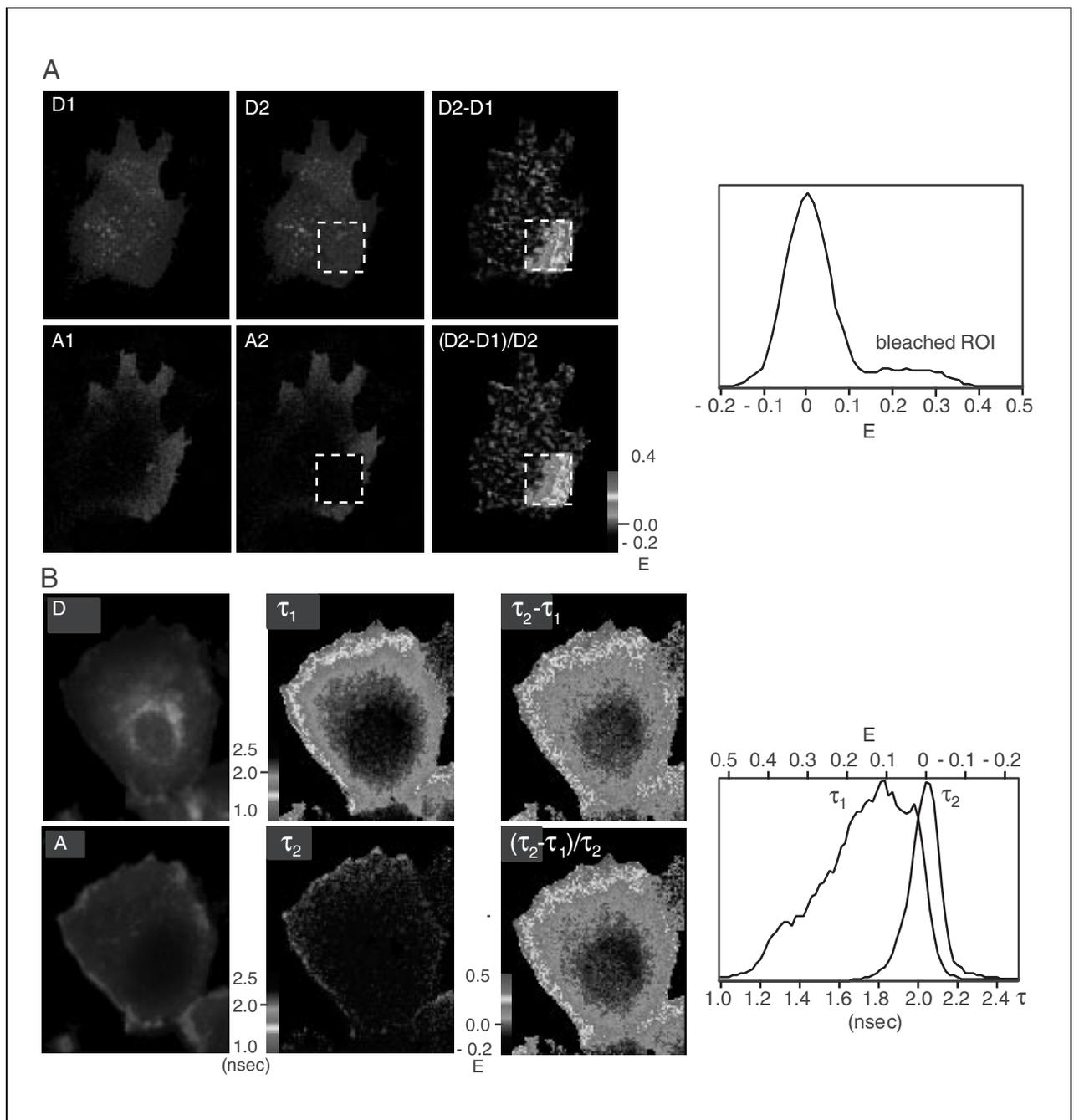


Figure 17.1.1 (A) Cy3-PY72 photobleaching releases FRET-quenched EGFR-GFP emission. The histogram shows the distribution of calculated FRET efficiencies in the cell. (B) FRET measured by fluorescence lifetime imaging microscopy. The histogram shows the distribution of measured lifetime (nsec) and corresponding calculated FRET efficiencies prior and after photobleaching of the acceptor. **See color figure.**

the photobleached area. The heterogeneity in this distribution contains information about variability in the formation of complexes of the proteins carrying the donor and acceptor fluorophores.

Figure 17.1.1 shows an MCF-7 mammary carcinoma cell expressing EGFR-GFP that was stimulated with 100 ng/ml EGF for 5 min. This cell was incubated with Cy3-labeled anti-phos-

photyrosine antibodies (e.g., 10 μ g/ml PY72 monoclonal) and EGFR-GFP tyrosine phosphorylation was measured by FRET using the unquenching of GFP fluorescence by acceptor photobleaching (Fig. 17.1.1A) and FLIM (Fig. 17.1.1B). The EGFR-GFP distribution is shown in the D1 image. Most EGFR-GFP is localized at the plasma membrane in addition to internalized (punctate) EGFR-GFP. The cor-

responding antibody-staining A1 shows phosphorylation at the plasma membrane. A rectangular region was subjected to acceptor photobleaching (white box). The GFP fluorescence in this region increased after photobleaching as shown in the difference image ($D2 - D1$), demonstrating positive FRET efficiencies [$(D2 - D1)/D2$]. Highest FRET efficiencies can be observed in the plasma membrane corresponding to fully phosphorylated receptor at this location. The large peak in the energy-transfer efficiency histogram that is distributed around zero originates from the area outside the photobleached region and indicates proper image registration. The additional population, ranging from 15% to 35% efficiency, corresponds to the photobleached region. Figure 17.1.1B shows the analogous result of a fluorescence lifetime measurement. The steady-state fluorescence distribution of EGFR-GFP is shown in (D) and the corresponding lifetime map in (τ_1). The anti-phosphotyrosine Cy3 immunofluorescence (A) is photobleached to obtain an intracellular reference lifetime in absence of acceptor (τ_2). As can be seen in the fluorescence-lifetime histogram, these values are homogeneously distributed around an average of ~ 2.0 nsec. The decrease in lifetime due to FRET is shown in the difference image ($\tau_2 - \tau_1$). The energy-transfer efficiency is given by the normalization of this difference to the reference EGFR-GFP lifetime [$E = (\tau_2 - \tau_1)/\tau_2$] and again shows highly phosphorylated receptor in the plasma membrane.

Time Considerations

From cell seeding, the entire procedure can be performed in 3 to 4 days. This period includes 1 to 2 days for expression of the GFP construct after transfection or microinjection; 1/2 day for treatment, fixation, and antibody incubation of cells; 1/2 day of data acquisition; and 1/2 day for data analysis. Removal of gelatin/BSA from commercial antibody takes ~ 3 hr. The most time-consuming step in the procedure for labeling antibodies is the buffer exchange using a Microcon concentration device. Depending on the amount of contaminating free amino groups in the original buffer, this can take between 2 and 5 hr. The labeling reaction and subsequent gel filtration chromatography takes ~ 1 hr.

There are a number of points where the procedure can be interrupted: (1) optimal expression after overnight incubation rather than a 3- to 4-hr incubation post microinjection can

be achieved by lowering the concentration of DNA; (2) after fixation and permeabilization, the cells can be stored overnight in PBS at 4°C before antibody incubation; (3) after mounting, the cells can be viewed immediately rather than the following day, by application of rubber cement instead of Mowiol; and (4) the samples can be stored at -20°C for weeks without appreciable loss of antibody staining or FRET.

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Identification of Protein Interactions by Far Western Analysis

UNIT 17.2

This unit describes far western blotting, a method of identifying protein-protein interactions. In a far western blot, one protein of interest is immobilized on a solid support membrane, then probed with a non-antibody protein. Far western blots can be used to identify specific interacting proteins in a complex mixture of proteins (see Basic Protocol). They are particularly useful for examining interactions between proteins that are difficult to analyze by other methods due to solubility problems or because they are difficult to express in cells. This method is performed totally *in vitro*, and the proteins of interest can be prepared in a variety of ways. Peptides can be used to determine the effects of specific residues or post-translational modifications on protein-protein interactions (see Alternate Protocol 2). In addition, many different detection techniques, either radioactive or nonradioactive, can be used. For example, the protein probe may be detected indirectly with an antibody, rather than being labeled radioactively (see Alternate Protocol 1). Thus, techniques and reagents already in hand can frequently be adapted for use with this assay.

CAUTION: Appropriate safety precautions must be taken when working with radioactive materials. Information on proper handling and disposal of radioactive compounds can be found in *APPENDIX 1D* and may be obtained from local radiation safety officials. Specific information on handling ³⁵S-labeled compounds can be found in *UNIT 7.1* and in *APPENDIX 1D*.

FAR WESTERN ANALYSIS OF A PROTEIN MIXTURE

The following is a basic method for detecting protein-protein interactions by far western blotting when one protein is contained within a simple or complex mixture of proteins. First, the protein sample is fractionated on an SDS-PAGE gel (*UNIT 6.1*). After electrophoresis, the proteins are transferred from the gels onto a solid support membrane by electroblotting (*UNIT 6.2*). Transferred membranes may be stained with Ponceau S to facilitate location and identification of specific proteins. Nonspecific sites on the membranes are blocked with standard blocking reagents, and the membranes are then incubated with a radiolabeled non-antibody protein probe. After washing, proteins that bind to the probe are detected by autoradiography (*UNIT 6.3*).

Materials

- Samples to be analyzed
- 1× SDS sample buffer (*APPENDIX 2A*)
- Ponceau S staining solution (see recipe)
- Blocking buffer I: 0.05% (w/v) Tween 20 in 1× PBS (see recipe for PBS); prepare fresh
- Blocking buffer II: dissolve 1 g bovine serum albumin (BSA; fraction V) in 100 ml 1× PBS (see recipe for PBS); prepare fresh
- Phosphate-buffered saline (PBS; see recipe), pH 7.9
- cDNA encoding protein of interest cloned into an *in vitro* expression vector
- In vitro* transcription/translation kit (Promega)
- 10 mCi/ml ³⁵S-methionine (1000 Ci/mmol)
- Probe purification buffer (see recipe)
- Probe dilution buffer (see recipe)

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PROTOCOL**

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Polyvinylidene difluoride (PVDF) or nitrocellulose membrane for protein transfer
Microfiltration centrifuge columns (e.g., Gelman Nanosep, Pall Filtron, or
Millipore Microcon)

Additional reagents and equipment for SDS-PAGE (UNIT 6.1), electrophoretic
transfer of proteins to a support membrane (UNIT 6.2), in vitro translation (UNIT
11.2), and autoradiography (UNIT 6.3)

NOTE: Always handle support membranes with gloves or membrane forceps.

Prepare protein blot

1. Prepare the protein sample to be analyzed by resuspending it in 1× SDS sample buffer.

UNIT 6.1 gives instructions on preparation of samples and amount of samples to load. In general, ~50 to 100 μg can be loaded in each lane for a complex mixture of proteins. A smaller amount, i.e., 10 to 20 μg, is loaded for less complex protein samples. The amount loaded may also need to be adjusted for the size of gel. (Usually 30 μg/mm² loading surface can be resolved without smearing.)

2. Separate the samples on an SDS-polyacrylamide gel (UNIT 6.1).
3. Transfer the proteins from the gel to a solid support membrane (e.g., PVDF or nitrocellulose) by semidry electroblotting (UNIT 6.2).

Either nitrocellulose or PVDF membranes can be used with good results. PVDF membranes are easier to handle and tend to give a slightly higher signal-to-noise ratio, probably due to increased protein retention by the membrane.

Stain with Ponceau S

4. After transfer, stain the membrane for 5 min in ~100 ml freshly diluted 1× Ponceau S staining solution. Stain the membrane in a plastic container large enough to hold the blot and use sufficient Ponceau S to cover the membrane completely.

This step is optional. When the protein samples consist of a few proteins, or when there are clearly visible bands that facilitate orientation of the blot, staining with Ponceau S can provide helpful landmarks. One can unequivocally identify interacting bands, mark the position of molecular weight standards, and trim away excess membrane more exactly.

5. Destain the membrane washing in several changes of deionized water until the proteins are clearly visible. Place light pencil marks adjacent to important protein bands to mark them for future reference. Trim away excess membrane.

The stain fades quickly so the marks must be placed immediately.

6. Destain an additional 5 min in water until the red staining fades.

Block membrane

7. Block blot for 2 hr in 200 ml blocking buffer I at room temperature with gentle agitation.
8. Decant and add 200 ml blocking buffer II. Incubate as in step 7.
9. Decant blocking buffer II and rinse the membrane briefly in 100 ml of 1× PBS.

At this point, the blot may be probed immediately or may be wrapped in plastic wrap and stored for up to 2 weeks at 4°C.

Prepare the probe

10. Following manufacturer's procedures, prepare a radiolabeled in vitro–translated probe of the protein of interest using ^{35}S methionine (also see *UNIT 11.2*).

The probe can be conveniently prepared during the blocking steps.

The authors routinely use the Promega TnT quick-coupled transcription/translation system for producing probes. For a small blot (e.g., $\leq 9 \times 9\text{-cm}$), one half of a standard in vitro transcription/translation reaction (i.e., 25 μl) is sufficient for the probe. For larger blots, an entire 50- μl reaction may be used. In the authors' laboratory it is considered essential that the transcription/translation lysate not be repeatedly frozen and thawed.

11. After translation, dilute the probe with 500 μl probe purification buffer, and purify by microcentrifuging 15 to 30 min at $10,000 \times g$, room temperature, in a microfiltration column. Save aliquots of the purified probe for analysis by SDS-PAGE (i.e., 2 μl), and for scintillation counting (i.e., 2 to 5 μl).

Check microfiltration column manufacturer's procedure for exact centrifugation times required by different columns. In practice, it is not always necessary to purify the probe through a microfiltration column; many probes give good signals without purification. However, if signal-to-noise ratio is low, probe purification may improve results. In addition, it is possible to quantitate the proportion of probe bound if the probe has been purified.

Bind probe

12. Preincubate blot for 10 min in 50 ml of $1\times$ probe dilution buffer (without probe) by gently agitating at room temperature.

13. Dilute the translated probe with $1\times$ probe dilution buffer in a volume sufficient to cover the membrane to be probed (typically 3 ml).

For small blots, i.e., $\leq 9 \times 9\text{-cm}$, a 50-ml conical tube makes a convenient incubation chamber. A volume of 3 ml is enough solution to cover the blot and tubes can be rotated on a mechanical rotator. In addition, the conical tube makes the radiolabeled probe easy to contain and dispose of. Larger blots can be incubated on a Nutator or orbital shaker in a heat-sealable bag, or rotated in a hybridization oven adjusted to room temperature.

14. Add the probe to the membrane and incubate 2 hr at room temperature. Rotate the tubes or agitate bags throughout the binding reaction.

Wash the membrane

15. Transfer the membrane to a plastic dish and wash the membrane with 200 ml $1\times$ PBS for 5 min, room temperature. Repeat for a total of four washes.

Background is generally quite low and extended washing does not substantially reduce background.

16. Air dry the membrane and expose to X-ray film (autoradiography) or phosphor imager screen (see *UNIT 6.3* for both techniques).

Do not cover the blots with plastic wrap as this will quench the ^{35}S signal. Overnight exposure to X-ray film is usually sufficient to detect positive interactions.

DETECTING INTERACTING PROTEINS BY IMMUNOBLOTTING

In vitro–translated probes have the advantages of being quickly produced, easily detected, and quantitated to give an estimate of relative binding. In addition, mutations in the protein probe can be generated by simple cloning procedures and can provide information on binding domains and their critical residues. A disadvantage of in vitro–translated probes is the need for multiple methionine or cysteine residues to obtain a well labeled probe. For the same reasons, small peptide fragments are often not suitable for use as in

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vitro–translated probes. [¹⁴C]leucine and [³H]leucine can also be used for in vitro translation of proteins; however, in the authors' laboratory [¹⁴C]leucine has not yielded probes suitable for use as far western probes.

There are many other ways to generate probes for far western blots. The protein probe may be labeled in vitro with ¹²⁵I (Schumacher and Tsomides, 1995) or enzymatically with ³²P (Kimball, 1998). Biotin-labeled probes may be detected with streptavidin-biotin detection schemes (Luna, 1996; Grulich-Henn et al., 1998; Kimball et al., 1998). Protein binding may be detected indirectly as well. If an antibody to the interacting protein is available, then an unlabeled protein probe can be bound to the blots as usual and then detected by western (immunoblot) analysis. This is especially useful when a tagged recombinant protein and antibody to the tag are available. The following procedure describes detection of an unlabeled protein probe with specific antibody. Many variations of immunoblotting exist; additional information and procedures may be found in *UNIT 6.2*.

Additional Materials (also see *Basic Protocol*)

- Recombinant protein or unlabeled in vitro translated–protein for probe
- 5% (w/v) non-fat instant dry milk in 1× TBST (see recipe for TBST)
- Primary antibody specific for protein probe
- TBST (see recipe)
- Alkaline phosphatase (AP)–conjugated secondary antibody against Ig of species from which specific antibody was obtained
- Alkaline phosphatase buffer (see recipe)
- Developing solution (see recipe)
- 100 mM EDTA, pH 8.0 (*APPENDIX 2A*)

Prepare blot and probe

1. Prepare and block the blot (see Basic Protocol, steps 1 to 9).
2. Prepare the probe protein by diluting in vitro–translated or recombinant protein in 3 ml of 1× probe dilution buffer.

The amount of recombinant protein must be empirically determined for each protein. Various researchers have used from 0.5 to 20 μg recombinant protein/ml of probe dilution buffer.

Expose blot to probe

3. Bind the probe to blot and wash (see Basic Protocol, steps 12 and 14 to 15). Do not dry the membrane after washing.
4. Incubate blot in 200 ml of 5% non-fat milk in 1× TBST for 1 hr, room temperature, with gentle rotation on an orbital shaker.

Expose to antibodies

5. Dilute the primary antibody in 5% milk in 1× TBST. Incubate blot in 5 to 10 ml diluted antibody at room temperature with gentle agitation to ensure blot is evenly covered with the antibody solution.

Incubations are usually carried out in heat-sealed plastic bags or hybridization bottles to minimize the volume necessary to completely cover the blot. A volume of 5 to 10 ml of diluted antibody is sufficient to cover most blots. Appropriate antibody concentrations vary for each antibody and must be determined empirically.

6. Wash for 10 min in ≥200 ml 1× TBST by agitating on an orbital shaker. Repeat an additional two times.

- Dilute the AP-conjugated secondary antibody in 5 to 10 ml of 5% milk in 1× TBST and incubate blot for 1 hr as in step 4.

Suppliers generally provide an estimate of appropriate dilution for the secondary antibody.

- Wash blot six times for 5 min each in ≥200 ml TBST, with agitation.

Detect antibodies

- Briefly, rinse blot in 50 ml alkaline phosphatase buffer.
- Incubate blot in 20 ml developing solution for 1 to 15 min and rinse blot with 100 ml water.
- Wash blot for 5 min with 100 ml of 100 mM EDTA, pH 8.0, to stop the development reaction. Rinse with 100 ml water, dry, and photograph.

An example of a far western blot of proteins is shown in Figure 17.2.1. Lane 1 shows a Coomassie blue stain of a protein sample enriched for histone proteins separated on a 22% SDS-PAGE gel. Lane 2 shows a far western autoradiogram of a parallel lane probed with the yeast Tup1 protein according to this protocol. Lane 3 shows a parallel lane probed with an unlabeled Tup1 protein and detected with antibody specific to Tup1 as in Alternate Protocol 2. Both protocols yield the same result—Tup1p interacts with H3 and H4 but not with H2A or H2B. Lanes 4 and 5 show immunoblots of parallel lanes using anti-H3 and anti-H4 antibodies to identify histories H3 and H4 unequivocally.

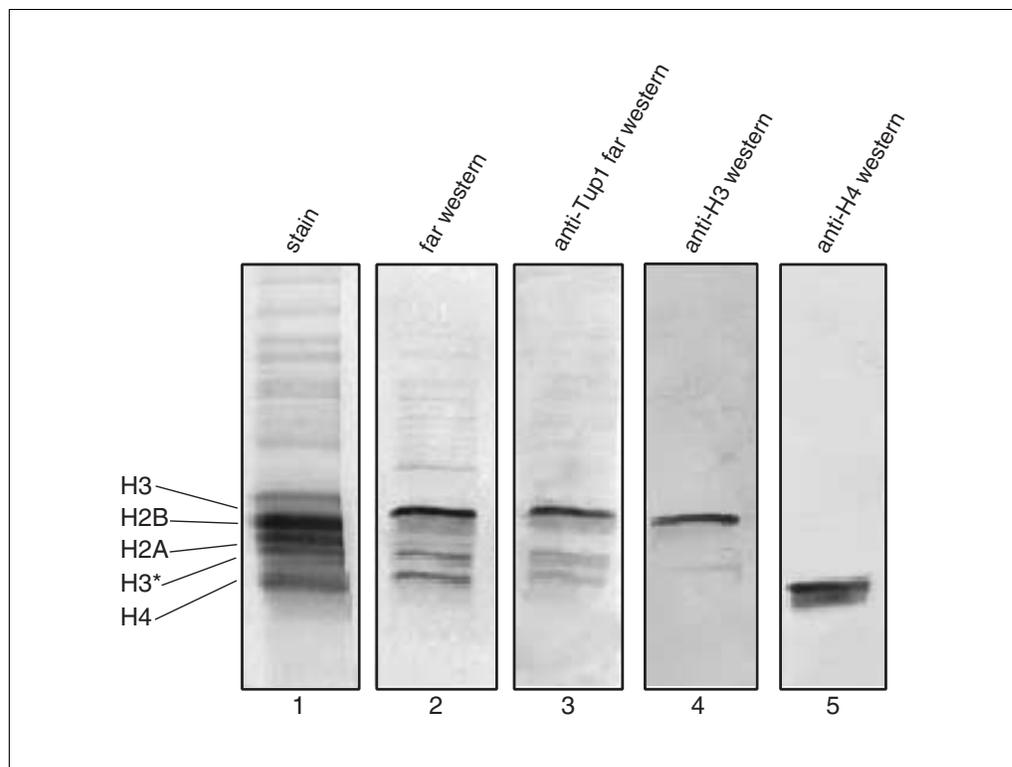


Figure 17.2.1 Far western of blotted SDS-PAGE gel. Lane 1, Coomassie blue–stained gel showing locations of histone bands. Lane 2, far western of a parallel lane using radiolabeled in vitro–translated probe. Lane 3, far western using unlabeled probe detected with probe-specific antibody. Lane 4, western blot using anti-histone H3 specific antibody. Lane 5, western blot using anti-histone H4 specific antibody.

**USING PEPTIDES TO IDENTIFY SPECIFIC INTERACTING SEQUENCES
IN A FAR WESTERN BLOT**

Synthetic peptides can also be used in far western analyses. The use of peptides enables the identification of specific interacting sequences. Specific post-translational modifications can be examined for their effect on protein-protein interactions. Peptides as small as 11 amino acids have been used successfully as far western targets.

Peptide far westerns differ from other far westerns only in the preparation of the blots. Peptide dilutions are prepared, then dot or slot blotted onto the support membrane. Blocking and probing of peptide blots are identical to procedures used for traditional far westerns. Duplicate blots are stained to verify that comparable quantities of different peptides have been loaded. Because Ponceau S staining is temporary, staining of duplicate blots with India ink is used to provide a permanent record for peptide blots.

In the authors' experience, only peptides that have been synthesized on MAP resins have worked well for peptide blots. MAP resins consist of branched lysine chains whose chemically active groups have been blocked. Although peptides prepared in other ways do give reproducible results, the peptide concentrations required are several orders of magnitude higher than those required for MAP peptides, making these blots very costly to perform. The reason why increased peptide is needed is unclear, but perhaps the MAP resin "presents" the peptide in such a way that it is more accessible for interaction.

Additional Materials (also see *Basic Protocol*)

Peptides

0.4% Tween 20/PBS (see recipe)

India ink solution (see recipe)

Slot or dot blot apparatus (e.g., Bio-Rad Bio-Dot SF or Schleicher & Schuell Minifold II)

1. Make dilutions of peptides between 5 ng and 5 μ g in a final volume of 100 to 200 μ l of distilled water.
2. Prepare slot or dot blotter and support membrane (PVDF or nitrocellulose) as described by the manufacturer. Load the peptide dilutions into wells. Prepare duplicate blots, one for far western and one for India ink staining. After all samples are loaded, apply vacuum to draw the peptide samples through the manifold device and onto the support membrane.
3. Block, bind, wash, and autoradiograph one blot for far western (see Basic Protocol, steps 7 to 16).
4. Incubate the second blot in 100 ml of 0.4% Tween 20/PBS for 5 min at room temperature with gentle agitation. Repeat incubation.
5. Stain blot by incubating 15 min to overnight with 100 ml India ink solution at room temperature.
6. Wash the filter for 2 hr in 4 changes of 1 \times PBS. Dry and store the membrane.

This stain is permanent.

Figure 17.2.2 shows an example of results from this alternate protocol, using peptides as a substrate for a far western. The right-hand panel is a blot stained with India ink verifying that comparable quantities of peptide were loaded on the blot. The left-hand panel is a far western of a duplicate blot demonstrating the effect of acetylation of lysine residues of histone peptides on Tup1p/histone interaction.

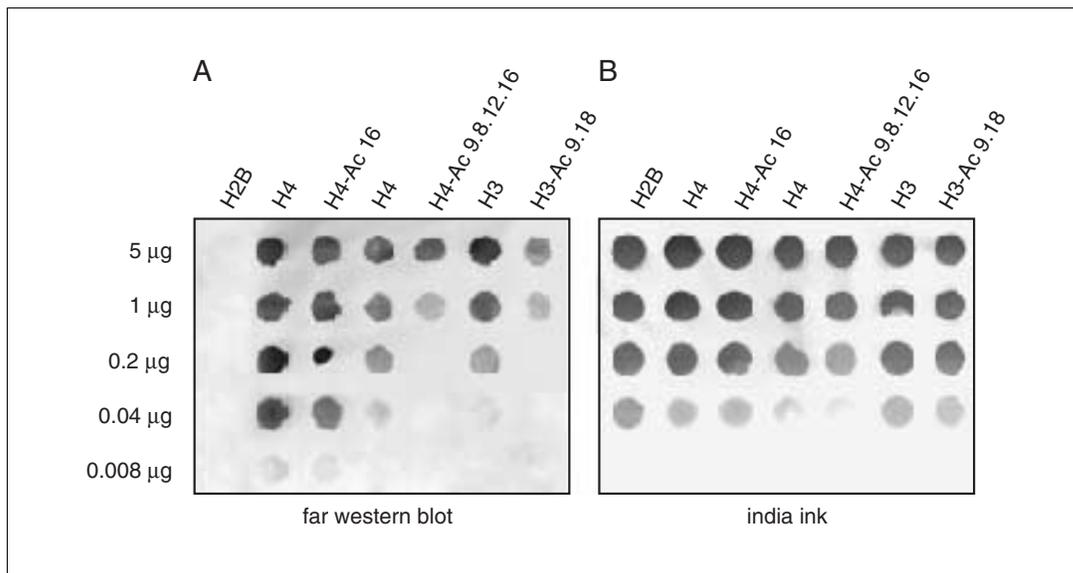


Figure 17.2.2 Far western blot of peptides (**A**) dot blotted onto PVDF membrane and (**B**) stained with India ink. Figure reproduced with permission of Cold Spring Harbor Laboratory Press.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

Alkaline phosphatase buffer

- 100 ml 1 M Tris-Cl, pH 9.5 (*APPENDIX 2A*)
- 20 ml 5 M NaCl (*APPENDIX 2A*)
- 5 ml 1 M MgCl₂ (*APPENDIX 2A*)
- Add H₂O to 1 liter
- Store up to 1 year at room temperature

5-Bromo-4-chloro-3-indolyl phosphate (BCIP) stock solution

Dissolve 0.5 g of BCIP in 10 ml of 100% dimethylformamide. Store at 4°C or in small aliquots at -20°C. Discard when solution turns color.

Developing solution

Add 66 µl of NBT stock (see recipe) to 10 ml of alkaline phosphatase buffer (see recipe). Mix well. Add 33 µl of BCIP stock solution (see recipe) and mix again. Prepare fresh.

India ink solution

Add 100 µl India ink (Pelikan or Higgins) to 100 ml 0.4% Tween 20/PBS (see recipe). Prepare fresh.

Nitroblue tetrazolium chloride (NBT) stock solution

Dissolve 0.5 g of NBT in 10 ml of 70% dimethylformamide. Store at 4°C or in small aliquots up to 6 months at -20°C.

Phosphate-buffered saline (PBS), 10×

- 80 g NaCl
- 2.2 g KCl
- 9.9 g Na₂HPO₄
- 2.0 g K₂HPO₄
- Add H₂O to 1 liter

continued

Adjust pH to 7.4
Store indefinitely at room temperature
Prior to use, dilute to 1× by mixing 1 part 10× PBS with 9 parts water
Leftover 1× PBS should be stored at 4°C to discourage bacterial growth.

Ponceau S staining solution, 10×

2 g Ponceau S
30 g trichloroacetic acid
30 g sulfosalicylic acid
Add H₂O to 100 ml
Store indefinitely at room temperature
Just prior to use dilute to 1× by mixing 1 part 10× Ponceau S with 9 parts water

Probe dilution buffer, 10×

3.0 g bovine serum albumin (BSA)
10 ml normal goat serum
10 ml 10× PBS (see recipe)
H₂O to 100 ml
Store indefinitely at -20°C
Just prior to use, dilute to 1× by mixing 1 part 10× stock with 9 parts 1× PBS

Probe purification buffer

400 μl 1 M HEPES, pH 7.4,
400 μl 1 M dithiothreitol (DTT)
9.2 ml H₂O
Prepare fresh

TBST, 10×

90 g NaCl
100 ml 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
10 g Tween 20
Add H₂O to 1 liter
For 1× TBST dilute 1 part 10× TBST with 9 parts water prior to use
Store indefinitely at room temperature

Tween 20/PBS, 0.4% (w/v)

Dissolve 0.4 g Tween 20 in 100 ml 1× PBS (see recipe). Store up to 1 week at room temperature.

COMMENTARY

Background Information

The far western blot (also called a west western and a ligand blot) has been widely used to examine the interactions of many diverse proteins. For example, it has been used to examine the interactions between the subunits of eukaryotic initiation factors (Kimball et al., 1998), to look at interactions between basic helix-loop-helix DNA-binding proteins (Chaudhary et al., 1997), and to examine the interactions of keratin intermediate filaments with desmosomal proteins (Kouklis et al., 1994). Far westerns have been particularly useful in examining interactions of histones with regulatory proteins. Far westerns have been

used to look at interactions of WD repeat proteins with histones (Edmondson et al., 1996; Palaparti et al., 1997), the interaction of Epstein-Barr virus nuclear antigen 2 with histone H1 (Grasser et al., 1993), and the interaction of histones with the *Xenopus* oocyte protein N1 (Kleinschmidt and Seiter, 1988). In addition, far westerns have been used to study receptor-ligand interactions and to screen libraries for interacting proteins (Grulich-Henn et al., 1998; Hsiao and Chang, 1999).

Sometimes, the nature of the proteins being examined is such that standard methods of studying protein-protein interactions are not possible. For example, some proteins are diffi-

cult to solubilize or to extract from cells except under conditions that disrupt protein-protein interactions and therefore, are difficult to assay by immunoprecipitation. Other proteins cannot be expressed in bacteria or yeast due to toxicity problems, thus making the production of recombinant proteins or the use of two-hybrid assays impossible. Far westerns are particularly useful in such cases. Since far westerns are performed totally *in vitro*, they circumvent these types of problems.

Another advantage of the far western blot is its flexibility. Proteins prepared in a variety of ways can be used for the assay. Cell extracts, recombinant proteins, and peptides can all be used as both probe and target proteins. For example, Palaparti et al. (1997) used cell extracts to probe a semipurified histone sample and detected bound proteins of interest with antibodies. Hsiao and Chang (1999) used phage-expressed proteins immobilized on filter lifts for a far western library screen. Unpurified *E. coli* extracts containing recombinant protein have been successfully used as probes (Fischer et al., 1997).

In addition, many different detection techniques, either radioactive or nonradioactive, can be used. Kimball and co-workers used recombinant protein probes that were labeled radioactively with kinases and recombinant proteins labeled with biotin and subsequently detected with a streptavidin-biotin detection scheme (Grulich-Henn et al., 1998; Kimball et al., 1998). Thus, techniques and reagents already in hand can frequently be adapted for use with this assay.

Finally, the far western can be modified to define the protein domains and amino acid residues that are important in protein-protein interactions. Mutagenized clones can be used to produce variants of protein probes. A single SDS-PAGE gel can be run with identical lanes and cut into strips, and a different *in vitro*-translated probe can be used for each strip. In this way, multiple variants of a protein can be tested simultaneously for their ability to interact with a target protein.

Non-SDS polyacrylamide gels can also be used to separate proteins for far westerns. For example, acid urea gels, which separate on the basis of both size and charge, have been successfully employed. Finally, peptides corresponding to specific interacting sequences can be synthesized with specific post-translational modifications to test their effects on protein-protein interactions.

Critical Parameters

Blocking nonspecific binding sites on the membranes is critical to achieving good results with far westerns. Too little blocking results in high background, while extended time in blocking solutions results in weakened or lost signal. The reason for the diminished signal is unclear, but protein renaturation apparently takes place during the blocking step, so an optimal renaturation may require limited blocking. The best time may well be different for each protein and require empirical optimization. In addition, different lots of BSA appear to result in diminished signal. Therefore, it is important to purchase high-quality BSA from a reputable manufacturer.

An important consideration is the inclusion of appropriate controls to rule out nonspecific interactions that might result in false positives. Suitable controls should be furnished for both the target proteins and the protein probe. When using a complex mixture of proteins, such as cell extracts, as target, “negative” control proteins are already present. However, when using a mixture of only a few proteins, it is important to provide a protein that does not interact to serve as a negative control for nonspecific binding. The ideal negative control should be similar to the protein of interest in charge and size. Appropriate controls should be subjected to SDS-PAGE and blotted in parallel with the samples of interest. Another important control is the use of an unprogrammed translation lysate as a probe. Translation of an unrelated protein as a control probe is also often helpful.

Troubleshooting

Precise conditions for far westerns vary from procedure to procedure and probably reflect the nature of the individual proteins being examined. Optimal conditions for each protein may need to be determined empirically. If background staining is too high, there are several possible remedies. The probe may be diluted or the sample concentration lowered. Other “blocking” reagents may be tested. The blocking reagent, nonfat dry milk, ranging in concentration from 1% to 5%, has been used successfully. Other detergents such as NP-40 and Triton X-100 are commonly employed for this procedure and may help to decrease background. Also, increasing the length of the blocking step may aid in background reduction. Most procedures that call for extended blocking times suggest incubation at 4°C.

If no specific staining is observed, confirm the quality of the radiolabeled probe by SDS-

PAGE and autoradiography. Make sure the reticulocyte lysate has not been repeatedly frozen and thawed. If using a recombinant protein/antibody scheme, confirm the affinity of that interaction by immunoblotting.

Sometimes a decrease in blocking time or use of a different blocking reagent will increase signal. Some proteins may interact more readily following a denaturation/renaturation cycle. In this case, the membrane is incubated for 1 hr in PBS-buffered 7 M guanidine or 8 M urea and renatured overnight. Renature in blocking buffer I (see Basic Protocol) without agitation at 4°C with several changes of buffer. Finally, the length of the binding reaction can be increased.

Anticipated Results

Sensitivity of the far western blot is dependent on the affinity of the protein-protein interactions being investigated and on the quality of the probe. Thus, extra attention given to the preparation of a high-quality probe is almost invariably worthwhile. When using a radiolabeled probe, a positive interaction can typically be visualized after overnight exposure to X-ray film.

Time Considerations

The basic protocol can be performed in 2 days. An SDS-PAGE gel can be set up and run in 4 to 6 hr. The rest of the procedure can be performed in 8 to 10 hr. It is often convenient to set up the SDS-PAGE gels on one day and run them slowly overnight. The rest of the basic protocol can then be performed the next day. Semidry transfer requires ~2 hr, the blocking steps take ~4 hr, binding of the probe takes ~2 hr, washing, drying, and setting up the autoradiography cassette takes ~1 hr. Detection of interactions using antibodies to detect the probe protein requires an additional day. Peptide blots can be performed in one day.

It is possible to store the blots after the blocking steps for up to 2 weeks at 4°C. The blots should be stored in airtight containers or wrappings so they do not dry out. In addition, *in vitro*-translated probes may be produced ahead of time and stored unpurified at -20°C, although the efficiency may be reduced for some probes.

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Interaction Trap/Two-Hybrid System to Identify Interacting Proteins

To understand the function of a particular protein, it is often useful to identify other proteins with which it associates. This can be done by a selection or screen in which novel proteins that specifically interact with a target protein of interest are isolated from a library. One particularly useful approach to detect novel interacting proteins—the two-hybrid system or interaction trap (see Figs. 17.3.1 and 17.3.2)—uses yeast as a “test tube” and transcriptional activation of a reporter system to identify associating proteins (see Background Information). This approach can also be used specifically to test complex formation between two proteins for which there is a prior reason to expect an interaction.

In the basic version of this method (see Fig. 17.3.2), the plasmid pEG202 or a related vector (see Fig. 17.3.3 and Table 17.3.1) is used to express the probe or “bait” protein as a fusion to the heterologous DNA-binding protein LexA. Many proteins, including transcription factors, kinases, and phosphatases, have been successfully used as bait proteins. The major requirements for the bait protein are that it should not be actively excluded from the yeast nucleus, and it should not possess an intrinsic ability to strongly activate transcription. The plasmid expressing the LexA-fused bait protein (see Table 17.3.1) is used to transform yeast possessing a dual reporter system responsive to transcriptional activation through the *LexA* operator. In one such example, the yeast strain EGY48 (see Table 17.3.2) contains the reporter plasmid pSH18-34. In this case, binding sites for LexA are located upstream of two reporter genes. In the EGY48 strain, the upstream activating sequences of the chromosomal *LEU2* gene—required in the biosynthetic pathway for leucine (Leu)—are replaced with *LexA* operators (DNA binding sites). pSH18-34 contains a *LexA* operator–*lacZ* fusion gene. These two reporters allow selection for transcriptional activation by permitting selection for viability when cells are plated on medium lacking Leu, and discrimination based on color when the yeast is grown on medium containing Xgal.

In Basic Protocol 1, EGY48/pSH18-34 transformed with a bait is characterized for its ability to express protein (Support Protocol 1), growth on medium lacking Leu, and for the level of transcriptional activation of *lacZ* (see Fig. 17.3.2A). A number of alternative strains, plasmids, and strategies are presented which can be employed if a bait proves to have an unacceptably high level of background transcriptional activation.

In an interactor hunt (Basic Protocol 2), the strain EGY48/pSH18-34 containing the bait expression plasmid is transformed (along with carrier DNA made as described in Support Protocol 2) with a conditionally expressed library made in the vector pJG4-5 (see Fig. 17.3.6 and Table 17.3.3). This library uses the inducible yeast *GAL1* promoter to express proteins as fusions to an acidic domain (“acid blob”) that functions as a portable transcriptional activation motif (act) and to other useful moieties. Expression of library-encoded proteins is induced by plating transformants on medium containing galactose (Gal), so yeast cells containing library proteins that do not interact specifically with the bait protein will fail to grow in the absence of Leu (see Fig. 17.3.2B). Yeast cells containing library proteins that interact with the bait protein will form colonies within 2 to 5 days, and the colonies will turn blue when the cells are streaked on medium containing Xgal (see Fig. 17.3.2C). The DNA from interaction trap positive colonies can be analyzed by polymerase chain reaction (PCR) to streamline screening and detect redundant clones in cases where many positives are obtained in screening (see Alternate Protocol 1). The plasmids are isolated and characterized by a series of tests to confirm specificity of the interaction with the initial bait protein (Support Protocols 3 to 5). Those found to be specific are ready for further analysis (e.g., sequencing).

Contributed by Erica A. Golemis, Ilya Serebriiskii, Russell L. Finley, Jr., Mikhail G. Kolonin, Jenő Gyuris, and Roger Brent

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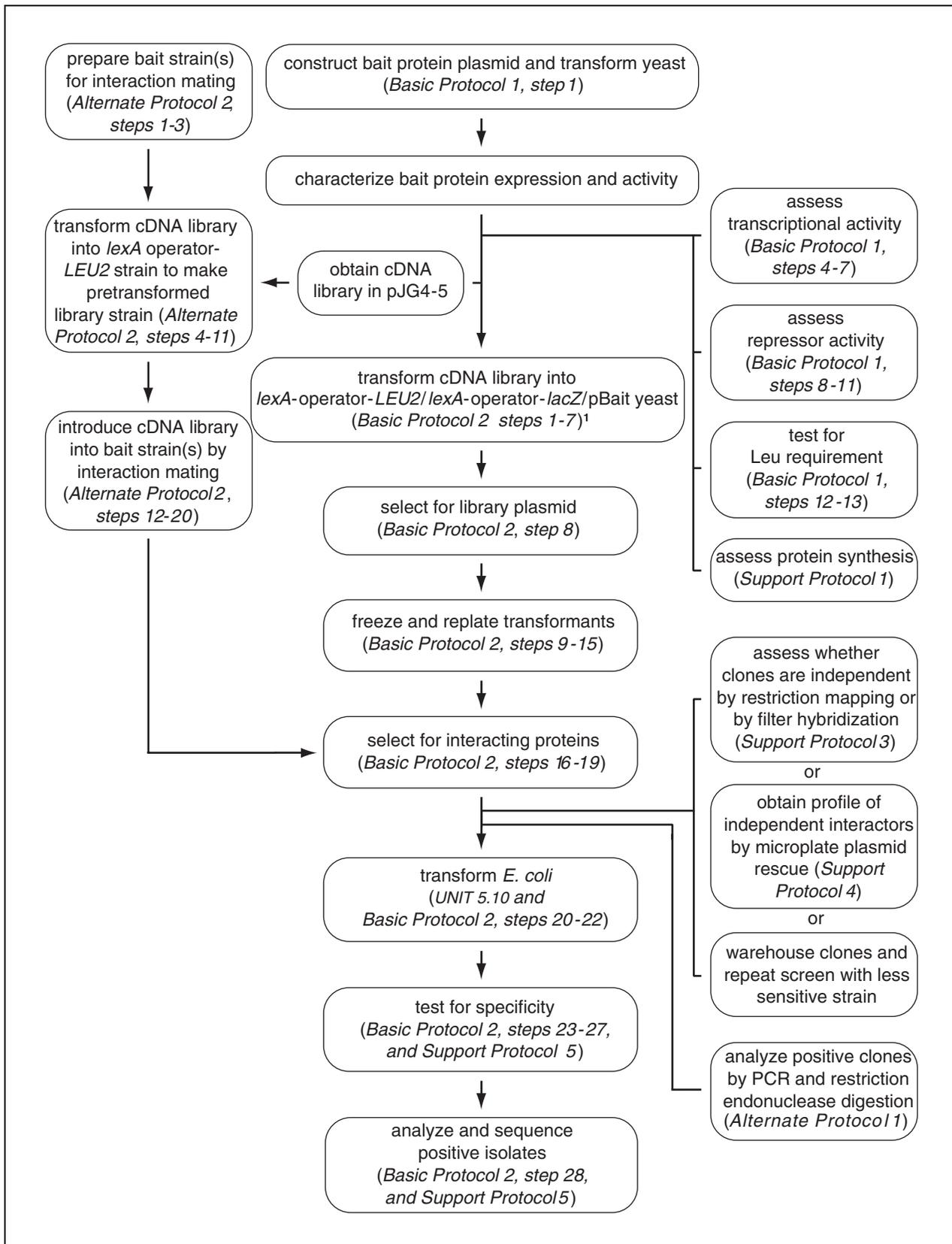


Figure 17.3.1 Flow chart for performing an interaction trap.

17.3.2

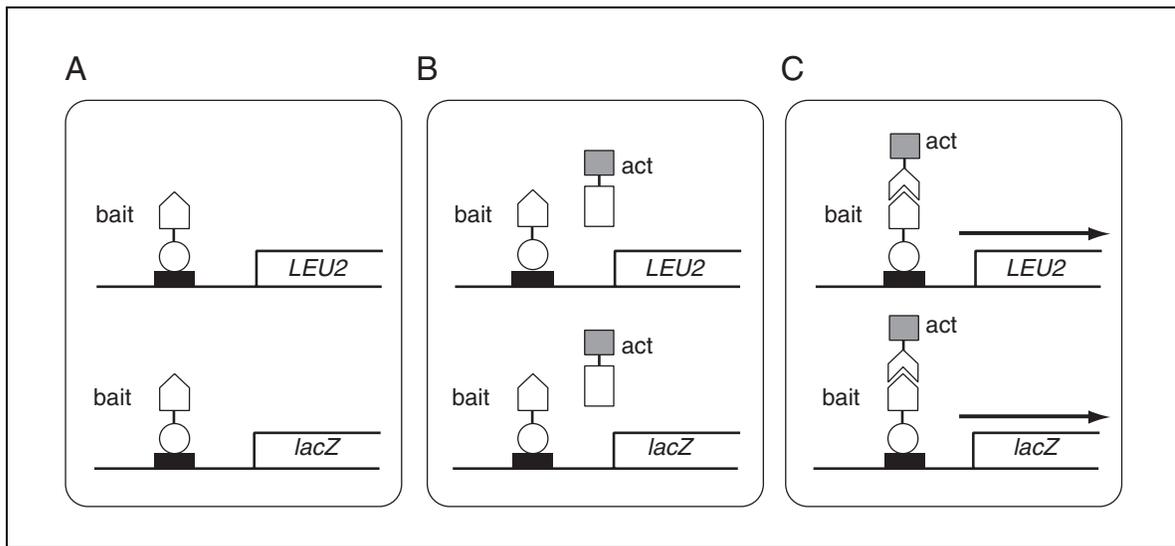


Figure 17.3.2 The interaction trap. (A) An EGY48 yeast cell containing two *LexA* operator–responsive reporters, one a chromosomally integrated copy of the *LEU2* gene (required for growth on –Leu medium), the second a plasmid bearing a *GAL1* promoter–*lacZ* fusion gene (causing yeast to turn blue on medium containing Xgal). The cell also contains a constitutively expressed chimeric protein, consisting of the DNA-binding domain of *LexA* fused to the probe or bait protein, shown as being unable to activate either of the two reporters. (B) and (C), EGY48/pSH18-34/pbait-containing yeast have been additionally transformed with an activation domain (act)–fused cDNA library in pJG4-5, and the library has been induced. In (B), the encoded protein does not interact specifically with the bait protein and the two reporters are not activated. In (C), a positive interaction is shown in which the library-encoded protein interacts with bait protein, resulting in activation of the two reporters (arrow), thus causing growth on medium lacking Leu and blue color on medium containing Xgal. Symbols: black rectangle, *LexA* operator sequence; open circle, *LexA* protein; open pentagon, bait protein; open rectangle, library protein; shaded box, activator protein (acid blob).

When more than one bait will be used to screen a single library, significant time and resources can be saved by performing the interactor hunt by interaction mating (see Alternate Protocol 2). In this protocol, EGY48 is transformed with library DNA and the transformants are collected and frozen in aliquots. For each interactor hunt, an aliquot of the pretransformed EGY48/library strain is thawed and mixed with an aliquot of a bait strain transformed with the bait expression plasmid and pSH18-34. Overnight incubation of the mixture on a YPD plate results in fusion of the two strains to form diploids. The diploids are then exposed to galactose to induce expression of the library-encoded proteins, and interactors are selected in the same manner as in Basic Protocol 2. The advantage to this approach is that it requires only one high-efficiency library transformation for multiple hunts with different baits. It is also useful for bait proteins that are somewhat toxic to yeast; yeast expressing toxic baits can be difficult to transform with the library DNA.

CHARACTERIZING A BAIT PROTEIN

The first step in an interactor hunt is to construct a plasmid that expresses *LexA* fused to the protein of interest. This construct is transformed into reporter yeast strains containing *LEU2* and *lacZ* reporter genes, and a series of control experiments is performed to establish whether the construct is suitable as is or must be modified, and whether alternative yeast reporter conditions should be used. These controls establish that the bait protein is made as a stable protein in yeast, that it is capable of entering the nucleus and binding *LexA* operator sites, and that it does not appreciably activate transcription of the *LexA* operator–based reporter genes. This last is the most important constraint on use of this system. The *LexA*–fused bait protein must not activate transcription of either re-

BASIC PROTOCOL 1

Macromolecular Interactions in Cells

17.3.3

Table 17.3.1 Interaction Trap Components^{a,b}

Plasmid name/source ^c	Selection		Comment/description
	In yeast	In <i>E. coli</i>	
<i>LexA fusion plasmids</i>			
pEG202 ^{d,e}	<i>HIS3</i>	Ap ^r	Contains an <i>ADH</i> promoter that expresses LexA followed by polylinker
pJK202 ^e	<i>HIS3</i>	Ap ^r	Like pEG202, but incorporates nuclear localization sequences between LexA and polylinker; used to enhance translocation of bait to nucleus
pNLexA ^e	<i>HIS3</i>	Ap ^r	Contains an <i>ADH</i> promoter that expresses polylinker followed by LexA; for use with baits where amino-terminal residues must remain unblocked
pGilda ^{d,e}	<i>HIS3</i>	Ap ^r	Contains a <i>GALI</i> promoter that expresses same LexA and polylinker cassette as pEG202; for use with baits whose continuous presence is toxic to yeast
pEE202I	<i>HIS3</i>	Ap ^r	An integrating form of pEG202 that can be targeted into <i>HIS3</i> following digestion with <i>KpnI</i> ; for use where physiological screen requires lower levels of bait to be expressed
pRFHM1 ^{e,f} (control)	<i>HIS3</i>	Ap ^r	Contains an <i>ADH</i> promoter that expresses LexA fused to the homeodomain of bicoid to produce nonactivating fusion; used as positive control for repression assay, negative control for activation and interaction assays
pSH17-4 ^{e,f} (control)	<i>HIS3</i>	Ap ^r	<i>ADH</i> promoter expresses LexA fused to GAL4 activation domain; used as a positive control for transcriptional activation
pMW101 ^f	<i>HIS3</i>	Cm ^r	Same as pEG202, but with altered antibiotic resistance markers; basic plasmid used for cloning bait
pMW103 ^f	<i>HIS3</i>	Km ^r	Same as pEG202, but with altered antibiotic resistance markers; basic plasmid used for cloning bait
pHybLex/Zeo ^{f,g}	Zeo ^r	Zeo ^r	Bait cloning vector compatible with interaction trap and all other two-hybrid systems; minimal <i>ADH</i> promoter expresses LexA followed by extended polylinker
<i>Activation domain fusion plasmids</i>			
pJG4-5 ^{c,d,e,f}	TRP1	Ap ^r	Contains a <i>GALI</i> promoter that expresses nuclear localization domain, transcriptional activation domain, HA epitope tag, cloning sites; used to express cDNA libraries
pJG4-5I	<i>TRP1</i>	Ap ^r	An integrating form of pJG4-5 that can be targeted into <i>TRP1</i> by digestion with <i>Bsu36I</i> (New England Biolabs); to be used with pEE202I to study interactions that occur physiologically at low protein concentrations
pYESTrp ^g	<i>TRP1</i>	Ap ^r	Contains a <i>GALI</i> promoter that expresses nuclear localization domain, transcriptional activation domain, V5 epitope tag, multiple cloning sites; contains f1 ori and T7 promoter/flanking site; used to express cDNA libraries (Invitrogen)
pMW102 ^f	<i>TRP1</i>	Km ^r	Same as pJG4-5, but with altered antibiotic resistance markers; no libraries yet available
pMW104 ^f	<i>TRP1</i>	Cm ^r	Same as pJG4-5, but with altered antibiotic resistance markers; no libraries yet available
<i>LacZ reporter plasmids</i>			
pSH18-34 ^{d,e,f}	<i>URA3</i>	Ap ^r	Contains 8 <i>LexA</i> operators that direct transcription of the <i>lacZ</i> gene; one of the most sensitive indicator plasmids for transcriptional activation
pJK103 ^e	<i>URA3</i>	Ap ^r	Contains two <i>LexA</i> operators that direct transcription of the <i>lacZ</i> gene; an intermediate reporter plasmid for transcriptional activation
pRB1840 ^e	<i>URA3</i>	Ap ^r	Contains 1 <i>LexA</i> operator that directs transcription of the <i>lacZ</i> gene; one of the most stringent reporters for transcriptional activation
pMW112 ^f	<i>URA3</i>	Km ^r	Same as pSH18-34, but with altered antibiotic resistance marker
pMW109 ^f	<i>URA3</i>	Km ^r	Same as pJK103, but with altered antibiotic resistance marker

continued

17.3.4

Table 17.3.1 Interaction Trap Components^{a,b}, continued

Plasmid name/source ^c	Selection		Comment/description
	In yeast	In <i>E. coli</i>	
pMW111 ^f	<i>URA3</i>	Km ^r	Same as pRB1840, but with altered antibiotic resistance marker
pMW107 ^f	<i>URA3</i>	Cm ^r	Same as pSH18-34, but with altered antibiotic resistance marker
pMW108 ^f	<i>URA3</i>	Cm ^r	Same as pJK103, but with altered antibiotic resistance marker
pMW110 ^f	<i>URA3</i>	Cm ^r	Same as pRB1840, but with altered antibiotic resistance marker
pJK101 ^{e,f} (control)	<i>URA3</i>	Ap ^r	Contains a <i>GALI</i> upstream activating sequence followed by two <i>lexA</i> operators followed by <i>lacZ</i> gene; used in repression assay to assess bait binding to operator sequences

^aAll plasmids contain a 2 μ m origin for maintenance in yeast, as well as a bacterial origin of replication, except where noted (pEE2021, pJG4.51).

^bInteraction Trap reagents represent the work of many contributors: the original basic reagents were developed in the Brent laboratory (Gyuris et al., 1993). Plasmids with altered antibiotic resistance markers (all pMW plasmids) were constructed at Glaxo in Research Triangle Park, N.C. (Watson et al., 1996). Plasmids and strains for specialized applications have been developed by the following individuals: E. Golemis, Fox Chase Cancer Center, Philadelphia, Pa. (pEG202); J. Kamens, BASF, Worcester, Mass. (pJK202); cumulative efforts of I. York, Dana-Farber Cancer Center, Boston, Mass. and M. Sainz and S. Nottwehr, U. Oregon (pNLexA); D.A. Shaywitz, MIT Center for Cancer Research, Cambridge, Mass. (pGilda); R. Buckholz, Glaxo, Research Triangle Park, N.C. (pEE2021, pJG4-51); J. Gyuris, Mitotix, Cambridge, Mass. (pJG4-5); S. Hanes, Wadsworth Institute, Albany, N.Y. (pSH17-4); R.L. Finley, Wayne State University School of Medicine, Detroit, Mich. (pRFHM1); S. Hanes, Wadsworth Institute, Albany, N.Y. (pSH18-34); J. Kamens, BASF, Worcester, Mass. (pJK101, pJK103); R. Brent, The Molecular Sciences Institute, Berkeley, Calif. (pRB1840). Specialized plasmids not yet commercially available can be obtained by contacting the Brent laboratory at (510) 647-0690 or brent@molsci.org, or the Golemis laboratory, (215) 728-2860 or EA_Golemis@fcc.edu.

^cMaps, sequences, and links for some of the plasmids are available at <http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html>.

^dPlasmids commercially available from Clontech and OriGene; for Clontech pEG202 is listed as pLexA, pJG4-5 as pB42AD, and pSH18-34 as p8op-LacZ.

^ePlasmids and strains available from OriGene.

^fIn pMW plasmids the ampicillin resistance gene (Ap^r) is replaced with the chloramphenicol resistance gene (Cm^r) and the kanamycin resistance gene (Km^r) from pBC SK(+) and pBK-CMV (Stratagene), respectively. The choice between Km^r and Cm^r or Ap^r plasmids is a matter of personal taste; use of basic Ap^r plasmids is described in the basic protocols. Use of the more recently developed reagents would facilitate the purification of library plasmid in later steps by eliminating the need for passage through KC8 bacteria, with substantial saving of time and effort. Ap^r has been maintained as marker of choice for the library plasmid because of the existence of multiple libraries already possessing this marker. These plasmids are the basic set of plasmids recommended for use.

^gPlasmids commercially available from Invitrogen as components of a Hybrid Hunter kit; this kit also includes all necessary positive and negative controls (not listed in this table). See Background Information for further details on commercially available reagents.

porter—the EGY48 strain (or related strain EGY191) that expresses the LexA fusion protein should not grow on medium lacking Leu, and the colonies should be white on medium containing Xgal. The characterized bait protein plasmid is used for Basic Protocol 2 to screen a library for interacting proteins.

Materials

DNA encoding the protein of interest

Plasmids (see Table 17.3.1): pEG202 (see Fig. 17.3.3), pSH18-34 (see Fig.

17.3.4), pSH17-4, pRFHM1, and pJK101 for basic characterization;

other plasmids for specific circumstances as described (Clontech, Invitrogen, OriGene, or R. Brent)

Yeast strain EGY48 (*ura3 trp1 his3 3LexA-operator-LEU2*), or EGY191 (*ura3 trp1 his3 1LexA-operator-LEU2*; Table 17.3.2)

Complete minimal (CM) medium dropout plates (see recipe), supplemented with 2% (w/v) of the indicated sugars (glucose or galactose), in 100-mm plates:

Glu/CM, –Ura, –His

Gal/CM, –Ura, –His

Gal/CM, –Ura, –His, –Leu

Z buffer (see recipe) with 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactosidase (Xgal)

Gal/CM dropout liquid medium (see recipe) supplemented with 2% Gal

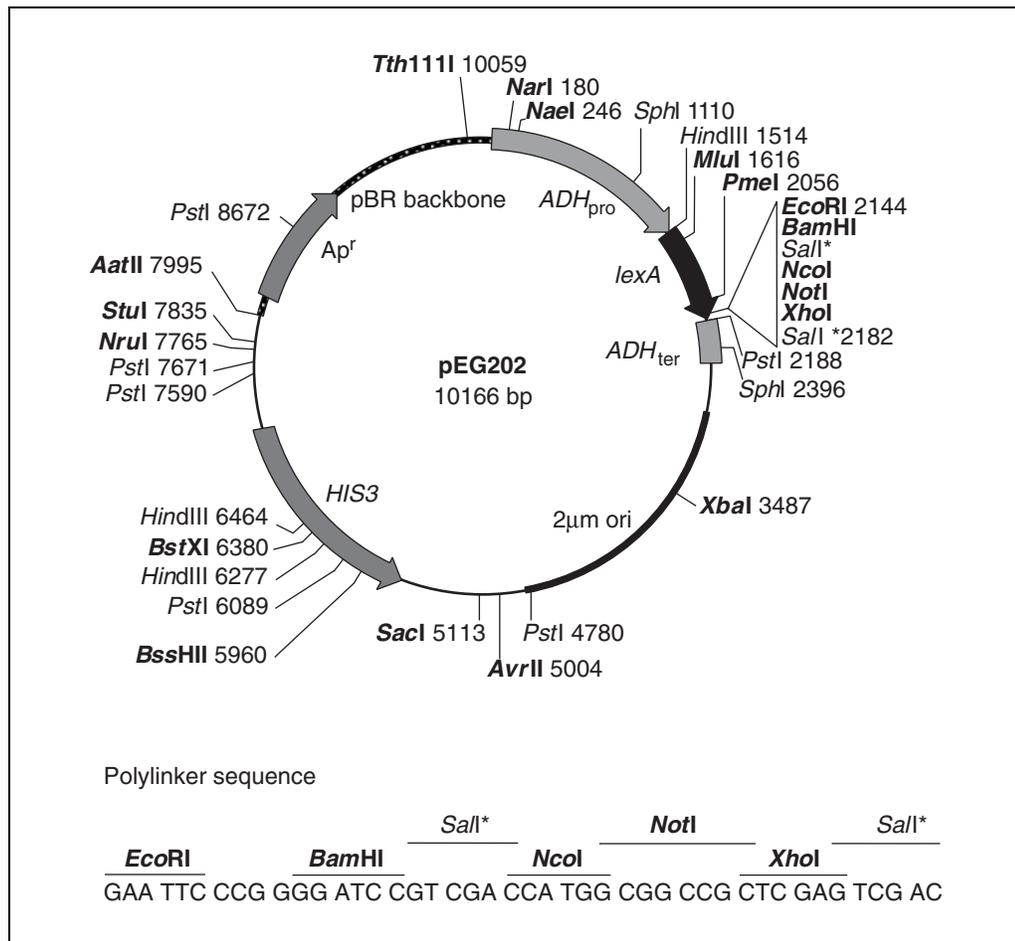


Figure 17.3.3 LexA-fusion plasmids: pEG202 (sequence: accession number U89960). The strong constitutive *ADH* promoter is used to express bait proteins as fusions to the DNA-binding protein LexA. Restriction sites shown in this map are based on recently compiled pEG202 sequence data and include selected sites suitable for diagnostic restriction endonuclease digests. A number of restriction sites are available for insertion of coding sequences to produce protein fusions with LexA; the polylinker sequence and reading frame relative to LexA are shown below the map with unique sites marked in bold type. The sequence 5'-CGT CAG CAG AGC TTC ACC ATT G-3' can be used to design a primer to confirm correct reading frame for LexA fusions. Plasmids contain the *HIS3* selectable marker and the 2μm origin of replication to allow propagation in yeast, and the *Ap^r* antibiotic resistance gene and the pBR origin of replication to allow propagation in *E. coli*. In the recently developed LexA-expression plasmids pMW101 and pMW103, the ampicillin resistance gene (*Ap^r*) has been replaced with the chloramphenicol resistance gene (*Cm^r*) and the kanamycin resistance gene (*Km^r*), respectively. Alternative bait plasmids are listed in Table 17.3.1, and maps, sequences, and links for some of them are available at <http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html>.

Antibody to LexA or fusion domain: monoclonal antibody to LexA (Clontech, Invitrogen) or polyclonal antibody to LexA (available by request from R. Brent or E. Golemis)

H₂O, sterile

30°C incubator

Nylon membrane

Whatman 3MM filter paper

Additional reagents and equipment for subcloning DNA fragments, lithium acetate transformation of yeast, liquid assay for β-galactosidase (APPENDIX 3), preparation of protein extracts for immunoblot analysis (see Support Protocol 1), and immunoblotting and immunodetection (UNIT 6.2)

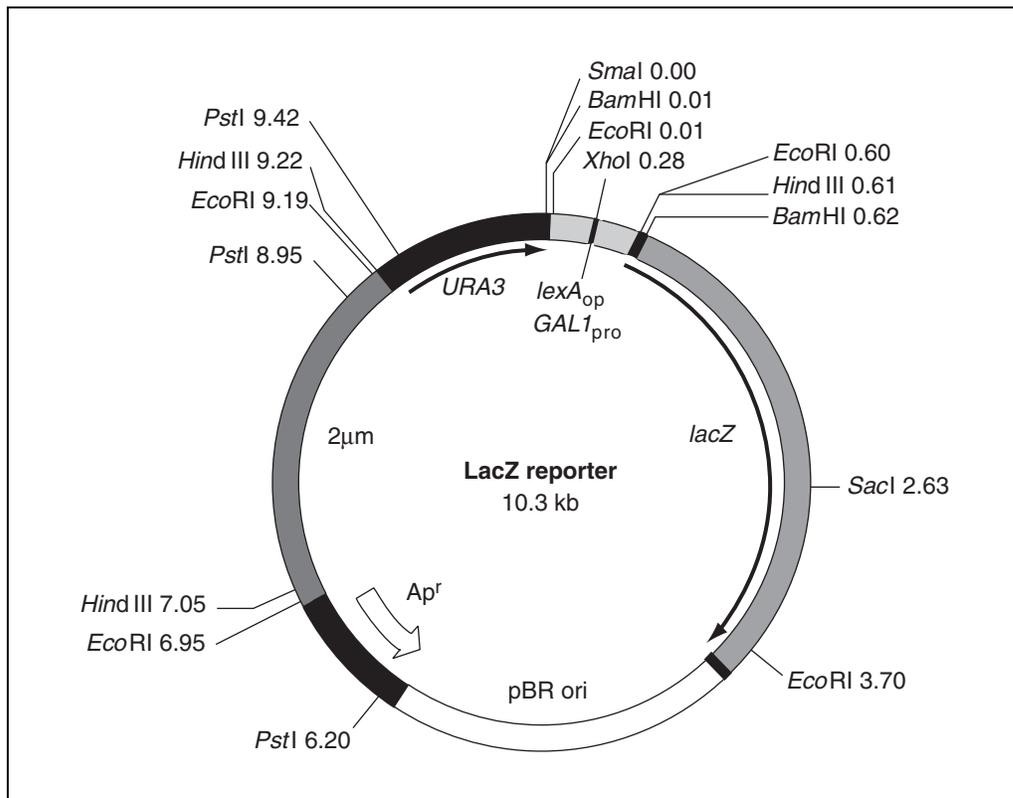


Figure 17.3.4 LacZ reporter plasmid. pRB1840, pJK103, and pSH18-34 are all derivatives of LR1Δ1 (West et al., 1984) containing eight, two, or one operator for LexA (*LexA_{op}*) binding inserted into the unique *XhoI* site located in the minimal *GAL1* promoter (*GAL1_{pro}*; 0.28 on map). The plasmid contains the *URA3* selectable marker, the 2µm origin to allow propagation in yeast, the ampicillin resistance (*Ap^r*) gene, and the pBR322 origin (*ori*) to allow propagation in *E. coli*. Numbers indicate relative map positions. In the recently developed derivatives, the ampicillin resistance gene (*Ap^r*) has been replaced with the chloramphenicol or kanamycin resistance genes.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

Transform yeast with the bait protein plasmid

- Using standard subcloning techniques (APPENDIX 3), insert the DNA encoding the protein of interest into the polylinker of pEG202 (see Fig. 17.3.3) or other LexA fusion plasmid to make an in-frame protein fusion.

The LexA fusion protein is expressed from the strong alcohol dehydrogenase (ADH) promoter. pEG202 also contains a HIS3 selectable marker and a 2µm origin for propagation in yeast. pEG202 with the DNA encoding the protein of interest inserted is designated pBait. Uses of alternative LexA fusion plasmids are described in Background Information.

- Perform three separate lithium acetate transformations (APPENDIX 3) of EGY48 using the following combinations of plasmids:

- pBait + pSH18-34 (test)
- pSH17-4 + pSH18-34 (positive control for activation)
- pRFHM1 + pSH18-34 (negative control for activation).

Use of the two LexA fusions as positive and negative controls allows a rough assessment of the transcriptional activation profile of LexA bait proteins. pEG202 itself is not a good negative control because the peptide encoded by the uninterrupted polylinker sequences is itself capable of very weakly activating transcription.

Table 17.3.2 Interaction Trap Yeast Selection Strains^a

Strain	Relevant genotype	Number of operators	Comments/description
EGY48 ^{b,c,d}	<i>MATα trp1, his3, ura3, lexAops-LEU2</i>	6	<i>lexA</i> operators direct transcription from the <i>LEU2</i> gene; EGY48 is a basic strain used to select for interacting clones from a cDNA library
EGY191 ^c	<i>MATα trp1, his3, ura3, lexAops-LEU2</i>	2	EGY191 provides a more stringent selection than EGY48, producing lower background with baits with intrinsic ability to activate transcription
L40 ^c	<i>MATα trp1, leu2, ade2, GAL4, lexAops-HIS3</i>	4	Expression driven from <i>GAL1</i> promoter is constitutive in L40 (inducible in EGY strains); selection is for <i>HIS</i> prototrophy.
	<i>MATα trp1, leu2, ade2, GAL4, lexAops-lacZ</i>	8	Integrated <i>lacZ</i> reporter is considerably less sensitive than pSH18-34 maintained in EGY strains

^aInteraction Trap reagents represent the work of many contributors: the original basic reagents were developed in the Brent laboratory (Gyuris et al., 1993). Strains for specialized applications have been developed by the following individuals: E. Golemis, Fox Chase Cancer Center, Philadelphia, Pa. (EGY48, EGY191); A.B. Vojtek and S.M. Hollenberg, Fred Hutchinson Cancer Research Center, Seattle, Wash. (L40).

^bStrains commercially available from Clontech.

^cStrains commercially available from Invitrogen as components of a Hybrid Hunter kit; the kit also includes all necessary positive and negative controls (not listed in this table). See Background Information for further details on commercially available reagents.

^dStrains commercially available from OriGene.

pSH18-34 contains a 2μm origin and a URA3 selectable marker for maintenance in yeast, as well as a bacterial origin of replication and ampicillin-resistance gene. It is the most sensitive lacZ reporter available and will detect any potential ability to activate lacZ transcription. pSH17-4 is a HIS3 2μm plasmid encoding LexA fused to the activation domain of the yeast activator protein GAL4. This fusion protein strongly activates transcription. pRFHM1 is a HIS3 2μm plasmid encoding LexA fused to the N-terminus of the Drosophila protein bicoid. This fusion protein has no ability to activate transcription.

3. Plate each transformation mixture on Glu/CM –Ura, –His dropout plates. Incubate 2 days at 30°C to select for yeast that contain both plasmids.

Colonies obtained can be used simultaneously in tests for the activation of lacZ (steps 4 to 7) and LEU2 (steps 12 to 13) reporters.

Assay lacZ gene activation by β-galactosidase assay

4. Streak a Glu/CM –Ura, –His master dropout plate with at least five or six independent colonies obtained from each of the three transformations in step 3 (test, positive control, and negative control) and incubate overnight at 30°C.

The filter assay described in Steps 5a to 7a (based on Breeden and Nasmyth, 1985) provides a rapid assay for β-galactosidase transcription. Alternatively, a liquid assay (APPENDIX 3) or a plate assay (described in Steps 5b to 7b) may be used. More information on β-galactosidase assays is available on <http://www.fccc.edu/research/labs/golemis/betagal.html>.

Perform filter assay for β-galactosidase activity:

- 5a. Lift colonies by gently placing a nylon membrane on the yeast plate and allowing it to become wet through. Remove the membrane and air dry 5 min. Chill the membrane, colony side up, 10 min at –70°C.

Whatman 3MM filters can be cut to the size of the yeast plate as a more economical alternative to nylon membranes for performing lifts. In addition, two or three 5-min temperature cycles (–70°C to room temperature) can be used instead of a single cycle to promote better lysis; this may be worth doing if there is difficulty visualizing blue color.

- 6a. Cut a piece of Whatman 3MM filter paper slightly larger than the colony membrane and soak it in Z buffer containing 1 mg/ml Xgal. Place colony membrane, colony side up, on Whatman 3MM paper, or float it in the lid of a petri dish containing ~2 ml Z buffer with 1 mg/ml Xgal.

Acceptable results may be obtained using as little as 300 µg/ml Xgal.

- 7a. Incubate at 30°C and monitor for color changes.

It is generally useful to check the membrane after 20 min, and again after 2 to 3 hr. Strong activators will produce a blue color in 5 to 10 min, and a bait protein (LexA fusion protein) that does so is unsuitable for use in an interactor hunt using this lacZ reporter plasmid. Weak activators will produce a blue color in 1 to 6 hr (compare versus negative control pRFHMI which will itself produce a faint blue color with time) and may or may not be suitable. Weak activators should be tested using the repressor assay described in steps 8 to 11.

Perform Xgal plate assay for lacZ activation:

- 5b. Prepare Z buffer Xgal plates.

For activation assays, plates should be prepared with glucose as a sugar source. For repression assays (steps 8 to 11), galactose should be used as a sugar source. In our experience, when patching from a master plate to Xgal plates, sufficient yeast are transferred that plasmid loss is not a major problem even in the absence of selection; this is balanced by the desire to assay sets of constructs on the same plate to eliminate batch variation in Xgal potency. Hence, plates should be made either with complete minimal amino acid mix, or by dropping out only uracil (–Ura), to make the plates universally useful.

- 6b. Streak yeast from master plate to Xgal plate and incubate at 30°C.

- 7b. Examine plates for color development at intervals over the next 2 to 3 days.

Strongly activating fusions should be visibly blue on the plate within 12 to 24 hr; moderate activators will be visibly blue after ~2 days.

When a bait protein appreciably activates transcription under these conditions, there are several recourses. The first and simplest is to switch to a less sensitive lacZ reporter plasmid; use of pJK103 and pRB1840 may be sufficient to reduce background to manageable levels. If this fails to work, it is frequently possible to generate a truncated LexA fusion that does not activate transcription.

Confirm fusion-protein synthesis by repression assay

For LexA fusions that do not activate transcription, confirm by performing a repression assay (Brent and Ptashne, 1984) that the LexA fusion protein is being synthesized in yeast (some proteins are not) and that it is capable of binding LexA operator sequences (Fig. 17.3.5). The following steps can be performed concurrently with the activation assay.

8. Transform EGY48 yeast with the following combinations of plasmids (three transformations):
- pBait + pJK101 (test)
 - pRFHM1 + pJK101 (positive control for repression)
 - pJK101 alone (negative control for repression).
9. Plate each transformation mix on Glu/CM –Ura, –His dropout plates or Glu/CM –Ura dropout plates as appropriate to select yeast cells that contain the indicated plasmids. Incubate 2 to 3 days at 30°C until colonies appear.
10. Streak colonies to a Glu/CM –Ura, –His or Glu/CM –Ura dropout master plate and incubate overnight at 30°C.
11. Assay β-galactosidase activity of the three transformed strains (test, positive control, and negative control) by liquid assay (using Gal/CM dropout liquid medium), filter

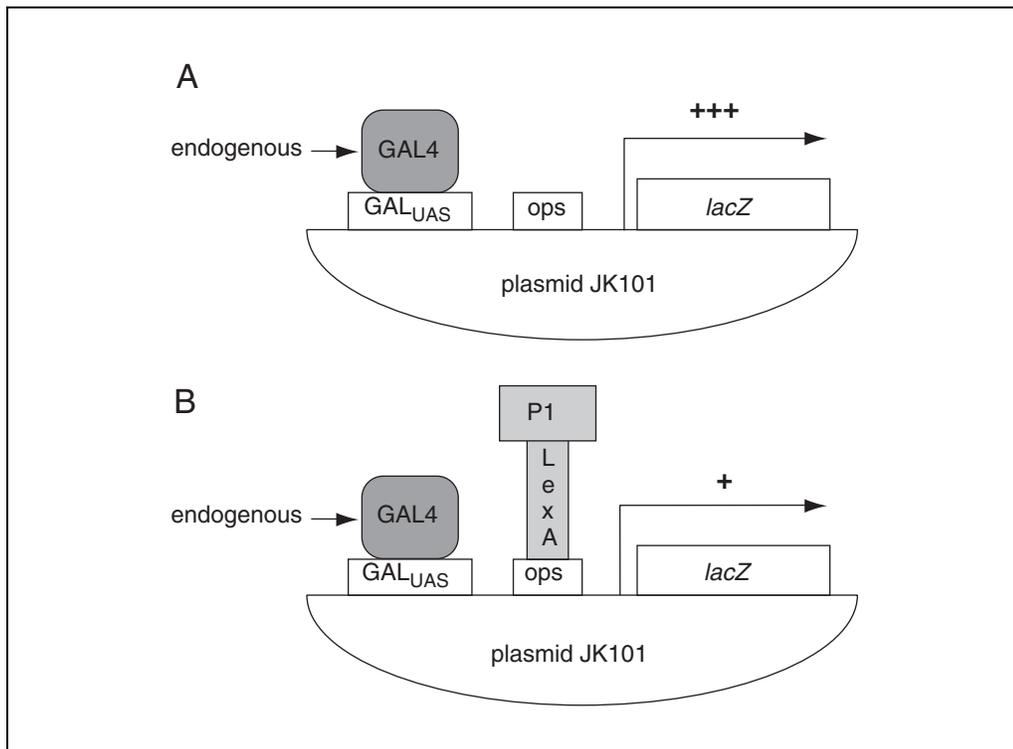


Figure 17.3.5 Repression assay for DNA binding. **(A)** The plasmid JK101 contains the upstream activating sequence (UAS) from the *GAL1* gene followed by *LexA* operators upstream of the *lacZ* coding sequence. Thus, yeast containing pJK101 will have significant β -galactosidase activity when grown on medium in which galactose is the sole carbon source because of binding of endogenous yeast GAL4 to the GAL_{UAS}. **(B)** LexA-fused proteins (P1-LexA) that are made, enter the nucleus, and bind the *LexA* operator sequences (ops) will block activation from the GAL_{UAS}, repressing β -galactosidase activity (+) 3- to 5-fold. On glucose/Xgal medium, yeast containing pJK101 should be white because GAL_{UAS} transcription is repressed.

assay (steps 5a to 7a, first restreaking to Gal/CM plates to grow overnight), or plate assay (steps 5b to 7b, using Gal/CM –Ura XGal plates).

This assay should not be run for more than 1 to 2 hr for membranes, or 36 hr for Xgal plates, as the high basal lacZ activity will make differential activation of pJK101 impossible to see with longer incubations. Use of Xgal plates, and inspection 12 to 24 hr after streaking, is generally most effective.

The plasmid pJK101 contains the galactose upstream activating sequence (UAS) followed by LexA operators upstream of the lacZ coding sequence. Thus, yeast containing pJK101 will have significant β -galactosidase activity when grown on medium in which galactose is the sole carbon source because of binding of endogenous yeast GAL4 to the GAL_{UAS}. LexA-fused proteins that are made, enter the nucleus, and bind the LexA operator sequences block activation from the GAL_{UAS}, repressing β -galactosidase activity 3- to 20-fold. Note that on Glu/Xgal medium, yeast containing pJK101 should be white, because GAL_{UAS} transcription is repressed.

12. If a bait protein neither activates nor represses transcription, perform immunoblot analysis by probing an immunoblot of a crude lysate with antibodies against LexA or the fusion domain to test for protein synthesis (see Support Protocol 1).

Even if a bait protein represses transcription, it is generally a good idea to assay for the production of full-length LexA fusions, as occasionally some fusion proteins will be proteolytically cleaved by endogenous yeast proteases. If the protein is made but does not repress, it may be necessary to clone the sequence into a LexA fusion vector that contains a nuclear localization motif, e.g., pJK202 (see Table 17.3.1), or to modify or truncate the

fusion domain to remove motifs that target it to other cellular compartments (e.g., myristoylation signals).

Test for Leu requirement

These steps can be performed concurrently with the *lacZ* activation and repression assays.

13. Disperse a colony of EGY48 containing pBait and pSH18-34 reporter plasmids into 500 μ l sterile water. Dilute 100 μ l of suspension into 1 ml sterile water. Make a series of 1/10 dilutions in sterile water to cover a 1000-fold concentration range.
14. Plate 100 μ l from each tube (undiluted, 1/10, 1/100, and 1/1000) on Gal/CM –Ura, –His dropout plates and on Gal/CM –Ura, –His, –Leu dropout plates. Incubate overnight at 30°C.

There will be a total of eight plates. Gal/CM –Ura, –His dropout plates should show a concentration range from 10 to 10,000 colonies and Gal/CM –Ura, –His, –Leu dropout plates should have no colonies.

Actual selection in the interactor hunt is based on the ability of the bait protein and acid-fusion pair, but not the bait protein alone, to activate transcription of the LexA operator-LEU2 gene and allow growth on medium lacking Leu. Thus, the test for the Leu requirement is the most important test of whether the bait protein is likely to have an unworkably high background. The LEU2 reporter in EGY48 is more sensitive than the pSH18-34 reporter for some baits, so it is possible that a bait protein that gives little or no signal in a β -galactosidase assay would nevertheless permit some level of growth on –Leu medium. If this occurs, there are several options for proceeding, the most immediate of which is to substitute EGY191 (see Table 17.3.2), a less sensitive screening strain, and repeat the assay.

As outlined in this protocol, the authors recommend the strategy of performing the initial screening using the most sensitive reporters and then, if activation is detected, screening with increasingly less sensitive reporters (see Critical Parameters for further discussion).

PERFORMING AN INTERACTOR HUNT

An interactor hunt involves two successive large platings of yeast containing LexA-fused probes and reporters and libraries in pJG4-5 (Fig. 17.3.6, Table 17.3.3) with a cDNA expression cassette under control of the *GAL* promoter. In the first plating, yeast are plated on complete minimal (CM) medium –Ura, –His, –Trp dropout plates with glucose (Glu) as a sugar source to select for the library plasmid. In the second plating, which selects for yeast that contain interacting proteins, a slurry of primary transformants is plated on CM –Ura, –His, –Trp, –Leu dropout plates with galactose/raffinose (Gal/Raff) as the sugar source. This two-step selection is encouraged for two reasons. First, a number of interesting cDNA-encoded proteins may be deleterious to the growth of yeast that bear them; these would be competed out in an initial mass plating. Second, it seems likely that immediately after simultaneous transformation and Gal induction, yeast bearing particular interacting proteins may not be able to initially express sufficient levels of these proteins to support growth on medium lacking Leu. Library plasmids from colonies identified in the second plating are purified by bacterial transformation and used to transform yeast cells for the final specificity screen.

A list of libraries currently available for use with this system is provided in Table 17.3.3. The protocol outlined below describes the steps used to perform a single-step screen that should saturate a library derived from a mammalian cell. For screens with libraries derived from lower eukaryotes with less complex genomes, fewer plates will be required.

Occasionally, baits that seemed well-behaved during preliminary tests produce unworkably high backgrounds of “positives” during an actual screen (see Background Informa-

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17.3.11

tion and Critical Parameters). To forestall the waste of time and materials performing a screen with such a bait would entail, an alternative approach is to perform a scaled-back screen when working with a new bait (e.g., 5 rather than 30 plates of primary transformants). The results can be assessed before doing a full screen; it is then possible to switch to lower-sensitivity reporter strains and plasmids, if appropriate. Although individual baits will vary, the authors' current default preference is to use the *lacZ* reporter pJK103 in conjunction with either EGY48 or EGY191. Polymerase chain reaction (PCR) can also be used in a rapid screening approach that may be preferable if a large number of positions are obtained in a library screen (see Alternate Protocol 1).

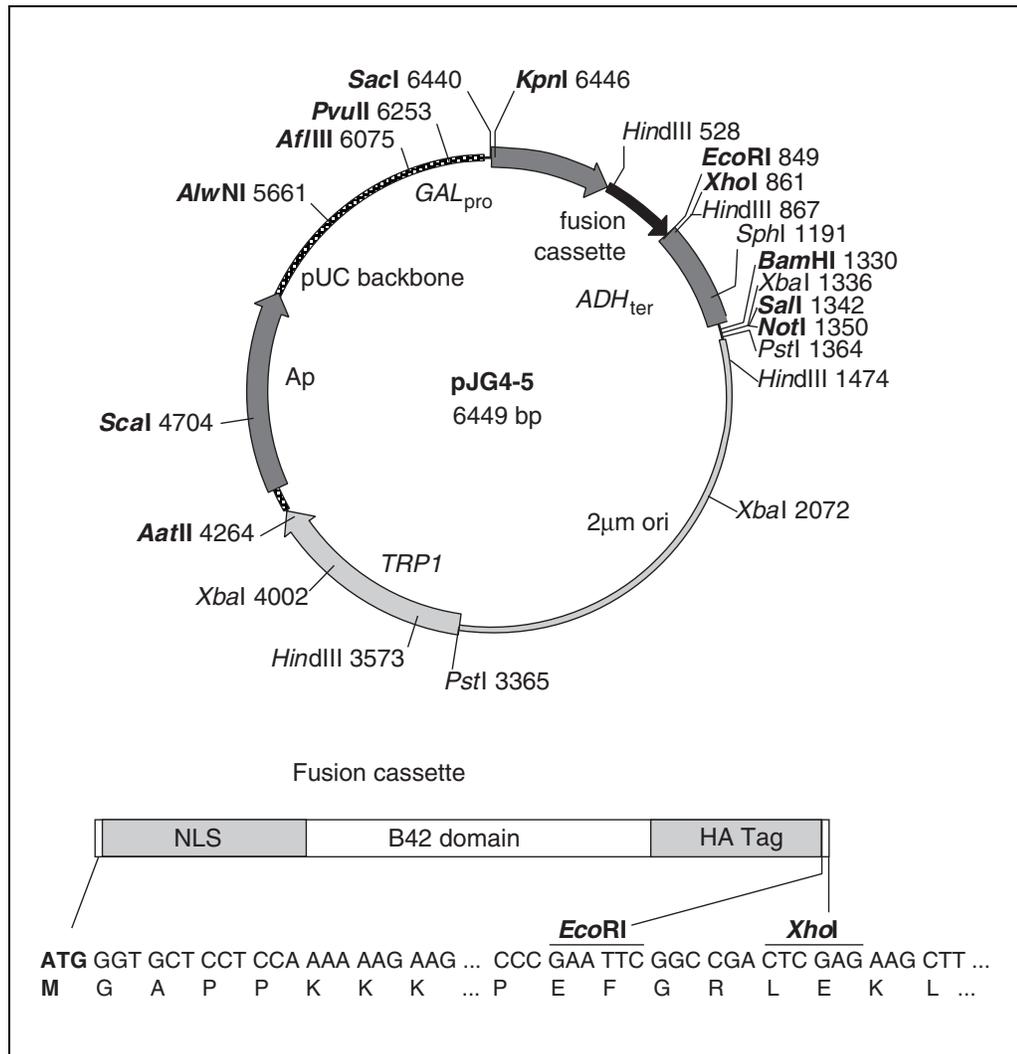


Figure 17.3.6 Library plasmids: pJG4-5 (sequence: accession number U89961). Library plasmids express cDNAs or other coding sequences inserted into unique *EcoRI* and *XhoI* sites as a translational fusion to a cassette consisting of the SV40 nuclear localization sequence (NLS; PPKKKRKVA), the acid blob B42 domain (Ruden et al., 1991), and the hemagglutinin (HA) epitope tag (YPYDVPDYA). Expression of cassette sequences is under the control of the *GAL1* galactose-inducible promoter. This map is based on the sequence data available for pJG4-5, and includes selected sites suitable for diagnostic restriction digests (shown in bold). The sequence 5'-CTG AGT GGA GAT GCC TCC-3' can be used as a primer to identify inserts or to confirm correct reading frame. The pJG4-5 plasmid contains the *TRP1* selectable marker and the 2 μ m origin to allow propagation in yeast, and the antibiotic resistance gene and the pUC origin to allow propagation in *E. coli*. Unique sites are marked in bold type. Alternative library plasmids are listed in Table 17.3.1 and maps, sequences, and links for some of them are available at <http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html>.

Materials

Yeast containing appropriate combinations of plasmids (see Table 17.3.1 and Table 17.3.2):

EGY48 containing *LexA*-operator-*lacZ* reporter and pBait (see Basic Protocol 1)

EGY48 containing *LexA*-operator-*lacZ* reporter and pRFHM-1

EGY48 containing *LexA*-operator-*lacZ* reporter and any nonspecific bait

Complete minimal (CM) dropout liquid medium (see recipe) supplemented with sugars (glucose, galactose, and/or raffinose) as indicated [2% (w/v) Glu, or 2% (w/v) Gal + 1% (w/v) Raff]:

Glu/CM –Ura, –His

Glu/CM –Trp

Gal/Raff/CM –Ura, –His, –Trp

H₂O, sterile

TE buffer (pH 7.5; APPENDIX 2A)/0.1 M lithium acetate

Library DNA in pJG4-5 (Table 17.3.3 and Fig. 17.3.6)

High-quality sheared salmon sperm DNA (see Support Protocol 2)

40% (w/v) polyethylene glycol 4000 (PEG 4000; filter sterilized)/0.1 M lithium acetate/TE buffer (pH 7.5)

Dimethyl sulfoxide (DMSO)

Complete minimal (CM) medium dropout plates (see recipe) supplemented with sugars and Xgal (20 µg/ml) as indicated [2% (w/v) Glu, and 2% (w/v) Gal + 1% (w/v) Raff]:

Glu/CM –Ura, –His, –Trp, 24 × 24-cm (Nunc) and 100-mm

Gal/Raff/CM –Ura, –His, –Trp, 100-mm

Gal/Raff/CM –Ura, –His, –Trp, –Leu, 100-mm

Glu/Xgal/CM –Ura, –His, –Trp, 100-mm

Gal/Raff/Xgal/CM –Ura, –His, –Trp, 100-mm

Glu/CM –Ura, –His, –Trp, –Leu, 100-mm

Glu/CM –Ura, –His, 100-mm

Gal/CM –Ura, –His, –Trp, –Leu, 100-mm

TE buffer (pH 7.5), sterile (optional)

Glycerol solution (see recipe)

E. coli KC8 (*pyrF leuB600 trpC hisB463*; constructed by K. Struhl and available from R. Brent)

LB/ampicillin plates: LB plates (APPENDIX 2A) with 50 µg/ml ampicillin

E. coli DH5α or other strain suitable for preparation of DNA for sequencing

Bacterial defined minimal A medium plates: 1× A medium plates containing 0.5 µg/ml vitamin B1 supplemented with 40 µg/ml each Ura, His, and Leu

30°C incubator, with and without shaking

Low-speed centrifuge and rotor

50-ml conical tubes, sterile

1.5-ml microcentrifuge tubes, sterile

42°C heating block

Glass microscope slides, sterile

Additional reagents and equipment for rapid miniprep isolation of yeast DNA, transformation of bacteria by electroporation, miniprep isolation of bacterial DNA, restriction endonuclease digestion (optional), and agarose gel electrophoresis (optional; APPENDIX 3)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

Table 17.3.3 Libraries Compatible with the Interaction Trap System^{a,b}

Source of RNA/DNA	Vector	Independent clones	Insert size (average) ^c	Contact information
Cell lines				
HeLa cells (human cervical carcinoma)	JG	9.6×10^6	0.3-3.5 kb (1.5 kb)	R. Brent, Clontech, Invitrogen, OriGene
HeLa cells (human cervical carcinoma)	Y	3.7×10^6	0.3-1.2 kb	Invitrogen
WI-38 cells (human lung fibroblasts), serum-starved, cDNA	JG	5.7×10^6	0.3-3.5 kb (1.5 kb)	R. Brent, Clontech, OriGene
Jurkat cells (human T cell leukemia), exponentially growing, cDNA	JG	4.0×10^6	0.7-2.8 kb (1.5 kb)	R. Brent
Jurkat cells (human T cell leukemia)	Y	3.2×10^6	0.3-1.2 kb	Invitrogen
Jurkat cells (human T cell leukemia)	Y	3.0×10^6	0.5-4.0 kb (1.8 kb)	Clontech
Jurkat cells (human T cell leukemia)	JG	5.7×10^6	(>1.3)	OriGene
Jurkat cells (human T cell leukemia)	JG	2×10^6	0.7-3.5 kb (1.2 kb)	S. Witte
Be Wo cells (human fetal placental choriocarcinoma)	Y	5.4×10^6	0.3-0.8 kb	Invitrogen
Human lymphocyte	JG	4.0×10^6	0.4-4.0 kb (2.0 kb)	Clontech
CD4 ⁺ T cell, murine, cDNA	JG	> 10^6	0.3-2.5 kb (>0.5 kb)	R. Brent
Chinese hamster ovary (CHO) cells, exponentially growing, cDNA	JG	1.5×10^6	0.3-3.5 kb	R. Brent
A20 cells (mouse B cell lymphoma)	Y	3.11×10^6	0.3-1.2 kb	Invitrogen
Human B cell lymphoma	JG	—	—	H. Niu
Human 293 adenovirus-infected (early and late stages)	JG	—	—	K. Gustin
SKOV3 human Y ovarian cancer	Y	5.0×10^6	(>1.4 kb)	OriGene
MDBK cell, bovine kidney	JG	5.8×10^6	(>1.2 kb)	OriGene
MDCK cells	JG	—	—	D. Chen
HepG2 cell line cDNA	JG	2×10^6	—	M. Melegari
MCF7 breast cancer cells, untreated	JG	1.0×10^7	(>1.5 kb)	OriGene
MCF7 breast cancer cells, estrogen-treated	JG	1.0×10^7	(>1.1 kb)	OriGene
MCF7 cells, serum-grown	JG	1.0×10^7	0.4-3.5 kb	OriGene
LNCAP prostate cell line, untreated	JG	2.9×10^6	(>0.8 kb)	OriGene
LNCAP prostate cell line, androgen-treated	JG	4.6×10^6	(>0.9 kb)	OriGene
Mouse pachytene spermatocytes	JG	—	—	C. Hoog
Tissues				
Human breast	Y	9×10^6	0.4-1.2 kb	Invitrogen
Human breast tumor	Y	8.84×10^6	0.4-1.2 kb	Invitrogen
Human liver	JG	> 10^6	0.6-4.0 kb (>1 kb)	R. Brent
Human liver	Y	2.2×10^6	0.5-4 kb (1.3 kb)	Clontech
Human liver	JG	3.2×10^6	0.3-1.2 kb	Invitrogen
Human liver	JG	1.1×10^7	(> 1 kb)	OriGene
Human lung	Y	5.9×10^6	0.4-1.2 kb	Invitrogen
Human lung tumor	Y	1.9×10^6	0.4-1.2	Invitrogen
Human brain	JG	3.5×10^6	0.5-4.5 kb (1.4 kb)	Clontech
Human brain	Y	8.9×10^6	0.3-1.2 kb	Invitrogen
Human testis	Y	6.4×10^6	0.3-1.2 kb	Invitrogen
Human testis	JG	3.5×10^6	0.4-4.5 kb (1.6 kb)	Clontech
Human ovary	Y	4.6×10^6	0.3-1.2 kb	Invitrogen

continued

Table 17.3.3 Libraries Compatible with the Interaction Trap System^{a,b}, continued

Source of RNA/DNA	Vector	Independent clones	Insert size (average) ^c	Contact information
Human ovary	JG	4.6 × 10 ⁶	(>1.3 kb)	OriGene
Human ovary	JG	3.5 × 10 ⁶	0.5-4.0 kb (1.8 kb)	Clontech
Human heart	JG	3.0 × 10 ⁶	0.3-3.5 kb (1.3 kb)	Clontech
Human placenta	Y	4.8 × 10 ⁶	0.3-1.2 kb	Invitrogen
Human placenta	JG	3.5 × 10 ⁶	0.3-4.0 kb (1.2 kb)	Clontech
Human mammary gland	JG	3.5 × 10 ⁶	0.5-5 kb (1.6 kb)	Clontech
Human peripheral blood leucocyte	JG	1.0 × 10 ⁷	(>1.3 kb)	OriGene
Human kidney	JG	3.5 × 10 ⁶	0.4-4.5 kb (1.6 kb)	Clontech
Human fetal kidney	JG	3.0 × 10 ⁶	(>1 kb)	OriGene
Human spleen	Y	1.14 × 10 ⁷	0.4-1.2 kb	Invitrogen
Human prostate	Y	5.5 × 10 ⁶	0.4-1.2 kb	Invitrogen
Human normal prostate	JG	1.4 × 10 ⁶	0.4-4.5 kb (1.7 kb)	Clontech
Human prostate	JG	1.4 × 10 ⁶	(>1 kb)	OriGene
Human prostate cancer	JG	1.1 × 10 ⁶	(>0.9 kb)	OriGene
Human fetal liver	JG	3.5 × 10 ⁶	0.3-4.5 kb (1.3 kb)	Clontech
Human fetal liver	Y	2.37 × 10 ⁶	0.3-1.2 kb	Invitrogen
Human fetal liver	JG	8.6 × 10 ⁶	(>1 kb)	OriGene
Human fetal brain	JG	3.5 × 10 ⁶	0.5-1.2 kb (1.5 kb)	R. Brent, Clontech, Invitrogen, OriGene
Mouse brain	JG	6.1 × 10 ⁶	(>1 kb)	OriGene
Mouse brain	JG	4.5 × 10 ⁶	0.4-4.5 kb (1.2 kb)	Clontech
Mouse breast, lactating	JG	1.0 × 10 ⁷	0.4-3.1 kb	OriGene
Mouse breast, involuting	JG	1.0 × 10 ⁷	0.4-7.0 kb	OriGene
Mouse breast, virgin	JG	1.0 × 10 ⁷	0.4-5.5 kb	OriGene
Mouse breast, 12 days pregnant	JG	6.3 × 10 ⁶	0.4-5.3 kb	OriGene
Mouse skeletal muscle	JG	7.2 × 10 ⁶	0.4-3.5 kb	OriGene
Rat adipocyte, 9-week-old Zucker rat	JG	1.0 × 10 ⁷	0.4-5.0 kb	OriGene
Rat brain	JG	4.5 × 10 ⁶	0.3-3.4 kb	OriGene
Rat brain (day 18)	JG	—	—	H. Niu
Rat testis	JG	8.0 × 10 ⁶	(>1.2 kb)	OriGene
Rat thymus	JG	8.2 × 10 ⁶	(>1.3 kb)	OriGene
Mouse liver	JG	9.5 × 10 ⁶	(>1.4 kb)	OriGene
Mouse spleen	JG	1.0 × 10 ⁷	(>1 kb)	OriGene
Mouse ovary	JG	4.0 × 10 ⁶	(>1.2 kb)	OriGene
Mouse prostate	JG	3.7 × 10 ⁶	0.3-4.0 kb	OriGene
Mouse embryo, whole (19-day)	JG	1.0 × 10 ⁵	0.2-2.5 kb	OriGene
Mouse embryo	JG	3.6 × 10 ⁶	0.5-5 kb (1.7 kb)	Clontech
<i>Drosophila melanogaster</i> , adult, cDNA	JG	1.8 × 10 ⁶	(>1.0 kb)	OriGene
<i>D. melanogaster</i> , embryo, cDNA	JG	3.0 × 10 ⁶	0.5-3.0 kb (1.4 kb)	Clontech
<i>D. melanogaster</i> , 0-12 hr embryos, cDNA	JG	4.2 × 10 ⁶	0.5-2.5 kb (1.0 kb)	R. Brent
<i>D. melanogaster</i> , ovary, cDNA	JG	3.2 × 10 ⁶	0.3-1.5 kb (800 bp)	R. Brent
<i>D. melanogaster</i> , disc, cDNA	JG	4.0 × 10 ⁶	0.3-2.1 kb (900 bp)	R. Brent
<i>D. melanogaster</i> , head	JG	—	—	M. Rosbash

continued

Table 17.3.3 Libraries Compatible with the Interaction Trap System^{a,b}, continued

Source of RNA/DNA	Vector	Independent clones	Insert size (average) ^c	Contact information
Miscellaneous				
Synthetic aptamers	pJM-1	>1 × 10 ⁹	60 bp	R. Brent
<i>Saccharomyces cerevisiae</i> , S288C, genomic	JG	>3 × 10 ⁶	0.8-4.0 kb	R. Brent
<i>S. cerevisiae</i> , S288C, genomic	JG	4.0 × 10 ⁶	0.5-4.0 kb	OriGene
Sea urchin ovary	JG	3.5 × 10 ⁶	(1.7 kb)	Clontech
<i>Caenorhabditis elegans</i>	JG	3.8 × 10 ⁶	(>1.2 kb)	OriGene
<i>Arabidopsis thaliana</i> , 7-day-old seedlings	JG	—	—	H.M. Goodman
Tomato (<i>Lycopersicon esculentum</i>)	JG	8 × 10 ⁶	—	G.B. Martin
<i>Xenopus laevis</i> embryo	JG	2.2 × 10 ⁶	0.3-4 kb (1.0 kb)	Clontech

^aMost libraries are constructed in either the pJG4-5 vector or the pYESTrp vector (JG or Y in the Vector column); the peptide aptamer library is made in the pJM-1 vector. Libraries available from the public domain were constructed by the following individuals: (1) J. Gyuris; (3) C. Sardet and J. Gyuris; (4) W. Kolanus, J. Gyuris, and B. Seed; (39) D. Krainc; (50-52) R. Finley; (55) P. Watt; (54) P. Colas, B. Cohen, T. Jessen, I. Grishina, J. McCoy, and R. Brent (Colas et al., 1996). All libraries mentioned above were constructed in conjunction with and are available from the laboratory of Roger Brent, (510) 647-0690 or brent@molsci.org. The following individual investigators must be contacted directly: (18) J. Pugh, Fox Chase Cancer Center, Philadelphia, Pa.; (8, 9) Vinyaka Prasad, Albert Einstein Medical Center New York, N.Y.; (57, 58) Gregory B. Martin, gmartin@dept.agry.purdue.edu; (11) Huifeng Niu, hn34@columbia.edu; (16) Christer Hoog, christer.hoog@cmb.ki.se; (12) Kurt Gustin, kgus@umich.edu; (6) Stephan Witte, Stephan.Witte@nimbus.rz.uni-konstanz.de.

^bThe most updated list of Interaction Trap compatible libraries is available at <http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html>.

^cInsert size ranges for pJG4-5 based libraries originally constructed in the Brent laboratory, which are now commercially available from Clontech, were reestimated by the company.

Transform the library

1. Grow an ~20-ml culture of EGY48 or EGY191 containing a *LexA*-operator-*lacZ* reporter plasmid and pBait in Glu/CM –Ura, –His liquid dropout medium overnight at 30°C.

For best results, the pBait and lacZ reporter plasmids should have been transformed into the yeast within ~7 to 10 days of commencing a screen.

2. In the morning, dilute culture into 300 ml Glu/CM –Ura, –His liquid dropout medium to 2 × 10⁶ cell/ml (OD₆₀₀ = ~0.10). Incubate at 30°C until the culture contains ~1 × 10⁷ cells/ml (OD₆₀₀ = ~0.50).
3. Centrifuge 5 min at 1000 to 1500 × *g* in a low-speed centrifuge at room temperature to harvest cells. Resuspend in 30 ml sterile water and transfer to 50-ml conical tube.
4. Centrifuge 5 min at 1000 to 1500 × *g*. Decant supernatant and resuspend cells in 1.5 ml TE buffer/0.1 M lithium acetate.
5. Add 1 μg library DNA in pJG4-5 and 50 μg high-quality sheared salmon sperm carrier DNA to each of 30 sterile 1.5-ml microcentrifuge tubes. Add 50 μl of the resuspended yeast solution from step 4 to each tube.

The total volume of library and salmon sperm DNA added should be <20 μl and preferably <10 μl.

A typical library transformation will result in 2 to 3 × 10⁶ primary transformants. Assuming a transformation efficiency of 10⁵/μg library DNA, this transformation requires a total of 20 to 30 μg library DNA and 1 to 2 mg carrier DNA. Doing transformations in small aliquots helps reduce the likelihood of contamination, and for reasons that are not clear, provides significantly better transformation efficiency than scaled-up versions.

Do not use excess transforming library DNA per aliquot of competent yeast cells because each competent cell may take up multiple library plasmids, complicating subsequent analysis.

6. Add 300 μ l of sterile 40% PEG 4000/0.1 M lithium acetate/TE buffer, pH 7.5, and invert to mix thoroughly. Incubate 30 min at 30°C.
7. Add DMSO to 10% (~40 μ l per tube) and invert to mix. Heat shock 10 min in 42°C heating block.
- 8a. *For 28 tubes:* Plate the complete contents of one tube per 24 \times 24-cm Glu/CM –Ura, –His, –Trp dropout plate and incubate at 30°C.
- 8b. *For two remaining tubes:* Plate 360 μ l of each tube on 24 \times 24-cm Glu/CM –Ura, –His, –Trp dropout plate. Use the remaining 40 μ l from each tube to make a series of 1/10 dilutions in sterile water. Plate dilutions on 100-mm Glu/CM –Ura, –His, –Trp dropout plates. Incubate all plates 2 to 3 days at 30°C until colonies appear.

The dilution series gives an idea of the transformation efficiency and allows an accurate estimation of the number of transformants obtained.

Collect primary transformant cells

Conventional replica plating does not work well in the selection process because so many cells are transferred to new plates that very high background levels inevitably occur. Instead, the procedure described below creates a slurry in which cells derived from $>10^6$ primary transformants are homogeneously dispersed. A precalculated number of these cells is plated for each primary transformant.

9. Cool all of the 24 \times 24-cm plates containing transformants for several hours at 4°C to harden agar.
10. Wearing gloves and using a sterile glass microscope slide, gently scrape yeast cells off the plate. Pool cells from the 30 plates into one or two sterile 50-ml conical tubes.

This is the step where contamination is most likely to occur. Be careful.

11. Wash cells by adding a volume of sterile TE buffer or water at least equal to the volume of the transferred cells. Centrifuge ~5 min at 1000 to 1500 \times g, room temperature, and discard supernatant. Repeat wash.

After the second wash, pellet volume should be ~25 ml cells derived from 1.5×10^6 transformants.

12. Resuspend pellet in 1 vol glycerol solution, mix well, and store up to 1 year in 1-ml aliquots at –70°C.

Determine replating efficiency

13. Remove an aliquot of frozen transformed yeast and dilute 1/10 with Gal/Raff/CM –Ura, –His, –Trp dropout medium. Incubate with shaking 4 hr at 30°C to induce the GAL promoter on the library.

Raffinose (Raff) aids in growth without diminishing transcription from the GAL1 promoter.

14. Make serial dilutions of the yeast cells using the Gal/Raff/CM –Ura, –His, –Trp dropout medium. Plate on 100-mm Gal/Raff/CM –Ura, –His, –Trp dropout plates and incubate 2 to 3 days at 30°C until colonies are visible.
15. Count colonies and determine the number of colony-forming units (cfu) per aliquot of transformed yeast.

In calculating yeast concentrations, it is useful to remember that 1 OD_{600} unit = $\sim 2.0 \times 10^7$ yeast cells. In general, if the harvest is done carefully, viability will be greater than 90%. Some intrepid investigators perform this step simultaneously with plating out on Leu selective medium (steps 16 and 17).

Screen for interacting proteins

16. Thaw the appropriate quantity of transformed yeast based on the plating efficiency, dilute, and incubate as in step 13. Dilute cultures in Gal/Raff/CM –Ura, –His, –Trp, –Leu medium as necessary to obtain a concentration of 10^7 cells/ml ($OD_{600} = \sim 0.5$), and plate 100 μ l on each of as many 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu dropout plates as are necessary for full representation of transformants. Incubate 2 to 3 days at 30°C until colonies appear.

Because not all cells that contain interacting proteins plate at 100% efficiency on –Leu medium (Estojak et al., 1995), it is desirable that for actual selection, each primary colony obtained from the transformation be represented on the selection plate by three to ten individual yeast cells. This will in some cases lead to multiple isolations of the same cDNA; however, because the slurry is not perfectly homogenous, it will increase the likelihood that all primary transformants are represented by at least one cell on the selective plate.

It is easiest to visually scan for Leu⁺ colonies using cells plated at $\sim 10^6$ cfu per 100-mm plate. Plating at higher density can contribute to cross-feeding between yeast, resulting in spurious background growth. Thus, for a transformation in which 3×10^6 colonies are obtained, plate $\sim 2 \times 10^7$ cells on a total of 20 selective plates.

17. Carefully pick appropriate colonies to a new Gal/Raff/CM –Ura, –His, –Trp, –Leu master dropout plate. Incubate 2 to 7 days at 30°C until colonies appear.

A good strategy is to pick a master plate with colonies obtained on day 2, a second master plate (or set of plates) with colonies obtained on day 3, and a third with colonies obtained on day 4. Colonies from day 2 and 3 master plates should generally be characterized further. If many apparent positives are obtained, it may be worth making master plates of the much larger number of colonies likely to be obtained at day 4 (and after). See Critical Parameters and annotation to step 19 for additional information about appropriate colony selection for the master plate.

If no colonies appear within a week, those arising at later time points are likely to be artifactual. Contamination that has occurred at an earlier step (e.g., during plate scraping) is generally reflected by the growth of a very large number of colonies (>500 /plate) within 24 to 48 hr after plating on selective medium.

Some investigators omit use of a Gal/Raff/CM –Ura, –His, –Trp, –Leu master plate, restreaking directly to a Glu/CM –Ura, –His, –Trp master plate as in step 19.

Test for Gal dependence

The following steps test for Gal dependence of the Leu⁺ insert and lacZ phenotypes to confirm that they are attributable to expression of the library-encoded proteins. The *GAL1* promoter is turned off and –Leu selection eliminated before reinducing.

18. Restreak from the Gal/Raff/CM –Ura, –His, –Trp, –Leu master dropout plate to a 100-mm Glu/CM –Ura, –His, –Trp master dropout plate. Incubate overnight at 30°C until colonies form.
19. Restreak or replica plate from this plate to the following plates:

Glu/Xgal/CM –Ura, –His, –Trp
Gal/Raff/Xgal/CM –Ura, –His, –Trp
Glu/CM –Ura, –His, –Trp, –Leu
Gal/Raff/CM –Ura, –His, –Trp, –Leu.

At this juncture, colonies and the library plasmids they contain are tentatively considered positive if they are blue on Gal/Raff/Xgal plates but not blue or only faintly blue on Glu/Xgal plates, and if they grow on Gal/Raff/CM –Leu plates but not on Glu/CM –Leu plates.

The number of positives obtained will vary drastically from bait to bait. How they are processed subsequently will depend on the number initially obtained and on the preference

of the individual investigator. If none are obtained using EGY48 as reporter strain, it may be worth attempting to screen a library from an additional tissue source. If a relatively small number (≤ 30) are obtained, proceed to step 20. However, sometimes searches will yield large numbers of colonies (>30 to 300, or more). In this case, there are several options. The first option is to warehouse the majority of the positives and work up the first 30 that arise; those growing fastest are frequently the strongest interactors. These can be checked for specificity, and restriction digests can be used to establish whether they are all independent cDNAs or represent multiple isolates of the same, or a small number, of cDNAs. If the former is true, it may be advisable to repeat the screen in a less sensitive strain background, as obtaining many different interactors can be a sign of low-affinity nonspecific background. Alternatively, if initial indications are that a few cDNAs are dominating the positives obtained, it may be useful to perform a filter hybridization with yeast (see Support Protocol 3) using these cDNAs as a probe to establish the frequency of their identification and exclude future reisolation of these plasmids. The second major option is to work up large numbers of positives to get a complete profile of isolated interactors (see Support Protocol 4). A third option is to temporarily warehouse the entire results of this first screen, and repeat the screen with a less sensitive strain such as EGY191, on the theory that it is most important to get stronger interactors first and a complete profile of interactors later. Finally, some investigators prefer to work up the entire set of positives initially obtained, even if such positives number in the hundreds. Particularly in this latter case, it is most effective to use Alternate Protocol 1 as a means to identify unique versus common positives.

Isolate plasmid from positive colonies by transfer into *E. coli*

- 20a. Transfer yeast plasmids directly into *E. coli* by following the protocol for direct electroporation (APPENDIX 3, Alternate Protocol 2). Proceed to step 22.
- 20b. Isolate plasmid DNA from yeast by the rapid miniprep protocol (APPENDIX 3) with the following alteration: after obtaining aqueous phase, precipitate by adding sodium acetate to 0.3 M final and 2 vol ethanol, incubate 20 min on ice, microcentrifuge 15 min at maximum speed, wash pellet with 70% ethanol, dry, and resuspend in 5 μ l TE buffer.

Cultures can be grown prior to the miniprep using Glu/CM –Trp to select only for the library plasmid; this may increase the proportion of bacterial colonies that contain the desired plasmid.

21. Use 1 μ l DNA to electroporate (APPENDIX 3) into competent KC8 bacteria, and plate on LB/ampicillin plates. Incubate overnight at 37°C.

Electroporation must be used to obtain transformants with KC8 because the strain is generally refractory to transformation.
22. Restreak or replica plate colonies arising on LB/ampicillin plates to bacterial defined minimal A medium plates containing vitamin B1 and supplemented with Ura, His, and Leu but lacking Trp. Incubate overnight at 37°C.

Colonies that grow under these conditions contain the library plasmid.

The yeast TRP1 gene can successfully complement the bacterial trpC-9830 mutation, allowing the library plasmid to be easily distinguished from the other two plasmids contained in the yeast. It is helpful to first plate transformations on LB/ampicillin plates, which provides a less stringent selection, followed by restreaking to bacterial minimal medium to maximize the number of colonies obtained (E. Golemis, unpub. observ.).

23. Purify library-containing plasmids using a bacterial miniprep procedure (APPENDIX 3).

Some investigators are tempted to immediately sequence DNAs obtained at this stage. At this point, it is still possible that none of the isolated clones will express bona fide interactors, and it is suggested that the following specificity tests be completed before committing the effort to sequencing (also see annotation to step 28).

Because multiple 2 μ m plasmids with the same marker can be simultaneously tolerated in yeast, it sometimes happens that a single yeast will contain two or more different library plasmids, only one of which encodes an interacting protein. The frequency of this occurrence varies in the hands of different investigators and may in some cases account for disappearing positives if the wrong cDNA is picked. When choosing colonies to miniprep, it is generally useful to work up at least two individual bacterial transformants for each yeast positive. These minipreps can then be restriction digested (APPENDIX 3) with EcoRI + XhoI to release cDNA inserts, and the size of inserts determined on an agarose minigel (APPENDIX 3) to confirm that both plasmids contain the same insert. An additional benefit of analyzing insert size is that it may provide some indication as to whether repeated isolation of the same cDNA is occurring, generally a good indication concerning the biological relevance of the interactor. See Background Information for further discussion.

Assess positive colonies with specificity tests

Much spurious background will have been removed by the previous series of controls. Other classes of false positives can be eliminated by retransforming purified plasmids into “virgin” LexA-operator-LEU2/LexA-operator-lacZ/pBait-containing strains that have not been subjected to Leu selection and verifying that interaction-dependent phenotypes are still observed. Such false positives could include mutations in the initial EGY48 yeast that favor growth on Gal medium, library-encoded cDNAs that interact with the LexA DNA-binding domain, or proteins that are sticky and interact with multiple biologically unrelated fusion domains.

24. In separate transformations, use purified plasmids from step 23 to transform yeast that already contain the following plasmids and are growing on Glu/CM –Ura, –His plates:

EGY48 containing pSH18-34 and pBait
EGY48 containing pSH18-34 and pRFHM-1
EGY48 containing pSH18-34 and a nonspecific bait (optional).

25. Plate each transformation mix on Glu/CM –Ura, –His, –Trp dropout plates and incubate 2 to 3 days at 30°C until colonies appear.
26. Create a Glu/CM –Ura, –His, –Trp master dropout plate for each library plasmid being tested. Streak adjacently five or six independent colonies derived from each of the transformation plates. Incubate overnight at 30°C.
27. Restreak or replica plate from this master dropout plate to the same series of test plates used for the actual screen:

Glu/Xgal/CM –Ura, –His, –Trp
Gal/Raff/Xgal/CM –Ura, –His, –Trp
Glu/CM –Ura, –His, –Trp, –Leu
Gal/CM –Ura, –His, –Trp, –Leu.

True positive cDNAs should make cells blue on Gal/Raff/Xgal but not on Glu/Xgal plates, and should make them grow on Gal/Raff/CM –Leu but not Glu/CM –Leu dropout plates only if the cells contain LexA-bait. cDNAs that meet such criteria are ready to be sequenced (see legend to Fig. 17.3.3 for primer sequence) or otherwise characterized. Those cDNAs that also encode proteins that interact with either RFHM-1 or another nonspecific bait should be discarded.

It may be helpful to cross-check the isolated cDNAs with a database of cDNAs thought to be false positives. This database is available on the World Wide Web as a work in progress at <http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html>. cDNAs reported to this database are generally those isolated only once in a screen in which obviously true interactive partners were isolated multiple times, cDNAs that may interact with more than one bait, or cDNAs for which the interaction does not appear to make biological sense

in the context of the starting bait. Although some proteins in this database may ultimately turn out in fact to associate with the bait that isolated them, they are by default unlikely to possess a unique and interesting function in the context of that bait if they are well represented in the database.

28. If appropriate, conduct additional specificity tests (see Support Protocol 5). Analyze and sequence positive isolates.

The primer sequence for use with pJG4-5 is provided in the legend to Figure 17.3.4.

DNA prepared from KC8 is generally unsuitable for dideoxy or automated sequencing even after use of Qiagen columns and/or cesium chloride gradients. Library plasmids to be sequenced should be retransformed from the KC8 miniprep stock (step 23) to a more amenable strain, such as DH5 α , before sequencing is attempted.

RAPID SCREEN FOR INTERACTION TRAP POSITIVES

Under some circumstances, it may be desirable to attempt the analysis of a large number of positives resulting from a two-hybrid screen. One such hypothetical example would be a bait with a leucine zipper or coiled coil known to dimerize with partner “A” that is highly expressed. In order to identify the rare novel partner “B”, it is necessary to work through the high background of “A” reisolates. This protocol uses the polymerase chain reaction (PCR) in a strategy to sort positives into redundant (multiple isolates) and unique classes prior to plasmid rescue from yeast, thus greatly reducing the number of plasmid isolations that must be performed. An additional benefit is that this protocol preidentifies positive clones containing one or multiple library plasmids; for those containing only one library plasmid, only a single colony needs to be prepared through KC8/DH5 α .

Additional Materials (also see Basic Protocol 2)

Yeast plated on Glu/CM –Ura, –His, –Trp master plate (see Basic Protocol 2, step 19)

Lysis solution (see recipe)

10 μ M forward primer (FP1): 5'-CGT AGT GGA GAT GCC TCC-3'

10 μ M reverse primer (FP2): 5'-CTG GCA AGG TAG ACA AGC CG-3'

Toothpicks or bacterial inoculating loops, sterile

96-well microtiter plate

Sealing tape, e.g., wide transparent tape

150- to 212- μ m glass beads, acid-washed (UNIT 3.8)

Vortexer with flat plate

Additional reagents and equipment for performing an interactor hunt (see Basic Protocol 2), PCR amplification of DNA, agarose gel electrophoresis, restriction endonuclease digestion, electroporation, and miniprep isolation of bacterial DNA (APPENDIX 3)

1. Perform an interactor hunt (see Basic Protocol 2, steps 1 to 19).
2. Use a sterile toothpick or bacterial inoculating loop to transfer yeast from the Glu/CM, –Ura, –His, –Trp master plate into 25 μ l lysis solution in a 96-well microtiter plate. Seal the wells of the microtiter plate with sealing tape and incubate 1.5 to 3.5 hr at 37°C with shaking.

The volume of yeast transferred should not exceed ~2 to 3 μ l of packed pellet; larger quantities of yeast will reduce quality of the DNA. DNA can be efficiently recovered from master plates that have been stored up to 1 week at 4°C. If yeast have been previously gridded on master plates, transfer to microtiter plates can be facilitated by using a multicolony replicator.

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3. Remove tape from the plate, add ~25 μ l acid-washed glass beads to each well, and reseal with the same tape. Firmly attach the microtiter plate to a flat-top vortexer, and vortex 5 min at medium-high power.

The microtiter plate can be attached to the vortexer using 0.25-in (0.64-cm) rubber bands.

4. Remove the tape and add ~100 μ l sterile water to each well. Swirl gently to mix, then remove sample for step 5. Press the tape back firmly to seal the microtiter plate and place in the freezer at -20°C for storage.
5. Amplify 0.8 to 2.0 μ l of sample by standard PCR (APPENDIX 3) in a ~30- μ l volume using 3 μ l each of the forward primer FP1 and the reverse primer FP2. Perform PCR using the following cycles:

Initial step:	2 min	94 $^{\circ}\text{C}$
31 cycles:	45 sec	94 $^{\circ}\text{C}$
	45 sec	56 $^{\circ}\text{C}$
	45 sec	72 $^{\circ}\text{C}$.

These conditions have been used successfully to amplify fragments up to 1.8 kb in length; some modifications, such as extension of elongation time, are also effective.

6. Load 20 μ l of the PCR reaction product on a 0.7% low melting temperature agarose gel (APPENDIX 3) to resolve PCR products. Based on insert sizes, group the obtained interactors in families, i.e., potential multiple independent isolates of identical cDNAs. Reserve gel until results of step 7 are obtained.

No special precautions are needed for storing the gel. Since HaeIII digests typically yield rather small DNA fragments, running the second gel does not take a lot of time. Usually, the delay does not exceed 45 to 60 min, during which time the first gel may be stored in a gel box at room temperature or wrapped in plastic wrap at 4 $^{\circ}\text{C}$.

7. While the gel is running, use the remaining 10 μ l of PCR reaction product for a restriction endonuclease digestion with HaeIII in a digestion volume of ~20 μ l (APPENDIX 3). Based on analysis of the sizes of undigested PCR products in the gel (step 6), rearrange the tubes with HaeIII digest samples so that those thought to represent a family are side by side. Resolve the digests on a 2% to 2.5% agarose gel (APPENDIX 3).

Most restriction fragments will be in the 200-bp to 1.0-kb size range so using a long gel run is advisable. This analysis should produce a distinct fingerprint of insert sizes and allow definition of library cDNAs as unique isolates or related groups.

A single positive yeast will sometimes contain multiple library plasmids. An advantage of this protocol is the ready detection of multiple library plasmids in PCR reactions; thus, following subsequent bacterial transformations, only a single TRP1 colony would need to be analyzed unless multiple plasmids were already known to be present.

8. Isolate DNA fragments from the low melting temperature agarose gel (step 6).

If inspection of the banding pattern on the two gels suggests that a great many reisolates of a small number of cDNAs are present, it may be worthwhile to immediately sequence PCR products representative of these clusters, but it is generally still advisable to continue through specificity tests before doing so. If the PCR products are sequenced, the FP1 forward primer works well in automated sequencing of PCR fragments, but the FP2 primer is only effective in sequencing from purified plasmid.

In general, priming from the AT-rich ADH terminator downstream of the polylinker/cDNA in library plasmid is less efficient than from upstream of the cDNA, and it is hard to design effective primers in this region.

9. Remove the microtiter plate of lysates from the freezer, thaw it, and remove 2 to 4 μ l of lysed yeast for each desired positive. Electroporate DNA into either DH5 α or KC8 *E. coli* as appropriate, depending on the choice of bait and reporter plasmids (see

Table 17.3.1 and see Background Information for further information). Refreeze the plate as a DNA reserve in case bacteria fail to transform on the first pass.

KC8 E. coli should be used for electroporation when the original reagents pEG202/pJG4-5/pJK101 are used for the interaction trap.

An additional strength of this protocol is that it identifies redundant clones before transfer of plasmids to bacteria, thus reducing the amount of work required in cases where plasmid identity can be unambiguously assigned. However, although restriction endonuclease digestion and PCR analysis are generally highly predictive, they are not 100% certain methods for estimating cDNA identity. Thus, if there is any doubt about whether two cDNAs are the same, investigators are urged to err on the side of caution.

10. Prepare a miniprep of plasmid DNA from the transformed bacteria (APPENDIX 3) and perform yeast transformation and specificity assessment (see Basic Protocol 2, steps 24 to 28).

PERFORMING A HUNT BY INTERACTION MATING

An alternative way of conducting an interactor hunt is to mate a strain that expresses the bait protein with a strain that has been pretransformed with the library DNA, and screen the resulting diploid cells for interactors (Bendixen et al., 1994; Finley and Brent, 1994). This “interaction mating” approach can be used for any interactor hunt and is particularly useful in three special cases. The first case is when more than one bait will be used to screen a single library. Interaction mating allows several interactor hunts with different baits to be conducted using a single high-efficiency yeast transformation with library DNA. This can be a considerable savings, since the library transformation is one of the most challenging tasks in an interactor hunt. The second case is when a constitutively expressed bait interferes with yeast viability. For such baits, performing a hunt by interaction mating avoids the difficulty associated with achieving a high-efficiency library transformation of a strain expressing a toxic bait. Moreover, the actual selection for interactors will be conducted in diploid yeast, which are more vigorous than haploid yeast and can better tolerate expression of toxic proteins. The third case is when a bait cannot be used in a traditional interactor hunt using haploid yeast strains (see Basic Protocol 2) because it activates transcription of even the least sensitive reporters. In diploids the reporters are less sensitive to transcription activation than they are in haploids. Thus, the interaction mating hunt provides an additional method to reduce background from transactivating baits.

In the protocol described below, the library DNA is used to transform a strain with a *LEU2* reporter (e.g., EGY48). This pretransformed library strain is then frozen in many aliquots, which can be thawed and used for individual interactor hunts. The bait is expressed in a strain of mating type opposite to that of the pretransformed library strain, and also bearing the *lacZ* reporter. A hunt is conducted by mixing the pretransformed library strain with the bait strain and allowing diploids to form on YPD medium overnight. The diploids are then induced for expression of the library-encoded proteins and screened for interactors as in Basic Protocol 2.

NOTE: Strain combinations other than those described below can also be used in an interaction-mating hunt. The key to choosing the strains is to ensure that the bait and prey strains are of opposite mating types and that both have auxotrophies to allow selection for the appropriate plasmids and reporter genes. Also, once the bait plasmid and *lacZ* reporter plasmid have been introduced into the bait strain, and the library plasmids have been introduced into the library strain, the resulting bait strain and library strain must each have auxotrophies that can be complemented by the other, so that diploids can be selected.

ALTERNATE PROTOCOL 2

Additional Materials (also see Basic Protocols 1 and 2)

Yeast strains: either RFY206 (Finley and Brent, 1994), YPH499 (Sikorski and Hieter, 1989; ATCC #6625), or an equivalent *MATa* strain with auxotrophic markers *ura3*, *trp1*, *his3*, and *leu2*

YPD liquid medium (UNIT 1.6)

Glu/CM –Trp plates: CM dropout plates –Trp (see recipe) supplemented with 2% glucose

pJG4-5 library vector (Fig. 17.3.6), empty

100-mm YPD plates (UNIT 1.6)

Additional reagents and equipment for lithium acetate transformation of yeast (APPENDIX 3)

Construct the bait strain

The bait strain will be a *MATa* yeast strain (mating type opposite of EGY48) containing a *lacZ* reporter plasmid like pSH18-34 and the bait-expressing plasmid, pBait.

1. Perform construction of the bait plasmid (pBait; see Basic Protocol 1, step 1).
2. Cotransform the *MATa* yeast strain (e.g., either RFY206 or YPH499) with pBait and pSH18-34 using the lithium acetate method (APPENDIX 3). Select transformants on Glu/CM –Ura, –His plates by incubating plates at 30°C for 3 to 4 days until colonies form. Combine 3 colonies for all future tests and for the mating hunt.

The bait strain (RFY206/pSH18-34/pBait or YPH499/pSH18-34/pBait) can be tested by immunoblotting to ensure that the bait protein is expressed (see Support Protocol 1). Synthesis and nuclear localization of the bait protein can also be tested by the repression assay (see Basic Protocol 1, steps 8 to 12).

3. *Optional:* Assay *lacZ* gene activation in the bait strain (see Basic Protocol 1, steps 4 to 7).

*If the bait activates the *lacZ* reporter, a less sensitive *lacZ* reporter plasmid (Table 17.3.1), or an integrated version of the *lacZ* reporter should be tried. A bait that strongly activates the *lacZ* reporters usually cannot be used in a hunt based on selection of interactors with the *LEU2* reporter, because the *LEU2* reporters are more sensitive than the *lacZ* reporters. However, both reporters are less sensitive to activation by a bait in diploid cells, as compared to haploid cells. Thus, a more important test of the transactivation potential of a bait is to test the leucine requirement of diploid cells expressing it, as described in steps 6 to 20, below.*

Prepare the pretransformed library strain (EGY48 + library plasmids)

4. Perform a large-scale transformation of EGY48 with library DNA using the lithium acetate method (see Basic Protocol 2, steps 1 to 8, except start with EGY48 bearing no other plasmids). To prepare for transformation, grow EGY48 in YPD liquid medium. Select library transformants on Glu/CM –Trp plates by incubating 3 days at 30°C.
5. Collect primary transformants by scraping plates, washing yeast, and resuspending in 1 pellet vol glycerol solution (see Basic Protocol 2, steps 9 to 12). Freeze 0.2- to 1.0-ml aliquots at –70° to –80°C.

The cells will be stable for at least 1 year. Refreezing a thawed aliquot will result in loss of viability. Thus, many frozen aliquots should be made, so that each thawed aliquot can be discarded after use.

Prepare the pretransformed control strain (EGY48 + pJG4-5)

6. Transform EGY48 grown in YPD liquid medium with the empty library vector, pJG4-5, using the lithium acetate method (APPENDIX 3). Select transformants on Glu/CM –Trp plates by incubating 3 days at 30°C.
7. Pick and combine three transformant colonies and use them to inoculate 30 ml of Glu/CM –Trp medium. Incubate 15 to 24 hr at 30°C (to OD₆₀₀ >3).
8. Centrifuge 5 min at 1000 to 1500 × g, room temperature, and remove supernatant. Resuspend in 10 ml sterile water to wash cells.
9. Centrifuge 5 min at 1000 to 1500 × g, room temperature, and remove supernatant. Resuspend in 1 pellet vol glycerol solution and freeze 100-μl aliquots at –70° to –80°C.

Determine plating efficiency of pretransformed library and pretransformed control strains

10. After freezing (at least 1 hr) thaw an aliquot of each pretransformed strain (from step 5 and step 9) at room temperature. Make several serial dilutions in sterile water, including aliquots diluted 10⁵-fold, 10⁶-fold, and 10⁷-fold. Plate 100 μl of each dilution on 100-mm Glu/CM –Trp plates and incubate 2 to 3 days at 30°C.
11. Count the colonies and determine the number of colony-forming units (cfu) per aliquot of transformed yeast.

The plating efficiency for a typical library transformation and for the control strain will be ~1 × 10⁸ cfu per 100 μl.

Mate the bait strain with the pretransformed library strain and the pretransformed control strain

In steps 12 through 20, an interactor hunt is conducted concurrently with testing *LEU2* reporter activation by the bait itself. For most baits, this approach will be the quickest way to isolate interactors. However, for some baits, such as those that have a high transactivation potential, or those that affect yeast mating or growth, steps 12 through 20 will serve as a pilot experiment to determine the optimal parameters for a subsequent hunt.

12. Grow a 30-ml culture of the bait strain in Glu/CM –Ura, –His liquid dropout medium to mid to late log phase (OD₆₀₀ = 1.0 to 2.0, or 2 to 4 × 10⁷ cells/ml).

A convenient way to grow the bait strain is to inoculate a 5-ml culture with approximately three colonies from a plate and grow it overnight at 30°C with shaking. In the morning, measure the OD₆₀₀, dilute into a 30-ml culture to a final OD₆₀₀ = 0.2, and grow at 30°C with shaking. The culture should reach mid to late log phase before the end of the day.

13. Centrifuge the culture 5 min at 1000 to 1500 × g, room temperature, to harvest cells. Resuspend the cell pellet in sterile water to make a final volume of 1 ml.

This should correspond to ~1 × 10⁹ cells/ml.

14. Set up two matings. In one sterile microcentrifuge tube mix 200 μl of the bait strain with 200 μl of a thawed aliquot of the pretransformed control strain from step 9. In a second microcentrifuge tube mix 200 μl of the bait strain with ~1 × 10⁸ cfu (~0.1 to 1 ml) of the pretransformed library strain from step 5.

The library mating should be set up so that it contains a ~2-fold excess of bait strain cfu over pretransformed library strain cfu. Because the bait strain was harvested in log phase, most of the cells will be viable (i.e., cells/ml = ~cfu/ml), and the number of cfu can be sufficiently estimated from optical density (1 OD₆₀₀ = ~2 × 10⁷ cells/ml). Under these conditions, ~10% of the cfu in the pretransformed library strain will mate with the bait

strain. Thus, a complete screen of 10^7 library transformants will require a single mating with at least 10^8 cfu of the pretransformed library strain and at least 2×10^8 cfu of the bait strain.

To screen more library transformants, set up additional matings. The number of pretransformed library transformants to screen depends on the size of the library and the number of primary transformants obtained in step 5. If the size of the library is larger than the number of transformants obtained in step 5, the goal will be to screen all of the yeast transformants. In this case, complete screening of the library will require additional transformations of EGY48 and additional interactor hunts. If the size of the library is smaller than the number of transformants obtained in step 5, the goal will be to screen at least a number of transformants equivalent to the size of the library.

15. Centrifuge each cell mixture for 5 min at 1000 to $1500 \times g$, pour off medium, and resuspend cells in $200 \mu\text{l}$ YPD medium. Plate each suspension on a 100-mm YPD plate. Incubate 12 to 15 hr at 30°C .
16. Add ~ 1 ml of Gal/Raff/CM –Ura, –His, –Trp to the lawns of mated yeast on each plate. Mix the cells into the medium using a sterile applicator stick.
17. Transfer each slurry of mated cells to a 500-ml flask containing 100 ml of Gal/Raff/CM –Ura, –His, –Trp dropout medium. Incubate with shaking 6 hr at room temperature to induce the *GAL1* promoter, which drives expression of the cDNA library.
18. Centrifuge the cell suspensions 5 min at 1000 to $1500 \times g$, room temperature, to harvest the cells. Wash by resuspending in 30 ml of sterile water and centrifuging again. Resuspend each pellet in 5 ml sterile water. Measure OD_{600} and, if necessary, dilute to a final concentration of $\sim 1 \times 10^8$ cells/ml.

This is a mixture consisting of haploid cells that have not mated and diploid cells. Under a microscope, the two cell types can be distinguished by size (diploids are $\sim 1.7\times$ bigger than haploids) and shape (diploids are slightly oblong and haploids are spherical). Because diploids grow faster than haploids, this mixture will contain $\sim 10\%$ to 50% diploid cells. The actual number of diploids will be determined by plating dilutions on –Ura, –His, –Trp medium, which will not support the growth of the parental haploids.

19. For each mating make a series of $1/10$ dilutions in sterile water, at least $200 \mu\text{l}$ each, to cover a 10^6 -fold concentration range. Plate $100 \mu\text{l}$ from each tube (undiluted, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} dilution) on 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu plates. Plate $100 \mu\text{l}$ from the 10^{-4} , 10^{-5} , and 10^{-6} tubes on 100-mm Gal/Raff/CM –Ura, –His, –Trp plates. Incubate plates at 30°C . Count the colonies on each plate after 2 to 5 days.
20. For the mating with the pretransformed library, prepare an additional 3 ml of a 10^{-1} dilution. Plate $100 \mu\text{l}$ of the 10^{-1} dilution on each of 20 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu plates. Also plate $100 \mu\text{l}$ of the undiluted cells on each of 20 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu plates. Incubate at 30°C . Pick Leu^+ colonies after 2 to 5 days and characterize them beginning with step 17 of Basic Protocol 2.

The number of Leu^+ colonies to pick to ensure that all of the pretransformed library has been screened depends on the transactivation potential of the bait protein itself. The transactivation potential is expressed as the number of Leu^+ colonies that grow per cfu (Leu^+/cfu) of the bait strain mated with the control strain, as determined in step 19 of this protocol. It can be calculated as the ratio of colonies that grow on Gal/Raff/CM –Ura, –His, –Trp, –Leu to the number of colonies that grow on Gal/Raff/CM –Ura, –His, –Trp for a given dilution of the mating between the bait strain and the control strain. A bait with essentially no transactivation potential will produce less than 10^{-6} Leu^+/cfu . For a bait to be useful in an interactor hunt it should not transactivate more than 10^{-4} Leu^+/cfu .

To screen all of the pretransformed library, it will be necessary to pick a sufficient number of Leu^+ colonies in addition to background colonies produced by the transactivation potential of the bait itself. Thus, the minimum number of Leu^+ colonies that should be picked in step 20 of this protocol is given by:

$$(\text{transactivation potential, } Leu^+/\text{cfu}) \times (\# \text{ library transformants screened}).$$

For example, if 10^7 library transformants were obtained in step 2 (and at least 10^8 cfu of these transformants were mated with the bait strain in step 14, since only ~10% will form diploids), and the transactivation potential of the bait is 10^{-4} Leu^+ /cfu, then at least 1000 Leu^+ colonies must be picked and characterized. In other words, if the rarest interactor is present in the pretransformed library at a frequency of 10^{-7} , to find it one needs to screen through at least 10^7 diploids from a mating of the library strain. However, at least 1000 of these 10^7 diploids would be expected to be Leu^+ due to the bait background if the transactivation potential of the bait is 10^{-4} . The true positives will be distinguished from the bait background in the next step by the galactose dependence of their Leu^+ and $lacZ^+$ phenotypes.

PREPARATION OF PROTEIN EXTRACTS FOR IMMUNOBLOT ANALYSIS

To confirm that the bait fusion protein constructed in Basic Protocol 1 is synthesized properly, a crude lysate is prepared for SDS-PAGE and immunoblot analysis (UNITS 6.1 & 6.2). The presence of the target protein is detected by antibody to LexA or the fusion domain.

Materials

Master plates with pBait-containing positive and control yeast on Glu/CM –Ura, –His dropout medium (see Basic Protocol 1, step 4)

Glu/CM –Ura, –His dropout liquid medium: CM dropout plates –Ura, –His (see recipe) supplemented with 2% glucose

2× Laemmli sample buffer (see recipe)

Antibody to fusion domain or LexA: monoclonal antibody to LexA (Clontech, Invitrogen) or polyclonal antibody to LexA (available by request from R. Brent or E. Golemis)

30°C incubator

100°C water bath

Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and immunoblotting and immunodetection (UNIT 6.2)

1. From the master plates, start a 5-ml culture in Glu/CM –Ura, –His liquid medium for each bait being tested and for a positive control for protein expression (i.e., RFHMI or SH17-4). Incubate overnight at 30°C.

For each construct assayed, it is a good idea to grow colonies from at least two primary transformants, as levels of bait expression are sometimes heterogenous.

2. From each overnight culture, start a fresh 5-ml culture in Glu/CM –Ura, –His at $OD_{600} = \sim 0.15$. Incubate again at 30°C.

3. When the culture has reached $OD_{600} = 0.45$ to 0.7 (~4 to 6 hr), remove 1.5 ml to a microcentrifuge tube.

For some LexA fusion proteins, levels of the protein drop off rapidly in cultures approaching stationary phase. This is due to a combination of the diminishing activity of the ADHI promoter in late growth phases and the relative instability of particular fusion domains. Thus, it is not a good idea to let cultures become saturated in the hopes of obtaining a higher yield of protein.

SUPPORT PROTOCOL 1

**SUPPORT
PROTOCOL 2**

4. Microcentrifuge cells 3 min at $13,000 \times g$, room temperature. When the pellet is visible, remove the supernatant.

Inspection of the tube should reveal a pellet ~1 to 3 μ l in volume. If the pellet is not visible, microcentrifuge another 3 min.

5. Working rapidly, add 50 μ l of 2 \times Laemmli sample buffer to the visible pellet in the tube, vortex, and place the tube on dry ice.

Samples may be frozen at -70°C .

6. Transfer frozen sample directly to a boiling water bath or a PCR machine set to cycle at 100°C . Boil 5 min.
7. Microcentrifuge 5 sec at maximum speed to pellet large cellular debris.
8. Perform SDS-PAGE (UNIT 6.1) using 20 to 50 μ l sample per lane.
9. To detect the protein, immunoblot and analyze (UNIT 6.2) using antibody to the fusion domain or LexA.

PREPARATION OF SHEARED SALMON SPERM CARRIER DNA

This protocol generates high-quality sheared salmon sperm DNA (sssDNA) for use as carrier in transformation (Basic Protocol 2). This DNA is also suitable for other applications where high-quality carrier DNA is needed (e.g., hybridization). This protocol is based on Schiestl and Gietz (1989). For more details of phenol extraction or other DNA purification methods, consult APPENDIX 3.

Materials

High-quality salmon sperm DNA (e.g., sodium salt from salmon testes, Sigma or Boehringer Mannheim), desiccated
TE buffer, pH 7.5 (APPENDIX 2A), sterile
TE-saturated buffered phenol
1:1 (v/v) buffered phenol/chloroform
Chloroform
3 M sodium acetate, pH 5.2 (APPENDIX 2A)
100% and 70% ethanol, ice cold
Magnetic stirring apparatus and stir-bar, 4°C
Sonicator with probe
50-ml conical centrifuge tube
High-speed centrifuge and appropriate tube
 100°C and ice-water baths

1. Dissolve desiccated high-quality salmon sperm DNA in TE buffer, pH 7.5, at a concentration of 5 to 10 mg/ml by pipetting up and down in a 10-ml glass pipet. Place in a beaker with a stir-bar and stir overnight at 4°C to obtain a homogenous viscous solution.

It is important to use high-quality salmon sperm DNA. Sigma Type III sodium salt from salmon testes has worked well, as has a comparable grade from Boehringer Mannheim. Carrier DNA is also commercially available from Clontech. Generally it is convenient to prepare 20- to 40-ml batches at a time.

2. Shear the DNA by sonicating briefly using a large probe inserted into the beaker.

The goal of this step is to generate sheared salmon sperm DNA (sssDNA) with an average size of 7 kb, but ranging from 2 to 15 kb. Oversonication (such that the average size is closer to 2 kb) drastically decreases the efficacy of carrier in enhancing transformation. The

original version of this protocol (Schiestl and Gietz, 1989) called for two 30-sec pulses at three-quarter power, but optimal conditions vary between sonicators. The first time this protocol is performed, it is worthwhile to sonicate briefly, then test the size of the DNA by running out a small aliquot alongside molecular weight markers on an agarose gel containing ethidium bromide. The DNA can be sonicated further if needed.

3. Once DNA of the appropriate size range has been obtained, extract the sssDNA solution with an equal volume of TE-saturated buffered phenol in a 50-ml conical tube, shaking vigorously to mix.
4. Centrifuge 5 to 10 min at $3000 \times g$, room temperature, or until clear separation of phases is obtained. Transfer the upper phase containing the DNA to a clean tube.
5. Repeat extraction using 1:1 (v/v) buffered phenol/chloroform, then chloroform alone. Transfer the DNA into a tube suitable for high-speed centrifugation.
6. Precipitate the DNA by adding $\frac{1}{10}$ vol of 3 M sodium acetate and 2.5 vol of ice-cold 100% ethanol. Mix by inversion. Centrifuge 15 min at $\sim 12,000 \times g$, room temperature.
7. Wash the pellet with 70% ethanol. Briefly dry either by air drying, or by covering one end of the tube with Parafilm with a few holes poked in and placing the tube under vacuum. Resuspend the DNA in sterile TE buffer at 5 to 10 mg/ml.

Do not overdry the pellet or it will be very difficult to resuspend.

8. Denature the DNA by boiling 20 min in a 100°C water bath. Then immediately transfer the tube to an ice-water bath.
9. Place aliquots of the DNA in microcentrifuge tubes and store frozen at -20°C . Thaw as needed.

DNA should be boiled again briefly (5 min) immediately before addition to transformations.

Before using a new batch of sssDNA in a large-scale library transformation, it is a good idea to perform a small-scale transformation using suitable plasmids to determine the transformation efficiency. Optimally, use of sssDNA prepared in the manner described will yield transformation frequencies of $>10^5$ colonies/ μg input plasmid DNA.

YEAST COLONY HYBRIDIZATION

This protocol is adapted from a modification of the classic protocol of Grunstein and Hogness (1975; Kaiser et al., 1994). It is primarily useful when a large number of putative interactors has been obtained, and initial minipreps and restriction digests have indicated that many of them derive from a small number of cDNAs; these cDNAs can then be used as probes to screen and eliminate identical cDNAs from the pool.

Materials

- Glu/CM –Trp plates: CM dropout plates –Trp (see recipe) supplemental with 2% glucose
- Master dropout plate of yeast positive for Gal dependence (see Basic Protocol 2, step 18)
- 1 M sorbitol/20 mM EDTA/50 mM DTT (prepare fresh)
- 1 M sorbitol/20 mM EDTA
- 0.5 M NaOH
- 0.5 M Tris-Cl (pH 7.5)/6× SSC (APPENDIX 2A)
- 2× SSC (see recipe)
- 100,000 U/ml β -glucuronidase (type HP-2 crude solution from *Helix pomatia*; Sigma)

SUPPORT PROTOCOL 3

Macromolecular
Interactions in
Cells

17.3.29

82-mm circular nylon membrane, sterile

Whatman 3MM paper

80°C vacuum oven *or* UV cross-linker

Additional reagents and equipment for bacterial filter hybridization (*APPENDIX 3*)

1. Place a sterile nylon membrane onto a Glu/CM –Trp dropout plate. From the master dropout plate of Gal-dependent positives, gently restreak positives to be screened onto the membrane and mark the membrane to facilitate future identification of hybridizing colonies. Grow overnight (~12 hr) at 30°C.

Growth for extended periods of time (i.e., 24 hr) may result in difficulty in obtaining good lysis. It is a good idea to streak positive and negative controls for the cDNAs to be hybridized on the membrane.

2. Remove membrane from plate. Air dry briefly. Incubate ~30 min on a sheet of Whatman 3MM paper saturated with 1 M sorbitol/20 mM EDTA/50 mM DTT.

Optionally, before commencing chemical lysis, membranes can be placed at –70°C for 5 min, then thawed at room temperature for one or more cycles to enhance cell wall breakage.

3. Cut a piece of Whatman 3MM paper to fit inside a 100-mm petri dish. Place the paper disc in the dish and saturate with 100,000 U/ml β -glucuronidase diluted 1:500 in 1 M sorbitol/20 mM EDTA (2 μ l glucuronidase per ml of sorbitol/EDTA to give 200 U/ml final). Layer nylon membrane on dish, cover dish, and incubate up to 6 hr at 37°C until >80% of the cells lack a cell wall.

The extent of cell wall removal can be determined by removing a small quantity of cells from the filter to a drop of 1 M sorbitol/20 mM EDTA on a microscope slide and observing directly with a phase-contrast microscope at $\geq 60\times$ magnification. Cells lacking cell wall are nonrefractile.

4. Place membrane on Whatman 3MM paper saturated with 0.5 M NaOH for ~8 to 10 min.
5. Place membrane on Whatman 3MM paper saturated with 0.5 M Tris-Cl (pH 7.5)/6 \times SSC for 5 min. Repeat with a second sheet of Whatman 3MM paper.
6. Place membrane on Whatman 3MM paper saturated with 2 \times SSC for 5 min. Then place membrane on dry Whatman paper to air dry for 10 min.
7. Bake membrane 90 min at 80°C in vacuum oven or UV cross-link.
8. Process as for bacterial filter hybridization (*APPENDIX 3*), hybridizing the membrane with probes complementary to previously isolated cDNAs.

When selecting probes, either random-primed cDNAs or oligonucleotides complementary to the cDNA sequence may be used. If the cDNA is a member of a protein family, it may be advantageous to use oligonucleotides to avoid inadvertently excluding genes related but not identical to those initially obtained.

SUPPORT PROTOCOL 4

**Interaction Trap/
Two-Hybrid
System to Identify
Interacting
Proteins**

17.3.30

MICROPLATE PLASMID RESCUE

In some cases, it is desirable to isolate plasmids from a large number of positive colonies (Basic Protocol 2, steps 18 and 19). The protocol described below is a batch DNA preparation protocol developed by Steve Kron (University of Chicago, Chicago, Ill.) as a scale-up of a basic method developed by Manuel Claros (Laboratoire de Génétique Moléculaire, Paris, France).

Materials

2× Glu/CM –Trp liquid medium: 2× CM –Trp liquid medium (see recipe) supplemented with 4% glucose
Master plate of Gal-dependent yeast colonies (see Basic Protocol 2, step 18)
Rescue buffer: 50 mM Tris·Cl (pH 7.5)/10 mM EDTA/0.3% (v/v) 2-mercaptoethanol (prepare fresh)
Lysis solution: 2 to 5 mg/ml Zymolyase 100T/rescue buffer *or* 100,000 U/ml β-glucuronidase (type HP-2 crude solution from *Helix pomatia*; Sigma) diluted 1:50 in rescue buffer
10% (w/v) SDS
7.5 M ammonium acetate (APPENDIX 2A)
Isopropanol
70% ethanol
TE buffer, pH 8.0 (APPENDIX 2A)
24-well microtiter plates
Centrifuge with microplate holders, refrigerated
Repeating micropipettor
37°C rotary shaker

Grow yeast cultures

1. Aliquot 2 ml of 2× Glu/CM –Trp medium into each well of a 24-well microtiter plate. Into each well, pick a putative positive colony. Grow overnight with shaking at 30°C.

The 2× minimal medium is used to maximize the yield of yeast. Four plates can generally be handled conveniently at once, based on the number that can be centrifuged simultaneously.

2. Centrifuge 5 min at 1500 × g, 4°C. Shake off supernatant with a snap and return the plate to upright.
3. Swirl or lightly vortex the plate to resuspend cell pellets in remaining liquid. Add 1 ml water to each well and swirl lightly.

Cell pellets can most easily be resuspended in residual liquid before adding new solutions. Addition of liquid can be accomplished using a repeating pipettor.

4. Centrifuge 5 min at 1500 × g, 4°C. Shake off supernatant and resuspend pellet. Add 1 ml rescue buffer.
5. Centrifuge 5 min at 1500 × g, 4°C. Shake off supernatant and resuspend pellet in the small volume of liquid remaining in the plate.

Lyse cells

6. To each well, add 25 μl lysis solution. Swirl or vortex to mix. Incubate (with cover on) on a rotary shaker ~1 hr at 37°C.

Lysis solution need not be completely dissolved before use. By 1 hr, lysis should be obvious as coagulation of yeast into a white precipitate.

Susceptibility of yeast strains to lytic enzymes varies. If lysis occurs rapidly, then less lytic enzyme should be used. If the lysis step is allowed to go too far, too much of the partially dissolved cell wall may contaminate the final material. Lysis can be judged by examining cells with a phase-contrast microscope. Living cells are white with a dark halo and dead cells are uniformly gray. Lysis leads to release of granular cell contents into the medium. Once cells are mostly gray and many are disrupted, much of the plasmid should have been released.

7. To each well, add 25 μl of 10% SDS. Mix gently by swirling to completely disperse the precipitates. Allow plates to sit 1 min at room temperature.

At this point, the wells should contain a clear, somewhat viscous solution.

Purify plasmid

8. To each well, add 100 μ l of 7.5 M ammonium acetate. Swirl gently, then incubate 15 min at -70°C or -20°C until frozen.

Addition of acetate should result in the formation of a massive white precipitate of cell debris and SDS. The freezing step appears to improve removal of inhibitors of E. coli transformation.

9. Remove plate from freezer. Once it begins to thaw, centrifuge 15 min at $3000 \times g$, 4°C . Transfer 100 to 150 μ l of the resulting clear supernatants to clean 24-well plates.

In general, some contamination of the supernatant with pelleted material cannot be avoided. However, it is better to sacrifice yield in order to maintain purity.

10. To each well, add ~ 0.7 vol isopropanol. Mix by swirling and allow to precipitate 2 min at room temperature.

A cloudy fine precipitate should form immediately after isopropanol is added.

11. Centrifuge 15 min at $3000 \times g$, 4°C . Shake off supernatant with a snap.

12. To each well, add ~ 1 ml cold 70% ethanol. Mix by swirling, centrifuge 5 min at $3000 \times g$, 4°C . Shake off supernatant with a snap, invert plates and blot well onto paper towel. Allow plates to air dry.

13. To each well, add 100 μ l TE buffer. Swirl well and allow to rest on bench several minutes, until the pellets appear fully dissolved. Transfer preps to microcentrifuge tubes or 96-well plates for storage at -20°C .

One to five microliters of each of the resulting preparations can be used to transform competent E. coli: for KC8, electroporation should be used (see Basic Protocol 2, step 21). Sometimes, the yield of transformants is low if E. coli carrying plasmids are not permitted time to increase the plasmid copy number above a critical threshold before the cells are placed on selective medium. Allow plenty of time for cells to express antibiotic resistance or the TRP1 gene before plating.

If insufficient numbers of colonies are obtained by this approach, the final plasmid preparation can be resuspended in 20 μ l instead of 100 μ l TE buffer to concentrate the DNA stock.

SUPPORT PROTOCOL 5

ADDITIONAL SPECIFICITY SCREENING

The three test plasmids outlined (pSH18-34, pRFHM1, and pEG202; see Basic Protocol 2, step 24) represent a minimal test series. If other LexA-bait proteins that are related to the bait protein used in the initial library screen are available, substantial amounts of information can be gathered by additional specificity tests. For example, if the initial bait protein was LexA fused to the leucine zipper of c-Fos, specificity screening of interactor-hunt positives against the leucine zippers of c-Jun or GCN4 in addition to that of c-Fos might allow discrimination between proteins that are specific for fos versus those that generically associate with leucine zippers.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

A medium

5 g $(\text{NH}_4)_2\text{SO}_4$
22.5 g KH_2PO_4
52.5 g K_2HPO_4
2.5 g sodium citrate $\cdot 2\text{H}_2\text{O}$

Before they are used, concentrated media should be diluted to 1× with sterile water and the following sterile solutions, per liter:

1 ml 1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
10 ml 20% carbon source (sugar or glycerol)
and, if required:
0.1 ml 0.5% vitamin B1 (thiamine)
5 ml L amino acids to 40 $\mu\text{g}/\text{ml}$ *or*
DL amino acids to 80 $\mu\text{g}/\text{ml}$
Antibiotic

Complete minimal (CM) dropout medium

1.3 g dropout powder (Table 17.3.4)
1.7 g YNB –AA/AS
5 g $(\text{NH}_4)_2\text{SO}_4$
20 g dextrose
(Alternatively, replace last three ingredients with 27 g minimal medium premix)

For CM plates, add 20 g agar and a pellet of NaOH per liter.

CM dropout powder, also known as minus or omission powder, lacks one or more nutrients but contains the other nutrients listed in Table 17.3.4. Complete minimal (CM) dropout medium is used to test for genes involved in biosynthetic pathways and to select for gene function in transformation experiments. To test for a gene involved in histidine biosynthesis one would determine if the yeast strain in question can grow on CM minus histidine (–His) or “histidine dropout” plates. It is convenient to make several dropout powders, each lacking a single nutrient, to avoid weighing each component separately for all the different dropout plates required in the laboratory.

It may be preferable to use a 10× solution of dropout powder (i.e., 13 g of dropout powder in 100 ml water) that has been “sterilized” separately (and added to the other ingredients after autoclaving) to improve the growth rate in this medium.

Glycerol solution

65% (v/v) glycerol, sterile
0.1 M MgSO_4
25 mM Tris-Cl, pH 8.0 (APPENDIX 2A)
Store up to 1 year at room temperature

Laemmli sample buffer, 2×

10% (v/v) 2-mercaptoethanol (2-ME)
6% (w/v) SDS
20% (v/v) glycerol
0.2 mg/ml bromphenol blue
0.025× Laemmli stacking buffer (see recipe; optional)
Store up to 2 months at room temperature

This reagent can conveniently be prepared 10 ml at a time.

Laemmli stacking buffer, 2.5×

0.3 M Tris-Cl, pH 6.8
0.25% (w/v) SDS
Store up to 1 month at 4°C

Lysis solution

50 mM Tris-Cl, pH 7.5 (APPENDIX 2A)
10 mM EDTA
0.3% (v/v) 2-mercaptoethanol (2-ME), added just before use

continued

Table 17.3.4 Nutrient Concentrations for Dropout Powders^a

Nutrient ^b	Amount in dropout powder(g) ^c	Final conc. in prepared media (μg/ml)	Liquid stock conc. (mg/100 ml) ^d
Adenine (hemisulfate salt)	2.5	40	500
L-arginine (HCl)	1.2	20	240
L-aspartic acid ^e	6.0	100	1200
L-glutamic acid (monosodium salt)	6.0	100	1200
L-histidine	1.2	20	240
L-leucine ^f	3.6	60	720
L-lysine (mono-HCl)	1.8	30	360
L-methionine	1.2	20	240
L-phenylalanine	3.0	50	600
L-serine	22.5	375	4500
L-threonine ^e	12.0	200	2400
L-tryptophan	2.4	40	480
L-tyrosine	1.8	30	180 ^f
L-valine	9.0	150	1800
Uracil	1.2	20	240

^aCM dropout powder lacks one or more nutrients but contains the other nutrients listed in this table. Nomenclature in this manual refers to, e.g., a preparation that omits histidine as histidine dropout powder. Conditions from Sherman et al., 1979.

^bAmino acids not listed here can be added to a final concentration of 40 μg/ml (40 mg/liter).

^cGrind powders into a homogeneous mixture with a clean, dry mortar and pestle. Store in a clean, dry bottle or a covered flask.

^dUse 8.3 ml/liter of each stock for special nutritional requirements. Store adenine, aspartic acid, glutamic acid, leucine, phenylalanine, tyrosine, and uracil solutions at room temperature. All others should be stored at 4°C.

^eWhile these amino acids can be used reliably when included in autoclaved media, they supplement growth better when added after autoclaving.

^fUse 16.6 ml/liter for L-tyrosine nutritional requirement.

2% (v/v) β-glucuronidase from *Helix pomatia* (Type HP-2; Sigma), added just before use

SSC (sodium chloride/sodium citrate), 20×

3 M NaCl (175 g/liter)

0.3 M Na₃citrate·2H₂O (88 g/liter)

Adjust pH to 7.0 with 1 M HCl (APPENDIX 2A)

Z buffer

16.1 g Na₂HPO₄·7H₂O (60 mM final)

5.5 g NaH₂PO₄·H₂O (40 mM final)

0.75 g KCl (10 mM final)

0.246 g MgSO₄·7H₂O (1 mM final)

2.7 ml 2-mercaptoethanol (50 mM final)

Adjust to pH 7.0 and bring to 1 liter with H₂O. Do not autoclave.

COMMENTARY

Background Information

Interaction-based cloning is derived from three experimental observations. In the first, Brent and Ptashne (1985) demonstrated that it was possible to assemble a novel, functional transcriptional activator by fusing the DNA-binding domain from one protein, LexA, to the activation domain from a second protein,

GAL4. This allowed the use of a single reporter system containing a single DNA-binding motif, the *LexA* operator, to study transcriptional activation by any protein of interest. In the second, Ma and Ptashne (1988) built on this work to demonstrate that the activation domain could be brought to DNA by interaction with a DNA-binding domain. In the third, Fields and Song

Table 17.3.5 Two-hybrid System Variants^{a,b}

System	DNA-binding domain	Activation domain	Selection	Reference
Two-hybrid	<i>GAL4</i>	<i>GAL4</i>	Activation of <i>lacZ</i> , <i>HIS3</i>	Chien et al., 1991
Interaction trap	<i>LexA</i>	<i>B42</i>	Activation of <i>LEU2</i> , <i>lacZ</i>	Gyuris et al., 1993
“Improved two-hybrid”	<i>GAL4</i>	<i>GAL4</i>	Activation of <i>HIS3</i> , <i>lacZ</i>	Durfee et al., 1993
Modified two-hybrid	<i>LexA</i>	VP16	Activation of <i>HIS3</i> , <i>lacZ</i>	Vojtek et al., 1993
KISS	<i>GAL4</i>	VP16	Activation of <i>CAT</i> , <i>hyg^r</i>	Fearon et al., 1992
Contingent replication	<i>GAL4</i>	VP16	Activation of T-Ag, replication of plasmids	Vasavada et al., 1991

^aAbbreviations: *CAT*, chloramphenicol transferase gene; *hyg^r*, hygromycin resistance gene; T-Ag, viral large T antigen.

^bAn extended version of this table is available on <http://www.fccc.edu/research/labs/golemis/betagal.html>.

(1989), working independently of Ma and Ptashne, used two yeast proteins, SNF1 and SNF4, to make an SNF1 fusion to the DNA-binding domain of *GAL4* and an SNF4 fusion to the *GAL4* activation domain. They demonstrated that the strength of the SNF1-SNF4 interaction was sufficient to allow activation through a *GAL4* DNA-binding site. From this, they suggested the feasibility of selecting interacting proteins by performing screens of cDNA libraries made so that library-encoded proteins carried activating domains.

Several groups have developed cDNA library strategies along these lines, with some systems using *LexA* and others using *GAL4* as the DNA-binding domain (Table 17.3.5). *LexA* and *GAL4* each have different properties that should be considered when selecting a system. *LexA* is derived from a heterologous organism, has no known effect on the growth of yeast, possesses no residual transcriptional activity, can be used in *GAL4*⁺ yeast, and can be used with a Gal-inducible promoter. Because *GAL4* is an important yeast transcriptional activator, it has the disadvantage that experiments must be performed in *gal4*⁻ yeast strains to avoid background due to activation of the reporter system by endogenous *GAL4*. Such *gal4*⁻ strains are frequently less healthy and more difficult to transform than wild-type strains, and either libraries must be constitutively expressed or alternate inducible systems must be used. By contrast, the *GAL4* DNA-binding domain may be more efficiently localized to the nucleus and may be preferred for some proteins (for a review of *GAL4*-based systems, see Bartel et al., 1993). Whichever system is used, it is important to remember that the bait protein constitutes a novel fusion protein whose prop-

erties may not exactly parallel those of the original unfused protein of interest. Although systems using the two-hybrid paradigm have been developed in mammalian cells (see Table 17.3.5), these have not been used effectively in library screens. It seems likely that the organism of choice for two-hybrid identification of novel partner proteins will remain yeast.

cDNAs that pass specificity tests are referred to as positives, or “true positives.” In interactor hunts conducted to date, anywhere from zero to practically all isolated plasmids passed the final specificity test. If no positives are obtained, the tissue source for the library originally used may not be appropriate, and a different library may produce better results. However, there are some proteins for which no positives are found. Various explanations for this are provided below. Conversely, some library-encoded proteins are known to be isolated repeatedly using a series of unrelated baits, and these proteins demonstrate at least some specificity. One of these, heat shock protein 70, might be explained by positing that it assists the folding of some *LexA*-fused bait proteins, or alternatively, that these bait proteins are not normally folded. This example illustrates the point that the physiological relevance of even quite specific interactions may sometimes be obscure.

Because the screen involves plating multiple cells to Gal/CM –Ura, –His, –Trp, –Leu dropout medium for each primary transformant obtained, multiple reisolates of true positive cDNAs are frequently obtained. If a large number of specific positives are obtained, it is generally a good idea to attempt to sort them into classes—for example, digesting minipreps of positives with *EcoRI*, *XhoI*, and *HaeIII* will

generate a fingerprint of sufficient resolution to determine whether multiple reisolates of a small number of clones or single isolates of many different clones have been obtained. The former situation is a good indication that the system is working well.

An important issue that arises in an interactor hunt is the question of how biologically relevant interacting proteins that are isolated are likely to be. This leads directly to the question of what K_d of association two molecules must have to be detected by an interactor hunt. In fact, this is not at all a simple issue. For the system described here, most fusion proteins appear to be expressed at levels ranging from 50 nM to 1 μ M (Golemis and Brent, 1992). Given the strength of the *GAL* promoter, it is likely that many library-encoded proteins are expressed at similarly high levels, $\geq 1 \mu$ M in the nucleus (Golemis and Brent, 1992). At this concentration, which is in considerable excess over the nuclear concentration of operator-bound bait protein, a cDNA library-encoded protein should half-maximally occupy the DNA-bound bait protein if it possesses a K_d of 10^{-6} M, making it theoretically possible that very-low-affinity interactions could be detected. Such interactions have been observed in some cases. In contrast, some interactions that have been previously established using other methods and are predicted by known K_d to be easily detected by these means, either are not detected or are detected only weakly (Finley and Brent, 1994; Estojak et al., 1995). Because of the conservation of many proteins between lower and higher eukaryotes, one explanation for this observation is that either one or both of the partners being tested is being sequestered from the desired interaction by fortuitous association with an endogenous yeast protein. A reasonably complete investigation of the degree of correlation between in vitro determinations of interaction affinity and apparent strength of interaction in the interaction trap is included in Estojak et al. (1995). The result of this investigation suggests it is important to measure the affinity of detected interactions under different conditions, using a second assay system, rather than to draw conclusions about affinity based on detection in the interaction trap.

A number of different plasmids can be used for conducting an interactor hunt. Their properties are summarized in Tables 17.3.1 and 17.3.2. Because of the generous and open scientific exchange between investigators using

the system, the number of available plasmids and other components has greatly expanded since the appearance of the initial two-hybrid reagents, facilitating the study of proteins inaccessible by the original system.

The original parent plasmid for generating LexA fusions, pEG202 is a derivative of 202 + PL (Ruden et al., 1991; see Fig. 17.3.3) that contains an expanded polylinker region. The available cloning sites in pEG202 include *EcoRI*, *BamHI*, *SalI*, *NcoI*, *NotI*, and *XhoI*, with the reading frame as described in the legend to Figure 17.3.3. Since the original presentation of this system, a number of groups have developed variants of this plasmid that address specialized research needs. Those currently available, as well as purposes for which they are suited, are listed in Table 17.3.1. pGilda, created by David A. Shaywitz, places the LexA-fusion cassette under the control of the inducible *GALI* promoter, allowing expression of the bait protein for limited times during library screening, reducing the exposure of yeast to toxic baits. pJK202, created by Joanne Kamens, adds nuclear localization sequences to pEG202, facilitating assay of the function of proteins lacking internal nuclear localization sequences. pNLexA, created by Ian York, places LexA carboxy-terminal in the fusion domain, allowing assay of interactions that require an unblocked amino-terminus on the bait protein. pEE202I, created by Mike Watson and Rich Buckholz, allows chromosomal integration of a pEG202-like bait, thus reducing expression levels so they are more physiological for bait proteins normally present at low levels intracellularly. All of these have been extensively tested by numerous researchers. pGilda, pJK202, and pEE202I work with complete reliability. pNLexA works effectively with ~50% of the fusion domains tried, but synthesizes only very low levels of protein (relative to expression of the same fusion domain as a pEG202 fusion) with the remaining 50%. Attachment of fusion domains amino-terminal either to *LexA* or *GAL4* has been generally problematic in the hands of many investigators; it may be that appending additional protein sequences to the amino termini of these proteins is destabilizing, although the problem has not been rigorously investigated.

A series of *lacZ* reporters of differing sensitivity to transcriptional activation can be used to detect interactions of varying affinity (see Table 17.3.2). These plasmids are *LexA* operator-containing derivatives of the plasmid

LR1Δ1 (West et al., 1984). In LR1Δ1, a minimal *GAL1* promoter lacking the *GAL1* upstream activating sequences (GAL_{UAS}) is located upstream of the bacterial *lacZ* gene. In pSH18-34, eight *LexA* operators have been cloned into an *XhoI* site located 167 bp upstream of the *lacZ* gene (S. Hanes, unpub. observ.). pJK103 and pRB1840 contain two and one operator, respectively.

pJK101 is similar to pSH18-34, except that it contains the *GAL1* upstream activating sequences (GAL_{UAS}) upstream of two *LexA* operator sites. A derivative of del20B (West et al., 1984), it is used in the repression assay (Brent and Ptashne, 1984; see Fig. 17.3.5) to assess LexA fusion binding to operator.

pSH17-4 is a *HIS3* 2μm plasmid that encodes LexA fused to the activation domain of the yeast activator GAL4. EGY48 cells bearing this plasmid will produce colonies in overnight growth on medium lacking Leu, and yeast that additionally contain pSH18-34 will turn deep blue on plates containing Xgal. This plasmid serves as a positive control for the activation of transcription.

pRFHM1 is a *HIS3* 2μm plasmid that encodes LexA fused to the N-terminus of the *Drosophila* protein bicoid. The plasmid has no ability to activate transcription, so EGY48 cells that contain pRFHM1 and pSH18-34 do not grow on -Leu medium and remain white on plates containing Xgal. pRFHM1 is a good control for specificity testing, because it has been demonstrated to be sticky—that is, to associate with a number of library-encoded proteins that are clearly nonphysiological interactors (R. Finley, Wayne State University, Detroit, Mich., unpub. observ.).

This protocol uses interaction libraries (Table 17.3.3) made in pJG4-5 or its derivatives (see Fig. 17.3.6). pJG4-5 was developed to facilitate isolation and characterization of novel proteins in interactor hunts (Gyuris et al., 1993). The pJG4-5 cDNA library expression cassette is under control of the *GAL1* promoter, so library proteins are expressed in the presence of galactose (Gal) but not glucose (Glu). This conditional expression has a number of advantages, the most important of which is that many false-positives obtained in screens can be easily eliminated because they do not demonstrate a Gal-dependent phenotype. The expression cassette consists of an ATG to start translation, a nuclear localization signal to extend the interaction trap's range to include proteins that are normally predominantly localized in the cyto-

plasm, an activation domain (acid blob; Ma and Ptashne, 1987), the hemagglutinin epitope tag to permit rapid assessment of the size of encoded proteins, *EcoRI-XhoI* sites designed to receive directionally synthesized cDNAs, and the alcohol dehydrogenase (*ADH*) termination sequences to enhance the production of high levels of library protein. The plasmid also contains the *TRP1* auxotrophy marker and 2μm origin for propagation in yeast. A derivative plasmid, pJG4-5I, was created by Mike Watson and Richard Buckholz to facilitate chromosomal integration of the activation domain fusion expression plasmid.

A series of recently developed derivatives of pEG202, pJG4-5, and *lacZ* reporter plasmids (MW101 to MW112) alter the antibiotic resistance markers on these plasmids from ampicillin (Ap^r) to either kanamycin (Km^r) or chloramphenicol (Cm^r ; Watson et al., 1996). Judiciously mixing and matching these plasmids in conjunction with Ap^r libraries would considerably reduce work subsequent to library screening, because the KC8 transformation, which involves *trpC* complementation in bacteria, could be omitted.

EGY48 and EGY191 (see Table 17.3.2) are both derivatives of the strain U457 (a gift of Rodney Rothstein, Columbia University, New York, N.Y.) in which the endogenous *LEU2* gene has been replaced by homologous recombination with *LEU2* reporters carrying varying numbers of *LexA* operators, using a procedure detailed in Estojak et al. (1995).

Interaction Trap-compatible reagents have recently become commercially available; Clontech and Invitrogen were the first to market such reagents and have recently been joined by OriGene. All suppliers use systems with the most sensitive reporters (EGY48 and pSH18-34), and provide their own positive and negative controls for testing activation or interaction between defined proteins. For expression of bait and library proteins, the Clontech Matchmaker LexA two-hybrid system and the OriGene Duplex-A system use some of the basic set of plasmids described here (see Table 17.3.1 for availability). Forward sequencing primers for bait and library plasmids are included in the Clontech kit, and Insert Screening Amplimer Sets for both plasmids can be acquired separately. Additional related products from Clontech include KC8 competent cells, anti-LexA monoclonal antibodies, a yeast transformation system, a yeast plasmid isolation kit, and an EGY48 partner strain for yeast

mating to facilitate the analysis of interaction specificity. OriGene has a generally similar product line to Clontech. In contrast, Invitrogen has substantially modified the Interaction Trap core reagents to develop its own bait and library plasmids. pHybLex/Zeo, a novel bait plasmid, is ~50% smaller than the original pEG202 (making it easier to clone into), and it has an enriched polylinker. Significantly, it replaces both the *Ap^r* and *HIS3* genes with a novel gene that confers resistance to the antibiotic Zeocin (supplied with the kit), which provides selection in both bacteria and yeast. This elimination of auxotrophic selection for the bait plasmid renders the LexA-fusion construct usable with libraries and strains from all existing two-hybrid systems and additionally facilitates the direct selection of library plasmid in strains other than KC8. Some changes, which are designed to make the vector easier to use, have also been introduced in the library vector pYESTrp (e.g., it uses a V5 epitope tag for protein detection). The Invitrogen kit, termed Hybrid Hunter, includes the bait/library/reporter plasmids and EGY48 yeast strain as noted, and additionally includes primer sets for bait and library plasmids and the L40 yeast strain, should an investigator wish to use a *HIS3* auxotrophy selection. Additional related products from Invitrogen include antibodies for detection of bait and prey fusion proteins (anti-LexA and anti-V5), pJG4-5 library vector primers, and a Transformation Kit.

A significant advantage of the entry of commercial entities into the Interaction Trap field is the rapid increase in the number of compatible cDNA libraries. A list of currently available premade libraries available from these companies is presented in Table 17.3.3, and custom-made libraries are also available upon request. Because new libraries and other related reagents are being constantly added to the line of two-hybrid related products, it is advisable to contact the companies or visit their Web sites (www.clontech.com, www.invitrogen.com, and www.origene.com) for the latest information.

Finally, over the last several years, a number of groups have adapted basic two-hybrid strategies to more specialized applications, and they have devised strategies to broaden their basic functionality. Interaction Mating (Finley and Brent, 1994) has been used to establish extended networks of targeted protein-protein interaction. In this approach, a panel of LexA-fused proteins is transformed into a *MAT α* haploid selective strain (such as RFY206), a panel of activation-domain fused proteins is trans-

formed into a suitable *MAT α* haploid (such as EG448), and the two panels are cross-gridded against each other for mating. Selected diploids are then screened by replica plating to selective medium. This approach complements library screening in large-scale applications, such as proposed definition of interaction maps for entire genomes (Bartel et al., 1996).

Interaction mating has also provided the basis for an alternative two-hybrid hunt protocol (see Alternate Protocol 2), useful in cases when a single library will be screened with different baits. In this approach (Bendixen et al., 1994; Finley and Brent, 1994; Kolonin and Finley, 1998), a library is introduced into a single strain, like EGY48, and aliquots are stored frozen. To conduct a hunt, an aliquot is thawed and mated with a strain expressing a bait. This allows one to avoid repeated high-efficiency transformations, since a single library transformation can provide enough pretransformed yeast to conduct dozens of interactor hunts. Moreover, some yeast strains pretransformed with libraries are becoming commercially available, which may eliminate altogether the need to conduct a high-efficiency library transformation for some researchers.

Two-hybrid approaches have been shown to be effective in identifying small peptides with biological activities on selected baits (Yang et al., 1995; Colas et al., 1996), which may prove to be useful as a guide to targeted drug design. Rapid screening protocols have been devised using custom-synthesized libraries expressing sheared plasmid DNA to facilitate rapid mapping of interaction interfaces (Stagljar et al., 1996). Osborne and coworkers have demonstrated the effectiveness of a tribrid (or tri-hybrid) approach, in which an additional plasmid expresses a tyrosine kinase to specifically modify a bait protein, allowing detection of SH2-domain-containing partner proteins that recognize specific phosphotyrosine residues (Osborne et al., 1995). A variety of more elaborate tribrid approaches, in which a DNA-binding domain fused protein is used to present an intermediate nonprotein compound for interaction with a library, have been developed and proven effective. These approaches have allowed the identification of proteins binding specific drug ligands (Chiu et al., 1994; Licitra and Liu, 1996), as well as the identification of proteins binding to RNA sequences (SenGupta et al., 1996; Wang et al., 1996). It is expected that the range of utility of these systems will continue to expand.

Critical Parameters and Troubleshooting

To maximize chances of a successful interactor hunt, a number of parameters should be taken into account. Before attempting a screen, bait proteins should be carefully tested to ensure that they have little or no intrinsic ability to activate transcription. Bait proteins must be expressed at reasonably high levels and must be able to enter the yeast nucleus and bind DNA (as confirmed by the repression assay). Optimally, integrity and levels of bait proteins should be confirmed by immunoblot analysis, using an antibody to either LexA or the fused domain. In particular, at this time, bait proteins that have extensive transmembrane domains or are normally excluded from the nucleus are not likely to be productively used in a library screen. Proteins that are moderate to strong activators will need to be truncated to remove activating domains before they can be used.

If a protein neither activates nor represses, the most likely reason is that it is not being made. This can be determined by immunoblot analysis of a crude lysate protein extract of EGY48 (UNIT 6.2; Samson et al., 1989) containing the plasmid, using anti-LexA antibodies as primary antiserum. If the full protein is not made, it may be possible to express truncated derivatives of the protein. If the protein is made, but still does not repress, it may not enter the yeast nucleus effectively, although this appears to be a relatively rare problem. In this case, introducing the coding sequence for the fused moiety into a LexA fusion vector containing a nuclear localization motif (e.g., pJK202; J. Kamens, BASF, Worcester, Mass., unpub. observ.) may solve the problem.

The test for the leucine (Leu) requirement is extremely important to determine whether the bait protein is likely to yield an unworkably high background. The *LEU2* reporter in EGY48 is more sensitive than the pSH18-34 reporter for some baits (Estojak et al., 1995). Therefore, it is possible that a bait protein demonstrating little or no signal in a β -galactosidase assay may nevertheless permit some growth on $-$ Leu medium. If this occurs, there are several options. First, a less sensitive strain can be used, as described in the text. Second, background can sometimes be reduced further by making the EGY strain diploid (e.g., D. Krainc, Harvard Medical School, Boston, Mass.; R. Finley and R. Brent, unpub. observ.) or by performing the hunt by interaction mating as described in Alternate Protocol 2. A third

option is to attempt to truncate the bait protein to remove activating function. In general, it is useful to extrapolate from the number of cells that grow on $-$ Leu medium to the number that would be obtained in an actual library screen, and determine if this is a background level that can be tolerated. For example, if two colonies arise from 100,000 plated cells on $-$ Leu medium, 200 to 400 would be expected in an actual screen of 10^6 cDNAs. Although this is a high initial number of positives, the vast majority should be eliminated immediately through easily performed controls. This is a judgment call. Finally, very rarely it happens that a bait that appears to be well behaved and negative for transcriptional activation through all characterization steps will suddenly develop a very high background of transcriptional activation following library transformation. The reason for this is currently obscure, and no means of addressing this problem has as yet been found: such baits are hence inappropriate for use in screens.

The protocols described in this unit use initial screening with the most sensitive reporters followed by substitution with less sensitive reporters if activation is detected. An obvious question is, why not start out working with extremely stringent reporters and know immediately whether the system is workable? In fact, some researchers routinely use a combination of pJK103 or pRB1840 with EGY191, and obtain proteins that to date appear to be biologically relevant partners from library screens. However, extensive comparison studies using interactors of defined *in vitro* affinity with different combinations of *LacZ* and *LEU2* reporters (Estojak et al., 1995) have indicated that although the most sensitive reporters (pSH18-34) may in some cases be prone to background problems, the most stringent reporters (EGY191, pRB1840) may miss some interactions that certainly are biologically relevant and occur inside cells. In the end, the choice of reporters devolves to the preference of individual investigators: the bias of the authors is to cast a broad net in the early stages of a screen, and hence to use more sensitive reporters when practicable.

It is important to move expeditiously through characterization steps and to handle yeast transformed with bait plasmids with care. In cases where yeasts have been maintained on plates for extended periods (e.g., 4 days at room temperature or >2 to 3 weeks at 4°C), unex-

pected problems may crop up in subsequent library screens.

The transformation protocol is a version of the lithium acetate transformation protocol described by Schiestl and Gietz (1989) and Gietz et al. (1992) that maximizes transformation efficiency in *Saccharomyces cerevisiae* and produces up to 10^5 colonies/ μg plasmid DNA. In contrast to *Escherichia coli*, the maximum efficiency of transformation for *S. cerevisiae* is $\sim 10^4$ to 10^5 / μg input DNA. It is extremely important to optimize transformation conditions before attempting an interactor hunt. Perform small-scale pilot transformations to ensure this efficiency is attained and to avoid having to use prohibitive quantities of library DNA. In addition, as for any effort of this type, it is a good idea to obtain or construct a library from a tissue source in which the bait protein is known to be biologically relevant.

In practice, the majority of proteins isolated by interaction with a LexA fusion turns out to be specific for the fused domain; a smaller number are nonspecifically sticky, and to date there appears to have been only one isolation from a eukaryotic library of a protein specific for LexA. However, it is generally informative to retest positive clones on more than one LexA bait protein; ideally, library-derived clones should be tested against the LexA fusion used for their isolation, several LexA fusions to proteins that are clearly unrelated to the original fusion, and if possible, several LexA fusions that there is reason to believe are related to the initial protein (e.g., if the initial probe was LexA-Fos, a good related set would include LexA-Jun and LexA-GCN4).

Colony selection for master plate production is one of the more variable parts of the procedure. For strong interactors, colonies will grow up in 2 days. However, if plates are left at 30°C , new colonies will continue to appear every day. Those that appear rapidly are most likely to reflect interactors that are biologically relevant to the bait protein. Those that appear later may or may not be relevant. However, many parameters can delay the time of colony formation of cells that contain valid interactions, including the strength of the interaction and the level of expression of the library-encoded protein.

Anticipated Results

Depending on the protein used as bait, anywhere from zero to hundreds of specific interactors will be obtained from 10^6 primary transformants.

Time Considerations

If all goes well, once the required constructions have been made it will take ~ 1 week to perform yeast transformations, obtain colonies, and determine whether bait proteins are appropriate. It will take a second week to perform library transformations, replat to selective medium, and obtain putative positives. A third week will be required to rescue the plasmid from the yeast, passage it through *E. coli*, transform fresh yeast, and confirm specificity.

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Key Reference

Gyuris et al., 1993. See above.

Initial description of interaction trap system.

Internet Resources

<http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork/html>

<http://cmmg.biosci.wayne.edu/finlab/finlabhomeb.html#Places>

Sources of two-hybrid information, protocols, and links; database for false positive proteins detected in interaction trap experiments; analysis of two-hybrid usage.

<http://www.clontech.com>

<http://www.invitrogen.com>

<http://www.origene.com>

Commercial sources for basic plasmids, strains, and libraries for interaction trap experiments.

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Sources of interaction trap plasmids for specialized interactions.

<http://xanadu.mgh.harvard.edu/brentlabhomepage4.html>

Database of interaction trap protocols and related issues.

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**Interaction Trap/
Two-Hybrid
System to Identify
Interacting
Proteins**

17.3.42

Mapping Protein-Protein Interactions with Phage-Displayed Combinatorial Peptide Libraries

UNIT 17.4

This unit describes the process and analysis of affinity selecting bacteriophage M13 from libraries displaying combinatorial peptides fused to either a minor or major capsid protein. The first protocol provided in this unit describes direct affinity selection using target protein bound to a microtiter dish, followed by purification of selected phage by ELISA (see Basic Protocol). A bead-based affinity selection using fusion protein targets is also described (see Alternate Protocol). With either protocol, one can readily isolate peptide ligands that bind to a target protein of interest and use the consensus sequence to search proteome databases for putative interacting proteins.

AFFINITY SELECTION USING PROTEIN TARGETS IN MICROTITER DISHES

BASIC
PROTOCOL

In this method, a target protein is immobilized on a microtiter plate and a phage-display combinatorial peptide library is added to the wells to select for phage that bind to the protein target. Selected (bound) phage are then recovered and amplified. The selection is repeated two additional times to enrich for the binding phage and reduce the recovery of nonspecifically bound phage. After the third selection, the binding specificity of individual clones is examined by ELISA using wells containing target protein and a negative control. Binding is detected with an HRP-conjugated anti-phage antibody and a chromogenic substrate. The sequences from specifically bound clones can then be analyzed by DNA sequencing and the predicted peptide ligand can be compared to available databases.

While peptides have been displayed on all six of the capsid proteins of bacteriophage M13, proteins III and VIII are the most popular fusion partners. Protein III (pIII) is a minor capsid protein, present in five copies at one end of the filamentous particle, and functions in infecting bacteria. Fortunately for molecular biologists, display of peptides (or proteins) at the N terminus of the mature protein generally does not interfere with the function of its three globular domains. Protein VIII (pVIII) is the major capsid protein, with ~2500 copies per particle. Due to the crystalline nature of this protein on the virus surface, mature pVIII tolerates only short (i.e., 9-mer) peptides at its N terminus. While pIII and pVIII display libraries are both useful in selecting peptide ligands that can be used to predict protein-protein interactions, peptides isolated from pIII libraries typically have low-micromolar (1 to 10 μM) dissociation constants, whereas peptides isolated from pVIII libraries have mid-micromolar (10 to 100 μM) dissociation constants. This quantitative difference reflects the difference in valency of the displayed peptides as well as the resulting avidity effects that occur when binding microtiter plate wells are densely coated with target. Libraries of phage displaying combinatorial peptide libraries at the N terminus of pIII can be purchased from New England Biolabs. Libraries of phage displaying combinatorial peptide libraries at the N terminus of pVIII can be obtained by request (see Felici et al., 1991).

Materials

- Purified protein target(s) to be analyzed
- 0.2 M sodium bicarbonate buffer (Na_2HCO_3), pH 8.5
- Control targets (see Troubleshooting)
- Blocking buffer: 1% (w/v) BSA in PBS (see recipe for PBS)
- Wash buffer: 0.05% (v/v) Tween 20 in PBS

Macromolecular
Interactions in
Cells

Contributed by Brian K. Kay and Luisa Castagnoli
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17.4.1

Supplement 17

Combinatorial phage-display library (New England Biolabs or Felici et al., 1991)

Acid elution buffer: 50 mM glycine-HCl, pH 2

Neutralization buffer: 0.2 M Tris-Cl, pH 7.5 (*APPENDIX 2A*)

Bacteria: fresh overnight culture of DH5 α F' (*APPENDIX 3A*)

2 \times YT culture medium and top and bottom agar (see recipe)

Negative control protein (fusion partner)

Anti-bacteriophage M13 monoclonal antibody coupled to horseradish peroxidase (HRP; Amersham Pharmacia Biotech), diluted 1:5000 (v/v) in wash buffer

Chromogenic substrate (see recipe)

ELISA-ready 96-well microtiter plates (Costar or Immunolon, high capacity)

Aerosol-resistant pipet tips

5-ml sterile tubes

Sterile toothpicks

Spectrophotometer capable of reading microtiter plates

Additional reagents and equipment for DNA purification and sequencing (*APPENDIX 3A*)

Coat microtiter plate wells

1. Dilute 1 to 3 μ g of purified protein target into 0.5 ml of 0.2 M sodium bicarbonate, pH 8.5, and add 150 μ l to three wells of a 96-well microtiter plate. Repeat for each protein target to be tested and for each control. Incubate without shaking for 2 hr at room temperature or, for less stable proteins, overnight at 4°C.

To prevent evaporation, keep the wells sealed with tape during all incubations.

2. Add 200 μ l blocking buffer to each well and incubate for 30 min at room temperature.
3. Flick the liquid into a sink and slap the plate two times against a paper towel.
4. Add 200 μ l wash buffer to each well, flick out the contents, and slap against a clean paper towel. Repeat two additional times.

Select interacting phage

5. Dilute combinatorial phage-display library to 1000 library equivalents per 150 μ l wash buffer and then add 150 μ l diluted phage particles to each test and control well. Incubate for 2 hr at room temperature.

To ensure selection of rare peptide sequences, the authors typically use 1000 library equivalents for the first selection step with a target protein. Thus, if a library has a complexity of 10^9 members, 10^{12} particles should be added to 150 μ l wash buffer.

To minimize the contamination of pipettors with phage particles, the authors strongly recommend using aerosol-resistant pipet tips when transferring liquids containing phage.

6. Flick the liquid into a sink and slap the plate two times against a paper towel.
7. Add 200 μ l wash buffer to each well, flick out the contents, and slap the plate against a clean paper towel. Repeat two additional times.

Recover bound phage

8. Add 150 μ l acid elution buffer to the washed, empty wells and incubate 10 min at room temperature. Pool the liquid from triplicate wells into a single clean 5-ml plastic tube containing 150 μ l neutralization buffer.

Generally, 10^3 to 10^8 phage particles are recovered from each well. Phage can also be eluted from the wells with 0.2 M ethanolamine, pH 12, and neutralized as above.

9. Add 200 μ l bacteria (fresh overnight culture) and incubate for 10 min at room temperature.

10. Add 3 ml of 2× YT top agar and pour onto a 100 × 15–mm petri dish containing 2× YT bottom agar (APPENDIX 3A). Allow plate to harden 5 min at room temperature, and then leave the plate overnight in a 37°C incubator.

Instead of plate amplification, it is also possible to amplify the phage in a liquid culture. To do this, transfer the solution containing the infected bacteria to 5 ml of 2× YT broth and incubate in a 50-ml tube, with vigorous aeration, overnight at 37°C.

11. Elute the phage particles from the top agar by covering with 5 ml wash buffer and incubating overnight at 4°C without shaking.
12. Transfer ~500 µl phage suspension to a clean tube and centrifuge 10 min at 16,100 × g (13,200 rpm) to pellet the bacteria. Transfer the supernatant to a clean tube.

If desired, the phage can be concentrated by precipitation. Add 400 µl of 20% (w/v) PEG/2.5 M NaCl to 1.6 ml of the cleared supernatant. Keep on ice for at least 15 min to allow precipitation of phage particles, centrifuge 5 min as above, and resuspend the pellet in 200 µl of wash buffer.

Repeat affinity selection process

13. Perform two additional rounds of screening by repeating steps 1 to 12 two times using the amplified phage supernatant from step 12. As in the first round, set up triplicate wells for selection (step 1) and pool the recovered phage before amplification (step 8).

The phage mixtures typically have titers of 10¹¹ plaque-forming units per milliliter (pfu/ml) after each round of selection and amplification.

14. After the third round of selection, make several serial dilutions at 1:100 in PBS. Then set up several sterile 5-ml tubes for infection (one for each dilution). To each tube add:

200 µl overnight culture of DH5αF' bacteria
1 to 10 µl diluted phage particles
3 ml 2× YT top agar.

15. Plate out each dilution on 2× YT bottom agar and incubate overnight at 37°C.
16. With sterile toothpicks, transfer phage from 20 well-isolated plaques to separate 5-ml sterile tubes containing 50 µl overnight culture of DH5αF' bacteria and 1 ml of 2× YT culture medium. Grow overnight at 37°C with vigorous aeration.

Repeat steps 14 and 15 at different dilutions if there are not twenty well-isolated plaques on one or more of the plates.

Perform ELISA

17. Coat one column of wells in a 96-well microtiter plate with the target protein and another column with a negative control protein (i.e., fusion partner), as described in steps 1 through 4. Set up enough column pairs to test the desired number of clones from step 16.
18. To each pair of wells (target and control), add 100 µl wash buffer and 25 µl of a culture supernatant prepared from a single bacteriophage clone (step 16). Incubate for 2 hr at room temperature.
19. Wash the wells as described in steps 6 and 7.
20. To each well, add 100 µl of anti-phage monoclonal antibody (HRP-conjugated) diluted 1:5000 in wash buffer. Incubate for 1 hr at room temperature.
21. Wash the wells as described in steps 6 and 7.
22. To each well, add 100 µl chromogenic substrate. Incubate ~30 min.

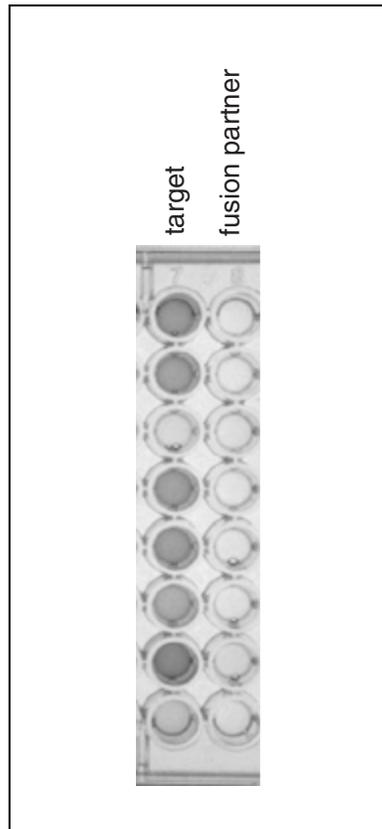


Figure 17.4.1
 Photograph of a phage ELISA result. Using eight different clones that were isolated with GST-SrcSH3 domain as the target, 25 μ l of culture supernatant were added to pairs of microtiter plate wells containing GST-SrcSH3 or GST. Retention of the phage in the wells was detected with anti-phage antibodies (coupled to HRP) through a colorimetric assay. Six of the clones were verified to bind the SrcSH3 domain specifically.

23. Measure the optical absorbance of the wells at 405 nm with a microtiter plate spectrophotometer.

Wells with bound phage yield optical density values of >0.6 , while wells without bound phage generate optical density values of ≤ 0.2 . See Figure 17.4.1 for typical results.

Typically, many of the output phage from the third round of selection bind to the target protein under these conditions. If not, one can perform an additional round of selection, perform filter lifts to identify binding clones in the output of the third round of selection (Sparks et al., 1996), or repeat the selection experiment with an alternative method of immobilizing the target (for example, see Alternate Protocol).

Characterize sequence of interacting phage

24. Purify and sequence the DNA inserts of binding phage, using either double-stranded DNA (prepared from the replicative form of DNA) or single-stranded DNA (prepared from secreted virus particles).

Purification kits for either application are commercially available (e.g., from Qiagen). Also see APPENDIX 3A for purification and sequencing methods.

Suitable sequencing primers upstream and downstream of the gene III cloning site are 5'-ATT CAC CTC GAA AGC AAG CTG-3' and 5'-CTC ATA GTT AGC GTA ACG-3', respectively. Suitable sequencing primers upstream and downstream of the gene VIII cloning site are 5'-GTT CCG ATG CTG TCT TTC GCT-3' and 5'-AAC CGA TAT ATT CGG TCG CTG AGG-3', respectively.

The authors often sequence from ten to twenty clones. If one sequence predominates, a heteroduplex mobility assay can be used to identify nonsibling clones for sequencing (Fack et al., 2000).

25. Convert the coding sequence to peptide sequence.

In the NNK coding scheme, there are 32 codons that encode one stop codon (TAG), which is suppressed in DH5 α F' with a glutamine, and all 20 amino acids, which are represented one (C, D, E, F, H, I, K, M, N, Q, W, Y), two (A, G, P, V, T), or three times (L, R, S).

AFFINITY SELECTION USING FUSION PROTEIN TARGETS ON BEADS

ALTERNATE PROTOCOL

If a fusion protein is used as a target, phage can be affinity selected with the target protein attached to a resin. In the authors' experience, glutathione-*S*-transferase (GST) fusion proteins can successfully select phage when bound to glutathione-Sepharose resin, which is commercially available. To avoid carry over of contaminant proteins that are bound nonspecifically to the resin, it is best to elute the fusion protein off the column with glutathione, dialyze the sample, and adsorb it to a fresh batch of glutathione-Sepharose resin. Other types of tags (i.e., maltose-binding protein or six-histidine tags) can be used to link the target protein to an affinity matrix; however, the corresponding affinity resin tends to have a background of nonspecifically adsorbed phage that is five to ten times higher than that obtained with glutathione-Sepharose. In addition, metal-chelating resins used for immobilized metal affinity chromatography tend to select for peptides rich in histidines from the phage-displayed combinatorial peptide libraries.

Additional Materials (also see *Basic Protocol*)

50% (w/v) slurry of glutathione-Sepharose resin 4B (Amersham Pharmacia Biotech, Sigma) in 0.5 M NaCl/20% (v/v) ethanol (capacity 5 mg bound protein/ml resin)

PBS (see recipe) with 0%, 1%, and 3% (w/v) BSA

Glutathione-*S*-transferase (GST) fusion protein of interest (target protein)

TBS (optional): 50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*), with 150 mM NaCl

Bind fusion protein target to resin

1. Microcentrifuge ~40 μ l of a 50% glutathione-Sepharose resin 4B slurry for 1 min at 800 \times g, room temperature. Remove the supernatant.
2. Wash the pelleted resin by adding 200 μ l PBS, microcentrifuging as in step 1, and removing the supernatant.
3. Resuspend beads in 200 μ l PBS, add 10 μ g GST fusion protein ($\sim 10^{14}$ molecules), and allow it to bind for 1 hr at 4°C with gentle mixing.
4. Wash the beads three times with 200 μ l ice-cold wash buffer (see step 2).
5. Resuspend the beads in 300 μ l PBS containing 3% BSA. Mix gently for at least 4 hr at 4°C to saturate the nonspecific binding sites on the resin.

Usually, it is convenient to prepare enough target-bound resin for the entire experiment (i.e., through the third round of selection in step 13). Prepared resin should be kept in PBS with 3% (w/v) BSA for no longer than 1 week at 4°C.

Bind phage to resin

6. Microcentrifuge the beads as above and add 100 μ l PBS (or TBS) containing 1% BSA and 10^{10} pfu of phage library.

This amount of phage particles represents 20 library equivalents of a library with a complexity of 5×10^8 clones.

7. Incubate for at least 1 hr at 4°C while mixing gently.

For convenience, overnight is also acceptable.

8. Centrifuge the beads as above and discard the supernatant.

9. Wash the beads five times with 1 ml wash buffer. While the resin beads are in the final wash, transfer the suspension to a fresh tube, centrifuge the beads, and remove the wash buffer.

The beads are transferred to a fresh tube to avoid carry-over of phage bound to the surface of the plastic tube.

Recover bound phage

10. Elute the bound phage by adding 200 μ l acid elution buffer. Let sit for 10 min at room temperature.
11. Microcentrifuge beads 30 sec at $800 \times g$ (2500 rpm), transfer the supernatant to a clean tube, and neutralize the solution by adding 50 μ l neutralization buffer.
12. Keep 10 μ l eluted phage in a sealed tube in the refrigerator (at 4°C) as a stock of affinity-selected phage. Amplify the titer of the remaining 100 μ l of bound phage as described above (see Basic Protocol, steps 9 to 12).

If necessary, the phage supernatant can be kept at 4°C with 0.02% (w/v) NaN_3 . Chloroform should not be used as this type of phage is sensitive to chloroform.

Repeat affinity selection

13. Perform two additional rounds of selection by repeating steps 6 to 12. Use 100 μ l ($\sim 10^{10}$ pfu) for the second round of selection. Add to a fresh 40- μ l aliquot of glutathione-Sepharose beads with the GST fusion protein bound to it.

To improve the isolation of high-affinity peptide ligands, the target protein can be adsorbed to the affinity resin at a much lower concentration (2 μ g total protein) and up to ten washes can be used.

This titer (10^{11} pfu/ml) is obtained consistently. The titer should only need to be checked when looking for the cause of a failure.

Characterize phage isolates

14. Perform a phage ELISA and sequence inserts of interest as described above (see Basic Protocol, steps 17 to 25).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Chromogenic substrate

Dissolve 220 mg of 2',2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) in 50 mM citric acid (10.5 g citrate monohydrate in 1 liter of sterile water), and adjust the pH to 4 with ~ 6 ml of 10 M NaOH. Filter sterilize the solution and store at 4°C (stable for at least 6 months). Immediately before use, add 30% H_2O_2 to a final concentration of 0.05%.

Phosphate-buffered saline (PBS), 10 \times

80 g NaCl
2.2 g KCl
9.9 g Na_2HPO_4
2.0 g K_2HPO_4
Add H_2O to 1 liter
Store up to 2 weeks at room temperature
Dilute to 1 \times before use

YT culture medium and top and bottom agar, 2×

Make up 2× YT medium:

10 g tryptone

10 g yeast extract

5 g NaCl

Bring to 1 liter with H₂O

Autoclave

Store up to 2 weeks at 4°C

For bottom agar: Before autoclaving, add Bacto-agar to a final concentration of 1.5% (w/v)

For top agar: Before autoclaving, add Bacto-agar to a final concentration of 0.8% (w/v)

COMMENTARY

Background Information

In phage display, a protein or peptide library is encoded within a phage genome and is expressed on the surface of the phage as a fusion with the phage coat protein. Phage may then be selected on the basis of the functionality of their surface proteins. This method allows one to screen large numbers of sequences based on protein function. In this unit, phage-displayed peptides are screened based on their ability to bind to a protein of interest (i.e., the target protein). Phage-display combinatorial peptide libraries are typically screened by affinity selection with a particular target protein. In many cases, it is possible to identify, from the affinity-selected peptides, members with a sequence that closely resembles segments (epitopes) of a natural interacting partner of the protein of interest. A practical consequence of the phenomenon known as convergent evolution (Kay et al., 2000) is that one can search whole genome databases for proteins containing segments that match consensus sequences shared by the selected peptides, and then determine experimentally whether or not the identified proteins interact with the target (Smothers and Henikoff, 2001). For example, combinatorial peptide libraries have proven useful in defining the optimal ligand preferences of protein interaction modules, such as EH (Salcini et al., 1997), PDZ (Songyang et al., 1997), SH2 (Songyang et al., 1993), SH3 (Tong et al., 2002), and WW domains (Linn et al., 1997), the heterodimeric G protein $\beta\gamma$ subunit (Scott et al., 2001), the catalytic subunit of protein phosphatase 1 (PP1c; Zhao and Lee, 1997), the estrogen receptor (Paige et al., 1999), and the ubiquitin ligase DM2 (Kay et al., 1998). Thus, a fruitful means of mapping protein-protein interactions is to isolate peptide ligands to various prokaryotic proteins and identify candidate

interacting proteins in the sequenced genome by computer analysis.

Critical Parameters

The most critical aspect of a successful experiment is to ensure that the protein targets retain their ability to interact with other proteins when immobilized (Stevens, 2001). In the Basic Protocol, the authors recommend immobilizing the protein target on microtiter plate wells. However, because the protein sometimes denatures when affixed to the plastic surface and fails to yield any target-binding phage, one frequently isolates plastic-binding phage instead (Adey et al., 1995). Thus, if the necessary reagents are available, the investigator should always confirm that the immobilized target protein retains its ability to interact with other protein. On the other hand, if the protein target is a GST fusion protein, the Alternate Protocol is recommended.

Troubleshooting

The authors recommend the following targets (with their motif among selected peptides) that are commercially available and can serve as positive controls: FLAG mAb M2 (DYKXXD), streptavidin [HP(Q/M)], Src SH3 domain (R_xLP_xLP or P_{xx}P_xR), and troponin C [(V/L)(D/E)_xLK_{xx}L_{xx}LA]. If the suggested positive control targets do not yield binding phage, the titer of selected and amplified phage should be checked. Titers of 10¹¹ pfu/ml are expected after amplification. The pH of elution and neutralization solutions should also be checked, as phage titers drop at low pH.

Anticipated Results

The authors have successfully isolated peptide ligands to ~50% of the target proteins tested. The K_d values of peptides recovered by

phage display, when chemically synthesized and tested in solution, range from 10 μ M to 500 nM (Grøn and Hyde-DeRuyscher, 2000; Hyde-DeRuyscher et al., 2000), and the peptides bind as surrogates (Pillutla et al., 2001) of interacting protein partners (i.e., the peptides compete off the interacting partner).

By inspecting the sequences of the selected peptides, one often finds a consensus or motif with which one can search sequence databases for potentially interacting proteins. Two Web sites that are useful for searching proteomes with short peptide motifs are <http://www.proteome.com/databases/> and http://www.arraygenetics.com/about_seqit.php3. Of course, given the large size of GenBank, a large number of computer matches will generally be found; thus, one must use some judgment in sifting through the best matches to choose the ones that might make biological sense. Such predictions can first be tested by chemically synthesizing peptide segments (i.e., 12-mers) corresponding to the matching segments and testing whether or not they bind *in vitro*. If antibodies are available to the candidate interacting proteins, positive results can then be followed by co-immunoprecipitation experiments. Thus, much like yeast two-hybrid screening (Fields and Song, 1989; Phizicky and Fields, 1995; *UNIT 12.1*), phage-displayed combinatorial peptide libraries can help in mapping protein-protein interactions (Tong et al., 2002).

Time Considerations

With a single target, it takes 1 to 2 weeks to complete three rounds of selection with a phage-displayed combinatorial peptide library. With a modest investment of time and effort, one can apply these relatively straightforward protocols toward isolating peptide ligands for many different targets. Much more time is spent in analyzing the selected peptides, evaluating their possible biological significance, and testing the hypotheses generated.

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Protein-Protein Interactions Identified by Pull-Down Experiments and Mass Spectrometry

The discovery of new interacting partners for a protein of interest can provide significant clues to its functional role. Identification of a binding protein with a defined cellular role can often present immediate new research directions. Several techniques have been developed to identify protein-protein interactions. Generally, these use a “bait” protein of interest to search a pool of cellular proteins for an interacting partner, coupled with some form of detection mechanism. One commonly used technique is the yeast two-hybrid system (UNIT 17.3), which utilizes transcriptional activation of a reporter gene in yeast as a readout of protein-protein interaction.

This unit describes the use of glutathione *S*-transferase (GST) fusion protein “pull-down” approaches to isolate interacting proteins and to then identify them by SDS-PAGE and mass spectrometry (MS). For more background on the use of GST fusion proteins and the pull-down technique, see Commentary. Protein-protein interaction partners are identified in a way that involves (and is dependent on) direct protein-protein interaction. The detection mechanism is protein staining of SDS-PAGE gels and is therefore not dependent on any secondary process, such as a cellular event or activation of enzyme activity. MS techniques for protein identification enable sensitive identification of proteins by comparison of spectral data obtained from tryptic protein digests of an unknown protein with theoretical *in silico* digests of entries within a DNA or protein sequence database.

The methods in this unit can be performed in a number of ways, and the advantages and disadvantages of some of these variations are presented in Strategic Planning, along with the options that are available within the protocols themselves. Some attention is given to long-term planning, after the pull-down experiments, to confirm and further study the protein-protein interactions identified. The first protocol presented (see Basic Protocol 1) details the use of GST fusion proteins coupled to glutathione (GSH)-agarose beads (as a support matrix) for affinity purification of interacting proteins from a tissue or cell lysate. The method involves the binding of GST fusion protein-coupled beads to proteins in a tissue lysate, recovery of the beads, washing away nonspecific proteins, elution of the bound proteins, and analysis by SDS-PAGE. The protocol details the application of spin columns to improve assay speed and reproducibility. A separate protocol (see Basic Protocol 2) describes MS-compatible methods of gel staining after SDS-PAGE, and sample preparation for analysis by MS. Methods of MS analysis are beyond the scope of this unit and are not described here (see Chapter 16 in Coligan et al., 2003, for an in-depth treatment of mass spectrometry; also see UNIT 5.6 in this manual). However, the samples are prepared to a stage that is suitable for shipping to an in-house or commercial MS service for analysis. One specific application of the pull-down approach (described in the Alternate Protocol) is for the identification of nucleotide-dependent binding partners of small GTPases, critical regulators of cellular signaling and function.

Three support protocols are described that detail preparatory procedures that are necessary before performing the pull-down experiment. Support Protocol 1 describes a method for preparation of GST fusion protein-coupled beads and Support Protocol 2 provides detail on cross-linking procedures to covalently couple the GST fusion protein to the GSH beads. Procedures for large-scale tissue lysate production are provided in Support Protocol 3, with a focus on sheep brain as a large-scale tissue source. Support Protocol 4 describes recycling procedures for the reuse of the large quantities of GSH-agarose beads that are necessary for the pre-clearing steps in tissue lysate preparation.

STRATEGIC PLANNING

This unit comprises several component protocols, some of which have alternative methods. This Strategic Planning section describes the planning steps required to link these together into a cohesive approach for performing pull-down experiments combined with mass spectrometry. A flow chart of the strategic planning and design stages involved, and how these link to the experimental steps, is shown in Figure 17.5.1. The left-hand column (design and analysis) is a representation of this Strategic Planning section; how it is linked to the experimental protocols is illustrated on the right. Some initial consideration must be given to the approach to be taken, based on the protein of interest, its cellular function, enzymatic or other activity, domain structure, and the properties to be investigated.

Achieving Specificity

One of the first considerations should be the question of how specificity can be achieved in the pull-down experiment. The conditions to be compared within the experiment should be carefully considered, as vector construction and mutation may be required well in advance of the experiment. Optimally, a pull-down experiment should encompass the target fusion protein as well as a set of truncations, mutations, deletions, or even a set of related proteins from other genes. For example, such an approach for a new SH3 domain might be to use five different SH3 domains (including the new one) to isolate binding proteins for each and determine which are unique or common. This may reveal which binding proteins are specific to the SH3 domain of interest. Another example might be the use of a set of characterized mutations from a particular disease to analyze differential protein-protein interactions in various pathological states. Another approach might use just one protein but in a number of different activation states. This produces internal controls for specificity and enables a more confident assessment of the significance of the results obtained. An example of the latter approach might be a phosphorylated protein with its phosphorylation site serine or threonine residue mutated to alanine (incapable of being phosphorylated) and/or glutamic acid (pseudophosphorylation). All three would be analyzed simultaneously to isolate potential phosphorylation-dependent binding proteins. Another example might be a GST-tagged small GTPase bound to GDP or GTP (as described in the Alternate Protocol) to isolate activation state-dependent binding proteins (effectors). It is clear from these examples that the choice of constructs required will be dependent on the protein of interest and its properties to be investigated in the pull-down experiment. Using truncations may have the extra advantage of narrowing down the protein-protein interaction site on the protein of interest and simultaneously increasing the specificity in identifying interacting proteins.

Appropriate external controls also need to be established for the constructs that will be used in the pull-down experiment. These will be analyzed by SDS-PAGE in parallel with the eluted proteins from the pull-down. The minimum set of controls includes a separate set of beads containing recombinant GST, as well as a pull-down with GSH beads alone (both to be used concurrently in the pull-down with lysate). The GSH bead pull-down control can be obtained by taking an aliquot of beads from those used to pre-clear the tissue lysates in Support Protocol 3 and Basic Protocol 1. Additionally, it may be necessary to concurrently analyze GST fusion protein beads that have not been combined with lysate—this is an essential control when the GST fusion protein beads have not been cross-linked.

Design of Expression Constructs

It is worth considering how recombinant fusion proteins will be attached to the beads, especially when designing the expression vectors from scratch. The main consideration is how the protein of interest will be fused to the tag. First, it needs to be determined if it will be an amino- or a carboxy-terminal tag. This choice will depend on where the functional regions of the protein of interest are located within the protein, as tagging close

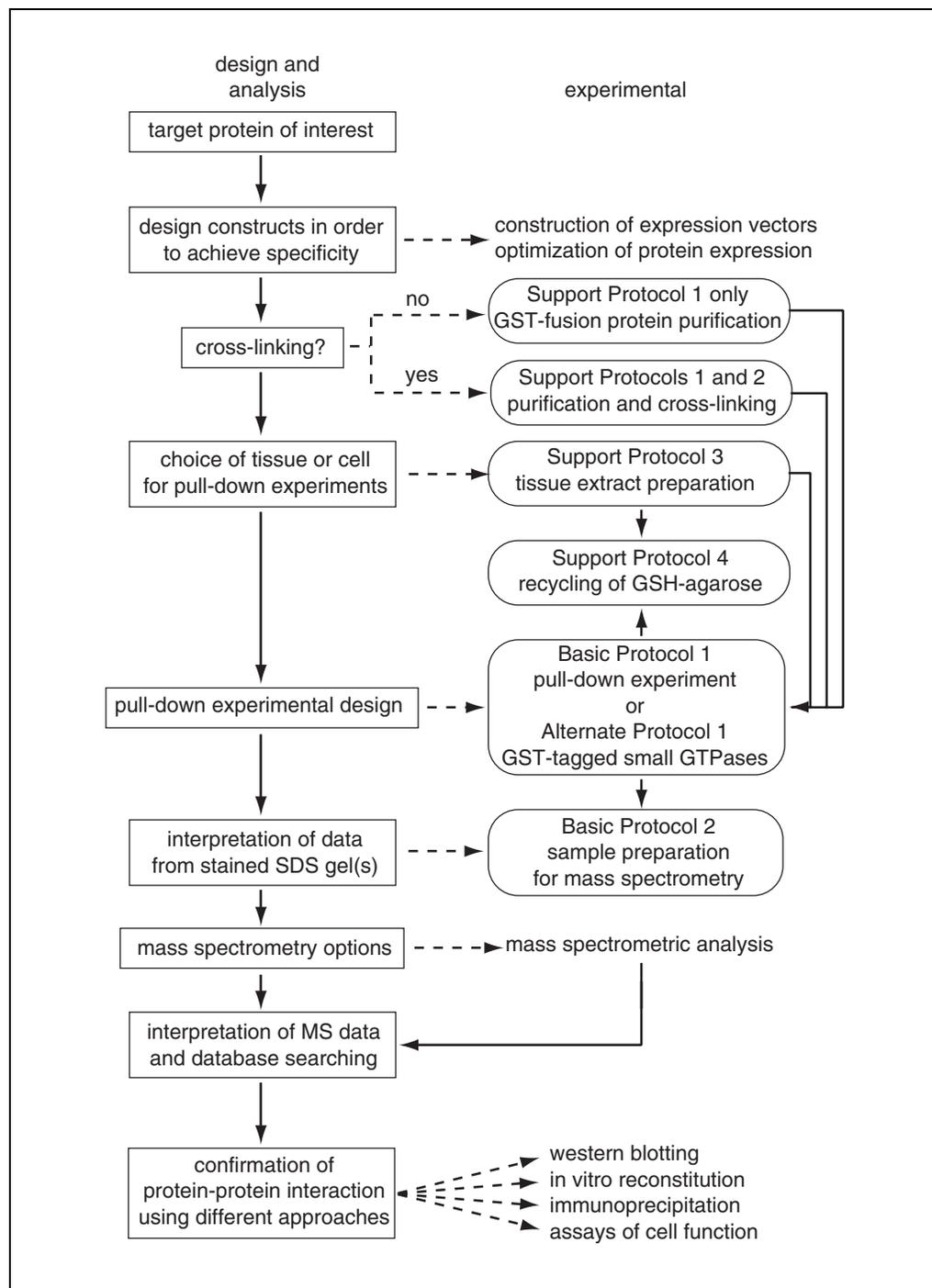


Figure 17.5.1 Flowchart for strategic design and the experimental processes described in this unit, showing how the planning and analysis stages (left-hand side; a summary of the Strategic Planning section) are linked with the experimental protocols (right-hand side). It is also a reference for how the protocols relate to one another.

to such regions could potentially interfere with the protein's function and thus with binding sites for other proteins. Secondly, the linker region between the fusion tag and the protein of interest may be significant. If this region is extensive or hydrophobic, the expressed protein could be insoluble (leading to poor yields in purification), and it may bind many proteins nonspecifically in pull-down experiments.

While this protocol is mainly concerned with GST fusion proteins, alternative tags can be used since the concepts are essentially the same. Alternatives include maltose binding protein (MBP) tags; purification of recombinant protein by whatever means, followed by coupling to a reactive matrix such as Affi-gel (Bio-Rad), may also be used. Some fusion protein tags are particularly unsuitable for use in pull-down experiments, particularly the six-histidine tag that is commonly used for recombinant protein purification as metal-affinity beads are known to bind many proteins nonspecifically. Whether or not cross-linking will be necessary needs to be considered, as recombinant proteins cannot be cross-linked to maltose or metal-affinity columns with DSS (as in Support Protocol 2) because both types of beads lack primary amine groups.

Cross-Linking

Next, consider whether to cross-link the protein of interest to the bead support. GST fusion proteins can be covalently attached to immobilized GSH-agarose beads using cross-linking reagents such as DSS (Support Protocol 2). Cross-linking a fusion protein of interest to the bead support is not essential, and significant results can be achieved without it. The advantage of coupling to the beads is the ability to elute only the interaction partners, leaving the GST fusion protein "bait" covalently attached to the bead. Thus, it will not be present in the subsequent extract or SDS acrylamide gel. The absence of GST fusion protein in the gel can be significant, because the presence of large amounts of fusion protein can make identification of specific binding proteins difficult, simply because of the number and size of the GST fusion protein-related bands that are present on the gel. This problem gets worse as the molecular weight of the fusion protein increases because many of the fusion protein-related bands migrate at smaller molecular weights than the fusion protein itself. Additionally, the presence of large quantities of GST fusion protein bands on the gel can sometimes seriously interfere with the identification of other proteins in the sample by MS peptide mass mapping (see below)—recombinant GST may seem to be present in every band, regardless of molecular weight, making identification of other proteins very difficult. Presumably, the large amount of fusion protein used does not resolve fully, and while the majority of the protein may be present as indicated by protein stain at the appropriate molecular weight, the traces that are present throughout the lane interfere with peptide mass mapping techniques. Other MS techniques of protein identification (e.g., peptide sequencing) may not be as susceptible to interference by the presence of GST fusion protein contamination in protein bands (see below). Non-MS techniques, such as Edman degradation, will also be relatively immune to this effect. While GST fusion protein contamination is common, it often proves to be inconsequential to achieving results, even from peptide mass mapping techniques like MALDI-TOF MS (see Anticipated Results and Brymora et al., 2001b).

While there are distinct advantages to using cross-linking in GST fusion protein pull-down experiments, there are also some caveats to consider. Loss of some or all biological activity of specific proteins (particularly enzymes) may occur as a result of the chemical modification that occurs during cross-linking. This loss of activity may be the consequence of a conformational change in the protein when primary amine groups (i.e., lysine residues) on the surface of the molecule react with an amine-directed cross-linking reagent (such as DSS). Alternatively, loss of activity may occur when specific lysine groups involved in binding a protein interaction partner or substrate (in the case of an enzyme) are modified

by the cross-linker. Therefore, it may be necessary to fully evaluate the influence of cross-linking reagents on any known functions of a protein of interest before using them in the pull-down approaches described herein. Alternatively, a pull-down experiment may be performed both with and without cross-linking of the GST fusion protein beads, particularly if the protein of interest has no known functions that can be tested outside of the pull-down. It may thus be possible to establish if cross-linking has any adverse influence on the protein-binding characteristics of a target protein.

Considerations in Tissue Source for Interacting Proteins

The source of protein for isolation of interacting proteins is often an obvious choice, decided on the basis of the investigator's research interests, but several factors should be kept in mind. Concerning mammalian systems, the tissue where the target protein is predominantly expressed can be an ideal starting point; presumably, if the protein is expressed there, then the interacting partner proteins must also be present. Also, a tissue could be chosen where the target protein is known to have a specific function, with the aim of determining the mechanism of how the protein carries out that function. However, the concentration of interacting partners may be higher in a tissue other than the tissue of interest (thus enabling easier isolation and identification) and yet may still represent a protein-protein interaction that is valid within the tissue of interest. In this way, it may initially be a useful approach to use a panel of different tissue sources (e.g., brain, lung, liver, kidney, and muscle) and establish which interacting proteins are unique to a particular tissue and which are common to all, and in which tissue they are present in the greatest abundance.

Likewise, it is worth considering a rational approach to how a lysate is to be prepared for use in pull-down approaches. This unit provides options for the preparation of whole-tissue lysate, as well as cytosolic, peripheral membrane, and membrane protein extracts (see Support Protocol 3). The whole tissue lysate, while having a higher concentration of potential interaction partners (and therefore being more likely to isolate them), is also more prone to higher levels of nonspecific protein binding. Fractionating proteins into the three described subcellular compartments reduces nonspecific binding but risks dilution of a protein of interest between three times the volume of lysate. It is usually also necessary to analyze the three extracts at once, which may lead to experiments that are too large and impractical (as three times the number of columns would be required). It is, however, possible that extracting proteins specifically from one of the subcellular compartments will concentrate that protein in that fraction and provide a clearer result than the use of a whole-tissue lysate. Given all of these factors, it can be worth considering the initial use of a panel of different tissue extracts to determine which gives the clearest result for isolation of interacting proteins.

Another consideration in the choice of tissue source is the abundance of endogenous GST proteins that can interfere with interpretation of results from pull-down experiments. After purification of recombinant GST fusion protein beads, the GSH beads still have free binding sites for other GSTs, and these can be specifically isolated on GSH beads if they are not fully removed from a tissue lysate. Some lysates contain high levels of endogenous GSTs, particularly cytosolic extracts and lysates from testis and liver. Further pre-clearing may be required for these lysates (see Critical Parameters and Troubleshooting).

The use of cultured cells as a source of interacting proteins has less utility for the procedures described in this unit, due to the limited quantity (and relatively high cost) of protein lysate that can be obtained. However, cells can be particularly of use in situations where a nonmammalian system is used as a source of interacting proteins (e.g., yeast), or when studying particular mammalian cell types (as opposed to whole tissues), provided

that enough lysate can be obtained. Cultured cells (e.g., thirty 7.7-ml flasks) can also be useful for a single pull-down experiment, without stringent control experiments. Any binding proteins identified may then be specifically targeted for more stringent analysis in scale-up experiments from larger tissue lysates. In this case, limited or no subcellular fractionation of cultured cells is recommended. A total cell lysate (using Triton X-100) is used in order to maximize protein recovery. Cultured cells may also be useful for further characterization of a protein-protein interaction detected from a tissue lysate (see below).

The final consideration for mammalian protein studies relates to scaling up tissue lysate size by switching to a physically larger species (e.g., ovine or bovine). The protein products of the same gene usually function similarly within different species including binding to similar proteins. Mass spectrometric methods for protein identification do not have any major difficulty adapting between species, since the vast majority of a particular protein's sequence is often identical between species (Larsen and Roepstorff, 2000). Mass spectrometric methods which rely on peptide mass mapping (see below) are thus usually little affected (except perhaps for small proteins that digest to only a few peptides), since the majority of peptides in a proteolytic digest are of identical molecular weight between species. Methods that directly provide primary sequence data for digested peptides are similarly little affected since amino acid sequence data can be used directly in database searches across species.

Mass Spectrometry Techniques

Modern advances in mass spectrometry (MS) have led to an increasingly complex number of approaches and types of instrumentation that can be utilized for identification of a protein of interest. While the sample preparation techniques are common to all (see Basic Protocol 2), the methods of analysis and the style of data obtained can vary widely between different techniques. The different instruments can also vary considerably in sensitivity, availability, and price, and often the identification of isolated proteins must be achieved using whatever instrument is available to the researcher. Proteins are generally identified by either one of two methods: peptide mass mapping (also known as peptide mass fingerprinting) or peptide sequencing (Coligan et al., 2003, Chapter 16).

Almost all types of MS instrumentation are capable of generating data suitable for analysis by peptide mass mapping. Peptide mass mapping uses a computer program to analyze a peptide mass spectrum for peaks that fit suitability criteria (e.g., of resolution and intensity). The masses of these peaks are then compared against a theoretical proteolytic digest of every protein in a selected database to identify a statistically significant match within a specified mass tolerance range (e.g., ± 0.01 Da, or 50 ppm). This whole procedure can be completely automated by software, where the mass spectrum is the input and the protein identification (ranked by some kind of statistical score) is the output. The power of this approach is that it is very fast and can lead to a positive identification even in the face of changes in some peptides (e.g., post-translational modification or species differences). The main disadvantage of this technique is its dependence on the purity of the initial sample—if there is more than one protein in the gel piece being analyzed, it becomes more difficult to reliably match the data to one specific protein. While it is possible to identify one or more proteins out of such a peptide mixture, it is generally more difficult to achieve reliable results from such samples. Thus, gel resolution and dexterity in band cutting become critical to ensure that only a single protein is present in a gel piece (see Basic Protocol 2). The most suitable instrument for peptide mass mapping (despite the fact that most mass spectrometers can be used) is the matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS). This suitability arises from the simplicity, cost, and speed of the MALDI-TOF technique—it is possible to analyze (and thereby identify) hundreds of prepared protein samples each day

using this instrument (with varying success rates). Therefore, the MALDI-TOF mass spectrometer is usually the instrument of first choice for protein identification.

Peptide sequencing is a self-descriptive title that applies to the ability of some of the higher-end mass spectrometers to isolate a specific peptide from a spectrum and fragment it, yielding data that can be interpreted to give a primary amino acid sequence for that selected peptide (often called “tandem MS” or “MS/MS”). This peptide sequence, combined with the peptide mass, is then used to search against a protein sequence database, enabling identification of the protein. These instruments are available in many different combinations of components, providing versatile methods of sample delivery and analysis; one example is electrospray ionization tandem MS (ESI-MS/MS), for liquid samples, perhaps in combination with a liquid chromatography step. This technique can therefore identify a protein or proteins that are present in a sample, irrespective of whether the sample is contaminated with other proteins. It is a very powerful technique and probably the best suited for unequivocal identification of proteins. Generally speaking, however, the technique is slow (a maximum of around five samples per day) and is perhaps more suited to a secondary role as an adjunct to a high-throughput peptide mass mapping technique such as MALDI-TOF MS. Protocols for protein identification by this method, further background, and comparisons of MS techniques can be found in *UNIT 5.6* (also see Coligan et al., 2003).

If it is not possible to perform mass spectrometric analysis in the laboratory (due to lack of access or instrumentation), a commercial service can be used. Alternatively, more traditional protein sequencing techniques can also be used, but they are generally limited to samples of high abundance. Edman sequencing of tryptic peptides (described in Coligan et al., 2003) is one possibility. Sample preparation for this technique is identical to the approach presented in Basic Protocol 2. Sequencing results can be compared directly to a protein sequence database.

Confirmation of Protein-Protein Interactions

After the pull-down experiments have been concluded and the specific protein-protein interaction partners have been identified, it is important to verify the obtained results with other complimentary techniques. These are required to confirm that the discovered interaction is not an artifact of the experimental conditions and that the protein identification is accurate, as well as to determine whether the interaction is direct. The following are all approaches that may be indicated for confirmation of an interaction:

1. Immunoblots (*UNIT 6.2*) may be performed with specific antibodies raised against the new interacting partner to probe smaller-scale pull-down experiments (e.g., from cultured cell lysates) and thus confirm identification. Also, probing with antibodies against known interaction partners of the target protein (if these are available) can show that the system is working as it should.
2. The protein-protein interaction may be reconstituted *in vitro* with expressed recombinant protein, in pull-down experiments where only the two interacting proteins are used. These experiments demonstrate that the interaction is direct and does not involve some other factor that is present in the original tissue lysate. Thorough controls are necessary, as artifacts arising from nonspecific binding are more common in a reconstituted system using recombinant proteins than in one using proteins from a tissue or cell lysate. This is often due to recombinant protein not being correctly folded or post-translationally modified in the same manner as the tissue-derived protein.

3. Coimmunoprecipitation experiments may be performed to cosediment the two proteins complexed to one another from a cell or tissue lysate. This can also be done from cells that are overexpressing epitope-tagged versions of the two proteins (with different tags on each), enabling immunoprecipitation of the tag. This approach eliminates the need to raise or obtain immunoprecipitating antibodies, particularly if the proteins in question are poorly characterized.

4. Genetic approaches (e.g., reporter gene assays) can also be used, particularly in a nonmammalian system like yeast.

5. Overexpression of mutations or truncations in the protein of interest in cells that are deficient in the protein-protein interaction in question may be exploited in the hope of eliciting a change in cellular response through alterations in the activity of the interacting protein.

BASIC PROTOCOL 1

THE PULL-DOWN EXPERIMENT

The large-scale approach to pull-down experiments described in this protocol uses recombinant GST fusion protein coupled to GSH agarose beads, with or without cross-linking (purified as described in Support Protocols 1 and 2) and tissue lysates (prepared as described in Support Protocol 3). It utilizes small spin columns for some of the steps, as these improve the efficiency. Specifically, improved assay speed, protein recovery, reproducibility, and decreased elution volumes are achieved when compared to traditional microcentrifuge tube approaches using centrifugation and aspiration of supernatant (Brymora et al., 2001a). There are several sizes of spin columns available that are more or less suitable for this protocol, the best being the MicroSpin columns from Amersham Biosciences. The different types, as well as the advantages and disadvantages of each, are described in Table 17.5.1.

It is possible (and often preferable) to perform the entire pull-down experiment (binding, washing, and elution steps) completely within the spin column (a schematic representation of this is shown in Fig. 17.5.2). This has the advantage of reducing any bead loss caused by repeated transfers between tubes, but it is dependent on the volume of lysate that is intended (as the columns are generally quite small: 0.5- to 1.5-ml) and the probable abundance of any interaction partners in that volume. Alternatively, the binding steps can be performed in larger volumes of tissue lysates (e.g., a 50-ml tube per pull-down), and the beads can later be transferred carefully to the spin columns for washing and elution. This latter approach is described in detail in this protocol, since it is more likely to yield successful results when combined with mass spectrometry. However, for subsequent characterization of protein-protein interactions, particularly for analysis by immunoblotting, smaller-scale pull-down experiments using the former technique are often preferable.

How much of the beads and recombinant GST-protein should be used? It is necessary to have an excess of recombinant protein relative to anything that may be in the lysate, but not so much that it will distort everything in the final analysis on the SDS acrylamide gel (if cross-linking is not used). The optimal amount of GST fusion protein to use needs to be determined empirically for a particular experiment.

For GST fusion proteins not cross-linked to GSH beads, an approximate place to start is 5 μ g recombinant protein on beads per 1 g of tissue in a whole tissue lysate.

For GST fusion proteins cross-linked to GSH beads, use 10 to 50 μ g recombinant protein on beads per 1 g of tissue in a whole tissue lysate.

Protein-Protein Interactions Identified by Pull-Down Experiments

17.5.8

Table 17.5.1 Advantages and Disadvantages of Different Types of Spin Columns

Name	Supplier	Maximum vol. (ml)	Advantage (+), Disadvantage (-)
MicroSpin Columns	Amersham Biosciences	0.6	+Minimal dead volume under frit +Reuseable (wash out beads with water and soak in 70% ethanol until needed; centrifuge tubes dry)
Micro Bio-Spin Chromatography Columns	Bio-Rad Laboratories	1	+Larger volume in a small profile that fits most microcentrifuges –Large dead volume in hollow plug (Luer-lok fitting) –Small volumes of elution buffers may need to be manually removed from plug after heating. Minimized by rapidly removing plug (within a few seconds) after removal from water bath. –Single-use only
Bio-Spin Chromatography Columns	Bio-Rad Laboratories	1.5	+Large volumes can be added (e.g., lysates) –Large dead volume in hollow plug (Luer-lok fitting), but this can be alleviated with custom-made plugs that penetrate into Luer fitting –Need to confirm that centrifuge has sufficient clearance above rotor to house spin column when it is sitting in its collection tube –Single-use only

The overall bead volume does not matter greatly, but it needs to be as small as possible (20 to 100 μ l). A larger bead volume will wash less efficiently and will bind more nonspecific proteins from the tissue lysate. The beads sometimes have a very high concentration of bound recombinant GST fusion protein; in these cases, dilute the beads with bead storage buffer (see Support Protocol 1), so that the small volumes can be pipetted accurately. Swirl the bead slurry or vortex very gently between each pipetting step to ensure uniform suspension.

When using multiple GST fusion proteins on different beads, the final bead volume used for each pull-down must be normalized or equalized. For example, if using five different GST constructs with 25 μ g of each attached to varying amounts of beads (as is normally the case), it is necessary to dilute all the beads with GSH agarose so they all match the same volume. In other words, use the same bead volume as well as the same protein amount in each pull-down that is intended to be used on the same gel. This ensures that any nonspecifically binding proteins are relatively constant between the different conditions and are therefore not mistaken for specific binding proteins.

Materials

Tissue lysate (see Support Protocol 3)

Bead storage buffer: 20 mM Tris·Cl, pH 7.4 (APPENDIX 2A)/50% (v/v) glycerol

GSH-agarose beads (e.g., glutathione-Sepharose 4B; Amersham Biosciences)

Wash buffers 1 or 2, and 3 (see recipes)

1 \times SDS sample buffer (UNIT 6.1)

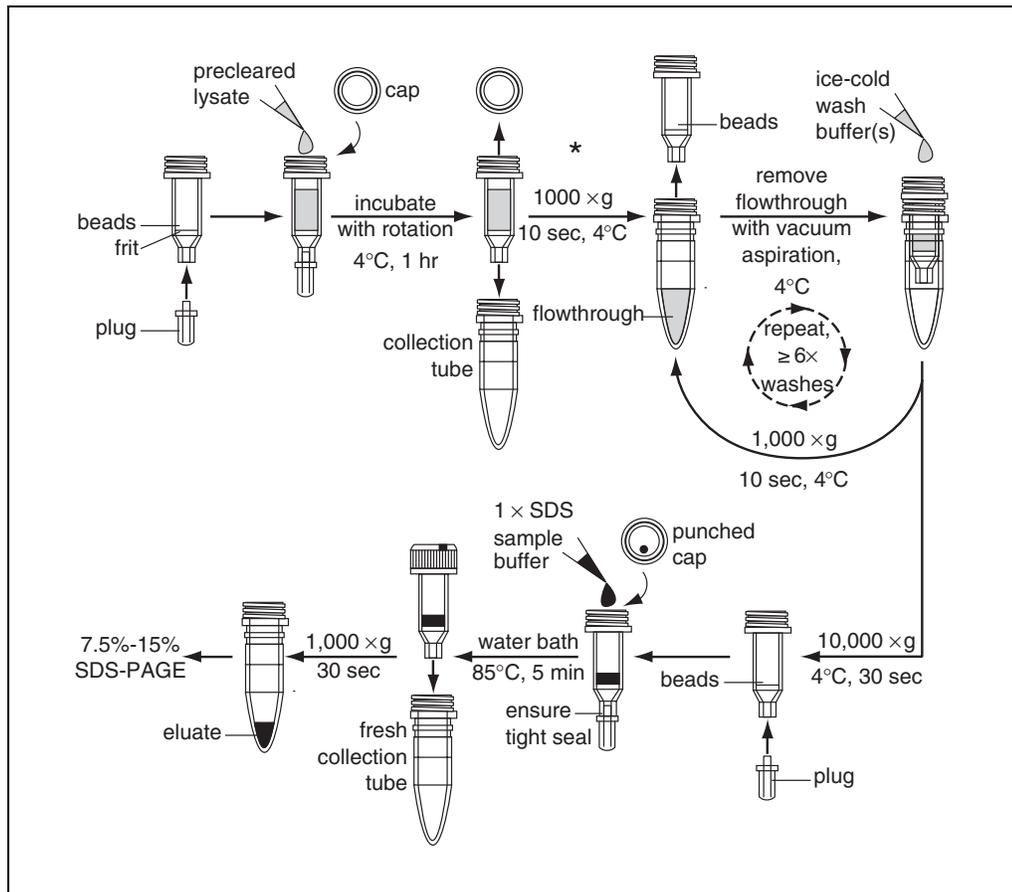


Figure 17.5.2 The spin column–based pull-down experiment. An illustrative description of the approach taken when an entire pull-down experiment is performed within the spin column. This differs from the protocol presented in Basic Protocol 1 and the Alternate Protocol, as it is designed for smaller-scale experiments. The large-scale experiments in the text intersect with this figure at the point marked with an asterisk (*), corresponding to step 12 in Basic Protocol 1 and the Alternate Protocol.

Benchtop centrifuge, 4°C

Appropriately sized tubes that can be centrifuged

End-over-end rotator

Empty plastic column (e.g., Poly-Prep from Bio-Rad; to suit lysate volume) *or* Miracloth (Calbiochem)

MicroSpin columns (Amersham Biosciences), or other spin columns (see Table 17.5.1)

85°C water bath

Additional reagents and equipment for preparing GST fusion proteins attached to GSH-agarose beads (see Support Protocol 1), cross-linking of fusion proteins (optional; see Support Protocol 2), preparing tissue lysate (see Support Protocol 3), and SDS-PAGE (UNIT 6.1)

Prepare GST fusion proteins and tissue lysates

1. Prepare recombinant GST fusion proteins attached to GSH-agarose beads (see Support Protocol 1). Cross-link if desired (see Strategic Planning and Support Protocol 2). Store at –20°C in bead storage buffer for up to 6 months.

2. Prepare a tissue lysate (see Support Protocol 3 or use other method).

Ensure that lysate is free from even small traces of particulate contamination. These will only block the columns later and result in large amounts of nonspecific protein on the gel. If present, it is very important to remove them by centrifugation at high speed (e.g., 18,000 × g) for 30 min, even if this has already been performed earlier.

Pre-clear lysates with GSH beads

If using tissue lysates prepared according to Support Protocol 3, the lysates have already been precleared with GSH-agarose: skip steps 3 to 8 and proceed starting with step 9. If using a different source of lysate, then continue with step 3.

3. Wash a batch of fresh or recycled GSH-agarose beads that have no recombinant protein attached as follows:

- a. For 2 g tissue use 100 μl of beads; for 200 g tissue use about 2 ml GSH beads.
- b. Add 1 ml (for 2 g tissue) or 20 ml (for 200 g tissue) of wash buffer (i.e., the base buffer that was used for tissue lysis in Support Protocol 3, or wash buffer 1) to the beads and centrifuge in a benchtop centrifuge 5 min at 500 × g, 4°C. Remove supernatant and collect the beads in a minimal volume of buffer.

Avoid centrifuging beads (until the elution step) much faster than 2000 × g, or the beads will be crushed.

4. Pre-clear the tissue lysate with the washed GSH beads for removal of endogenous GSTs present in the lysate and nonspecifically binding proteins, by combining the washed GSH beads (from step 3) with the tissue lysate and incubating at 4°C on an end-over-end rotator for 30 min.

This will remove most of the endogenous cellular GST. The addition of a large volume of GSH-agarose still does not fully remove all endogenous GST proteins from a tissue lysate (~80% are removed with each clearing step).

5. Sediment the beads by centrifuging the mix in a benchtop centrifuge, 5 min at 500 × g, 4°C.
6. Carefully pour off the supernatant into fresh, cooled tubes on ice. Keep the supernatant as “precleared tissue lysate” for addition to the GST fusion protein beads at step 10.

Optionally, keep an aliquot (of an equivalent bead volume to that of the recombinant protein beads to be added to the lysate) of these beads to run in parallel on the gel in the final analysis. The remaining bulk GSH beads can later be recycled (see Support Protocol 4).

7. Repeat this pre-clearing twice more, if required, to remove more endogenous tissue GST proteins.

This is especially important for tissue lysates that are rich in endogenous GSTs (particularly cytosolic extracts and lysates of specific tissues, e.g., testis or liver).

8. Pass the total precleared tissue lysate through an empty, clean plastic column—e.g., a Bio-Rad Poly-prep column of an appropriate size for the volume of lysate used: use a 10-ml column (10-cm length, 20-mm top diameter) for ≤100 ml lysate—or four layers of Miracloth.

This will remove traces of beads that may have been missed earlier but which cannot readily be seen. It is essential to prevent even minor contamination of individual samples with even a small number of beads (as they likely have bound large amounts of endogenous GSTs). A larger column (e.g., 40-ml) will be faster than a smaller column; with larger volumes of lysate, the Miracloth is preferable.

Bind tissue lysates to the GST fusion protein beads

9. Divide the precleared tissue lysate into equal portions, depending on the number of conditions to be examined.
10. Add from 0.5 to 50 ml of lysate to 10 to 200 μ l of GST fusion protein beads (from step 1) in appropriately sized tubes that can be later centrifuged. Cap the tubes and incubate 1 hr at 4°C on an end-over-end rotator (preferably in a cold room).

The beads should not be allowed to settle.

If concerned about adding any of the components of the bead storage buffer to the tissue lysates, then centrifuge the beads 15 to 20 sec at 1000 \times g, 4°C, carefully remove the supernatant, and resuspend them in the base buffer of the lysate before adding them to the precleared tissue lysate.

Recover and wash beads

11. Centrifuge the lysate and bead mixture 5 min at 500 \times g, 4°C, then carefully decant (or remove by vacuum aspiration) most (but not all) of the supernatant, leaving a minimal volume of lysate above the bead pellet, without disturbing the bead bed.

IMPORTANT NOTE: *Be extremely careful not to disturb the bed of beads. From here on, it is essential to recover as much of the beads as possible, minimizing accidental losses. It is important to inspect the lid and sides of the tube before centrifugation to ensure that the beads are within the liquid. Beads that are trapped on the walls or lid (not in the liquid) are not adequately pelleted by the low-speed centrifugation and will result in bead loss. Ensure that all these beads are collected by thorough washing in step 13 below.*

12. Transfer the beads plus remaining small volume of tissue lysate to a prechilled MicroSpin column and microcentrifuge the remaining tissue lysate into a collection tube for 10 sec at 1000 \times g, 4°C.

Try not to centrifuge beads so fast or so long that they totally dry out, as determined by visual inspection of the beads. The beads should be visible, but should not appear to be loose and crystalline.

Note that Figure 17.5.2 is marked with an asterisk () at the point equivalent to step 12, so the remaining steps can be followed diagrammatically.*

13. Remove the tissue lysate in the collection tube by vacuum aspiration, replace the MicroSpin column, and add 0.5 ml of ice-cold wash buffer 1 to the tube that had originally contained the lysate. Rinse the tube well to recover any traces of beads. Transfer this wash to the MicroSpin column.

If the lysate used above was a membrane or whole-tissue lysate, substitute wash buffer 2 for wash buffer 1 in this step and in step 15.

The aim is to recover any residual beads from the bottom and sides of the tube and pool them with the others that are now in the MicroSpin column. This eliminates variability between the various conditions. Visually inspect the initial tube for beads; if any are present, repeat this step.

14. Spin the wash into the collection tube by microcentrifuging for 10 sec at 1000 \times g, 4°C. Remove the flowthrough in the collection tube to waste by vacuum aspiration, then replace the column in the collection tube.

This step is faster if the collection tubes are left in the prechilled rotor.

15. Wash the beads (now containing the target binding proteins) six times, each time by adding 0.5 ml of ice-cold wash buffer 1 (or 2, see annotation to step 13), microcentrifuging 10 sec at 1000 \times g, 4°C, and removing flowthrough from the collection tube to waste by vacuum aspiration.

16. Wash three additional times with 0.5 ml of wash buffer 3, using the technique described in step 15.
17. For the very last wash, spin the beads dry by microcentrifuging 30 sec at $10,000 \times g$, 4°C .

Elute proteins from MicroSpin columns for SDS-PAGE analysis

18. Plug the bottom of the column thoroughly to ensure a tight seal. Add $1\times$ SDS sample buffer to the beads.

Use a volume of $1\times$ SDS sample buffer appropriate for the bead volume and analysis by SDS-PAGE, e.g., for 50 μl of bead volume use 50 to 80 μl of sample buffer. Try to keep the volume to a minimum. It is essential to dilute the SDS sample buffer to a final $1\times$ (i.e., 2% SDS) before use; do not use the concentrated SDS sample buffer found in most laboratories until it is diluted.

19. Cap the MicroSpin column with a punched cap and place in an 85°C water bath for 5 min.

A single hole in the cap, punched with a heavy-gauge needle, is essential to prevent pressure buildup during heating, which would cause the sample to leak out the bottom. Under no circumstances should the tube be heated at 100°C , or the white plastic frit may buckle, causing leakage of sample and beads. If using columns other than the MicroSpin, ensure in advance that they will tolerate heating at 85°C .

20. After heating, dry the outside of the column with a tissue, remove the bottom plug, and centrifuge the $1\times$ SDS sample buffer (now containing the proteins for analysis) into a fresh collection tube by microcentrifuging 30 sec at maximum speed. Store the samples on ice (if loading gels immediately) or in the freezer (if the gel will be run at a later date).

If small volumes of $1\times$ SDS (relative to the bead volume) are used, the elutions will likely lose some blue color. This is acceptable, as the beads retain bromophenol blue dye. Load gel using the normal technique (UNIT 6.1).

21. Analyze the samples by SDS-PAGE (UNIT 6.1) on a long gradient gel run overnight at 10°C .

For optimal protein resolution (essential for analysis by mass spectrometry) the gels must be long gels (e.g., 20-cm gels; preferably gradient gels of 7.5% to 15% acrylamide), not minigels. Additionally, the gels should be 1 mm in thickness for optimal strength and ease of processing for mass spectrometry. Gels of 1.5-mm thickness are too thick for in-gel digestion (see Basic Protocol 2), and 0.75-mm gels tear far too easily. The controls suggested above (see Strategic Planning) should be run on the same gel.

22. Stain the gel by an appropriate technique suitable for mass spectrometry (see Basic Protocol 2).

Analyze results

23. Compare bands between the lanes of the gel for differential binding between the conditions used and controls. Refer to Anticipated Results for more detail.

EFFECTOR ISOLATION WITH GST-TAGGED SMALL GTPASES

This protocol is specifically aimed at the study of nucleotide-dependent binding proteins (e.g., Ras, Rab, or Rho family GTPases). For other GST fusion proteins, such as isolated domains or enzymes, use Basic Protocol 1. Small GTPases bind guanine nucleotides (GTP and GDP) in a nucleotide-binding pocket on their surface. In so doing they undergo changes in conformation which allow them to bind to specific proteins in a GTP- or GDP-dependent manner (Takai et al., 2001). The GTP-bound form of the small GTPase is termed the active state, and GTP-dependent binding proteins are termed effectors since activation of many signaling pathways leads to increased GTP binding of the small GTPase, promoting further signaling. These proteins are particularly significant in cell biology investigations because of their role as regulators of a multitude of cellular processes. In humans there are around 150 small GTPases known to be expressed.

The main differences between this alternate protocol and Basic Protocol 1 are the presence of Mg^{2+} in the buffers (for maintenance of nucleotide binding and stability of the small GTPase) and the additional nucleotide-binding steps; there are also additional small changes in other steps. It is advisable to be familiar with the details of Basic Protocol 1 before following this protocol.

For small GTPases, it is most interesting to isolate specific binding proteins for each of the two states of activation, i.e., to load the GST–small GTPase columns respectively with GDP and GTP. Depending on the intrinsic GTP hydrolysis rate of the small GTPase to be used, it may be necessary to use a GTP analog such as GTP γ S [guanosine 5'-O-(3-thiotriphosphate)] instead of GTP. For instance, the small GTPase Rac1 has a GTP hydrolysis rate that is 40 times higher than H-Ras (Menard et al., 1992). The authors have used GTP quite successfully for the isolation of effectors for the small GTPase RalA (Brymora et al., 2001b). This is possible because RalA has a relatively low GTPase activity (Frech et al., 1990). It may be preferable to use GTP rather than costly GTP γ S for purely economical reasons, but it must be justified according to the criteria above.

Additional Materials (also see Basic Protocol 1)

- 1 M $MgCl_2$ (APPENDIX 2A)
- Bead storage buffer (20 mM Tris·Cl, pH 7.4 (APPENDIX 2A)/50% (v/v) glycerol containing 2.5 mM $MgCl_2$ (added from 1 M $MgCl_2$ stock; APPENDIX 2A)
- Small GTPase loading buffer (see recipe)
- 100 mM GDP and GTP (or GTP γ S) stock solutions (prepare fresh on the day of the experiment and keep cold):
 - 100 mM GDP (mol. wt. 463.2) = 1 mg in 21.6 μ l H_2O
 - 100 mM GTP (mol. wt. 567.1) = 1 mg in 17.6 μ l H_2O
 - 100 mM GTP γ S (mol. wt. 627.2) = 1 mg in 15.9 μ l H_2O
- Wash buffers 4 or 5, and 6 (see recipes)

Prepare GST fusion proteins and tissue lysate

1. Prepare recombinant GST-tagged small GTPase proteins attached to GSH-agarose (Support Protocols 1 and 2) with the following modifications:
 - a. Purify small GTPases with 1 to 5 mM Mg^{2+} present in all buffers (added from 1 M $MgCl_2$ stock), including those used in the cross-linking steps.
 - b. Omit EDTA from any buffers that come into contact with the small GTPase throughout the protocol (apart from the small GTPase loading buffer) and replace with 2.5 mM $MgCl_2$ (added from 1 M $MgCl_2$ stock).
 - c. Store at $-20^\circ C$ in bead storage buffer (including 2.5 mM $MgCl_2$ added from 1 M $MgCl_2$ stock) for up to 4 to 6 months.

2. Prepare tissue lysate (Support Protocol 3) with addition of 2.5 mM MgCl₂ (added from 1 M MgCl₂ stock) to all buffers. Pre-clear with GSH-agarose (Basic Protocol 1, steps 3 to 8), if necessary.

Ensure that the tissue lysate is free from even small traces of particulate contamination.

If using tissue lysates prepared according to Support Protocol 3, the lysates have already been pre-cleared with GSH-agarose; in this case proceed to nucleotide loading (step 3 of this protocol). If using a different source of lysate, the lysate must be pre-cleared with GSH-agarose beads.

Load GST fusion protein beads with the appropriate nucleotides

If the pre-clearing steps are being performed, the beads may be loaded with nucleotides while the pre-clearing beads are being incubated (see Basic Protocol 1, step 4). The following steps are based on the protocol used by Cantor et al. (1995), which works on the principle that, in the absence of Mg²⁺, the small GTPases bind nucleotides with low affinity and cannot hydrolyze GTP. Therefore, brief incubation in EDTA (present in the small GTPase loading buffer) promotes nucleotide exchange, and subsequent addition of an excess of Mg²⁺ loads the small GTPase with whichever guanine nucleotide is present. It is essential to load the GST–small GTPase beads with nucleotides as close as possible to the point at which they will be added to the tissue lysate.

3. Pipet the required amount of GST fusion protein beads (as a well suspended slurry) into two MicroSpin columns (or use a microcentrifuge tube if the binding steps are not going to be performed within the spin column): two equal lots for GDP versus GTP.

It is important to use precisely equal amounts of beads for the different nucleotide loading procedures.

4. Remove the storage buffer (i.e., remove most of the Mg²⁺) by microcentrifuging the MicroSpin column 10 sec at 1000 × g into a collection tube. Plug the base of the column.
5. Add an equal volume (or 50 μl, if the bed volume is <50 μl) of small GTPase loading buffer; ensure that there is an excess of EDTA present, so as to chelate all the Mg²⁺ in the protein beads (i.e., if not using MicroSpin columns, increase the added EDTA to 5 mM, for a final concentration of 2.5 mM).
6. Add the required nucleotide (GDP, GTP, or GTPγS) from a 100 mM stock, to a final concentration of 2 mM.

GTP stocks must be prepared fresh, as GTP hydrolyzes to GDP in solution.

7. Mix the GST fusion protein beads by gently flicking the tube (avoid vigorous vortexing and try to avoid spreading the beads on the sides of the tube), then incubate at 37°C for 20 min in a water bath to induce nucleotide binding and exchange.
8. Cool the tubes on ice. After ~1 min, add 1 M MgCl₂ to achieve a final concentration of 10 mM Mg²⁺ (1.2 μl for a 100-μl reaction), and gently mix.

The beads are now ready for pull-down experiments. They should be kept on ice and not left too long before use (not more than 30 min). The buffer that is currently in the beads (nucleotide and Mg²⁺) can remain.

Bind tissue extracts to the GST fusion protein

9. Divide the precleared tissue lysate into precisely equal portions, depending on the controls included (at minimum this is three aliquots: one for GST beads and one each for GDP and GTP). Add GDP or GTP to the appropriate aliquot, from a 100 mM stock, to achieve a final concentration of 0.1 mM.

There should already be Mg^{2+} present; if not, add a sufficient quantity of 1 M $MgCl_2$ to achieve 2.5 mM.

The addition of nucleotides to the precleared tissue lysate will reduce competition and exchange with the preloaded nucleotides on the GST-small GTPase beads to be added in the next step.

10. Directly add the nucleotide-loaded beads to the precleared tissue lysate, including the buffer in which they are contained (anywhere from 10 to 200 μ l of beads are added to 0.5 to 50 ml of lysate in suitably sized capped tubes). Incubate at 4°C for 1 hr on an end-over-end rotator (in a cold room).

Recover and wash beads

11. Centrifuge the lysate and bead mixture 5 min at $500 \times g$, 4°C, then carefully decant (or remove by vacuum aspiration) most (but not all) of the supernatant, leaving a minimal volume of lysate above the bead pellet, without disturbing the bead bed.

IMPORTANT NOTE: *Be extremely careful not to disturb the bed of beads. From here on, it is essential to recover as much of the beads as possible, minimizing accidental losses. It is important to inspect the lid and sides of the tube before centrifugation to ensure that the beads are within the liquid. Beads that are trapped on the walls or lid (not in the liquid) are not adequately pelleted by the low-speed centrifugation, and will result in bead loss. Ensure that all these beads are collected by thorough washing in step 13 below.*

12. Transfer the beads plus remaining small volume of tissue lysate to a prechilled MicroSpin column and microcentrifuge the remaining tissue lysate into a collection tube for 10 sec at $1000 \times g$, 4°C.

Try not to centrifuge the beads so fast or so long that they totally dry out, as determined by visual inspection of the beads. The beads should be visible but should not appear to be loose and crystalline.

Note that Figure 17.5.2 is marked with an asterisk () at the point equivalent to step 12, so the remaining steps can be followed diagrammatically.*

13. Remove the tissue lysate in the collection tube by vacuum aspiration, replace the MicroSpin column, and add 0.5 ml ice-cold wash buffer 4, to the tube that had originally contained the lysate. Rinse the tube well to recover any traces of beads. Transfer this volume to the MicroSpin column.

If membrane or whole tissue lysates were used above, substitute wash buffer 5 in this step and step 15.

The aim is to recover any residual beads and pool them with the others that are now in the MicroSpin column. This eliminates variability between the samples (particularly between GDP- and GTP-loaded samples). Visually inspect the initial tube for beads; if any are present, repeat this step.

14. Spin the wash into the collection tube by microcentrifuging for 10 sec at $1000 \times g$, 4°C. Remove the flowthrough in the collection tube to waste by vacuum aspiration, then replace the column in the collection tube.

This step is faster if the collection tubes are left in the prechilled rotor.

15. Wash the beads (now containing the target binding proteins) six times, each time by adding 0.5 ml of ice-cold wash buffer 4 (or 5, see annotation to step 13), microcentrifuging 10 sec at $1,000 \times g$, 4°C , and removing the flowthrough from the collection tube to waste by vacuum aspiration.
16. Wash three additional times with 0.5 ml of ice-cold wash buffer 6, using the technique described in step 15.
17. For the very last wash, spin the beads dry by microcentrifuging 30 sec at $10,000 \times g$, 4°C .

Elute proteins from MicroSpin columns for SDS-PAGE analysis

18. Plug the bottom of the column thoroughly to ensure a tight seal. Add $1 \times$ SDS sample buffer to the beads.

Use a volume of $1 \times$ SDS sample buffer appropriate for the bead volume and analysis by SDS-PAGE, e.g., for 50 μl of bead volume use 50 to 80 μl of sample buffer. Try to keep the volume to a minimum. It is essential to dilute the SDS sample buffer to a final $1 \times$ (i.e., 2% SDS) before use; do not use the concentrated SDS sample buffer found in most laboratories until it is diluted.

19. Cap the MicroSpin column with a punched cap and place in an 85°C water bath for 5 min.

A single hole in the cap, punched with a heavy-gauge needle, is essential to prevent pressure buildup during heating, which would cause the sample to leak out the bottom. Under no circumstances should the tube be heated at 100°C , or the white plastic frit may buckle, causing leakage of sample and beads. If using columns other than the MicroSpin, ensure in advance that they will tolerate heating at 85°C .

20. After heating, dry the outside of the columns with a tissue, remove the bottom plug, and centrifuge the $1 \times$ SDS sample buffer (now containing the proteins for analysis) into a fresh collection tube by microcentrifuging 30 sec at maximum speed. Store the samples on ice (if loading gels immediately) or in the freezer (if the gel will be run at a later date).

If small volumes of $1 \times$ SDS (relative to the bead volume) are used, the elutions will likely lose some blue color. This is acceptable, as the beads retain bromophenol blue dye. Load gel using the normal technique.

21. Analyze the samples by SDS-PAGE (UNIT 6.1) on a long-gradient gel run overnight at 10°C .

For optimal protein resolution (essential for analysis by mass spectrometry) the gels must be long gels (e.g., 20-cm gels; preferably gradient gels of 7.5% to 15% acrylamide), not minigels. Additionally, the gels should be 1 mm in thickness for optimal strength and ease of processing for mass spectrometry. Gels of 1.5-mm thickness are too thick for in-gel digestion (see Basic Protocol 2), and 0.75-mm gels tear far too easily. The controls suggested above (see Strategic Planning) should be run on the same gel.

22. Stain the gel by an appropriate technique suitable for mass spectrometry (see Basic Protocol 2).

Analyze results

23. Compare the protein stain in both lanes (GDP-loaded versus GTP-loaded) to find the proteins that bind in a nucleotide-dependent manner, and which do not bind to the various controls. See Anticipated Results for more details.

GST FUSION PROTEIN PURIFICATION

This procedure describes a technique for expression and purification of GST fusion proteins. There are many different protocols available for purification of GST fusion proteins, and the one provided here is a fairly standard protocol that works well for many GST fusion proteins. It is not, however, universally applicable, and modifications may be required to increase yield of functional product. Such modifications are many and varied (there are almost as many different protocols as there are GST fusion proteins), and as such they will not be discussed in this unit, since there are many other suitable references available that specifically cover such optimization procedures (see, e.g., Harper and Speicher, 1997). Some of these are provided by the manufacturers of expression vectors or GSH-agarose beads (e.g., see *The Recombinant Protein Handbook* from Amersham Biosciences).

Materials

Glycerol stock of *E. coli* cells containing GST fusion protein expression vector
(see *APPENDIX 3A* and Coligan et al., 2003)

LB medium and LB agar plates (see *APPENDIX 2A*) containing 100 µg/ml ampicillin
(or other appropriate antibiotic selection for GST expression vector used)

100 mM isopropyl-1-thio-β-D-galactoside (IPTG), filter sterilized

Bleach (e.g., Clorox)

Bacterial lysis buffer: 20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)/250 mM NaCl

100 mg/ml lysozyme

100 mM PMSF (*APPENDIX 2A*)

20 mg/ml leupeptin

Liquid nitrogen and appropriate storage canister

1 mg/ml DNase I

10% (v/v) Triton X-100 (*APPENDIX 2A*)

Bacterial lysis buffer (see above) containing 1% (v/v) Triton X-100

1% (w/v) SDS

Wash buffer 1 (see recipe)

Bead storage buffer: 20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)/50% (v/v) glycerol

1× and 3× SDS sample buffer (*UNIT 6.1*)

Bovine serum albumin (BSA; optional)

Shaking bacterial incubator

Culture tubes, sterile

1-liter conical flasks (Pyrex or autoclavable plastic), sterilized by autoclaving or
baking

Bugstoppers (Whatman), sterilized by autoclaving, *or* other sterile, gas-permeable
flask closures

Probe sonicator (e.g., Branson)

Refrigerated high-speed centrifuge and benchtop centrifuge

15-ml and 50-ml polypropylene screw-cap tubes (e.g., Falcon)

Pipet tip cut to increase opening to >1 mm

85°C water bath

MicroSpin columns (Amersham Biosciences)

Additional reagents and equipment for growing bacterial cells (see *APPENDIX 3A*),
SDS-PAGE (*UNIT 6.1*), Coomassie blue staining of gels (*UNIT 6.6*)

Culture bacterial cells and induce protein expression

1. *Day 1 (evening)*: Streak out glycerol stock of an *E. coli* strain containing GST fusion protein expression vector on an LB agar plate containing 100 µg/ml ampicillin (or other appropriate antibiotic selection for GST-expression vector), and grow overnight (~16 hr) at 37°C.

See APPENDIX 3A for references on growing bacterial cells.

2. *Day 2 (morning)*: Remove plates from incubator and store inverted at 4°C for up to a month.
3. *Day 2 (evening)*: Inoculate an appropriate volume of LB medium containing 100 µg/ml ampicillin (or other appropriate antibiotic) with a single colony from the LB agar plate. Incubate overnight at 37°C with shaking at 200 to 250 rpm.

Typically, a 1:50 to 1:100 dilution into 0.5 to 2 liters will be required the next day, so ensure that sufficient overnight culture is inoculated.

4. *Day 3 (morning)*: Dilute 7.5 ml of the overnight culture into 500 ml of LB medium containing 100 µg/ml ampicillin (or other appropriate antibiotic) in a 1-liter conical flask, and cap flask with an autoclaved Bugstopper (or another suitable gas-permeable closure). Incubate at 37°C with shaking at 200 to 250 rpm for 3 to 4 hr. Measure the optical density (OD) at 600 nm against LB medium (or water) as a blank.

Induce the bacteria (step 5) when the OD is ~0.8 to 1.2 (up to 2).

5. Remove a 1-ml aliquot of the OD 0.8 to 1.2 bacterial culture to a sterile 1.5-ml microcentrifuge tube and grow in parallel with the remainder of the culture (this is the noninduced control sample, “-IPTG”). To the remainder of the culture, add 100 mM IPTG for a final concentration of 0.1 mM to induce protein expression, and incubate 2 to 6 hr at 25° to 30°C.

The optimal induction temperature, OD, and duration will vary depending on the fusion protein of interest and may need to be determined systematically on a small scale (e.g., in 5- to 10-ml cultures) prior to large-scale protein production. Lower-temperature induction and shorter induction times at higher OD may increase the yield of folded, soluble protein (also see Coligan et al., 2003).

6. At the end of the incubation, place a 1-ml aliquot of the culture in a microcentrifuge tube (this is the induced sample, +IPTG). Centrifuge the bulk culture for 20 min at 18,000 × g (or maximum speed in a high-speed centrifuge), room temperature. Add bleach for a concentration of 2% to 4% (v/v) to the supernatant in the flasks, let stand for a minimum of 2 hr, and discard.

Some plastic flasks may not be tolerant to bleach; these should be washed well in detergent and disinfected by autoclaving. Do not bleach Bugstoppers, or soak them in detergent; instead, autoclave them.

7. Resuspend bacterial pellet in a volume of bacterial lysis buffer equal to 5% of the total induced culture volume (i.e., 50 ml per liter of culture), and freeze this bacterial suspension at -20°C overnight or until purification. Microcentrifuge the 1-ml -IPTG and +IPTG control samples at maximum speed, carefully remove and discard the supernatant, and freeze these control pellets at -20°C.

The samples can be stored in this way for up to 6 months.

Purify GST fusion proteins

8. Thaw the bacterial suspension (use cool water if necessary, but do not allow the samples to warm above $\sim 4^{\circ}\text{C}$). Once thawed, add a final concentration of 1 mg/ml lysozyme (from 100 mg/ml stock), 1 mM PMSF (from 100 mM stock), and 20 $\mu\text{g}/\text{ml}$ leupeptin (from 20 mg/ml stock), and vortex well. Incubate on ice for ~ 5 min.
9. Pass the bacterial suspension through two freeze/thaw cycles by sequential dropping of the tubes into liquid nitrogen for about 20 to 30 sec (or longer on dry ice if liquid nitrogen is not available), removing with tongs, and thawing in cool water (again ensuring that the samples do not warm much beyond 4°C).

CAUTION: When handling liquid nitrogen, ensure adequate eye protection and ventilation. Also, ensure that the tubes can withstand liquid nitrogen without shattering before dropping in any samples. There may be a slight color change (creamy white to a slightly more yellow/brown color) and a viscosity change after the freeze/thaw cycles, indicating that lysis has occurred.

10. Sonicate on ice for 30 sec with a probe sonicator to further rupture the cells and break up any DNA.

Do not oversonicate, as this may denature the proteins. This step can be omitted if protein degradation or denaturation occurs.

11. Add 1 mg/ml DNase I stock to a final concentration of 5 $\mu\text{g}/\text{ml}$ and 10% Triton X-100 to a final concentration of 1% (v/v). Incubate at 4°C (in a cold room), with slow rotation on an end-over-end rotator for 30 min.

12. Centrifuge samples in a high-speed centrifuge 30 min at $18,000 \times g$, 4°C .

13. While centrifugation is in progress, pipet an appropriate quantity of well-suspended GSH-agarose slurry (as supplied by the manufacturer) into a 50-ml polypropylene screw-cap tube using a pipet tip that has been cut to increase the opening to >1 mm.

How much of the GSH-agarose beads should be used? The manufacturers of GSH-agarose usually evaluate each batch of beads for binding capacity (and this is usually around 8 mg/ml for GST). Some prior estimation of protein expression level is useful, and an initial small-scale purification can be useful to assess this. For 0.5 to 1 liter of bacterial culture, a 0.5- to 1-ml bed volume of GSH-agarose yields a relatively high incorporation of protein to the beads (~ 1 to 2 mg/ml bed volume or greater). Some highly expressing soluble proteins may require even more GSH-agarose (e.g., the GST that is used for controls).

14. Centrifuge in a benchtop centrifuge 5 min at $500 \times g$, then remove and discard the supernatant (preferably by vacuum aspiration), being extremely careful not to lose any beads. Add 10 bed volumes of bacterial lysis buffer including 1% Triton X-100, resuspend the beads well and centrifuge 5 min at $500 \times g$, then carefully remove the supernatant and discard.

Do not centrifuge beads much faster than $2000 \times g$ at any point, or the beads may be crushed. Crushed beads are harder to isolate and bind more nonspecific proteins.

15. When the centrifugation of the bacterial lysate (from step 12) is finished, mark the level of the liquid in the centrifuge tube with a marker (the soluble bacterial fraction is the supernatant). Take a 20- μl sample (for gel; label as soluble), then decant the rest of the supernatant into the 50-ml tube containing the beads (step 14). Incubate at 4°C for 1 hr with rotation on an end-over-end rotator (preferably in a cold room) to bind the GST fusion proteins to the beads.

The beads should remain suspended at all times during incubation.

16. While the beads are binding, resuspend the pellet from the centrifugation of the bacterial lysate in a volume of 1% SDS solution equivalent to that indicated by the mark on the centrifuge tube. When it is relatively homogenous, take a 20- μ l sample (for the gel; label as pellet) and discard the remainder.

Considerable agitation (vortexing and trituration) will be required to disrupt the pellet. It will likely not fully solubilize, the aim is to homogenize and reduce the particle size. Full solubilization is not required, as it is intended as a control on SDS-PAGE—the heating and additional SDS added will make it homogenous enough for the gel.

17. After the binding step (step 15) is complete, centrifuge the 50-ml tube 5 min at 500 \times g, 4°C, to pellet the beads. Carefully decant the supernatant from the beads, taking care not to lose any beads. Ideally, leave a small volume of lysate behind at the end to avoid disturbing the bead bed. Take a 20- μ l sample of the supernatant (for the gel; label as through).

18. Add 10 to 20 bed volumes of wash buffer 1 to the beads, then centrifuge 5 min at 500 \times g, 4°C, and carefully remove the supernatant by decanting or vacuum aspiration. Repeat this wash three to four times, and in the process carefully transfer all of the beads to a 15-ml tube for storage, ensuring that all beads are transferred.

One of the best tubes for storage of beads are 15-ml Falcon 2059 polypropylene tubes, as the beads do not adhere to the walls and resuspend easily from the round base.

19. Wash the beads once by resuspending them in 5 bed volumes of bead storage buffer, centrifuging 5 min at 1000 \times g, 4°C, and removing the supernatant.

20. Add 2 bed volumes of bead storage buffer to the bead volume to make a 33% (v/v) slurry, and store at -20°C .

The beads should never be allowed to freeze, which will cause the beads to crack. The presence of 50% glycerol in the bead storage buffer is essential to prevent this.

21. Perform SDS-PAGE (UNIT 6.1) and stain with Coomassie blue (UNIT 6.6) to analyze purification and quantitate amount of protein bound to GSH beads. Prepare samples and lay out gel as follows:

- a. *Molecular weight standards:* Serially dilute to provide a reference for estimation of protein on beads after gel is stained (see step 22). Alternatively, load four lanes with 2, 5, 10, and 20 μ g BSA as a standard.
- b. *–IPTG and +IPTG induction controls:* Resuspend control pellets (step 7) in 80 μ l of 1 \times SDS sample buffer, heat to 85°C for 5 min, and microcentrifuge 1 min at maximum speed. Load 10 μ l of the supernatant on the gel.
- c. *Soluble, pellet, and through samples:* Add 10 μ l of 3 \times SDS sample buffer to the 20 μ l of sample, vortex, and heat to 85°C for 5 min. Load 10 μ l on the gel.
- d. *Beads:* Using a cut-off pipet tip with an opening 1 to 2 mm in diameter, pipet 5, 10, or 20 μ l (or all three to quantitate more accurately) of the slurry prepared in step 20 (ensuring the beads are well suspended by swirling or vortexing gently for \sim 10 sec) into a fresh MicroSpin column. Place in a collection tube and microcentrifuge 30 sec at 10,000 \times g to remove bead storage buffer. Cap the bottom of the column well, add 30 μ l of 1 \times SDS sample buffer, and cover with a punched cap. Heat at 85°C in a water bath for 5 to 10 min. Dry the outside of the column, and place in a fresh microcentrifuge tube. Microcentrifuge 30 sec at maximum speed to collect sample buffer and eluted protein. Load the entire volume on the gel.

Normalize the protein between different GST fusion proteins

Prior to performing the pull-down experiment (in Basic Protocol 1 or the Alternate Protocol) or the cross-linking (Support Protocol 2) it is necessary to approximate the amount of protein attached to the beads (milligram protein per milliliter beads).

22. Analyze the gel bands from 5, 10, and 20 μl of bead slurry on the gel (see step 21) by visual (or densitometric) comparison with the serially diluted markers to estimate the protein concentration on the beads.

This is important even for a single GST fusion protein, but it is essential for multiple GST fusion proteins. When comparing a number of different GST fusion proteins in the same pull-down experiment, the same amount of protein must be used for each condition on the same amount of beads (see Basic Protocol 1).

23. Before performing pull-down experiments (Basic Protocol 1 or the Alternate Protocol) or cross-linking (Support Protocol 2), dilute each of the batches of beads to be used in the experiment with GSH beads that have been freshly washed as in step 13, to adjust them all to about the same protein concentration per milliliter of beads. If desired, confirm equal protein concentration by loading an equal small volume of all beads on SDS-PAGE (UNIT 6.1).

The target is around 1 mg/ml, however the range can be rather large. A protein concentration of 0.05 mg/ml (5 μg on 100 μl beads) is about the minimum, while there is probably no maximum concentration; once the bed volume added to a pull-down becomes too small, it can become difficult to recover the beads from a large volume (unless they never leave the column, such as in the spin column-based pull-down experiment in Figure 17.5.2).

SUPPORT PROTOCOL 2

CROSS-LINKING GST FUSION PROTEINS TO GSH-AGAROSE

Perform this protocol only if the intention is to covalently couple a GST fusion protein to GSH beads. Cross-linking has a number of benefits and caveats, and these are discussed in Strategic Planning. The main chemical cross-linking agents used for this purpose are disuccinimidyl suberate (DSS) and bis(sulfosuccinimidyl) suberate (BS3). DSS is a water-insoluble, homobifunctional *N*-hydroxysuccinimide ester (NHS-ester), and BS3 is its water-soluble analog (both are available from Pierce). The main targets for NHS esters are primary amines. For proteins, cross-linking usually involves reaction with the side chains of amino acids. Only the ϵ -amine of lysine reacts significantly with NHS esters, as the *N*-terminal α -amine groups on a protein are not available for cross-linking. A covalent amide bond is formed when the NHS ester reacts with the primary amine, releasing *N*-hydroxysuccinimide. When cross-linking GST fusion proteins to GSH beads, the reaction must be between the primary amine of GSH (the α -amine of the γ -glutamyl residue is the only one available) and lysine residues in either recombinant GST or the attached fusion protein. Due to the length of the DSS molecule (11.4 Å), the former seems more likely, though the latter is not impossible. Due to reactivity with amines, cross-linking reactions should be performed in non-amine buffers such as phosphate, carbonate/bicarbonate, HEPES, or borate buffers to maximize coupling efficiency. Other buffers can also be used provided they do not contain primary amines; Tris buffers contain primary amines and must be avoided. A large excess of Tris at neutral-to-basic pH is added at the end of the reaction to quench it.

Since DSS is insoluble in aqueous solvent, it is first dissolved in an organic solvent (e.g. DMSO), then added to the aqueous reaction mixture. Cross-linkers fall out of aqueous solutions at the concentrations used for cross-linking, and a milky, turbid solution can be observed. Cross-linking will still proceed effectively. Storage of stock solutions for long periods (hours) is not recommended for either class of compounds, because NHS esters hydrolyze rapidly. The solvent DMSO is hygroscopic and absorbs water, which will

promote hydrolysis. BS3 is a more efficient cross-linker than DSS, primarily because it is not insoluble in aqueous solution; however, it is considerably more expensive.

This procedure is designed for the coupling of an ~0.3- to 1.0-ml bed volume of GST fusion protein beads.

Materials

GST fusion protein beads (prepared as in Support Protocol 1, with the protein concentration determined)

PBS (*APPENDIX 2A*)

Disuccinimidyl suberate (DSS), 13.2 mg in screw-cap tube (see recipe)

bis(sulfosuccinimidyl) suberate (BS3; see recipe)

Dimethylsulfoxide (DMSO; H₂O-free, i.e., fresh)

Quenching buffer: 1 M Tris·Cl, pH 8.2 (*APPENDIX 2A*), room temperature

Glutathione wash buffer (see recipe), room temperature

Wash buffer 1 (see recipe), ice-cold

Cross-linking wash buffer: 25 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)/1.5 M NaCl, ice-cold

Bead storage buffer: 20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)/50% (v/v) glycerol

Appropriately sized columns (e.g., Bio-Rad 2-ml Bio-Spin columns or 10-ml Poly-Prep columns)

Cross-link beads

1. Pipet the required volume of GST fusion protein beads to be cross-linked into a suitably sized plastic column (which can also be capped at both ends, e.g., Bio-Rad Bio-Spin 2-ml column), and allow to drain. Keep a 10- μ l bed volume aliquot of starting beads for SDS-PAGE analysis of cross-linking efficiency at the end of the protocol (step 14).

If the beads have been stored in 50% glycerol (i.e., bead storage buffer in Support Protocol 1), the column will not drain, due to the viscosity. It can be assisted by the use of a vacuum line or gentle centrifugation into a collection tube (5 min at 500 to 1000 \times g). Make sure that the beads do not dry out.

2. Wash the column with four 2-ml washes of PBS. Let all the solution drain out.

This is required to thoroughly remove any traces of amines, such as Tris.

3. Plug the bottom of the column and add 0.5 ml PBS. Flick gently to resuspend the beads.

4. Dissolve 13.2 mg of DSS in 1 ml of dry DMSO to make a 35.8 mM solution. Dilute this 1 ml of DSS solution into a clean tube containing 1.5 ml of PBS at room temperature.

The DSS solution must be made fresh, immediately before use; do not store this reagent.

The 35.8 mM DSS stock solution is a good place to start in most cases, but it may not always be ideal. Generally, if the exact amount of protein is known, a 20- to 50-fold molar excess of the cross-linker is appropriate. The final cross-linker concentration in the reaction should be between 0.25 and 5 mM.

BS3 is added to a cross-linking reaction at the same molar concentration as DSS. Due to its aqueous solubility, it can be added directly to a protein solution. If desired, a 35.8 mM stock solution can be prepared by dissolving 20.5 mg of BS3, immediately before use, in 1 ml of 5 mM sodium citrate, buffer pH 5.0. This stock solution should be added in a dropwise fashion to the reaction.

5. Immediately add the diluted DSS solution to the prepared GST fusion protein bound to the column (step 3), at room temperature. Plug both ends of the column and suspend the beads by repeated gentle inversions and by tapping the gel-bed end of the column (alternatively, gently mix with a fine spatula). Incubate 45 min at room temperature, with continuous mixing or inversion.

When DSS is added to the aqueous buffer it may become very cloudy. This is normal, and cross-linking will still proceed.

6. After the reaction is complete, remove the top cap first, then the bottom cap. Place the column inside a collection tube. Drain the column, but do not allow the beads to run completely dry, then plug the bottom.

Block amine-reactive sites on beads

7. Add 2 ml quenching buffer to the column. Mix it in and gently invert the column for 10 min at room temperature.

This blocks all amine-reactive sites that may still be present after the cross-linking reaction, preventing any other proteins from being covalently coupled to the beads.

8. Drain the column, but do not allow the gel to run completely dry, then plug the bottom.
9. Wash the column four times, each time by adding 3 ml freshly prepared glutathione wash buffer at room temperature, and allowing it to pass through. Keep an aliquot of the eluate to run on a gel later (step 14).

This step removes GST fusion protein that is not covalently coupled to the glutathione beads. Keeping the eluate allows the assessment of any eluted GST fusion protein, and thus gives a crude index of the coupling efficiency.

Wash beads

Switch back to 4°C for the washing steps.

10. Wash the column by adding 4 ml of ice-cold wash buffer 1 and allowing it to pass through.

The column can be on the laboratory bench (at room temperature) for the washing, but keep the buffers ice cold.

11. Wash the column twice, each time with 4 ml of ice-cold cross-linking wash buffer.
12. Wash the column twice, each time with 4 ml of ice-cold wash buffer 1.
13. Add twice the bed volume of bead storage buffer, resuspend the beads by gently flicking the column, and store capped at -20°C up to 4 to 6 months.
14. Analyze 10 µl of starting beads, 20 µl of the GSH elutions (step 9), and 10 µl of the final cross-linked beads on an SDS-PAGE mini-gel.

Ideally, there should be no or little protein on the gel from the GSH elutions, and no protein from the final cross-linked beads. Practically, there are often faint bands in the elutions and occasionally a trace in the final beads. This level of protein can still be acceptable for use in pull-down experiments, depending on the initial reason for performing the cross-linking. Greater levels of protein in either the elutions or the final beads indicates that the reaction was inefficient and probably insufficient for use.

PREPARATION OF LARGE-SCALE TISSUE LYSATES

This method is for preparation of two liters of sheep brain cytosol, peripheral membrane, and integral membrane lysates for use with GST fusion–protein pull-down experiments. Alternatively, a whole-tissue lysate (Triton X-100 extract) can be prepared. The samples are prepared and frozen as fifty 40-ml aliquots in 50-ml screw-cap tubes, ready to thaw, centrifuge, and then use immediately. The aliquots provide a rapid source of protein that can be stored in the freezer until required. Lysates are stored at -20°C for up to 6 months and could be stored longer at -70°C . Generally, it is best to use the lysates when they are as fresh as possible. For other tissues, this method may need to be adapted or scaled up or down. However the protocol below gives a good indication of the optimal tissue-derived protein amounts required for identification of the major proteins that bind to a particular GST fusion protein. Using less tissue will work, but this amount is optimal for obtaining the largest number of interacting proteins.

Materials

200 g sheep brain (see recipe)
Buffers A, B, C, and D (see recipes; prepare in advance and cool overnight to 4°C)
GSH-agarose beads (e.g., glutathione-Sepharose 4B; Amersham Biosciences)
10% (v/v) Triton X-100

Colander or tea strainer
1-liter plastic beakers
Ultra-Turrax tissue homogenizer (IKA Labor Technik) with 25-mm-diameter blade/tip
Refrigerated centrifuge and 0.5-liter centrifuge bottles
Miracloth (Calbiochem)
50-ml screw-cap plastic tubes

NOTE: Sometimes precipitates accumulate during freeze/thaw of these samples, and this is particularly true for the peripheral membrane extract (see note above). All frozen aliquots must be centrifuged after defrosting at $35,000 \times g$ (or maximum speed) for 30 min at 4°C , before incubation with recombinant GST fusion protein beads. It is essential to clear any debris before adding the extract to the beads. It may also be useful to pre-clear the cytosol extracts a third time before use, to remove any remaining traces of endogenous GST (this is optional; see Basic Protocol 1 or the Alternate Protocol). All volumes of beads in this protocol refer to bed volumes.

Extract cytosolic protein

1. Defrost 200 g frozen sheep brain by placing the pieces in 1 liter of buffer A that is being stirred at 10°C , (add the brains to the buffer, rather than vice versa) and leaving them until they start to sink (after ~ 7 min). Strain the brains with a colander or tea strainer and place them in 2 liters of ice-cold buffer B.

Sheep brain can be replaced with rat brain (one rat brain weights ~ 1.85 g; 40 rats = 74 g), but at considerable expense.

Alternatively, for a whole-tissue lysate, add 0.5 liter buffer D (instead of buffer B) and skip directly to step 17. Make sure the homogenate is free of particulate matter by an additional centrifugation in step 17, followed by filtration through Miracloth, before proceeding to step 18.

2. Homogenize brains in four 500-ml lots of buffer B (in 1-liter plastic beakers) with an Ultra-Turrax (i.e., an homogenizer with sharp blades, not a Teflon/glass homogenizer), using a 25-mm diameter blade/tip and a speed setting of 19,000 rpm.

This should be performed in 1-liter plastic beakers (to minimize risk of breaking glass).

3. Centrifuge homogenate in 0.5-liter centrifuge bottles 30 min at $18,000 \times g$, 4°C .

If the available centrifuge cannot manage large volumes at $18,000 \times g$, then use the maximum speed wherever this is indicated in this protocol.

4. Collect the supernatant and filter through Miracloth to remove floating particulate matter.

Keep the pellet on ice for step 10.

5. Centrifuge the filtrate again as in step 3 and filter through fresh Miracloth again.

6. Wash 8 ml of GSH-agarose beads in a 50-ml screw-cap tube by centrifuging 5 min at $500 \times g$, 4°C , and carefully remove the supernatant. Resuspend the pellet in 50 ml of buffer B and centrifuge again 5 min at $500 \times g$, 4°C . Discard the supernatant, and pre-clear the cytosol extract by incubating 20 min with 2 ml of the prewashed beads at 4°C with continuous stirring. Filter the pre-cleared cytosol extract through clean Miracloth.

Do not use a stir bar to stir the extract with beads, as the beads may be crushed and will then pass through the Miracloth.

7. Repeat the pre-clearing step with an additional 2 ml of fresh GSH-agarose beads.

The multiple pre-clearing steps are performed to remove the enormous amounts of endogenous GST protein from the brain (particularly the brain cytosol), which would interfere with the subsequent pull-down experiments.

8. Filter the precleared cytosolic protein extract through clean Miracloth to collect the GSH-beads.

Pool the GSH beads with the previous volume for later recycling (see Support Protocol 4).

9. Divide the cytosol into fifty 40-ml aliquots in 50-ml plastic tubes with screw-cap lids, freeze on dry ice, and store up to 6 months at -20°C .

Extract peripheral membrane protein

10. *Wash 1:* Rehomogenize the pellet from step 4 in 2 liters of buffer B (see step 2) and centrifuge (see step 3).

This is simplified by leaving the homogenate in the same centrifuge bottle and rehomogenizing within that bottle.

11. *Wash 2:* Remove and discard the supernatant, then repeat the homogenization and centrifugation.

12. Remove and discard the supernatant. Rehomogenize the pellet in 500 ml of buffer C using the technique described in step 2. Stir in a beaker in the cold room for 20 min, then centrifuge 30 min at $35,000 \times g$, 4°C . Remove the supernatant and centrifuge it again for 30 min at $35,000 \times g$, 4°C .

13. Collect the supernatant as peripheral membrane extract and dilute to 2 liters with buffer B. Add 10% Triton X-100 solution to a final concentration of 0.1%.

Keep the pellet for step 16.

Since no extra salt is added beyond that in the buffer C that was added in step 12, the final NaCl concentration is reduced to 125 mM, which is more appropriate for pull-downs than 0.5 M. The addition of Triton X-100 is required to prevent the formation of excessive protein precipitates that occur with this peripheral membrane extract during pre-clearing steps and during pull-down experiment incubations. This precipitation only occurs with this extract and seems to be triggered by freeze/thawing and incubation with beads. It is not removed by prior centrifugation, but it is prevented by addition of trace amounts of Triton X-100 (0.1% v/v final concentration). It is worth keeping in mind that the presence of this detergent may interfere with some protein-protein interactions.

14. *Pre-clearing*: Incubate the peripheral membrane extract with 2 ml prewashed (step 6) GSH-agarose beads for 20 min at 4°C with continuous stirring. Filter the precleared peripheral membrane protein extract through Miracloth.

For the peripheral membrane, it is not necessary to repeat the pre-clearing, since there is much less endogenous GST in this fraction than there is in the cytosol.

Pool the GSH beads with the others (from steps 6 to 8) for recycling.

15. Divide the peripheral membrane extract into fifty 40-ml aliquots in 50-ml tubes with screw-cap lids, freeze on dry ice, and store at –20°C for up to 6 months.

Extract membrane protein

16. Wash the pellet from step 13 once with 1 liter buffer C by rehomogenizing (as in step 2) and centrifuging as in step 3. Discard the supernatant.
17. Rehomogenize the pellet in 500 ml of buffer D using the technique described in step 2. Stir in the cold room for 20 min in a beaker, then centrifuge 30 min at 35,000 × g, 4°C.

18. Collect the supernatant as membrane extract and immediately dilute to 2 liters with buffer B, to match the volume of the cytosol. Discard the pellet.

Since no extra Triton X-100 is added beyond that present in the buffer D, the Triton X-100 is diluted to 0.25%.

19. *Pre-clearing*: Incubate the membrane extract with 2 ml pre-washed (step 6) GSH-agarose beads for 20 min at 4°C (keep stirring). Filter the precleared peripheral membrane protein extract through clean Miracloth to collect the beads.

For the membrane, it is not necessary to repeat the pre-clearing, since there is much less endogenous GST in this fraction than there is in the cytosol.

Pool the GSH beads with those from the other extracts, and recycle them for reuse as described in Support Protocol 4.

20. Divide the membrane extract into fifty 40-ml aliquots in 50-ml tubes with screw-cap lids, freeze on dry ice, and store up to 6 months at –20°C.

SAMPLE PREPARATION FOR MASS SPECTROMETRY

Enzymatic in-gel digestion of SDS-PAGE-separated proteins followed by mass spectrometry (MS) analysis and database searching is now a common approach for identifying unknown proteins from a semipurified system such as a pull-down experiment. The following is a step-by-step protocol to take protein samples from an unstained SDS-PAGE gel through proteolytic digestion to a peptide mixture ready for analysis by MS. First, an SDS-PAGE gel, prepared and run in Basic Protocol 1 or the Alternate Protocol, is stained and analyzed for protein bands that bind specifically to the target protein (see Anticipated Results). The bands of interest are excised and completely destained to remove any stain that might interfere with subsequent proteolysis and MS analysis. Treatment with protease (usually trypsin) digests the protein within the gel piece, and the resulting peptides are extracted, concentrated, and analyzed by MS. Techniques for performing MS analysis are not described here, as they are beyond the scope of this unit (some techniques are described in *UNIT 5.6*; see Chapter 16 in Coligan et al., 2003, for a full treatment). It is envisaged that many researchers would utilize a service (institutional or commercial) for their MS analysis; therefore, suitable methods for shipping SDS acrylamide gels and gel pieces are outlined.

BASIC PROTOCOL 2

Macromolecular Interactions in Cells

17.5.27

Two different MS-compatible options are presented for staining of SDS acrylamide gels: colloidal Coomassie blue and zinc-imidazole stain. Either staining technique is particularly suitable for routine use in this protocol. There are other MS-compatible stains not described in this protocol. One of these is the silver stain, a highly sensitive stain that enables detection of low nanogram quantities of protein. One must ensure that the chosen silver stain protocol does not contain a glutaraldehyde fixation step, as this interferes heavily with MS analyzes. The most widely used compatible silver stain is that described by Shevchenko et al. (1996). It is important to convert the silver in the excised gel piece to a soluble form and remove it from the gel piece completely for MS analysis. Another staining method utilizes the fluorescent dye SYPRO Ruby, which is highly suitable for MS as it requires no destaining (Berggren et al., 2002; *UNIT 6.6*). This has the disadvantage that it requires specialized imaging equipment to observe the stained bands, but it is a very simple technique that is highly sensitive (comparable with silver stain).

The colloidal Coomassie blue stain (Neuhoff et al., 1988) is a rapid gel staining method that is slightly superior in sensitivity and reliability of quantitation when compared with traditional noncolloidal Coomassie brilliant blue stain (which is usually dissolved in acetic acid/methanol or trichloroacetic acid). The colloidal stain is faster than other stains since there is no need for strong destaining steps. In fact, destaining of the whole gel is not required at all, though it does help to better visualize the lower-intensity protein bands. Destaining is performed only briefly in high-quality water, thus avoiding acids and improving protein identification by mass spectrometric techniques. Acidic residues of proteins can become methylated when SDS-PAGE gels are kept for prolonged periods in acetic acid, making it harder to identify the protein. Additionally, colloidal Coomassie can be easily destained from a small gel piece. Coomassie blue has a distinct appearance in mass spectra, a variable peak of $m/z \sim 800$ to 850 Da (often 804 or 832 Da). If this is a strong peak in the spectra, it can significantly interfere with detection of weaker samples, particularly in MALDI-TOF MS. Additionally, the presence of Coomassie in a gel piece can inhibit trypsin's proteolytic activity, so that digestion may not proceed efficiently. Therefore, effective destaining of a Coomassie-stained gel piece is essential for analysis by MS.

The zinc-imidazole negative staining method is fast, simple, sensitive, and highly compatible with MS (Castellanos-Serra and Hardy, 2001). It is also gentle, as there are no acids, dyes or chemical modifiers. The sensitivity of the method (>10 ng protein) is better than Coomassie blue staining (>100 ng protein) and approaches that of silver staining (1 to 10 ng protein/band). Therefore, this negative stain often reveals bands not stained with Coomassie (Fernandez-Patron et al., 1995). It is also completely reversible. The stain is a negative, or reverse stain: it relies on precipitation of a zinc salt in the part of the gel matrix that is not occupied by proteins. Imidazole reacts with Zn^{2+} to produce insoluble zinc imidazolate, which is a deep white. Proteins inhibit the formation of zinc imidazolate and therefore appear clear. Protein bands are visualized by placing the gel above a dark surface, e.g., black paper or plastic. Negative staining techniques do not offer as much contrast as a positively stained gel, and they are therefore not ideal for quantification purposes. Also, it should be noted that the zinc-imidazole stain is not retained when the gel is dried. On the positive side are the great speed of this method and the fact that no stain is attached to protein, which avoids unwanted chemical modifications. Finally, one should note that not all staining methods will detect all proteins, and some may be missed by any of these approaches.

It is also possible to excise and in-gel digest bands from dried gels for analysis by MS (but not from zinc-imidazole negatively stained gels). However, a small reduction in peptide recovery after in-gel tryptic digestion is observed when compared with wet gels

that have not been dried. Therefore, it is recommended that wet gels be used as a first preference for the procedures described in this protocol. If dried gels are to be used, begin from step 10, carefully excising the band with a sharp scalpel blade. The dried gel piece will reswell upon washing with water (step 13a or b). At this point it is important to remove any and all pieces of cellulose membrane (commonly used for gel drying procedures), which will detach from the gel as it swells, then proceed with destaining and in-gel digestion in the protocol as described.

The protocols for in-gel protein digestion are well established, fairly simple, and straightforward. Very often, key handling procedures during the sample preparation steps are overlooked because they seem easy and nonchallenging. Weeks and months of work can easily be jeopardized because the sample was contaminated or wasted in the final steps before MS analysis. Therefore, several general experimental considerations apply to all stages in this protocol. Precautions need to be taken, mainly to avoid contamination of the samples with various substances that will interfere with MS analysis. These precautions are:

1. Lab coats and powder-free gloves must be worn at all times. Use the highest-grade purity chemicals available, including the highest-grade laboratory water.
2. Take particular care to avoid protein contamination from skin, hair, and saliva (one of the most common interfering substances for MS protein identification is keratin) at all steps, including scanning and drying of SDS acrylamide gels and cutting and washing of stained bands. Avoid talking, coughing, sneezing, or breathing directly over the sample.
3. Keep samples covered or capped at all times unless being processed. Use cleaned tools and containers for anything that will come in contact with the samples.
4. Always use high-quality microcentrifuge tubes and pipet tips to avoid polymer contamination and loss of peptide sample through binding to plastic. Either siliconized, hydrophobically coated or highly polished plastics are recommended.
5. Heating gel pieces or peptide samples should be avoided. When using the Speedvac evaporator, the heat setting is not recommended.
6. Check with the mass spectrometry service first, to obtain additional advice on how to prepare, store, and ship the samples for analysis, and strictly follow their instructions.

Two different protocols for staining SDS acrylamide gels are presented, the colloidal Coomassie and zinc-imidazole negative stain. Either protocol is appropriate for the purposes of this unit, so both are described.

Materials

- Unstained SDS acrylamide gel (Basic Protocol 1 or Alternate Protocol)
- Colloidal Coomassie stain (see recipe)
- 1% (v/v) acetic acid
- Zinc stain solutions I and II (see recipes)
- 50% (v/v) and 100% acetonitrile
- 25 and 50 mM ammonium bicarbonate (prepare from 1 M stock; see recipe)
- 25 mM ammonium bicarbonate in 50% (v/v) acetonitrile (prepare from 1 M ammonium bicarbonate stock; see recipe)
- Zinc destain solution (see recipe)
- Digestion buffer (see recipe), ice-cold
- 5% (v/v) formic acid
- 50% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA)

Sealable plastic containers of appropriate size for gel
Platform shaker
Flatbed scanner (preferably with transparency adapter for Coomassie stain)
Computer running graphics software, e.g., Adobe Photoshop
Light box
Scalpel (with new blades) or fine-gauge needles
Siliconized, hydrophobically coated, or highly polished plastic microcentrifuge tubes (e.g., Axygen Maxymum recovery clear 0.6-ml tubes), pipet tips (Axygen Maxymum recovery pipet tips), and gel-loading tips (e.g., Sorenson Multi Miniflex round sterile gel loading pipet tips, 0.1 to 10 μ l)
Stainless steel mortar and pestle (e.g., Quantum Scientific)
Heat-sealable plastic sleeves and heat sealer
Pieces of cardboard and large sturdy flat box to accommodate gels
50-ml screw-cap tubes
Heavy-gauge needle
Thermomixer/shaker (to fit microcentrifuge tubes)
Bath sonicator

Stain the gel

For colloidal Coomassie staining

- 1a. After running the gel, immediately rinse the whole gel with water at room temperature at least three times (each time for 5 min) before proceeding with the stain.

This step is to remove any traces of SDS remaining on the gel, which could lead to high background and reduced protein staining.

- 2a. In a container with a cover that can be sealed, cover gel with enough colloidal Coomassie stain so that gel can float freely. Seal the container (to avoid methanol evaporation or contamination) and incubate 1 hr to 3 days with gentle shaking or rocking at room temperature.

The gel must float freely, or it may tear during staining.

Most proteins are detectable after ~1 hr, even while still in the stain solution. Weaker bands show up better within a few hours (normally 3 hr). For quantitative staining, the gel should be left in the solution for ~24 hr (up to 3 days), but this is not usually required.

It is not possible to overstain the gel, and over 1 to 3 days the staining increases slightly in sensitivity.

- 3a. Destain by immersing in two to three changes of Milli-Q purified water for 15 to 30 min each.

Faint bands often become much clearer after destaining. Very little background staining is encountered with this protocol.

- 4a. If destaining goes too far, or if the gel was not left in the initial stain for >3 hr, restain as in step 2a.

In many cases restaining actually enhances band detection after a second round of staining. Restaining can be done at any time.

- 5a. Store gels up to 2 weeks in water at 4°C (to prevent bacterial or fungal growth) or for longer periods in 1% (v/v) acetic acid.

It is highly desirable to excise the target protein bands within a few days of gel staining (see step 10) to avoid artifacts arising from storage.

For zinc-imidazole negative staining of SDS gels

- 1b. After running the gel, immediately rinse it twice in Milli-Q purified water for up to 5 min each time.

Free glycine can bind to zinc and interfere with the staining process.

- 2b. Cover the gel with zinc stain solution I and leave on a shaker for 15 min.
- 3b. Pour off the solution and rinse once quickly with distilled water for 10 to 30 sec.

This rinse step must be very brief. Do not leave the gel in the rinse even for a couple of minutes or the stain will wash out.

- 4b. Cover the gel with in zinc stain solution II for 35 sec.

The gel background will rapidly appear as a milky precipitate. If the gel is left in this solution longer than 35 sec the stain will begin to fade. To prevent overstaining, proceed quickly to the next step. The timing here also depends somewhat on the acrylamide percentage. Thus, at low acrylamide (~7.5%), <35 sec may be slightly better, while for higher acrylamide (~15%), 40 to 45 sec may be better.

- 5b. Pour off the solution and immediately rinse three times in Milli-Q purified water (each time for ~1 min), then leave the gel in Milli-Q purified water.

A transparent container will enable the bands to be visualized when placed above a dark background like black paper.

Analyze the stained gel

6. Search the gel for bands that appear to be specific for a particular condition and are absent from the controls, as described in Anticipated Results.

Scan the stained gel

7. For documentation purposes, obtain a quality scan (or a photograph) of gels before excising the bands of interest. For Coomassie-stained gels, use a flatbed scanner (preferably with a transparency adaptor, i.e., a light box in the lid) to record the gel stain. For zinc-imidazole negative staining, record an image by placing the gel onto a flatbed scanner and covering it with a black piece of paper or plastic.

For some scanners it is necessary only to simply leave the lid of the scanner open while recording the zinc image.

For large gels, scan at least at 600 dpi, TIFF format, in 8-bit color. Use LZW compression to keep the file size to a minimum.

8. Using graphics software like Photoshop, convert a copy of the TIFF file to the much smaller JPEG format and then insert the JPEG image into a drawing program (e.g., Microsoft Powerpoint, or CorelDraw). Draw rectangular shapes around the bands of interest and place a simple, unique label next to each one. Use exactly the same labels on the microtubes with the samples as in the computer file. Cover the labels with tape so the writing does not rub off during subsequent steps.

This serves as a laboratory record, and a copy can be sent to an MS service as well. If it is intended to send the whole, intact gel to an MS service, skip to step 13a.

Excise bands

9. Inspect the hydrophobically coated or highly polished pipet tips and microcentrifuge tubes for dust prior to use. If unsure, rinse the microcentrifuge tubes with 50% acetonitrile, followed by water, prior to use.

10. Rinse the whole gel with water and (wearing powder-free gloves) place on a cleaned glass plate over a light box.

For band cutting, work in a clean filtered forced-air-flow hood if possible. If not, clean the bench area thoroughly with ethanol or methanol.

11. Excise the band of interest with a clean scalpel or needle, cutting as close to the edge of the band as possible.

Remember to change scalpels and needles between samples to avoid cross-contamination. Alternatively, rinse the scalpel after each band first with ethanol, then with water.

It is important to reduce the volume of the background gel that is excised. Keep the amount of excess acrylamide included around the band to a minimum. For very large or fat bands, a piece from the center or side is often all that is required.

12. Carefully transfer the gel piece to a clean, labeled microcentrifuge tube.

Store and ship intact gels and gel pieces

It is preferable to not store gels or gel pieces for prolonged periods, but the following guidelines are provided to prevent unwanted spoilage if this is necessary. Additionally, if the researcher is using a commercial or institutional MS service, these often require that the intact gels or excised gel pieces are shipped to them for analysis. Therefore, appropriate handling techniques for shipping these are described here, and the remainder of this protocol does not need to be followed. If it is intended to perform in-gel protein digestion and peptide extraction, then continue with step 14.

- 13a. *For intact gels:* Store intact gels at 4°C submerged in water for no more than 2 weeks, in a covered container to avoid drying out or contamination. Do not store the gels on the bench or they will grow mold. Alternatively, heat seal them in a strong thoroughly rinsed plastic sleeve for storage at 4°C. Ship intact gels in a few milliliters of water in a heat-sealed plastic bag, packaged in such a way as to avoid tearing or having the gel fold over on itself—e.g., place the heat-sealed bag between rigid pieces of cardboard to keep the gel flat, then place the assembly in a large sturdy flat box.
- 13b. *For excised gel pieces:* Store excised gel pieces (sometimes referred to as gel plugs) in sealed microcentrifuge tubes either at 4°C submerged in a small volume of water or at –20°C without water for months. Ship gel pieces, each in a separately labeled microcentrifuge tube, submerged in a small volume of water (e.g., 50 µl) to prevent the gel slices from drying out; place the microcentrifuge tubes in a 50-ml screw-cap tube to protect them from being crushed in transit.

Wash and destain the excised gel pieces

For destaining colloidal Coomassie–stained gel pieces

- 14a. Discard the storage solution. Rinse gel pieces three times, each time by adding 100 µl water and incubating 10 min at room temperature with shaking, then removing the liquid.

Coomassie blue interferes with the MS analysis and inhibits tryptic digestion. Therefore it is removed prior to tryptic digestion. The number and the duration of the following washing steps depends on the sample itself. Some bands destain easily and may only need a few rinses with water and a single step with 100% acetonitrile, while others are more stubborn and may need several rounds of ammonium bicarbonate/acetonitrile (CH₃CN). Under no circumstances should one proceed with trypsin digestion until the gel pieces are completely clear and colorless.

The use of a mixer/shaker (suitable for microcentrifuge tubes) or occasional vortexing at room temperature is recommended for all washing and destaining steps. Use gel-loading tips, to prevent aspirating the gel particles when removing and discarding the liquid after each change of solution during the wash steps. Change tips between different samples. A

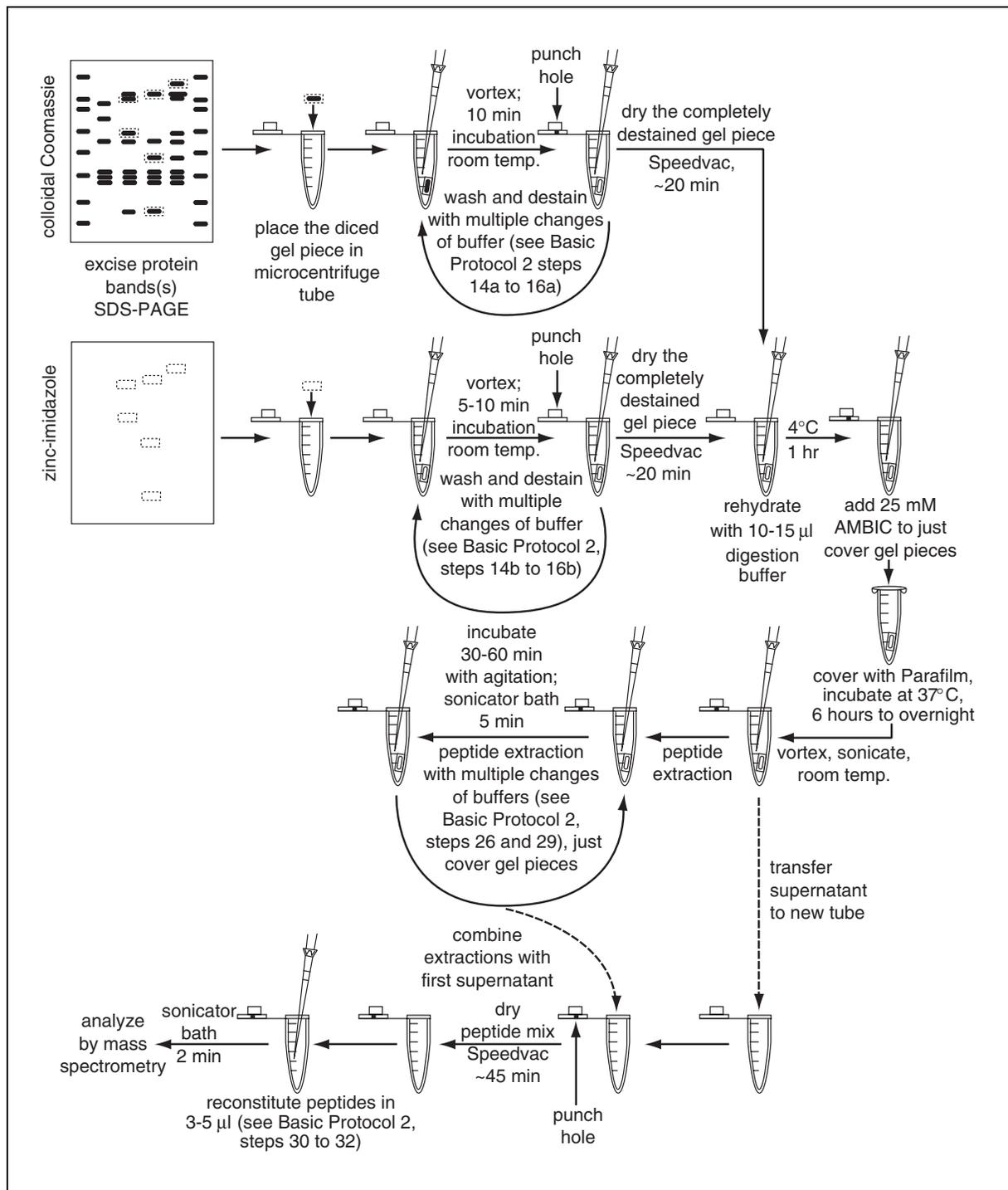


Figure 17.5.3 Schematic of Basic Protocol 2 describing the in-gel digestion technique and peptide extraction steps. For graphical reasons, the gel pieces are depicted as whole (to show the state of the stain), but would in practice be diced. Refer to the main body of the text for more details. Abbreviation: AMBIC, ammonium bicarbonate, pH 8.0.

schematic diagram of the remainder of this protocol can be found in Figure 17.5.3, though it is best to use the figure while paying close attention to the text of the following steps.

- 15a. Add 100 µl of 25 mM ammonium bicarbonate in water to equilibrate the gel particles. Incubate for 30 min to overnight with shaking. Remove liquid. Wash gel pieces three times, each time by adding 100 µl of 25 mM ammonium bicarbonate in 50% acetonitrile, incubating 10 min at room temperature with shaking, then removing the liquid.

- 16a. Add enough 100% acetonitrile to just cover the gel particles (which should become white and stick together). Change the acetonitrile once after 5 min. Remove the liquid. Add 100 μ l of 50 mM ammonium bicarbonate, leave for 5 min, then add an equal volume of 100% acetonitrile (to make a final concentration of 25 mM ammonium bicarbonate in 50% acetonitrile). Incubate for an additional 5 to 10 min with shaking. Remove liquid.

In this step the gel particles are completely dehydrated with neat acetonitrile to allow the subsequent aqueous solution to better access the center of the gel piece, giving a better chance of removing any stain.

For destaining zinc-imidazole negative stained gel pieces

- 14b. Discard the storage solution. Rinse gel pieces three times, each time by adding 100 μ l water and incubating 10 min at room temperature with shaking, then removing the liquid.
- 15b. Add 200 μ l of zinc destain solution per gel piece, vortex, and incubate with shaking for 5 to 10 min at room temperature until gel piece is completely clear. Remove the liquid.

A zinc chelator is required to destain the gel. Glycine (present in the zinc destain solution) is suitable for this purpose. The pH must be kept in the neutral to basic range to avoid precipitation of the protein in the gel.

- 16b. Wash gel pieces four times, each time by adding 1 ml water and incubating 10 min at room temperature with shaking, then removing the liquid.

This step is to dilute the glycine, which will interfere with subsequent staining or analysis.

Grind and dehydrate the gel pieces

17. Using a clean stainless steel mortar and pestle, grind each of the excised bands into smaller particles inside the tube. Alternatively, use a long needle to carefully cut it into cubes (~1 mm to a side) inside the tube.

This step is to increase the surface area in contact with buffer and to minimize the volumes of the solutions used in the subsequent in-gel digestion and extraction of the resulting peptide mixture.

18. Add sufficient 100% acetonitrile to just cover the gel particles and leave for 5 min to dehydrate. Remove the liquid.
19. Pierce a hole, if one is not already present, in the lid of the microcentrifuge tube using a heavy-gauge needle to allow for evaporation. Completely dry the gel particles in a Speedvac evaporator (~20 min).

The gel pieces should shrink, become white and stick together. At this point the destained gel pieces can be stored at 4°C for months.

Perform in-gel tryptic digestion

20. Rehydrate the dried gel particles in 10 to 15 μ l of freshly prepared ice-cold digestion buffer (use just enough to cover the sample).

Gel pieces should expand and change from white dry pellets back to transparent gel pieces.

21. Incubate the samples at 4°C (sitting on ice) for about 1 hr.

This will allow the trypsin to enter the gel pieces, while it remains inactive at 4°C.

22. After 1 hr, if the initial liquid is fully absorbed, add 10 to 15 μ l of 25 mM ammonium bicarbonate in water (without trypsin), or just enough to cover the gel particles.

It is necessary to have a small excess of solution covering the gel piece, so that the gel piece does not dry out during the subsequent 37°C overnight incubation (step 23).

23. Cap the tubes and wrap the tops with Parafilm to seal them. Transfer the tubes to an incubator at 37°C for overnight digestion.

At 37°C, the trypsin (which had previously entered the gel piece during rehydration) is active and digests the proteins from within the gel piece.

Most proteins should be completely digested by trypsin after 4 to 6 hr. However samples are usually incubated overnight for convenience. The extraction of the resulting peptide mixture could then take place on the following day.

Extract the peptides

24. Next day, microcentrifuge the tubes briefly at maximum speed to get the condensation to the bottom of the tubes. Vortex the tubes or sonicate (in a mild sonicator bath) for 1 to 5 min.

This step is to assist the peptides to diffuse from the gel piece into the solution.

25. Microcentrifuge briefly at maximum speed (to force all the sample to the bottom of the tube) and transfer the supernatant to a fresh microcentrifuge tube.

The supernatant should contain most of the tryptic peptides. If performing MS on-site, then take a small aliquot (0.5 µl) for immediate analysis.

This first extraction step has a lower extraction efficiency but is quick, and there is less sample loss because the samples are not dried in a Speedvac evaporator. Subsequent extractions in organic phase are needed for recovery of hydrophobic or long peptides.

26. Wash gel pieces by adding ~25 µl (or enough) 25 mM ammonium bicarbonate in water to just cover them, then removing the liquid and combining it with the supernatant from step 25.

27. Add 20 µl of 5% formic acid, incubate 10 min, then add 20 µl of 100% acetonitrile (final concentration of 2.5% v/v formic acid and 50% v/v acetonitrile) to the gel pieces and incubate for 20 to 30 min at room temperature with shaking.

28. Vortex or sonicate the gel particles and supernatant for 1 to 5 min. Microcentrifuge briefly at top speed and collect the supernatant. Pool with the previous extract from step 25.

29. Add 20 µl of 100% acetonitrile for a single extraction of 20 min with shaking. Vortex or sonicate the gel particles and supernatant for 1 to 5 min. Microcentrifuge briefly at maximum speed, collect the supernatant, and pool it with the previous extracts.

30. Concentrate the pooled extracts in an Speedvac evaporator to near-complete dryness (5 µl).

The aim is to reduce the sample volume and to remove volatile salts (such as ammonium bicarbonate).

31. Subject the samples to MS analysis (see remaining steps) or freeze them and store at –20°C for shipment to an MS facility.

Alternatively, lyophilize the samples and store them at –80°C for long periods of time.

Reconstitution of tryptic peptides

- 32a. *For MS analysis by MALDI-TOF MS:* Resuspend the dried peptides in 3 to 5 µl of 50% acetonitrile/0.1% TFA.

- 32b. *For analysis by ESI-MS:* Resuspend the dried peptides in 0.1% v/v formic acid.

The choice of reconstitution solvent system depends on the MS method to be used (see Strategic Planning). Some solvents (e.g., TFA) may not be universally compatible between approaches.

33. Sonicate for 2 to 5 min by immersion of the tubes in a bath sonicator, then microcentrifuge the tubes briefly (1 min) at maximum speed.

The optimal resuspension conditions may vary, depending on the chosen method of MS analysis (see Strategic Planning). The above techniques are suitable for general resuspension of dried peptides.

34. Analyze the reconstituted peptides by the MS method of choice (see, e.g., Coligan et al., 2003, Chapter 16).

SUPPORT PROTOCOL 4

RECYCLING OF GSH AGAROSE

GSH-agarose beads can be regenerated and reused, preferably for the same purpose (for example, preclearing of tissue lysates). In this support protocol, a method is described which removes bound endogenous GSTs from the GSH beads. Since large amounts of these beads are used in the preclearing steps (see Basic Protocol 1, the Alternate Protocol, and Support Protocol 3), it can be economical to recycle them for use in the next preparation of tissue lysates. It should be noted, however, that it is not recommended to recycle any beads that have had their bound proteins eluted for analysis by SDS-PAGE.

It is also worth noting that the GSH-agarose regeneration protocol described by the manufacturer (Amersham Biosciences) proved ineffective at removing the large quantities of bound proteins that are present after preclearing tissue lysates. The authors have found that many other treatments will remove bound endogenous GSTs from GSH agarose, including reduced glutathione, acidic glycine, and denaturants (such as SDS or guanidine). All of these treatments will remove an equivalent amount of GST from the beads (~95% after two washes); however, the order in which they are applied influences the ability to remove the remaining bound protein. Specifically, if denaturants are used initially, the protein remaining on the beads is rendered insensitive to removal by glutathione. Therefore, the optimal protocol is described herein—reduced GSH, followed by acidic glycine. After many cycles of reuse, the beads may lose some binding capacity from accumulated protein and hydrophobically bound material, so additional steps are included to remove these substances using detergents.

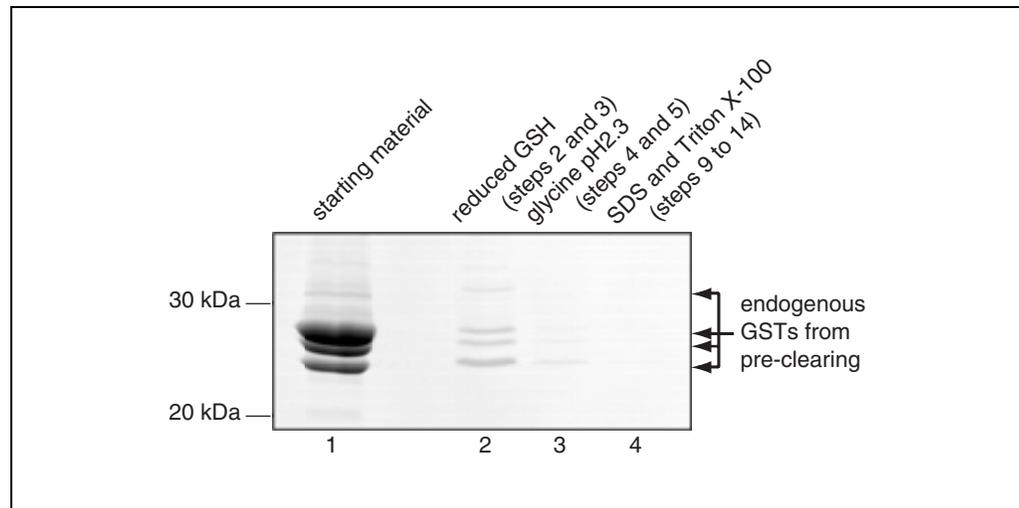


Figure 17.5.4 Regeneration of GSH agarose. The expected results from Support Protocol 4 are shown. Lane 1 shows the stained endogenous GST proteins present bound to a batch of GSH agarose that has been used for preclearing a tissue lysate. Lanes 2 to 4 show the sequential treatments which remove protein from the beads, and enable their reuse for preclearing procedures.

The sequential removal of bound endogenous GSTs that occurs from the utilization of this protocol is illustrated in Figure 17.5.4. After all the washes are performed, almost none of the endogenous GST protein remains bound to the beads.

Materials

Beads for recycling (from pre-clearing steps in Basic Protocol 1, the Alternate Protocol, or Support Protocol 3)
Glutathione wash buffer (see recipe), room temperature
Bead recycling buffer: 100 mM glycine hydrochloride, pH 2.3, room temperature
Phosphate buffered saline (PBS; APPENDIX 2A), room temperature
20% (v/v) ethanol
1% (w/v) SDS (see APPENDIX 2A)
0.1% (v/v) Triton X-100 (see APPENDIX 2A)
End-over-end rotator

1. Estimate or measure the bed volume empirically from the settled mass of beads. Take a small sample of the beads (20 to 40 μ l bed volume) before recycling for later analysis by SDS-PAGE.
2. Elute the GST proteins from the GSH beads by adding ten times the bed volume of glutathione wash buffer and incubating 1 hr at room temperature on an end-over-end rotator. Centrifuge 5 min at $500 \times g$, room temperature, in a benchtop centrifuge, then carefully remove the supernatant by vacuum aspiration or decanting.

This is a competitive elution of GST with glutathione.

3. Repeat step 2, for another 1-hr incubation with glutathione wash buffer.
4. Repeat step 2, but use bead recycling buffer in place of glutathione wash buffer.

This step uses pH 2.3 glycine to effect greater elution. The authors have noted that bound GSTs are not eluted from GSH beads at pH 2.5, hence attention to pH is essential.

5. Repeat step 4, for another 1-hr incubation with bead recycling buffer.
6. Wash the GSH beads by resuspending them in ten bed volumes of room temperature PBS. Centrifuge 5 min at $500 \times g$, room temperature, in a benchtop centrifuge, then carefully remove the supernatant by vacuum aspiration or decanting. Repeat two additional times.
7. Wash once more as in step 6, but use ten bed volumes of 20% ethanol in place of the PBS.

The ethanol is included as a preservative.

8. Store at 4°C in 20% ethanol as a 25% to 50% slurry.

At this point the beads are largely clear of specifically bound endogenous GSTs. However, it is useful to analyze the beads for the absence of bound proteins by taking a small sample (20- to 40- μ l bed volume) and analyzing the bound proteins, both before and after recycling by SDS-PAGE and Coomassie staining. If the beads show no protein bands (or very faint bands) after the above steps, they are suitable for reuse. If significant protein remains bound to the beads, continue with step 9.

After multiple cycles of recycling, the GSH beads may lose some binding capacity and become unable to efficiently extract endogenous GSTs from tissue lysates. This may be due to an accumulation of precipitated, denatured or nonspecifically bound proteins and hydrophobically bound substances. If this is found to be the case, carry out the remaining steps of this protocol to remove these.

9. Incubate the beads twice, for 1 hr each (as in step 2), but with 10 bed volumes of 1% SDS.

Note that GSH-agarose beads are stable to 1% SDS for up to 14 days at room temperature, without any loss in binding capacity.

10. Wash briefly with ten bed volumes of PBS, centrifuge 5 min at $500 \times g$, room temperature, and remove supernatant.
11. Wash beads as in step 6, but use 0.1% Triton X-100 (in water) in place of PBS.

The Triton X-100 wash is included to remove more hydrophobic material, and to complex with any residual SDS that is present on or around the beads.

12. Wash the GSH-agarose beads three times with PBS, using the technique described in step 6 except without the 1-hr incubation.
13. Wash once more as in step 6 (without the incubation), but use ten bed volumes of 20% v/v ethanol in place of the PBS.
14. Store at 4°C in 20% ethanol as a 25% to 50% slurry.

It may be necessary to re-evaluate the amount of protein bound to the beads by taking a sample (20- to 40- μ l bed volume) and analyzing by SDS-PAGE.

REAGENTS AND SOLUTIONS

Use Milli-Q purified water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Ammonium bicarbonate, 1 M

Prepare a 1 M stock solution, pH 8.0, by dissolving 0.79 g of ammonium bicarbonate in 10 ml water; there is no need to adjust the pH because it should be pH 8.0.

Store at room temperature for up a few weeks.

Bis(sulfosuccinimidyl) suberate (BS3)

Weigh out 20.5-mg aliquots of bis(sulfosuccinimidyl) suberate (B53; Pierce) into screw-capped tubes, flush with N₂ gas, and cap tightly. Store desiccated at -20°C. Immediately before use, dissolve one 20.5-mg aliquot in 1 ml of 5 mM sodium citrate buffer, pH 5.0 (38.5 mM final). Add the solution dropwise to the reaction.

Buffer A

20 mM Tris·Cl, pH 7.5
1 mM EGTA

Prepare 10 liters by dissolving 24.22 g Tris base in 10 liters water and adding 50 ml of 200 mM EGTA stock (see recipe), then adjusting the pH to 7.5 with concentrated HCl. Prepare fresh before use, store at 4°C.

Buffer B

20 mM Tris·Cl, pH 7.5
1 mM EGTA
7 μ g/ml leupeptin
1 mM PMSF

To 9 liters buffer A (see recipe), add 3.15 ml of 20 mg/ml leupeptin stock. Prepare fresh and store at 4°C without PMSF. Add 5 ml of 100 mM PMSF stock (APPENDIX 2A) per 500-ml volume to each aliquot of buffer immediately before use.

PMSF is added immediately before use because it does not last more than 45 min in solution.

Buffer C

20 mM Tris·Cl, pH 7.5
500 mM NaCl
1 mM EGTA
7 µg/ml leupeptin
1 mM PMSF

To 1.5 liters buffer A (see recipe), add 43.8 g NaCl, 525 µl of 20 mg/ml leupeptin stock. Prepare fresh before use and store at 4°C without PMSF. Add 5 ml of 100 mM PMSF stock (*APPENDIX 2A*) per 500-ml volume to each aliquot of buffer immediately before use.

Buffer D

20 mM Tris·Cl, pH 7.5
1% v/v Triton X-100
1 mM EGTA
7 µg/ml leupeptin
1 mM PMSF

To 500 ml buffer A (see recipe), add 50 ml of 10% (v/v) Triton X-100 (*APPENDIX 2A*) and 175 µl of 20 mg/ml leupeptin stock. Prepare fresh before use and store at 4°C without PMSF. Add 5 ml of 100 mM PMSF stock (*APPENDIX 2A*) immediately before use.

Colloidal Coomassie Blue stain

Mix the following components in the order indicated. Weigh 170 g ammonium sulfate into a beaker. Add 340 ml of methanol, then 36 ml of concentrated phosphoric acid, and bring to 1 liter with water. Dissolve completely using magnetic stirring and heating to 60° to 70°C for 30 min or more (it is essential to fully dissolve the ammonium sulfate before proceeding). Add 1 g of Coomassie G-250 and again stir well to dissolve (to retain the colloidal dye particles this solution must not be filtered). Store up to 6 months at room temperature.

It is extremely helpful to let this stain settle in the bottle (i.e., by leaving the bottle to sit on the bench) for a day or more before use, and then to slowly decant the stain to a clean bottle, leaving the insoluble material behind. This removes/reduces the fine particulate matter which can adhere to the gel during staining. Do not recycle this stain, discard after each use.

Final concentrations: 17% (w/v) (NH₄)₂SO₄, 3% (v/v) phosphoric acid, 34% (v/v) methanol, and 0.1% (w/v) Coomassie Brilliant Blue G-250.

Digestion buffer

Reconstitute lyophilized trypsin (sequencing-grade porcine trypsin or Trypsin Gold MS grade, Promega) in 50 mM acetic acid. Divide into aliquots and store reconstituted trypsin at -70°C.

For short periods of time, the reconstituted trypsin may be stored at -20°C, with no more than five freeze/thaw cycles.

Final concentrations: 25 mM ammonium bicarbonate, pH 8.0/12.5 ng/µl trypsin.

Disuccinimidyl suberate (DSS)

Weigh out 13.2-mg aliquots of disuccinimidyl suberate (DSS; Pierce) into screw-cap tubes, flush with N₂ gas, and cap tightly. DSS is moisture-sensitive, so store it desiccated at 4°C. Warm vials to room temperature before opening. Once opened, store under nitrogen gas (stable up to 2 years, but only if stored correctly). Dissolve DSS in DMSO (see Support Protocol 2, step 4) immediately before use, and do not keep in solution for more than 1 hr.

EGTA, 200 mM

Combine 76.08 g EGTA (acid form; mol. wt. 380.4) with 1 liter of water (or 15.22 g with 200 ml of water). Adjust pH to 7.4 with ~26 ml saturated Tris base.

The EGTA takes a long time to dissolve and to equilibrate at the correct pH; EGTA will not fully dissolve until pH is increased.

To prepare saturated Tris base, note that maximum Tris solubility at 0°C is 2.4 M, or 290.6 g/liter.

Glutathione wash buffer

Dissolve 184 mg of reduced glutathione (GSH; mol. wt. 307.3) in 8 ml TBS (see recipe for wash buffer 1), adjust to pH 8.0 with ~300 µl saturated Tris base, and make up to 12 ml with TBS (final glutathione concentration, 50 mM). Make fresh before use.

To prepare saturated Tris base, note that maximum Tris solubility at 0°C is 2.4 M, or 290.6 g/liter.

Sheep brain

One sheep brain is ~76 g. The authors normally collect about 12 brains (900 g) on each abattoir visit. At the abattoir, brains must be obtained within 1 min of sacrifice, rinsed very briefly in cold PBS (*APPENDIX 2A*), diced into 2- to 5-g cubes (~1 to 2 cm per side) on a cutting board, and frozen immediately in liquid nitrogen, before returning to the laboratory. Store frozen at -70°C and use as soon as possible (within ~3 months).

Small GTPase loading buffer

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)

2 mM EDTA

25 mM NaCl

The small GTPase loading buffer must be made fresh the same day it is to be used. It is not stable whether frozen or kept at room temperature for these experiments, as the EDTA breaks down in solution (Glebska et al., 2002).

Wash buffer 1 (TBS)

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)

150 mM NaCl

Store up to 1 year at room temperature

Wash buffer 2

20 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)

150 mM NaCl

0.2% (v/v) Triton X-100

Store up to 1 year at room temperature

Wash buffer 3

20 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
Store up to 1 year at room temperature

Wash buffer 4

20 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
150 mM NaCl
2.5 mM MgCl₂
Store up to 1 year at room temperature

Wash buffer 5

20 mM Tris·Cl pH, 7.4 (APPENDIX 2A)
150 mM NaCl
0.2% v/v Triton X-100
2.5 mM MgCl₂
Store up to 1 year at room temperature

Wash buffer 6

20 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
2.5 mM MgCl₂
Store up to 1 year at room temperature

Zinc destain solution

25 mM Tris·Cl, pH 8.3 (APPENDIX 2A)
192 mM glycine
Combine 3.03 g of Tris base and 14.4 g glycine and make up to 1 liter with water.
Do not adjust pH, it will be pH 8.3.

Zinc stain solution I

Add 10 ml of 10% (w/v) SDS (APPENDIX 2A) to 13.62 g of imidazole, and make up to 1 liter with water. Store up to 1 year at room temperature.

Final concentrations: 0.2 M imidazole, 0.1% (w/v) SDS.

Zinc stain solution II

Bring 57.51 g ZnSO₄·7H₂O to 1 liter with water (final concentration, 0.2 M). Store up to 1 year at room temperature.

COMMENTARY**Background Information**

Glutathione (γ -glutamyl cysteinyl glycine; GSH) contains a free thiol group that provides protection for cells against oxidative stress by maintaining a reducing intracellular environment. In conjunction with glutathione *S*-transferases (GSTs), glutathione also participates in detoxification of many reactive exogenous chemical substances by forming adducts through the thiol group. Glutathione-agarose (GSH-agarose, or GSH beads) are prepared by immobilizing glutathione through the thiol group (S-linked) onto beaded agarose, using a 12-atom carbon spacer to link the bead to the glutathione. GSTs bind glutathione with high affinity, and this is the basis for the use of *Schistosoma japonicum* GST in recombinant

fusion proteins. A simple purification procedure therefore utilizes the GST fusion protein's specific coupling to GSH agarose, isolation of beads, washing, and competition from GSH beads with free glutathione for elution. This is commonly used as a method for purification of GST fusion proteins, but the affinity of GST fusion proteins for GSH agarose has also been employed as a way to couple a protein of interest to a solid support for affinity chromatographic approaches.

Pull-down experiments are based on affinity chromatography. The use of GST fusion proteins for this technique is common. As an affinity chromatography technique, therefore, the pull-down experiment is ideally suited to specifically isolate protein-protein interaction

Table 17.5.2 Troubleshooting Guide for GST Fusion Protein Pull-Down Experiments

Problem	Probable/possible causes	Possible solutions
Many or all bands are identified as glutathione <i>S</i> -transferase (<i>Schistosoma japonicum</i>) Intense staining of four to five proteins of 20-30 kDa (in all lanes), which interfere with the detection of other proteins	Very large amount of GST recombinant protein used as bait The very large amount of endogenous tissue GST present in tissue lysates, binding to the remaining free glutathione sites of the GSH beads (multiple mammalian isoforms give rise to the band pattern); see Figures 17.5.4 and 17.5.5 for examples of brain GSTs	Cross-link the GST fusion protein to the GSH beads with DSS or BS3 (see Support Protocol 2). Refer to Strategic Planning for more details. Reduce the amount of GST fusion protein used. Rigorously preclear lysates with GSH beads (at least three times). Alternatively, if the GST fusion protein is cross-linked to the beads (Support Protocol 2): wash the beads (after binding to lysate) 2 to 3 times with glutathione wash buffer, followed by three washes of wash buffer 2 to specifically elute the GSTs before elution in SDS sample buffer. <i>CAUTION:</i> Washing with GSH may also elute other specifically binding proteins, due to its reducing potential, and should therefore be carefully tested.
No specific binding proteins are observed for the protein of interest relative to control	Bacterially produced GST fusion proteins are not correctly folded or post-translationally modified Tissue source for lysate inappropriate for protein of interest Washing or binding buffers may be too stringent (salt, detergent concentration interferes with binding)	Refer to literature on protein of interest and on bacterial expression systems, and change expression system accordingly. Use a nonbacterial expression system (e.g., baculovirus, in vitro translation), as long as sufficient GST fusion protein for pull-downs can be obtained. Change tissue source. Use a tissue that is known to express the protein of interest, or screen several tissues and analyze simultaneously by SDS-PAGE, to compare binding proteins from different tissues. This has the advantage of revealing binding proteins that are specific to the function of the protein of interest in a particular tissue as contrasted with other tissues. Scale up chosen tissue 10-fold. Try tissues isolated from animals of different developmental stages. Dilute out salt or detergent further (for peripheral membrane and membrane extracts). Homogenize pellets in smaller volumes (e.g., 250 ml instead of 500 ml), and then dilute to 2 liters with buffer B. Alternatively, use buffers for tissue extraction that have different properties (e.g., CHAPS, NP-40, or saponin).

continued

Table 17.5.2 Troubleshooting Guide for GST Fusion Protein Pull-Down Experiments, *continued*

Problem	Probable/possible causes	Possible solutions
Spin columns blocked and any added solutions will not flow through	Particulate contamination is present in lysate, which blocks the pores of the column's frit (10- μ m pore size)	Ensure that the lysate used is free of particulate contamination. Sometimes the particles cannot be seen with the naked eye, so it is important to thoroughly centrifuge the lysate before use at maximum speed in a high-speed centrifuge, $\geq 10,000 \times g$ for 30 min at 4°C, even if this has already been done. It is possible to obtain temporary flow in the column by the use of a thin, flat spatula to stir the beads within the column. However, such samples do not usually wash efficiently and are not likely to yield useful results.
SDS sample buffer leaks out of MicroSpin columns during heating in the 85°C water bath, resulting in lost samples	A sufficiently tight seal was not obtained when the bottom of the column was plugged	Ensure that: (1) The plug is not loose. It may be necessary to exchange and try different plugs until one is sufficiently tight. It can help to maintain a small collection of washed plugs, in case one cannot be found in the current batch that adequately seals the column. (2) The column is not cracked or unevenly broken at the base. Care must be taken early on when snapping the columns from the plugs that the plastic does not break unevenly or crack the column. It may be necessary, if it is too uneven, to trim the base of the column with a scalpel blade to ensure a flush seal.
Large amounts of nonspecific proteins are eluted from GST fusion protein beads after pull-down (particularly from membrane protein extracts)	The top cap for the column was not punched with a needle. When heated, pressure built up inside the column, forcing the SDS sample buffer out through the bottom Hydrophobic (largely membrane) proteins may adhere to the beads when washed without detergent	Punch the top screw caps of the columns with a heavy gauge needle (22-G or wider). Keep a collection of punched caps (they never contact the sample) to avoid having to punch them every time. After the binding step, wash beads with wash buffer 1 plus 0.1% Triton X-100 for the first round of washing. It is essential to subsequently wash thoroughly (at least three times) with wash buffer 2 (no Triton) to eliminate the chance of SDS-PAGE distortions caused by Triton X-100. This detergent also can interfere with MS analysis if present in the sample loaded onto the gel.
High-molecular-weight bands appear in samples eluted from non-cross-linked GST fusion protein beads after storage (e.g., the bands marked G on Figure 17.5.5)	Many GST fusion proteins (by unknown mechanisms) form polymers upon prolonged storage at -20°C that are resistant to reducing SDS-PAGE	Purify fresh GST fusion-protein beads, and perhaps cross-link beads to prevent recurrence.

continued

Table 17.5.2 Troubleshooting Guide for GST Fusion Protein Pull-Down Experiments, *continued*

Problem	Probable/possible causes	Possible solutions
Beads stick to the walls of the tubes and cannot be easily removed by washing	Agarose beads often stick to walls of tubes, particularly when using small volumes of bead/liquid mix in large tubes	<p>Polypropylene tubes (particularly 15-ml Falcon 2059 tubes) bind fewer beads than equivalent tubes of other plastics.</p> <p>Use tubes which are of a suitable volume for the volume containing the beads, or increase volume to match the tubes.</p> <p>The inclusion of a detergent (e.g., Triton X-100) may prevent beads adhering to the walls of the tubes.</p> <p>With small volumes of bead slurry, avoid vigorous vortexing when resuspending the settled beads.</p>
<i>In the Alternate Protocol:</i> Known GTP-dependent binding proteins (effectors) do not bind, or bind in a fashion that is not GTP-dependent	The GTP used for preloading of GST-small GTPase beads has been hydrolyzed to GDP, or GST-small GTPase beads were inefficiently loaded with nucleotides	<p>Ensure that:</p> <p>(1) GTP stock is sufficiently fresh (sometimes it is hydrolyzed before the expiration date).</p> <p>(2) All the Mg²⁺ was complexed with freshly prepared EDTA in the loading step. Any free Mg²⁺ that is present will promote hydrolysis of GTP at 37°C.</p> <p>Use a nonhydrolyzable analog of GTP, such as GTPγS, instead of GTP.</p>
There is still GST fusion protein eluted from the beads after chemical cross-linking, which interferes with pull-down experiment	<p>The cross-linking reaction was incomplete or inefficient, or cross-linker has degraded</p> <p>GSH washing to remove small amounts of non-cross-linked fusion protein was inadequate, or inefficient</p>	<p>Ensure that sufficient cross-linker is used; requantitate the level of protein bound to GSH beads.</p> <p>Wash more extensively with PBS before cross-linking to ensure absence of amine buffers. Cross-link for longer durations (up to 2 hr). Use freshly purchased DSS (if stored for some time).</p> <p>Switch to the more soluble cross-linker BS3.</p> <p>Wash the column after cross-linking with two 4-ml washes of bead recycling buffer, and allow the solution to pass through.</p> <p>Note that 100 mM glycine at pH 2.3 is relatively harsh, and may damage some proteins. At this pH it elutes GST from GSH, and thus can be a useful adjunct to the GSH wash. Keep the eluate (pH-neutralized) for a gel to see if any protein eluted.</p>
Poor digestion (seen as few peptides in mass spectrum, i.e., poor sequence coverage)	<p>Protein substrate has many oxidized cysteine residues, and/or is internally disulfide-bonded</p> <p>The protein of interest is not a good trypsin substrate (i.e., too few Lys and Arg residues)</p>	<p>Reduction and alkylation of the substrate can lead to improved sequence coverage (an example protocol is found in <i>UNIT 5.6</i>).</p> <p>Chose a different protease, such as LysC. This is particularly necessary for highly basic, or particularly small (<15-20-kDa) proteins with few Arg or Lys residues.</p>

continued

Table 17.5.2 Troubleshooting Guide for GST Fusion Protein Pull-Down Experiments, *continued*

Problem	Probable/possible causes	Possible solutions
	Too short or too long incubation	Need to optimize digestion time. Most proteins are completely digested after 4-6 hr. Exhaustive digestion of up to 48 hr, with an addition of a second batch of digestion buffer, could be performed in some cases to achieve a higher sequence coverage.
	Trypsin has been stored or reconstituted inappropriately, has expired, or has lost activity	Check storage conditions and expiration date Wrong reconstitution buffer. Resuspend trypsin according to manufacturer's recommendations. Multiple freeze/thaw will affect enzyme activity. Check pH of digestion buffer. Trypsin works best at pH 7-9. May need different enzyme-to-substrate ratio: add more digestion buffer for larger (more intensely stained) protein bands.
	Coomassie Blue stain interferes with digestion. (In this case it will also appear in the spectra, most likely as the strongest peak, $m/z = 800-850$ Da.)	More thorough destaining required. Under no circumstances should one proceed with trypsin digestion unless Coomassie-stained gel pieces are completely clear and colorless.
No solution remains around the gel piece after the digestion period	Insufficient rehydration with digestion buffer, before 37°C incubation The temperature difference between the bottom of the tube and the lid may result in evaporation of the liquid	Add more (10-20 μ l) of 25 mM ammonium bicarbonate, pH 8.0, before 37°C incubation Incubate longer at 4°C before digestion. Use of a 37°C oven or incubator is better than a heating block or water bath.
Polymers, detergents, and other impurities lead to poor quality MS spectra	Check all stages of experimental and sample preparatory protocols for possible sources of contamination Many brands of plastics may leach small amounts of contaminants that interfere with high-sensitivity MS techniques	In general, minimize or completely avoid the use of detergents throughout all protocol steps. Triton X-100 particularly interferes with MS. This detergent may even be present from the gel piece at sufficient levels to interfere with MS analysis. Ensure that Triton X-100 is not present in any samples for SDS-PAGE (it may also originate from an adjacent lane in the same gel). Remake all buffers from scratch. Use high-quality plastics: siliconized, highly polished, or hydrophobic-coated microcentrifuge tubes and pipet tips. Avoid screw-cap microcentrifuge tubes, as the rubber O-ring may leach plasticizers. Colored tubes and tips may also leak dyes. Pierce holes on the top of the microcentrifuge tube from the inside out to avoid contamination of the sample with plastic fragments from the lid.
	Salt and detergents in sample	Use Millipore Zip-Tips or other microcolumns to concentrate and purify the peptide samples. Follow the manufacturer's protocols.

continued

Table 17.5.2 Troubleshooting Guide for GST Fusion Protein Pull-Down Experiments, *continued*

Problem	Probable/possible causes	Possible solutions
Trypsin autolytic products interfere with and suppress other peaks in mass spectrum (note that peaks at 842 Da and 2211 Da are expected autolytic products of trypsin)	Some autolytic peaks are expected; excessive peaks may be the result of incorrect trypsin use	Sequencing-grade trypsin is modified for maximal specificity. When stored according to instructions, it is highly stable and resistant to autolysis, as the Lys residues have been reduced and methylated. Autolytic products are expected as a result of a autocleavage at Arg residues, which are not protected. Use sequencing-grade trypsin (see recipe for digestion buffer).
	Too much trypsin during digestion. Excessive volume of digestion buffer used (inappropriate for small gel pieces)	Use less: 10-15 μ l or just enough to cover gel particles. Change trypsin-to-substrate ratio. 12.5 ng/ μ l is usually enough for an average-sized protein band that has been diced to pieces and properly destained.
	Trypsin stored inappropriately, or stock freeze/thawed too often	Store according to manufacturer's recommendations. Aliquot trypsin stock and freeze/thaw no more than five times.
MALDI-TOF MS sample spots do not dry well, forming a glossy surface; crystallization is poor	Wrong buffer used to reconstitute the trypsin	Use 50 mM acetic acid, or the optimal buffer recommended by the manufacturer.
	Impurities in the sample, such as detergents or glycine from zinc-imidazole stained gels interferes with crystallization	More extensive gel washing steps are required to thoroughly remove glycine before preparing gel bands for MS analysis. Use Millipore Zip-Tips or other microcolumns to purify the peptides from the impurities. Follow the manufacturer's protocols.
High-intensity peak in mass spectrum of 832 Da suppresses all other peptide signals	The peak is most likely Coomassie blue	Destain gel piece more thoroughly before proceeding to tryptic digestion. Samples can be recovered by micropurification using Millipore Zip-Tips or other microcolumns. Follow manufacturer's protocols.
Many or all bands are identified by MS as keratin	Common contamination from skin, hair, or dust	Review and follow the recommendations and precautions as described in the introduction to Basic Protocol 2.

Protein-Protein Interactions Identified by Pull-Down Experiments

17.5.46

partners with a GST fusion of a protein of interest. Traditionally, the pull-down experiment has been used as a secondary confirmatory approach to some other protein-protein interaction detection technique, such as a yeast two-hybrid screen. Partly this has been due to the lack of a sufficiently sensitive detection technique for the identification of stained bands on an SDS-PAGE gel. Immunoblots are sensitive enough for this purpose, so once an interaction is discovered, it is relatively simple to confirm it using a pull-down experiment and a specific antibody. Rapid advances in mass spectrometry (MS) techniques have led to it becoming standard institutional or laboratory instrumentation. The sensitivity of these instruments is now sufficient to identify the proteins in a stained gel piece at the sensitivity of the staining

technique. Therefore, the pairing of GST fusion protein pull-down experiments with MS creates a new and powerful technique that is readily able to identify protein-protein interaction partners from SDS acrylamide gels (Graves and Haystead, 2002).

Some of the main advantages of the pull-down experiment in combination with MS as a means of identifying protein-protein interactions are:

1. The isolation of a protein-protein interaction partner roughly correlates to the strength of the particular protein-protein interaction. That is, the more intense a stained, identified band on SDS-PAGE, the greater the likely affinity of the interaction. It can therefore give some notion of the major (highest-affinity) interaction in a particular lysate, based on the

intensity of band staining. This is only an approximate correlation, since the intensity of staining may also be influenced by the abundance of the binding protein, or its activation state.

2. Isolation and identification of the protein-protein interaction are not dependent on any third-party detection system (e.g., transcriptional activation of a reporter gene, or selection by cellular viability).

3. The target gene is, by the nature of the approach, cloned into a suitable GST vector, and thus is immediately available for further experiments to map the interaction site using deletion or site-directed mutagenesis.

4. The assay can be internally controlled for specificity, and can simultaneously isolate protein-protein interaction partners dependent on target protein structure and/or function. This is determined by the experimental design (see Strategic Planning for more details).

5. The use of MS techniques draws the identification of a protein-protein interaction partner from an entire sequence database, and it is possible to use protein sequence databases (e.g., nonredundant or Swiss-Prot) or translated DNA sequence databases (nucleotide, EST, or even genomic) for this purpose. Therefore, if one database does not contain a match to the obtained data, other possibilities can be entertained through comparison with other databases, particularly if a binding protein is a novel protein product.

As described in Strategic Planning, MS-based techniques for protein identification are largely species-independent, at least within a group of closely related species (e.g., mammals).

Some of the limitations/disadvantages of the pull-down experiment are:

1. It is dependent on MS analysis (therefore initially expensive and technically demanding); however, results may be achieved collaboratively with MS labs or through the use of a commercial MS service.

2. The entire approach is dependent on recombinant GST fusion proteins and therefore requires optimization of expression, solubility within the bacteria, and achieving appropriate folding. Not all proteins will be compatible for expression of functional product in bacteria.

3. Not all protein-protein interactions are stable enough, or are of sufficient affinity, to be isolated by pull-down experiment. Interference can also arise from detergents, salts, and pre-existing cellular complexes, among other factors.

4. The approach can perform poorly for identification of low-abundance protein-protein interaction partners. This is particularly true for some nuclear proteins (e.g., telomerase), which exist at very low levels in cells. In this case, a good interaction may be achieved, but it may not be detected by stained SDS-PAGE gels.

5. Not all proteins isolated are direct binding partners of the protein of interest. It is common to isolate entire complexes of proteins in the pull-down experiment, where only a subset of the constituent proteins interact directly with the GST fusion protein. An example of this can be observed in Figure 17.5.5, where the entire exocyst complex is isolated with GTP-loaded GST-RalA (see Anticipated Results for more detail). It was later shown that two out of the eight exocyst complex proteins, Sec5 and Exo84, are the binding partners for RalA (Moskalenko et al., 2002; Sugihara et al., 2002; Moskalenko et al., 2003). The authors have observed several other examples where binding proteins of the partners of the target protein are isolated by pull-down experiment. Additional experiments are required to clarify the mode of binding for proteins which are isolated in this way (see Strategic Planning).

Critical Parameters and Troubleshooting

There are many factors that will contribute towards a successful outcome when using the methods in this unit. Most of these are discussed throughout the protocols in the appropriate steps. The pull-down experimental design is heavily dependent on the Support Protocols in this unit, which are the preparatory techniques required for achieving successful outcomes. The commonly encountered problems, their possible cause, and possible solutions are outlined in Table 17.5.2, in the form of a troubleshooting guide. The solutions described are remedies for the major interfering factors in these experiments; however, since this unit contains a relatively complex set of protocols, this list is by no means exhaustive. Considerations that are specific to a particular protein of interest should also be taken into account when troubleshooting any problems that are encountered. Other sources of reference should also be consulted, particularly for troubleshooting GST fusion protein expression and purification, a subject that is better and more comprehensively covered elsewhere (see, e.g., Harper and Speicher, 1997).

Anticipated Results

The intention of this unit is to isolate specific binding proteins that are specific to a protein of interest through the use of GST fusion proteins coupled to GSH-agarose beads and to prepare them for identification by mass spectrometry. An example of the type of results that can be achieved is shown in Figure 17.5.5, which illustrates a sample pull-down experiment using

GST-RalA to isolate nucleotide-specific binding proteins, by the approach described in the Alternate Protocol. Pull-down experimental results can often be interpreted in more than one way, so there are several possibilities that should be considered.

Generally speaking, the most important results are those protein bands that bind specifically to the internal controls (e.g., to one mu-

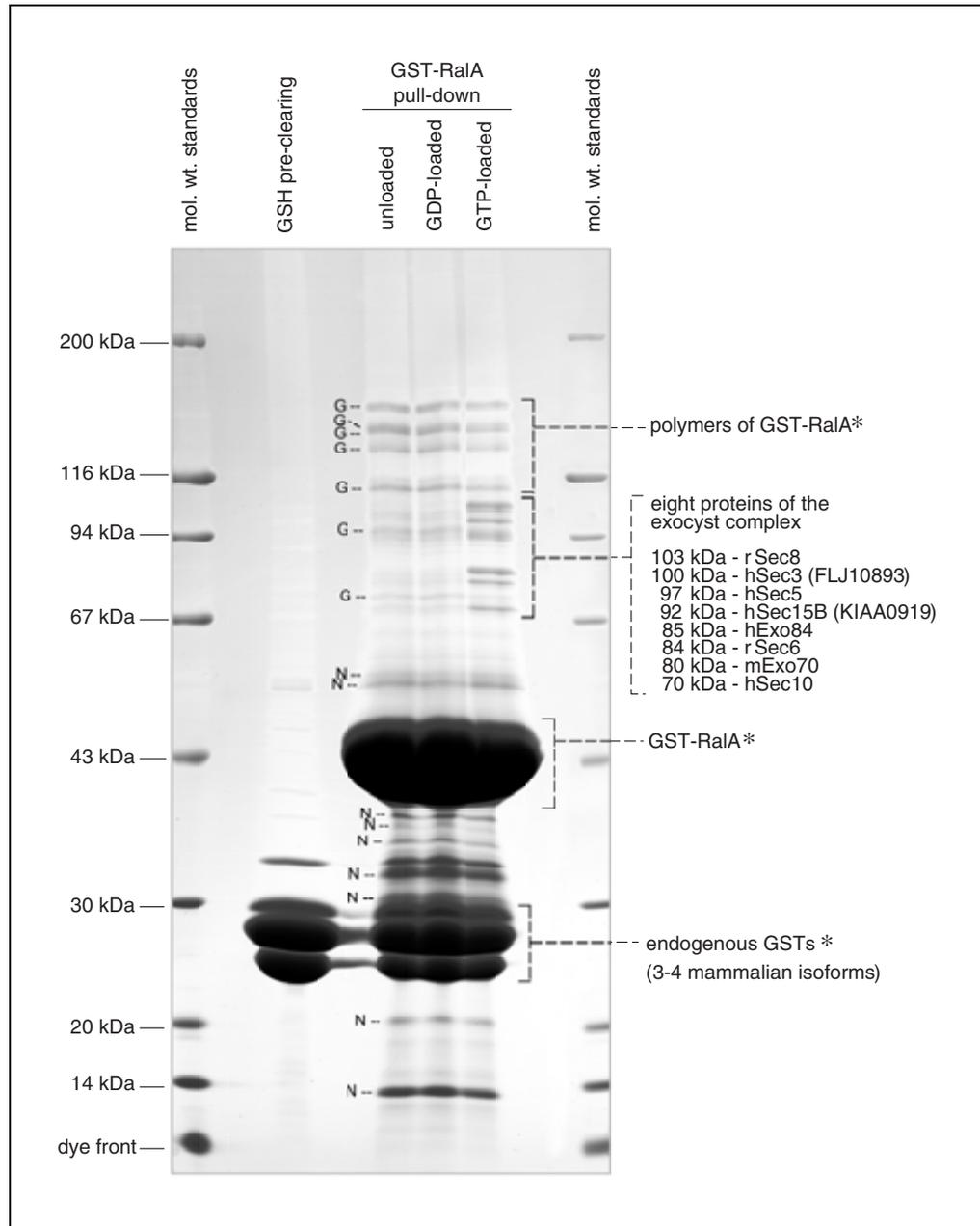


Figure 17.5.5 Sample results from a GST-RalA pull-down experiment. Shown is a Coomassie blue-stained gel of a pull-down from sheep brain cytosol using GST-tagged RalA. For a full discussion, see Anticipated Results. Stained protein bands that have been identified by MALDI-TOF MS are indicated on the right. Features marked with an asterisk (*) are interfering species. Bands that have been identified as erroneously migrating GST-RalA are marked G. The bands marked N are either nonspecific sheep proteins, fragments of GST-RalA, copurified bacterial contaminants, or real nucleotide-independent binding proteins.

Table 17.5.3 Time Considerations for GST Fusion Protein Pull-Down Experiments

Protocol	Step(s)	Action	Total time	Hands-on time
Support Protocol 1	1–2	Streak out glycerol stock	Overnight ^a	20 min
	3	Inoculate overnight culture	Overnight	20 min
	4–7	Dilute bacteria and induce GST- fusion–protein expression	1 day ^a	1–2 hr
	8–21	Purification of recombinant GST fusion proteins	1 day (including SDS-PAGE)	1 day (with breaks)
	22–23	Normalize protein concentration on beads	3–4 hr (including SDS-PAGE) ^a	1–2 hr
Support Protocol 2	All	Cross-linking of beads	5–6 hr (including SDS-PAGE) ^a	3–4 hr (with incubations)
Support Protocol 3	— ^b	Abattoir and tissue collection	1–2 hr	1–2 hr
	1–20	Tissue lysate preparation	1 (long) day, less with help ^a	1 day (with some breaks)
Basic Protocol 1 or Alternate Protocol	All, except gels	Pull-down experiment	1 day ^a	1 day (with breaks)
	21 (Basic Protocol 1 and Alternate Protocol)	Large-format SDS-PAGE	Overnight	1 hr
Basic Protocol 2	1a–5a	Staining gels with colloidal Coomassie stain	1–3 days	20 min
	1b–5b	Staining gels with zinc-imidazole negative stain	1 hr	30 min
	6–12	Gel scanning and band excision	2–4 hr ^a	2–4 hr
13a–19a	12a–18	Coomassie gel piece destaining	2–3 hr ^a	1–2 hr
13b–19b	12b–18	Zinc-imidazole gel piece destaining	1–2 hr ^a	1 hr
	20–23	In-gel digestion	6 hr to overnight	1–2 hr
	24–30	Peptide extraction	2–4 hr ^a	1–3 hr
Support Protocol 4	All	Recycling GSH beads	6–7 hr (including SDS-PAGE)	2–3 hr

^aProduct can be stored for prolonged period of time at conclusion of steps.

^bSee recipe for sheep brains in Reagents and Solutions.

tant/domain/activation state of the protein relative to others), but at the same time do not bind to the external controls (e.g., GSH or GST beads). These proteins are likely to be functionally relevant protein-protein interaction partners, since their interaction is specific to one state of the GST fusion protein under study. In Figure 17.5.5, the most striking results are the eight bands that bind specifically to GTP-loaded GST-RalA beads, between 70 and 103 kDa. These were identified by MALDI-TOF MS as the eight components of the mammalian exocyst complex (Brymora et al., 2001b). The

proteins were identified by peptide mass mapping and comparison with the mammalian non-redundant database, and it can be seen that this approach has identified the proteins—regardless of the fact that they are all from sheep lysate—as being identical to exocyst sequences from human, rat, and mouse sources. Two of the identified proteins were identified as novel human proteins (FLJ10893 and KIAA0919) and subsequently through bioinformatic analysis as new exocyst complex proteins (hSec3 and hSec15B, respectively). It is apparent that the pull-down approach, combined with the power

of mass spectrometric analysis, enabled the identification of a new, GTP-dependent binding complex for the small GTPase RalA. It should also be noted that even though this gel lacks most of the required external controls (e.g., recombinant GST beads, GST-RalA beads alone), it is still possible to assign specificity of binding to the exocyst bands, since they do not bind to the other internal states of GST-RalA (i.e., unloaded and GDP-loaded GST-RalA).

Proteins that bind to all states of the GST fusion protein beads, but not the external controls, may also be relevant binding proteins that are not regulated under the conditions used in the pull-down experiment. Also, the issue may simply be that the protein-protein interaction is a more stable, less dynamic interaction that fulfills a different cellular purpose. This type of interaction requires more characterization to establish its relevance (see Strategic Planning). It is possible that the correct conformational change for an alteration in binding could not be achieved *in vitro* with the GST fusion protein, but it might be achieved in a different system. It is also possible that they are nonspecifically binding proteins, so it is necessary to run sufficient controls to determine if this is so. In Figure 17.5.5, the protein bands marked “N” are a good example of where insufficient controls limit data interpretation. It is impossible to determine from this experiment (without using MS analysis to identify every protein band) if they represent nonspecific binding of sheep brain proteins to the recombinant GST part of GST-RalA, fragments or polymers of GST-RalA (e.g., the bands marked G), copurified bacterial contaminants, or specifically binding proteins that bind in a nucleotide-independent manner. This experiment could have been strengthened considerably (for the interpretation of these bands) by the addition of a recombinant GST with lysate control (to eliminate recombinant GST-binding proteins), and also by running an equal amount of lysate-untouched GST-RalA (to see which bands originate from GST-RalA or bacterial contamination). After these control conditions have been taken into account, it probably could be determined whether these bands are specific binding proteins.

Proteins that are present throughout all lanes (external controls and pull-down lanes) are not likely to be genuine interactions and are most likely to be nonspecific. In Figure 17.5.5, the bands marked as endogenous GSTs are a prime example of this (the level of endogenous GST

in this experiment could have been reduced significantly by repeated preclearing; see Critical Parameters and Troubleshooting). However, if there is a difference in the intensity of staining of these nonspecific bands that is disproportionate with the level of GST fusion protein coupled to the beads, there may also be a specifically binding protein that is comigrating on SDS-PAGE with the nonspecific protein. Identification of such bands requires careful analysis.

Additionally, it should be noted that identification of specific types of proteins should be viewed with caution. Cytoskeletal proteins and chaperones—notably tubulin, actin, myosin heavy chain, and hsc70—are often found in pull-down experiments to some degree. Be careful when ascribing specific binding to any of these proteins, as they are commonly present in pull-down experiments and are mostly bound nonspecifically or trapped in the spin column by filtration.

Time Considerations

An estimation of the time required for each group of steps is described in detail in Table 17.5.3. Several steps have been given a relatively wide time estimate (e.g., 4 to 6 hr), due to the number of samples being processed, experience of the investigator, and other experimental variables. Time taken to perform these experiments may actually be longer than described herein when particularly large numbers of samples need to be processed. Steps that include the time required for performing SDS-PAGE (indicated) can be shortened within 1 day by ~2 hr by freezing the samples in SDS sample buffer at -20°C and running the gels at a later time.

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Measuring Protein Interactions by Optical Biosensors

Optical evanescent wave biosensors, such as surface plasmon resonance, resonant mirror, or waveguide biosensors, have been increasingly used during the last several years for the characterization of protein-protein interactions (Margulies et al., 1993, 1996; Malmberg and Borrebaeck, 1995; Garland, 1996; van der Merwe and Barclay, 1996; Schuck, 1997a). This includes both the determination of equilibrium binding constants, which reveal the magnitude of the binding energy, as well as the determination of the bimolecular rate constants describing the kinetics of the interaction.

Studying protein interactions at a surface, as opposed to solution methods, has at least two fundamental advantages. First, the magnitude of the signal involved is independent of the affinity of the interaction over a wide range. Because one binding partner is confined to the surface (in the following referred to as ligand), the number of free binding sites that are under observation in an experiment is determined predominantly by the surface immobilization procedure (requiring usually only very small amounts of material). Therefore, the sensitivity of the method is limited essentially by the availability of the soluble binding partner (the analyte) in a well-defined state, at concentrations in the order of the inverse affinity constant. This allows studies of interactions with affinities spanning a range of at least 10^5 to 10^{10} M^{-1} , and makes this method very attractive, in particular, for high-affinity interactions. Second, the separation of the surface-immobilized sites from the mobile binding partners in solution, if combined with an appropriate fluidic handling system, is virtually ideally suited for the real-time observation of the kinetics of the binding processes. Chemical on-rate constants of up to 10^5 /Msec to 10^6 /Msec and off-rate constants of between 10^{-5} /sec and 10^{-1} /sec are generally accessible. Information on the chemical on-rate constants, and on the lifetime of the complexes formed, can be of great value, for example, in the study of interactions involved in cellular signaling processes.

However, these fundamental advantages are gained at the expense of additional experimental difficulties intrinsic to surface binding studies. These include the need for immobilization of one reactant at a relatively high local surface density (and the execution of binding studies under these conditions), the problems of nonspecific surface binding, possible multiple attachments to the surface through multivalent interactions, the problem of mass transport in kinetic experiments, and finally the possibility of surface potentials influencing the thermodynamics of the interaction (Leckband et al., 1995; Leckband, 1997).

Although the different commercial instruments for optical biosensing are based on slightly different physical principles, they all exploit surface-confined electromagnetic fields for the precise and real-time measurement of the refractive index of the medium in the immediate vicinity of the sensor surface (Garland, 1996; Knoll, 1998; Lukosz, 1991). When ligands are immobilized to the sensor surface, reversibly interacting analytes will accumulate at the sensor surface when brought into the solution above. By virtue of the difference of the refractive index increments of proteins and most aqueous buffer solutions, an increase of the total refractive index at the surface will then be reported (Fig. 17.6.1). Removal of the analyte from the solution causes dissociation of the reversibly bound molecules from the surface sites, which is accompanied by a decrease in the refractive index at the sensor surface, generating a decrease in the signal. Since the changes in signal are, to a good approximation, proportional to the changes in the mass of surface-bound analyte, the steady-state signals can be analyzed in terms of the law of

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mass action and Langmuir isotherms. The time course of the signal can be interpreted in the context of chemical binding kinetics. This basic principle, and consequently the derived strategies for studying protein-protein interactions, as well as most of the experimental procedures and potential complications, are the same in the different commercial instruments. Among the manufacturers are Biacore (<http://www.biacore.com>), Affinity Sensors (<http://www.affinity-sensors.com>), Intersens Instruments (Amersfoort, Netherlands), BioTul (<http://www.biotul.com>), and Artificial Sensing Instruments

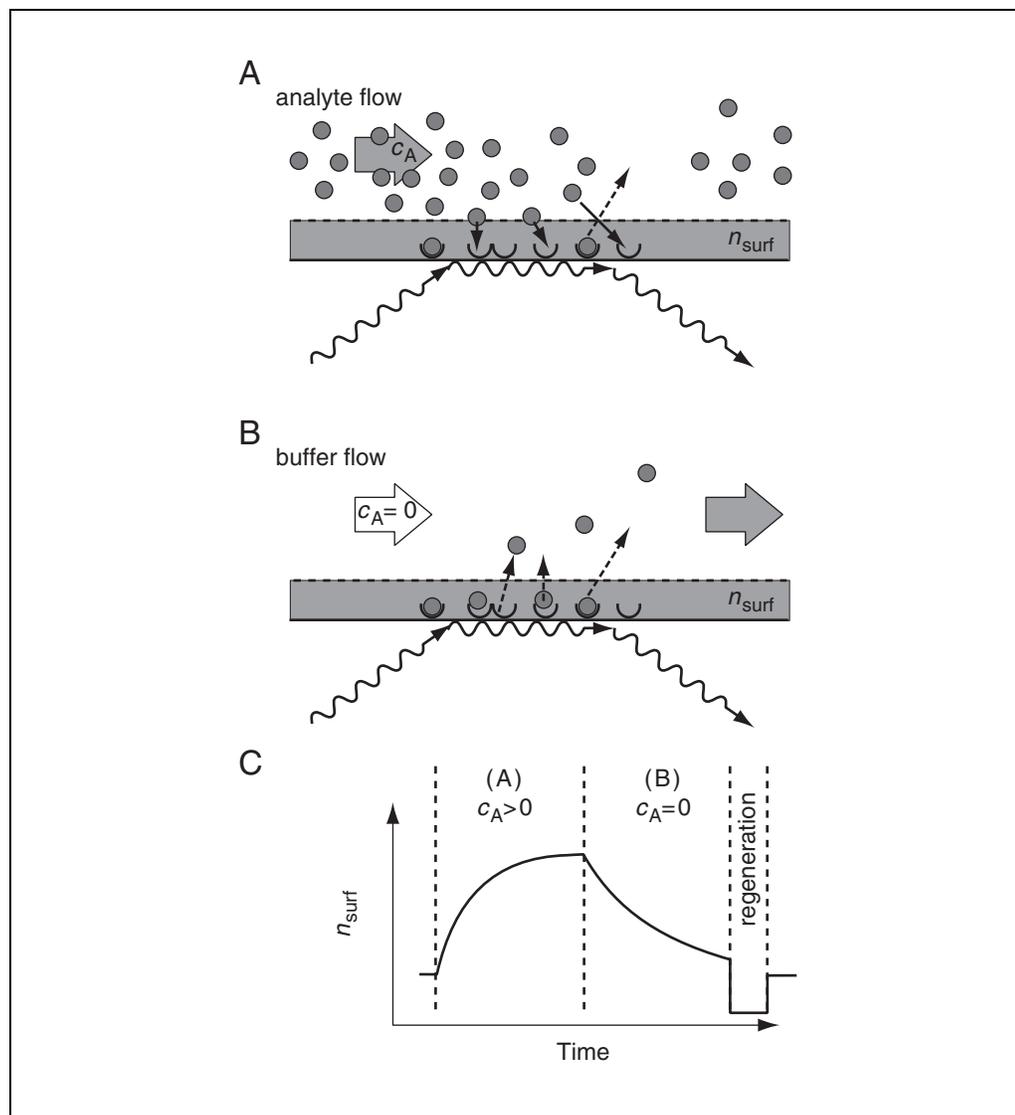


Figure 17.6.1 Schematic presentation of a typical optical biosensor experiment. Light is coupled into a structure that allows generation of surface-confined electromagnetic waves (e.g., surface plasmons in a gold film, or modes of a planar waveguide), which are sensitive to the refractive index of the solution in the range of the evanescent field, n_{surf} . Typical penetration depths of the sensitive volume into the solution are in the order of 100 nm. Ligands are attached to the sensor surface, as indicated by half-circles. **(A)** When analytes (full circles) are introduced into the solution above the surface, reversible interactions with the ligand lead to association events at a rate governed by $k_{\text{on}}c_A c_{\text{L,free}}$ (solid arrows), and dissociation events at a rate $c_{\text{complex}}k_{\text{off}}$ (dashed arrows). In the association phase, association events outnumber dissociation events until a steady state is reached. **(B)** When the surface is washed with running buffer in the absence of analyte, only dissociation events take place. **(C)** Signal obtained from probing the refractive index n_{surf} during the sequential application of the configurations depicted in A and B, given in arbitrary units. Following the association phase (A) and the dissociation phase (B), usually a regeneration procedure is applied for removing all remaining analyte from the surface before a new experimental cycle takes place.

(ASI AG, Zürich, Switzerland). The purpose of this unit is to provide a brief introduction to these techniques for the quantitative characterization of biomolecular interactions.

At the time of the introduction of optical biosensors in biomedical research, these instruments were thought to offer a particularly simple technique for the rapid quantitative measurement of equilibrium binding constants as well as binding kinetics. The reader is cautioned that the apparent simplicity and rapidity of the method is deceptive, and that the ease of using some of the commercial data analysis software packages can be highly misleading. The reliability of many of the initially used approaches in this field could not be verified in subsequent critical assessments of the methods. However, the technique has evolved significantly during the last few years. This is mainly due to the refinement and increasing variety of immobilization strategies, the identification of sources of artifacts, the use of control experiments, and the maturation of the analytical techniques (most notably global modeling). Emphasis is given in this unit to introducing the reader to robust approaches that will result in reliable results. Although the technique is frequently presented as being label-free, one of the interacting proteins has to be attached in an active form to the sensor surface. Most frequently, this is accomplished through covalent coupling, which can introduce all the problems well-known in chromophoric labeling and related covalent protein modifications. For all these reasons, the use of this technique requires careful planning and the execution of a number of different experiments, with time and effort more or less comparable to any of the other techniques available for the study of protein-protein interactions.

Optical biosensors are very versatile for the study of protein interactions. However, as described below (see Strategic Planning), the choice of the experimental strategy is usually dictated by the proteins under investigation. Only general guidelines can be given for this stage of the experiment. Detailed procedures for some of the most commonly employed immobilization procedures are described, followed by an introduction to the different strategies for the binding studies, and a brief description of some of the most commonly encountered problems. Next, the need for control experiments is emphasized, and the execution of a variety of such control experiments is described. Finally, some ideas are given for simple troubleshooting. Obviously, this introductory unit cannot be even remotely complete, or cover many of the more advanced techniques such as assembly of protein complexes (Andersen et al., 1999; Schuster et al., 1993). For many of these, the reader will be referred to the specialized literature cited throughout this unit.

STRATEGIC PLANNING

The first step in planning a biosensor experiment for the measurement of the interaction of two proteins requires the choice of the binding partner to be immobilized and the type of binding experiment that will be employed (steady-state, kinetic, or competition experiment, see Binding Experiments and Data Analysis). This involves the consideration of several factors related to size, purity, and chemical properties of the protein, as well as the stoichiometry of the interaction. In general, this will require the protein samples to be well characterized with respect to these properties.

Size, Concentration, and Volume

Optical biosensors detect changes in the refractive index at the sensor surface due to analyte binding. This signal is superimposed by an offset from the bulk refractive index differences of the buffers used). Because the refractive index increment of proteins is very similar in units of weight concentration, the signal obtained from saturation of a given number of surface sites will increase with increasing molar mass of the soluble protein

(or other analyte). In some of the commercial instruments, the binding of an analyte with a molar mass of only 1000 Da can be detected without difficulty, given the commonly achievable surface densities of sites. There is virtually no upper limit for the size of the analyte. Size consideration is important before setting up the experiment if the two binding partners to be studied are of significantly different size. The choice of a larger species as the analyte is generally preferable because this provides a larger signal. However, the immobilization of a very small peptide is usually difficult to control, and in practice frequently leads to unsuitably high density of surface sites, which can introduce problems of steric hindrance and mass transport limitation (see discussion of Mass Transport Limitation). In the absence of any other considerations, it seems advantageous to immobilize the larger binding partner, in order to diminish the probability of steric hindrance problems brought about by binding to surface sites in close vicinity. This will also help to minimize the probability of the immobilization affecting the conformation of the binding epitope. On the other hand, peptide ligands often are far more resistant to the denaturing effects of repeated cycles of binding and regeneration, and can be expected to provide a more stable binding surface than most proteins.

If one of the proteins under study is available only in small quantities, it is advantageous to immobilize this species, because during the course of a study usually only a few functionalized surfaces are needed, each requiring usually only a few micrograms of material. The analytes are used in larger amounts; in one complete set of kinetic experiments volumes of the order of 100 μl at concentrations of ten times the K_D are typically required. In competition experiments, similar quantities of the immobilized species are additionally required. The active concentration of the analyte should be well known, because errors in this value will directly translate into proportional errors of both the binding constant K_D and the chemical on-rate constant k_{on} , resulting from analysis of the experiment (see Binding Experiments and Data Analysis).

Purity

The sample purity requirements in biosensor experiments can be relatively low. In principle, the criterion for sufficient purity is the absence of cross-reaction between the two samples, aside from the interaction of the proteins under study—i.e., that no binding occurs between any contaminating species in the ligand sample and in the analyte sample. In practice, this means that at least either one of the samples should be highly purified. However, very strict requirements have to be met to ensure the absence of contaminating multimeric aggregates of the mobile species (see discussion of Analyte Aggregates, below, and Davis et al., 1998).

Oligomeric Structure, Stoichiometry, and Multivalent Binding

It is important to avoid multiple attachment of a single analyte molecule to two or more surface sites. If this occurs, the lifetime of the surface-bound complex is greatly enhanced. This avidity effect depends strongly on the local density of surface sites (Muller et al., 1998), and the binding data obtained under these conditions are governed to a large extent by the specific properties of the functionalized surface, much more than the surface binding properties of the analyte. (It cannot be assumed that the avidity effect of multivalent binding to the sensor surface is similar to possible avidity effects in binding to cell surface receptors.) Therefore, if the proteins under study are subject to multimeric binding, the multivalent species must be chosen for immobilization. For example, in antibody-antigen interactions, the antibody should be immobilized and the antigen should be the mobile analyte (Fig. 17.6.2). For unknown stoichiometries, appropriate control experiments should be performed that can identify the presence of multivalent species. For the same reason, the presence of even very small amounts of reversibly formed

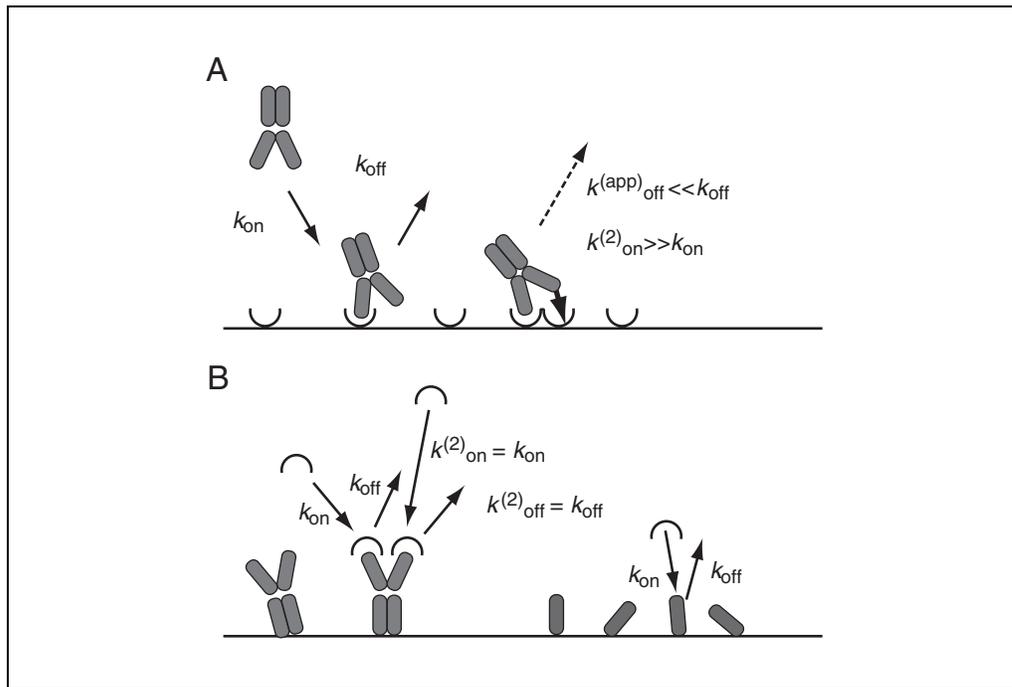


Figure 17.6.2 (A) The binding of a bivalent analyte can take place through the interaction with a single ligand molecule (left), or with two ligand molecules (right), depending on the local ligand density. If an unoccupied immobilized ligand molecule is within an accessible distance to an already singly bound analyte molecule, then the on-rate constant of the second interaction is strongly enhanced because of the entropic colocalization constraints. The overall dissociation rate constant of the double attached analyte is substantially reduced (usually by orders of magnitude), because it requires the virtually simultaneous dissociation of both interactions, which is much more improbable than a single dissociation event. The overall binding kinetics obtained from multivalent analytes is highly complex. (B) If the configuration is reversed, with the bivalent binding partner immobilized to the sensor surface, then both valencies act independently (left), and (in the absence of cooperativity of the sites) lead to binding kinetics that are identical to that of the monovalent interactions (right).

oligomers (self-association) or irreversibly formed oligomers can significantly interfere with the analysis of surface binding equilibria or kinetics. Because of the difficulties in detecting relatively weak or rapidly reversible oligomerization, solution techniques like analytical ultracentrifugation can be preferable to chromatographic or electrophoretic techniques.

Reactive Properties: Susceptibility to Immobilization, Nonspecific Binding, and Regeneration

The key to successful biosensor experiments is the immobilization of one protein species in an active, and, if possible, uniformly oriented state to the sensor surface. The ease of immobilization can be the major consideration for the choice of the immobilized species. Examples are the presence of biotin, histidine, or other tags on the molecule that allow specific capture, or the presence of a single accessible cysteine suitable for employing specific cross-linking chemistry. If a protein is produced by recombinant methods, appropriate tags can frequently be introduced.

Analogously, the protein that exhibits less nonspecific surface binding is the preferable choice for an analyte. Many sensor surfaces are slightly hydrophobic, and it is highly recommended to measure the extent of binding of both proteins to the nonfunctionalized surface at the maximal concentrations to be used in the binding experiments (e.g., at concentrations equal to 10-fold the expected dissociation equilibrium constant, K_D).

Significant degrees of nonspecific binding can make the analysis of the surface binding kinetics very difficult.

Most analytical strategies require a series of multiple association/dissociation cycles to be executed with the same surface. Therefore, conditions need to be established that allow removal of all surface-bound analyte, without permanently altering the conformation and activity of the immobilized ligand (but tolerating possible destruction of the analyte). Naturally, the choice of the regeneration procedure depends very much on the proteins. Frequently, helpful hints can be obtained from the procedure used to purify the ligand—e.g., conditions employed during ion-exchange or affinity chromatography. Examples of common regeneration procedures include exposure to low-pH glycine or HCl buffer for 1 or 2 min. For some sensitive ligands that interact weakly with their respective analytes, an extended washout with the binding buffer is the gentlest and most reliable regeneration procedure. After selection of a regeneration procedure, control experiments for the integrity of the surface and for the reproducibility of the observed signal should be performed. These should consist of several identical association/dissociation/regeneration cycles. Insufficient regeneration can be identified by residual signal increase after each cycle and/or a negative drift of the baseline. The loss of binding sites through too-harsh regeneration conditions is indicated by a maximal signal during the association phase, which decreases with each cycle. Regeneration is not required for the equilibrium-titration techniques (Myszka et al., 1998; Schuck et al., 1998).

IMMOBILIZATION PROTOCOLS

The goal of immobilization is the stable coupling of the ligand to the sensor surface in an active form. In addition to the obvious functional test of analyte binding during the course of the interaction study, probing the conformation with monoclonal antibodies against ligand epitopes can be highly useful. If possible, it is desirable to reverse the role of ligand and analyte to rule out artifacts of immobilization on the interaction. It is important to note that the immobilization methods differ in their requirement for the exposure of the ligand to relatively harsh conditions, such as pH values below the pI in the commonly employed amine coupling. A related consideration in the choice of the immobilization strategy is the orientation of the surface-immobilized ligand. Random coupling techniques, such as amine coupling, may create different subpopulations of differently reactive ligand molecules at the surface, while specific orientation techniques (e.g., through specific cysteines or through specifically attached biotin moieties) create uniform surfaces. An elegant method for allowing uniform surface attachment is the introduction of specific tags (such as cysteine or histidine) into recombinantly produced or synthetic ligands.

Below are basic descriptions of three of the most commonly used immobilization techniques, which can be applied to the most commonly employed carboxymethylated dextran sensor surfaces. In any of these methods it is very important to control the number of surface sites, since several surface-related artifacts are invoked if the surface density of the proteins under study is too high. The density of immobilized protein can be measured as the difference of the signal before and after immobilization, which can be transformed into the maximal analyte binding capacity by multiplication with the molar mass ratio of the analyte and ligand. For kinetic experiments, generally a relatively low density of immobilized protein should be obtained—i.e., a binding capacity of 50- to 200-fold of the instrumental noise [~50 to 200 units (RU) on a Biacore system]. For competition and for certain control experiments (see Competition Analysis), a larger binding capacity can be desirable. In practice, for initial orientation on the behavior of a particular system of interacting proteins, it is best to prepare several different surfaces of different ligand densities and to compare the analysis of each. This will be helpful for

diagnosing the regimes where mass transport limitations may be present (see Mass Transport Limitations), or where high surface coverage may lead to steric hindrance of analyte binding to neighboring surface sites (leading to biphasic surface binding kinetics).

Instruments that allow the on-line observation of a reference surface can be optimally utilized if the reference surface is treated as similarly as possible to the functionalized surface. This is important predominantly because of possible nonspecific interactions of the analyte with the sensor surface and the possible changes in surface properties due to immobilization. For amine coupling, this can include the application of the activation/deactivation cycle to the reference surface, but without cross-linking of any protein, or with cross-linking of a nonfunctional control protein (for example an unrelated antibody of the same isotype). For the same reason, if using biotin-streptavidin coupling, it is advantageous to immobilize streptavidin to both surfaces.

A variety of additional immobilization techniques are available in addition those described below, such as hydrazide coupling (O'Shannessy et al., 1992), Ni²⁺ chelate coupling (Gershon and Khilko, 1995; O'Shannessy et al., 1995; Sigal et al., 1996), coupling of hydrophobic groups (Stein and Gerisch, 1996), the use of self-assembled monolayers (Sigal et al., 1996) or supported lipid or hybrid bilayers (Plant et al., 1995; Ramsden et al., 1996), and immobilization to aminosilane-derivatized surfaces (Edwards et al., 1995; Buckle et al., 1993). For details on chemical modifications of the proteins, see Hermanson (1996). Additionally, capturing strategies can be used for reversible attachment of the ligand. More specific information on commercially available sensor surfaces, and step-by-step protocols for the corresponding immobilization techniques, can be found in the commercial instrument handbooks, such as the Biacore applications handbook or the IAsys applications notes.

Amine Coupling

Amine coupling is most frequently carried out using a sensor surface that is modified with carboxymethylated dextran. It relies on the electrostatic preconcentration of the ligand to the sensor surface, and therefore requires that the proteins be positively charged. The coupling buffer should be 1 to 2 pH units below the pI of the protein, of low ionic strength (to avoid charge screening), and not contain any primary amines. Effective pre-concentration can be tested before the activation of the surface. It is frequently necessary to test immobilization with several different buffers of slightly different pH; an adjustment by only one half pH unit can be crucial for a successful immobilization.

In a typical protocol, the carboxyl groups of the sensor surface are activated by exposure for 5 to 7 min with 50 mM *N*-hydroxysuccinimide (NHS) and 200 mM *N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide (EDC). Next, the ligand is injected in 10 mM sodium acetate pH 4.5 buffer. To control the amount of cross-linked ligand, it is usually better to adjust the reaction time as compared to adjustment of the ligand concentration. Repeated injection/wash cycles can be used to differentiate the covalently attached ligand from electrostatically surface-bound ligand. Finally, the surface is deactivated with 1 M ethanolamine-HCl, pH 8.5, for 7 min. Biacore and IAsys provide kits and prepared buffers for this procedure. The NHS and EDC should be kept frozen separately, in aliquots, at -20°C until use.

Avidin- or Streptavidin-Biotin Coupling

Covalent immobilization of avidin or streptavidin allows the capture of biotinylated proteins. This method avoids the need to expose the protein to a pH lower than the pI, and therefore can better preserve activity. Procedures for biotinylating the analyte are found in Hermanson (1996). The affinity of biotin-avidin interaction is very high and leads to a

very stable surface. It should be noted that streptavidin-coated surfaces tend to be more sticky and lead to an increased level of nonspecific binding of the analyte to the surface. Therefore, good blank control surfaces are important.

The amine coupling protocol described above and a carboxymethylated dextran surface can be used for this procedure. The surface is activated with NHS/EDC (see discussion of Amine Coupling), the streptavidin (~100 µg/ml in buffer of pH 4.5 to 5.0) or avidin (~50 µg/ml in pH 4.5 buffer) is immobilized, and the surface is deactivated with ethanolamine. Note that, if commercially prepared streptavidin surfaces are used, this activation step can be omitted. The biotinylated protein is then injected; usually concentrations of 10 to 100 µg/ml and low flow rates are sufficient for efficient capture. Typical times for exposure of the surface are 10 to 30 min.

Thiol-Disulfide Exchange Coupling

This immobilization technique is frequently used for acidic proteins, for small peptides, and for cases when immobilization in a specific orientation is important. It also serves as a highly efficient coupling procedure that can be used if amine coupling or avidin-biotin coupling is unsuccessful. In this method, the immobilization is accomplished either by the introduction of an active disulfide group onto the sensor surface, which can react with thiols on the protein to be immobilized, or, vice versa, by the modification of the sensor surface with a thiol group, which then can react with active disulfides on the protein. The choice between these alternative procedures is dependent on the protein, i.e., the presence of accessible thiol groups, or the potential for introducing activated disulfide groups. The latter procedure may be accomplished, e.g., using 2-(2-pyridinyldithio)ethaneamine (PDEA) to derivatize carboxyl groups or succinimidyl- α -methyl- α -(2-pyridinyldithio)toluene (SMPT) for derivatizing amino groups. It should be noted that, in general, thiol-disulfide exchange coupling cannot be used for studies in the presence of reducing agents. However, the use of covalent thioester coupling—e.g., with succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), sulfo-SMCC, or *N*-(γ -maleimidobutyryloxy)succinimide ester (GMBS)—produces a surface that is stable against reducing agents, and allows one to specifically choose the site of coupling. In addition, the latter technique provides a small spacer between the protein and the surface, which may allow better accessibility (Khilko et al., 1995).

BINDING EXPERIMENTS AND DATA ANALYSIS

Optical biosensors provide data on the time course of analyte binding, and allow for a number of different experimental and analytical strategies for interaction analysis. Most rely on a repeated cycle of association, dissociation, and surface regeneration (Fig. 17.6.1). Among the most important analysis strategies are the kinetic analysis, which takes advantage of the real-time observation to obtain the kinetic rate constants of the reaction; the steady state analysis for measuring the affinity of the reaction; and the competition analysis for measuring the affinity of the interaction in solution. The choice may be constrained by the affinity and kinetics of the proteins under study. If possible, multiple approaches should be taken and the results should be compared for their consistency. Their optimal application requires approximate knowledge of the order of magnitude of the affinity of the interaction. This may make it necessary to perform these studies in several steps.

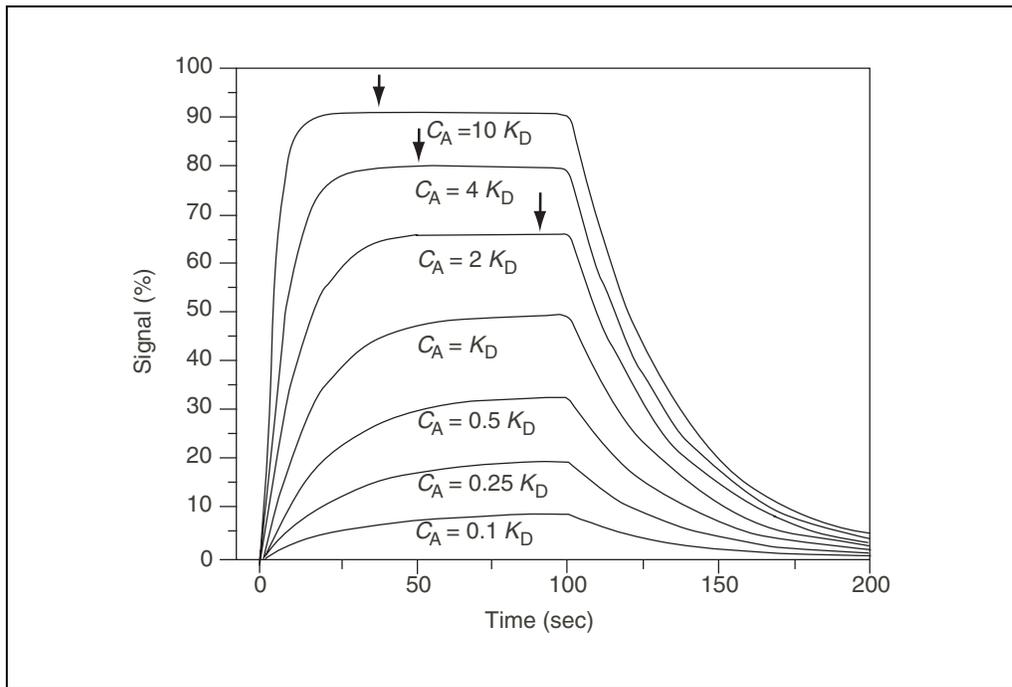


Figure 17.6.3 Schematic presentation of an ideal 1:1 pseudo first-order reaction. Shown is a superposition of the binding progress curves that should be obtained in a series of experiments at different analyte concentrations. The data are given in percent of the signal at maximal binding, but the units of the signal do not affect the results of the data analysis. The arrows on the curves at the higher concentrations indicate that steady-state binding is attained, whereas, for the lower curves, longer association times would be required as part of a steady-state analysis.

Kinetic Analysis

Experimental

The experiment consists of repeated injections of the analyte at different concentrations (Fig. 17.6.3). It should be conducted at relatively low surface density of sites, to avoid kinetic artifacts arising from mass transport limitation. An analyte concentration range as wide as possible should be covered (at least from 1/10 of the K_D to 10 times the K_D), using two-fold or three-fold dilutions. The time allowed for the surface binding phase (injection time) should be long enough for the observation of significant curvature in the binding progress, since it is only the curvature that contains the information about the reaction rate constants. It is highly desirable that steady-state binding be attained, at least for the highest concentrations used. The observation time for the dissociation phase should at least be similar to the injection time.

Data analysis

If the analyte-ligand interaction follows a pseudo first-order reaction, $L + A \rightleftharpoons LA$ then the measured binding progress (proportional to $[LA]$) is described by:

$$\frac{dR}{dt} = k_{\text{on}} c_A (R_{\text{max}} - R) - k_{\text{off}} R$$

Equation 17.6.1

where c_A denotes the analyte concentration, R denotes the signal (in arbitrary units), and R_{max} denotes the signal at maximum saturation of the surface sites. The goal is the calculation of the chemical on-rate and off-rate constants, k_{on} and k_{off} , which relate to the equilibrium constant as:

$$K_D = \frac{k_{\text{off}}}{k_{\text{on}}}$$

Equation 17.6.2

While it is possible, in principle, to transform the data as dR/dt versus R in the association phase and as $\log(R)$ versus time in the dissociation phase, where $k_{\text{obs}} = (k_{\text{on}}c_A + k_{\text{off}})$ and k_{off} , respectively, would represent the slopes of the obtained straight lines, this procedure is very unreliable. Instead, it is state-of-the-art procedure to directly fit Equation 17.6.1, or its integrated form:

$$R_{\text{assoc}}(t) = \frac{R_{\text{max}}}{1 + \frac{k_{\text{off}}}{k_{\text{on}}c_A}} [1 - \exp(-(k_{\text{on}}c_A + k_{\text{off}})t)]$$

Equation 17.6.3

$$R_{\text{dissoc}}(t) = R_{\text{assoc}}(t_d) \exp[-k_{\text{off}}(t - t_d)]$$

Equation 17.6.4

directly to the data (with t_d denoting the end of the association and the beginning of the dissociation phase, where $c_A = 0$; O'Shannessy et al., 1993). This should be done without truncation of the data beyond possible regions of artifacts from buffer changes or injections. Global analysis should be employed, which allows one to analyze all experimentally observed curves at all analyte concentrations simultaneously. This results in unambiguous best-parameter estimates for the binding constants, and provides residuals that should be inspected for comparison of the model and the data. Software needed for global analysis can be obtained from the manufacturer or as shareware. It should be noted that exclusion of specific data regions that may be poorly described by the model will introduce a bias into the analysis.

If the global analysis cannot be performed, and separate analyses of the individual binding curves of each experiment according to Equations 17.6.3 and 17.6.4 are applied, then tests for consistency of the results are crucial (Schuck and Minton, 1996a). Important tests are: (1) the estimated extrapolated values of $R_{\text{assoc}}(t \rightarrow \infty)$ at different concentrations should be consistent with a Langmuir isotherm for the estimated K_D (e.g., approaching half-saturation at $c_A = K_D$; see Figure 17.6.2); and (2) the extrapolated values of $(k_{\text{assoc}}c_A + k_{\text{off}})$ from the association phases should be consistent with the value of k_{off} as estimated in the dissociation phase. An analysis of the accuracy of the kinetic rate constants can be found in Ober and Ward (1999a).

Possible problems

The data may be only poorly described by Equations 17.6.3 and 17.6.4 (leading to a poor fit and systematically distributed residuals) and instead show multiphasic behavior. Although it is in principle possible to apply global modeling with multiple binding-sites models, this is not recommended, because it is very difficult and can easily lead to very large errors (up to several orders of magnitude). Such modeling is usually not very robust, and many different models may fit the same data equally well (Glaser and Hausdorf, 1996). The reader is also advised to be extremely cautious with some of the implemented models in commercial or shared software, because they may not be valid descriptions of real surface binding processes (for example, mass transport models or models for bivalent analytes). Frequently, the deviations of the data from the single exponential binding

progress predicted in Equations 17.6.3 and 17.6.4 are signatures of experimental artifacts, such as heterogeneity of the ligand (subpopulations of ligand with different binding properties through nonspecific immobilization), steric hindrance of binding to neighboring sites, or the presence of mass transport limitations or multivalent aggregates of the analyte (see discussion of Common Experimental Obstacles).

Steady-State Experiments and Binding Isotherm Analysis

This approach can yield both equilibrium constants and, if appended by a dissociation analysis, kinetic constants. Its main advantage with respect to the previously described technique (see discussion of Kinetic Analysis) is that it is much more robust and reliable because it is less affected by problems of potential multiphasic binding.

Experimental

If in the course of the association experiments a steady-state signal is observed, which is generally possible for interactions with relatively high k_{off} , then a binding isotherm can be constructed (Figs. 17.6.3 and 17.6.4). In an experimental procedure similar to the kinetic analysis, a concentration range as wide as possible should be covered, with two-fold concentration steps starting from both far below ($\leq 1/10$) K_D up to almost complete saturation of the surface sites, far above the K_D (≥ 10 times the K_D). The injection times required to attain steady state at the lower concentrations are longer (see Equations 17.6.3 and 17.6.4), and exponential extrapolation may be used, in particular in the Biacore system, since the association times may be limited in the by the limited volume in the injection loop of the microfluidics. Exponential extrapolation is not advised, however, for concentrations above K_D .

For interactions that do not yield steady-state binding within a tolerable time interval for the association phase, or where the ligand does not withstand a regeneration procedure, equilibrium titration represents an experimental variant (Fig. 17.6.4). In this approach, the analyte concentration is increased in two-fold steps, allowing for attainment of steady state in each step, avoiding regeneration between the steps. This can be achieved by addition of a small aliquot of the analyte into a fixed volume of solution above the sensor surface (in cuvette-based sensors; Hall and Winzor, 1997), into a loop of recycling sample (slightly modified Biacore X; Schuck et al., 1998), or into the running buffer reservoir of a standard Biacore (Myszka et al., 1998). The fixed-volume techniques require far less sample volume.

After the highest analyte concentration has been applied, the dissociation phase can be observed and analyzed in terms of Equation 17.6.4. This can give an estimate of k_{off} , which can be used in conjunction with Equation 17.6.2 to calculate k_{on} as k_{off}/K_D .

Data analysis

Although the data analysis could in principle be performed by Scatchard analysis, this is not recommended for statistical reasons. A better method is a plot of $R_{\text{steady-state}}$ versus $\log(c_A)$, in which the K_D can be easily estimated as the inflection point at half saturation of the typical sigmoid isotherm (Fig. 17.6.4). The functional form for modeling is:

$$R_{\text{steady-state}}(c_A) = \frac{R_{\text{max}}}{1 + K_D/c_A}$$

Equation 17.6.5

Even if the data cannot be fitted well with this expression, the 50% saturation will still represent a good estimate of the order of magnitude of an average K_D . It is important, however, in such an approach, to have enough data points at high analyte concentrations

for a reliable estimate of R_{\max} and the 50% saturation level, respectively. Then the robustness will be greater than that of the kinetic analysis of Equations 17.6.3 and 17.6.4.

Competition Analysis

Competition experiments can be performed in different variations, but conceptually all use a calibration of the sensor signal as a function of free analyte concentration, followed by experiments with mixtures of analyte and soluble ligand in different molar ratios. The basic presumption is that the soluble ligand competes with the immobilized ligand for the

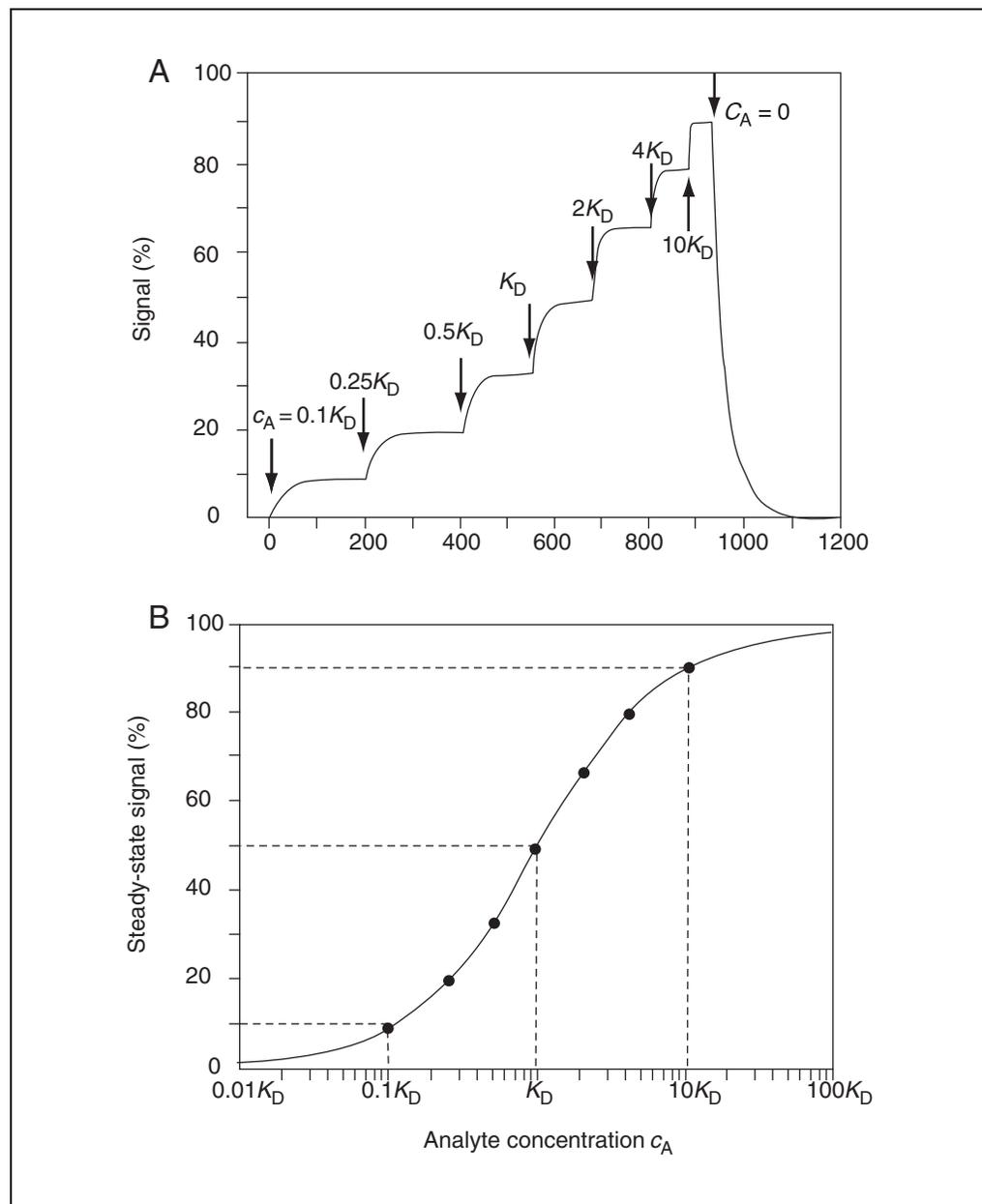


Figure 17.6.4 (A) Time course of an equilibrium titration experiment. Arrows indicate the time-points of the step-wise increase in the analyte concentration. (B) Steady-state binding analysis according to Equation 17.6.5. In a plot of the steady-state signal versus the base-10 logarithm of the analyte concentration, the binding isotherm exhibits a typical sigmoid shape. The inflection point at 50% saturation determines K_D , and the width of the curve is characterized by ~10% saturation at $1/10 K_D$ and ~90% saturation at $10 K_D$ (dotted lines). Visible inspection of the data in this representation already allows a robust parameter estimation.

free analyte, which leads to a reduction of the surface binding. This gives information on the solution affinity of ligand and analyte, while the freedom from the need to explicitly model the surface binding drastically diminishes the influence of potential surface artifacts on the results (Nieba et al., 1996; Schuck et al., 1998). This analysis can be extended to any other competitor that completely inhibits ligand binding of the analyte when bound to the analyte.

Experimental

For low-affinity systems with high chemical off-rate constants, in general, steady-state conditions can be achieved. Accordingly, the binding isotherm can be taken as a “calibration” of the sensor signal as a function of the free analyte concentration. Experiments to derive the surface binding constant can be conducted as described above (see Steady-State Experiments and Binding Isotherm Analysis). In a second variation, for high-affinity systems, mass-transport limited conditions are established by using a large immobilization density. Under these conditions, the initial binding rate constant will be directly proportional to the free analyte concentration ($dR/dt = k_t c_A$). The initial binding rate constant should be measured in equidistant concentration intervals for analyte concentrations up to a few times (two-fold) the K_D . After either one of these sets of calibration experiments, a set of experiments is performed using a constant concentration of analyte, $c_{A,tot}$ (which should be taken in the range of K_D), premixed and equilibrated with different concentrations of the soluble form of the ligand. The ligand concentrations, c_L , should be taken in two-fold steps, spanning the range from far below to far above the K_D . Choice of the fixed analyte concentration is important, since too high a value for $c_{A,tot}$ would show competition only in the range of stoichiometric binding. For systems that do not withstand regeneration, a recycling competitive titration procedure has been developed (Schuck et al., 1998).

Data analysis

For the high-affinity variant of the experiment with a linear initial rate of binding, $dR/dt = k_t c_A$, a plot of dR/dt versus c_A can be used to graphically calculate the amount of free analyte, $c_{A,free}$, during the competition experiment, or $c_{A,free}$ is calculated according to the analytical inversion $c_{A,free}(c_L) = dR/dt(c_L)/k_t$. Similarly, in place of the linear function, an empirical calibration function for $dR/dt(c_A)$ could be used. The obtained concentration of free analyte $c_{A,free}$ in experiments at different soluble ligand concentrations c_L can be plotted as $c_{A,free}$ versus $\log(c_L)$, which gives a competition isotherm with the characteristic sigmoid shape. It can be fitted with Equation 17.6.6, the solution of the quadratic equation of the mass action law combined with mass balance:

$$c_{A,free} = c_{A,tot} - \frac{1}{2} \left(c_{A,tot} + c_L + K_D^{sol} - \sqrt{(c_{A,tot} + c_L + K_D^{sol})^2 - 4c_{A,tot}c_L} \right)$$

Equation 17.6.6

which gives the solution binding constant of analyte and ligand, K_D^{sol} . If the low-affinity variant of the experiment was used, the steady-state analysis of the sensor response in the absence of soluble ligand can be performed according to Equation 17.6.5, giving R_{max} and $K_D^{surface}$. This can be used in a direct analysis of the competition steady-state response using:

$$R(c_L) = \frac{R_{max}}{1 + K_D^{surf} / \left[c_{A,tot} - \frac{1}{2} \left(c_{A,tot} + c_L + K_D^{sol} - \sqrt{(c_{A,tot} + c_L + K_D^{sol})^2 - 4c_{A,tot}c_L} \right) \right]}$$

Equation 17.6.7

Alternatively, both titrations can be analyzed simultaneously in a global fit to both isotherms, assuming the binding constants to the surface sites and in solution to be identical.

COMMON EXPERIMENTAL OBSTACLES

Two of the most important experimental problems are mass transport limitations and the effect of aggregates on the binding kinetics. The first difficulty is found most frequently (but not exclusively) in systems of medium to high affinity with high k_{on} , whereas the second is observed predominantly in systems with lower affinity, where higher analyte concentrations are required. Other obstacles can be steric hindrance effects of binding to adjacent surface sites (O'Shannessy and Winzor, 1996), or the contribution of second-order kinetics to the binding process in cuvette systems (Edwards et al., 1998). Most of these processes scale with the immobilization level of the ligand. For this reason, a low ligand density is generally advantageous in minimizing potential problems. Special considerations for working at very low ligand densities are described in Ober and Ward (1999a,b). The use of control experiments (e.g., comparison of the K_D from solution competition experiments with the K_D obtained from surface binding kinetic measurements) is very useful in verifying the absence of artifacts. The role of mass transport and analyte aggregates will be explained in detail below.

Mass Transport Limitation

If the supply of analyte to the sensor surface is the limiting factor in the surface binding rate, the observed binding kinetics are mass transport limited. This will depend directly on both the density of the immobilized sites, and on the chemical on-rate constant of the interaction. In the association phase, this will produce a zone of depleted analyte concentration in the vicinity of the surface, while in the dissociation phase, this will lead to a zone of nonvanishing free analyte concentration, which is subject to rebinding-retention effect (Silhavy et al., 1975; Fig. 17.6.5). The quantitative influence of this process on the observed surface binding kinetics is governed by a highly complex reaction/diffusion/convection process (Yarmush et al., 1996; Schuck, 1996). Although the processes in the association and dissociation phase are conceptually closely related, it cannot be assumed that the ratio of the “apparent” rate constants still reflects a good estimate of the equilibrium constant. Also, it is strongly recommended that the nonspecialist reader refrain from modeling using mass-transport-corrected kinetic models.

Instead, the primary goal is to identify mass transport limitations, and to establish experimental conditions avoiding transport effects. The following is a list of diagnostic indicators, in the order of reliability, for mass transport limited binding.

1. *Dependence on surface capacity.* This is a very good indicator of mass transport limitation, since the transport limit always directly scales with the density of active surface sites. For this reason, variations of the immobilization density of the ligand are recommended as routine tests demonstrating the absence of transport limitation and other surface-related artifacts. This test can fail in the presence of significant non-specific analyte binding to the surface.
2. *Increased dissociation rate when a soluble form of the ligand is injected (Fig. 17.6.5D).* This always strongly indicates rebinding (mass transport limitation) if detected. Since the ligand by itself should not interact with the surface, its effect is the binding to analyte near the sensor surface, preventing it from rebinding and allowing diffusion and washout from the surface (Fig. 17.6.5C). Unfortunately, this

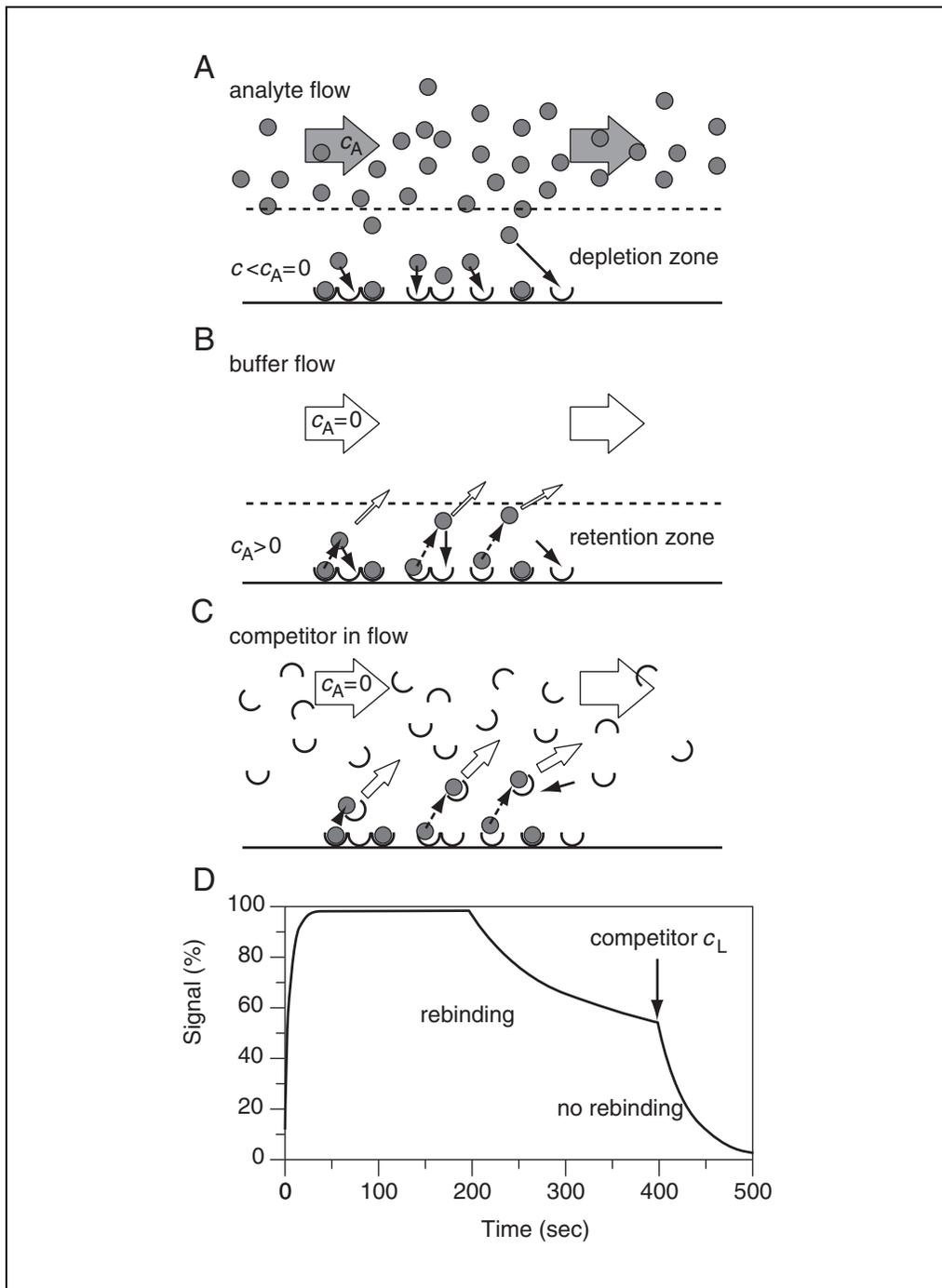


Figure 17.6.5 Mass transport effects on the surface binding process. **(A)** In the association phase, insufficient transport does not fully replenish the free analyte in a zone near the sensor surface (depletion zone). The inability to maintain the concentration c_A in the depletion zone leads to a limited surface binding rate. **(B)** The corresponding effect of mass transport limitation in the dissociation phase is that the surface is insufficiently washed and dissociated analyte is insufficiently removed. This generates a zone near the surface in which free analyte (after dissociation from the ligand) can rebind to empty ligand sites before diffusing into the bulk flow. This zone is indicated as retention zone. **(C)** Introduction of an excess of the soluble form of the ligand as a competitor into the buffer of the dissociation phase helps prevent rebinding to the surface. Soluble ligand can bind to dissociated analyte before rebinding takes place, and the soluble complex can diffuse into the buffer flow. This can allow the measurement of k_{off} free of mass transport effects. **(D)** Time course of dissociation during a mass transport induced rebinding situation (as depicted in panel B), and after introduction of a soluble competitor into the washing buffer (arrow). In the presence of the competitor, the signal reflects the chemical off-rate constant k_{off} , while in the rebinding situation, the apparent rate governing the signal is reduced.

effect may not be present for large ligands or ligands/analytes with high nonspecific binding if they exhibit limited diffusivity.

3. *Double exponential dissociation phase.* This will be seen only after dissociation from more than 50% occupation of all available surface sites.
4. *Weak dependence on the flow rate (stirring speed in cuvette-based systems).* Since the transport parameters only change with the cube root of the flow rate, i.e., generating only a factor of 2 when changing the flow rate by a factor of 10, this flow rate dependency can be difficult to detect. This is particularly true if the flow rate is only varied by a factor of 2, and the reaction is only partially transport influenced. Then, only a ~10% change in the apparent k_{off} would be expected in cases where the true k_{off} is 100% larger than the apparent k_{off} .
5. *Linear association phase.* This effect may not always be present, in particular at substantial transport limitation (Schuck, 1996; Schuck, 1997b; Schuck and Minton, 1996b).

Generally, tests 1 and 2 are the most reliable. They also lead to experimental techniques that can be utilized to reduce or eliminate mass transport artifacts. The most effective way for reducing mass transport influence is lowering the surface density of the immobilized ligand. Higher flow rates give only comparatively very small improvements, but are connected with strongly increased sample volume requirements. If the surface density of the ligand cannot be reduced further without leading to an insufficient signal-to-noise ratio, then switching from kinetic experiments to steady-state or competition steady-state experiments is the best solution. This will give information on the equilibrium constant. The kinetic rate constants can then be estimated best from a saturation experiment (approaching complete saturation of all surface sites), followed by a dissociation phase during which soluble ligand is coinjected. The soluble ligand will minimize rebinding and allow the estimation of the chemical off-rate constant, from which the chemical on-rate constant can be determined via Equation 17.6.3.

Analyte Aggregates

Oligomeric aggregates of analyte can be troublesome in biosensor experiments in two different ways. First, if trace amounts of higher oligomers are present in the analyte sample, this will lead, in the association phase, to a slow accumulation at the sensor surface, which can be visible as a slower second phase of binding. As depicted in Figure 17.6.2, these multimeric analyte aggregates can have multiple interactions with immobilized ligand molecules, and therefore they will dissociate much more slowly than the monomeric analyte. Consequently, they will appear in the dissociation phase as a submoiety with a very low off-rate constant (Davis et al., 1998). The troublesome trace amounts of oligomers can be eliminated by careful chromatographic purification, or their influence can be minimized by exchanging the role of analyte and ligand (Davis et al., 1998; Andersen et al., 1999). This is illustrated in the example of Figure 17.6.6, which demonstrates the importance of both the sample preparation and the choice of ligand and analyte.

The second potentially problematic form of aggregation is a surface-induced multimerization of the analyte. Because the local macromolecular concentrations at the sensor surface are very high (e.g., in the order of 10 mg/ml at a signal of 1000 RU in Biacore instruments), local crowding effects combined with non-specific interactions of the analyte can promote oligomer formation at the sensor surface (Fig. 17.6.7A; Minton, 1995, 1998). As with the influence of preformed aggregates, this process will lead to biphasic association and dissociation profiles, with the slower phase resulting from oligomer accumulation and dissociation, respectively. This process will be favored by

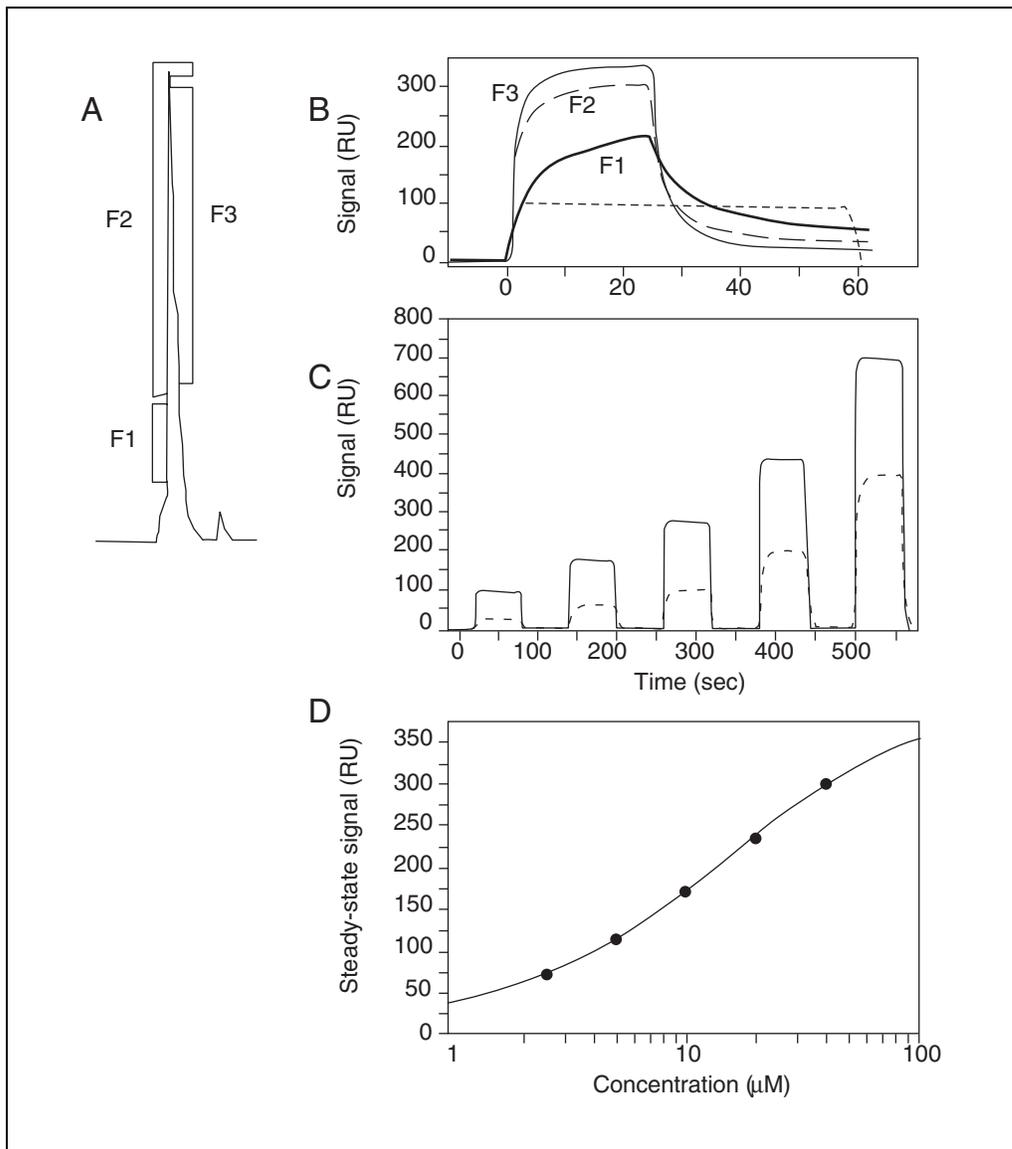


Figure 17.6.6 Exemplary data of the interaction of TCR with superantigen (for details of this interaction see Andersen et al., 1999). **(A)** Elution profile of the TCR from size-exclusion chromatography, indicating the fractions used for kinetic analysis of the interaction with immobilized superantigen, as shown in **(B)**. It should be noted that fraction 1 (bold solid line), fraction 2 (dashed line), and fraction 3 (solid line) all exhibit multiphasic binding, with different relative magnitudes of the slower component. It should also be noted that despite the lower concentration of fraction 1, which results in the lowest response in the association phase, the signal in the dissociation phase is highest. This slower kinetic component in the binding progress curve reflects different amounts of aggregates bound to the sensor surface. The aggregates have a slower dissociation because of their multivalent attachment. For comparison, the same interaction is shown in the reverse orientation, with immobilized TCR and soluble superantigen (dotted line). **(C)** Sequence of association-dissociation curves at different superantigen concentrations, binding to immobilized TCR. In this orientation, the association and the dissociation is much faster, virtually monophasic, and the binding is completely reversible, which provides further evidence that the slow kinetic components introduced in the different fractions of the soluble TCR sample in Panel B are artifacts. For quantitative analysis, the signal measured at a nonfunctionalized surface (dotted line) is subtracted in order to remove the bulk refractive index contribution of the analyte. **(D)** A plot of the net steady-state binding signal versus superantigen concentration allows the measurement of the affinity of the interaction free from artifacts due to TCR aggregation that would be introduced in the configuration of Panel B.

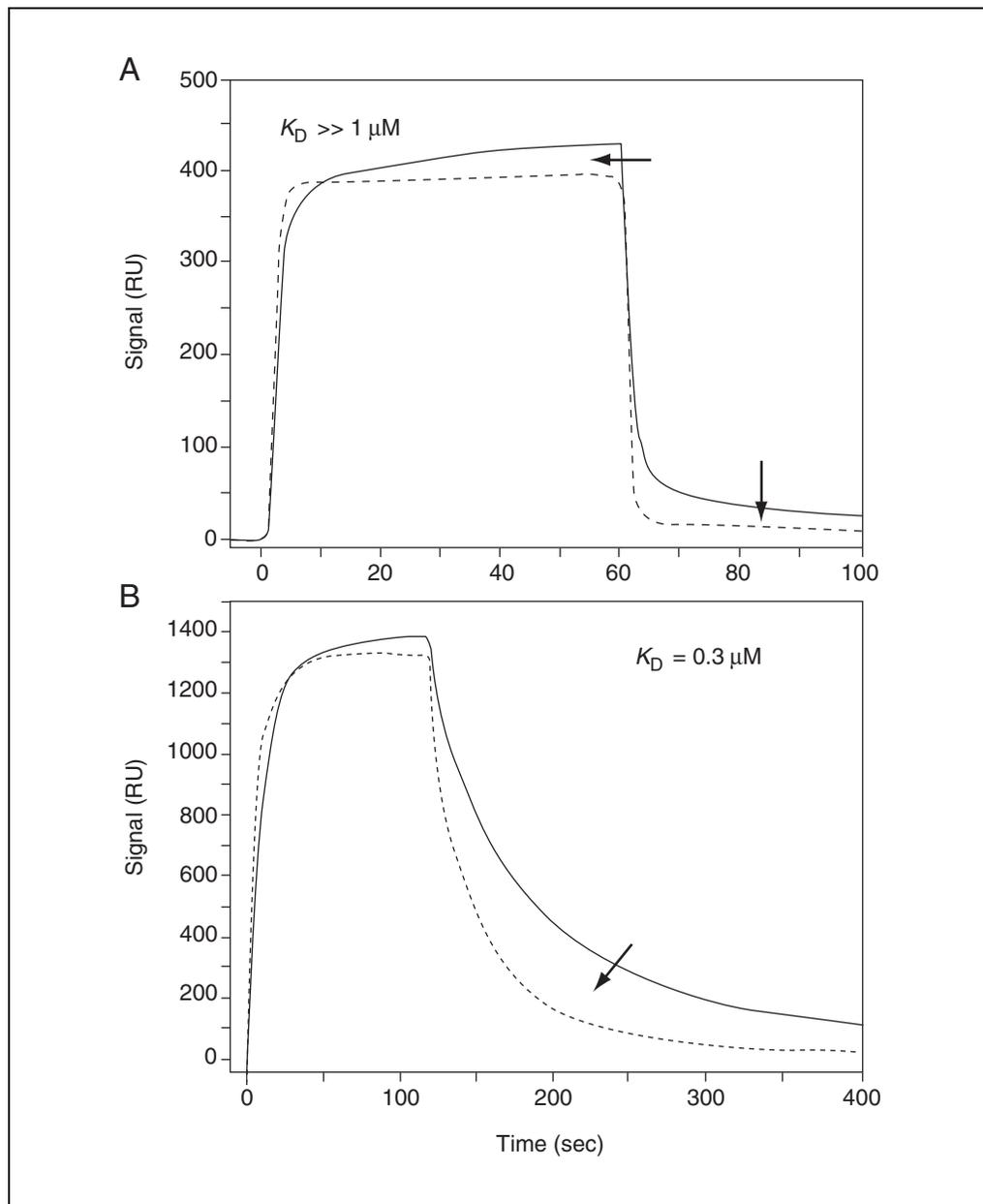


Figure 17.6.7 Effects of the ligand density on the binding kinetics for interactions with relative low affinity (**A**) and medium affinity (**B**). Each panel shows the interaction of immobilized superantigen with single-chain TCR, observed at high ligand surface density (1700 RU, solid lines) and low ligand surface density (700 RU, dashed lines). For easier comparison, the signal obtained at the lower density surface was scaled proportionally. In panel A, a slow phase of binding in the association phase and a residual binding (possible slow dissociation of multivalently bound aggregates) is introduced at high ligand density, under otherwise identical conditions. For the interaction in panel B, the chemical off-rate constant is smaller than for the low-affinity interaction shown above. Nevertheless, from comparison of the binding curves at different ligand surface densities (under otherwise identical conditions) it is obvious that an increased ligand density has significant effects on the surface binding kinetics. This observation could be due to aggregation or to mass transport limitations, both of which are more likely at higher ligand densities.

Table 17.6.1 Troubleshooting Guide for Measuring Protein Interactions by Optical Biosensors

Problem	Solution
No electrostatic preconcentration achieved; poor immobilization	Desalt protein (e.g., using spin column or microdialysis); decrease pH of buffer used for immobilization (should be below pI of protein).
Nonspecific binding is high	Analyte may be too hydrophobic, or there may be electrostatic interaction with surface. Increase salt or detergent concentration in running buffer. ^a
High signal from buffer; refractive index changes	If this does not result from high analyte concentrations, dialyze the analyte against running buffer, or use a spin column for buffer exchange.
Analyte does not come off after regeneration	Use increasingly harsher conditions for regeneration; test procedures used in affinity chromatography; check for strong nonspecific binding of the analyte; check for possible incomplete blocking of activated surface sites after immobilization.
No binding of biotinylated sample to streptavidin surface	Check for presence of free biotin in sample. Biotin on the analyte may not be accessible by surface-immobilized streptavidin; in this case try biotinylated linker.
Kinetics does not follow 1:1 binding	Check for mass-transport artifacts; check for possible traces of aggregates and for formation of aggregates at the surface; change immobilization method (avoid random coupling, avoid large surface densities). If this is not successful, go to steady-state analysis methods.
No steady-state binding is reached in a flow system	Use longer injections by increasing injection volume and/or decreasing flow rate. If sample volume is limiting, try an equilibrium titration.
Mass transport limitation	Decrease immobilization density. ^b If immobilization density cannot be lowered further, go to steady-state analysis, combined with establishment of lower limit of k_{dis} from dissociation after saturation.
Increasing slope in the association phase	Potential signature of mass transport; lower the immobilization density.
Increasing signal in the dissociation phase	Signature of mass transport limitation; lower the immobilization density.

^aBe aware of effects of nonspecific binding on the binding kinetics, which substantially decreases the diffusivity of the analyte across the sensor surface, potentially leading to mass transport artifacts that cannot be detected through change of the ligand density.

^bIncreasing the flow rate affects transport much less, but consumes much more sample.

higher surface concentrations—i.e., higher density of immobilized ligand, and by higher ligand affinity (Fig. 17.6.7). As with mass transport limitations, they can be detected by variation of the surface density of the ligand. They also can be reduced by lowering the surface density of the immobilized ligand, by size-exclusion chromatography immediately prior to the experiment, or by exchanging the role of ligand and analyte.

Troubleshooting

As with other complex biophysical techniques and all investigations involving biological samples, it is impossible to give guidelines which are even nearly complete or generally helpful for troubleshooting. Nevertheless, Table 17.6.1 presents a small list of possible solutions to potential problems, given in the hope that some readers may find them helpful.

SUMMARY

Binding studies with optical biosensors can be very powerful and versatile. Among the most important virtues are their high sensitivity and utility for a broad range of affinities, real-time detection allowing studies of binding kinetics, and relatively low requirements of sample volume. We have outlined some general strategies and described some of the most commonly used techniques. The work with protein interactions at a surface can introduce additional experimental difficulties as compared to solution methods. As a general rule in experiments with optical biosensors, it is highly recommended that analyses be performed in different ways so that the consistency of the results can be tested. Biosensors can be an excellent tool in the study of protein interactions, and become particularly powerful if combined with other methods.

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KEY REFERENCES

Davis et al, 1998. See above.

Contains a detailed description of analyte aggregation effects on the measured surface binding.

Nieba et al., 1996. See above.

Demonstration how competition approaches can be used to circumvent kinetic artifacts.

O'Shannessy et al., 1992. See above.

Collection of immobilization techniques.

Schuck, 1997b. See above.

General review of the method and its application.

INTERNET RESOURCES

<http://www.biacore.com>

Web site for Biacore; extensive list of published biosensor applications

<http://www.affinity-sensors.com>

Web site for Affinity Sensors; extensive list of published biosensor applications.

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Chromatin Immunoprecipitation for Determining the Association of Proteins with Specific Genomic Sequences In Vivo

Chromatin immunoprecipitation (ChIP) is a powerful and widely applied technique for detecting the association of individual proteins with specific genomic regions in vivo. In this technique, live cells are treated with formaldehyde to generate protein-protein and protein-DNA cross-links between molecules in close proximity on the chromatin template in vivo. A whole-cell extract is prepared, and the cross-linked chromatin is sheared by sonication to reduce average DNA fragment size to ~500 bp. The resulting material is immunoprecipitated with an antibody against a desired protein, modified (e.g., acetylated, phosphorylated, methylated) peptide, or epitope (in situations where the protein of interest is epitope-tagged). DNA sequences that directly or indirectly cross-link with a given protein (or modified variant) are selectively enriched in the immunoprecipitated sample. Thus, the method is not restricted to sequence-specific DNA-binding proteins. Reversal of the formaldehyde cross-linking by heating permits the recovery and quantitative analysis of the immunoprecipitated DNA. The amounts of specific genomic regions in control and immunoprecipitated samples are determined individually by quantitative PCR. The fold enrichment of certain chromosomal sequences (e.g., presumed binding sites) relative to other chromosomal sequences (e.g., presumed nonbinding sites) provides quantitative information about the relative level of association of a given protein with different genomic regions. Protein association with specific genomic regions can be performed under a variety of conditions (e.g., environmental change, cell-cycle status) and/or in wild-type versus mutant strains. Furthermore, as formaldehyde inactivates cellular enzymes essentially immediately upon addition to cells, ChIP provides snapshots of protein-protein and protein-DNA interactions at a particular time point, and hence is useful for kinetic analysis of events occurring on chromosomal sequences in vivo. In addition, ChIP can be combined with microarray technology to identify the location of specific proteins on a genome-wide basis (see Commentary). This unit describes the ChIP protocol for cells of the baker's yeast *Saccharomyces cerevisiae* (see Basic Protocol); however, it is also applicable to other organisms, although some organism-specific modifications related to cell lysis and sonication are necessary. A protocol for eluting immunoprecipitated protein-DNA complexes is also provided (see Alternate Protocol 1). As an alternative to gel electrophoretic analysis of the PCR products, a quantitative PCR analysis in real time with SYBR Green is also provided (see Alternate Protocol 2).

CHROMATIN IMMUNOPRECIPITATION

Materials

Saccharomyces cerevisiae cells to be studied
 37% formaldehyde: store up to 1 year at room temperature
 2.5 M glycine, heat sterilized
 TBS (APPENDIX 2A), ice cold
 FA lysis buffer with and without 2 mM PMSF (see recipe), ice cold
 ChIP elution buffer (see recipe)
 20 mg/ml Pronase (Roche) in TBS; store up to 1 year at -20°C
 TE buffer, pH 7.5 (APPENDIX 2A)
 20 mg/ml DNase-free RNase A (see recipe)
 10 \times loading buffer (see recipe)

BASIC PROTOCOL

Macromolecular Interactions in Cells

Contributed by Oscar Aparicio, Joseph V. Geisberg, and Kevin Struhl

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17.7.1

Primary antibody against protein or epitope of interest
50% (v/v) protein A-Sepharose beads (Amersham Pharmacia Biotech) or equivalent in TBS
FA lysis buffer (see recipe), room temperature
FA lysis buffer (see recipe)/0.5 M NaCl
ChIP wash buffer (see recipe)
Primers (see Critical Parameters and Troubleshooting)
3000 Ci/mmol [³²P]dATP (optional; see annotation to step 30)

2-ml screw-cap microcentrifuge tubes with (relatively) flat bottoms
~0.5-mm-diameter silica-zirconia (BioSpec; preferred) or glass beads
Mini bead beater (BioSpec; preferred) or individual or multivortexer
5-ml syringe
15-ml conical tubes, disposable
25-G needles
Sonicator with microtip probe (e.g., Branson Sonifier 250)
End-over-end rotator
0.5-ml PCR tube
Spin-X centrifuge-tube filter (e.g., Corning)
65°C water bath
PCR-purification spin column (Qiagen)
Software for analyzing PCR primers and products

Additional reagents and equipment for growth of *Saccharomyces cerevisiae* cultures (APPENDIX 3A), phenol/chloroform extraction and ethanol precipitation (APPENDIX 3A), PCR (APPENDIX 3F), agarose gel electrophoresis (APPENDIX 3A), and nondenaturing acrylamide gel electrophoresis (UNIT 6.5)

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see APPENDIX 1D).

Cross-link protein-DNA complexes in vivo

1. For each sample, grow 200 ml *Saccharomyces cerevisiae* to OD₆₀₀ = 0.6 to 0.8.

CAUTION: *Keep cultures covered or work in a fume hood to avoid noxious formaldehyde fumes.*

The volumes of culture can be reduced (20 ml is a reasonable minimum) or increased depending on need. Typically, 20 to 40 ml yeast is used for an individual immunoprecipitation, so the 200-ml volume permits multiple immunoprecipitations from the same cells. This is particularly useful for experiments involving the analysis of multiple factors or for carrying out independent immunoprecipitations involving the same factor for data reproducibility.

2. Add 5.5 ml of 37% formaldehyde (1% final). Cross-link 15 to 20 min at room temperature by occasionally swirling flask or shaking slowly on a platform.
3. Add 30 ml heat-sterilized 2.5 M glycine and incubate an additional 5 min at room temperature.

Glycine stops the cross-linking by reacting with formaldehyde.

Harvest cells

4. Centrifuge cells 5 min at 2500 × g, 4°C. Discard supernatant into a chemical waste container and resuspend pellet in 50 to 200 ml ice-cold TBS. Repeat once.

5. Centrifuge cells for a third time 5 min at $2500 \times g$, 4°C . Discard supernatant and resuspend cells in 10 ml ice-cold FA lysis buffer.
6. Pellet cells by centrifuging in a benchtop centrifuge 5 min at 3000 rpm, 4°C . Discard supernatant.

The cells can remain on ice for a few hours while other samples are being collected so that all samples may be processed as a group from this point onward. Alternatively, the cells may be frozen in liquid nitrogen or a dry ice/ethanol bath and stored up to several months at -80°C . This is particularly helpful if multiple samples are being generated during a time-course experiment. If cells are frozen, they must be thawed on ice before continuing with the procedure.

Lyse cells and isolate chromatin

For lysis using a mini bead beater (preferred)

- 7a. Resuspend the cell pellet in 1 ml ice-cold FA lysis buffer/2 mM PMSF. Fill three-quarters of a 2-ml flat-bottomed screw-cap microcentrifuge tube with ~ 0.5 -mm-diameter silica-zirconia or glass beads. Add cells, taking care to avoid introduction of bubbles, and screw the cap on tightly. Make sure there are no leaks.

The mini bead beater is recommended because it is more efficient at breaking cells (multiple samples can be broken simultaneously). Silica-zirconia beads are more efficient at breaking cells than glass beads and are also recommended. To facilitate cell breakage with the mini bead beater, it is important that the final suspension nearly fill the tube. Do not break $>160 \text{ OD}_{600}$ units of cells (i.e., $<5 \times 10^9$ cells) in a single 2-ml tube; for larger cultures, split the cells into multiple tubes.

- 8a. Lyse cells 3 min with a mini bead beater at maximum speed. Remove sample and incubate 1 min in an ice-water bath. Repeat five times for a total breakage time of 18 min.

This step assumes breakage with silica-zirconia beads. The cell breakage time with glass beads may be longer.

For lysis using an individual or multivortexer

- 7b. Resuspend in 250 μl FA lysis buffer/2 mM PMSF. Add 350 μl silica-zirconia or glass beads to a 2-ml microcentrifuge tube with relatively flat bottom. Add cells.

When using a multivortexer (or standard vortexer), it is important to keep the volume small as this improves cell breakage. Do not break $>160 \text{ OD}_{600}$ units of cells (i.e., $<5 \times 10^9$ cells) in a single 2-ml tube; for larger cultures, split the cells into multiple tubes.

- 8b. Vortex continuously on an individual or multivortexer 30 min at full speed, 4°C .

Success and reproducibility of the ChIP procedure is aided by complete (or near-complete) breakage of cells. In this regard, formaldehyde-cross-linked cells are considerably harder to break than untreated cells. The use of 1.5-ml microcentrifuge tubes with conical bottoms should be avoided because the narrow shape constricts bead movement, resulting in unequal lysis among samples. The 2-ml microcentrifuge tubes have a nearly flat bottom that allows the beads to vortex vigorously. The indicated vortexing or bead-beating conditions should be tested if a different device is used.

Isolate lysate

9. Cut a 5-ml syringe ~ 1 cm below the flared opening (i.e., where the plunger is inserted) with a razor. Insert the smaller portion into a 15-ml disposable conical tube so that the flared portion of the truncated syringe rests on top of the conical tube opening, forming a microcentrifuge-tube holder.
10. Invert the sample tube and punch a hole in the bottom with a 25-G needle. Place the sample tube into the syringe/conical tube and punch a hole in the top cover with the same needle.

11. Spin the assembly in a benchtop centrifuge 1 min at 1000 rpm, 4°C. Place the conical tube on ice. Discard the 2-ml centrifuge tube containing the dry beads after confirming the sample has been transferred to the 15-ml tube.

Occasionally, beads will clog the pierced hole and prevent complete transfer of the sample. If this occurs, pierce the tube one or two more times and repeat the step in the same 15-ml tube. No additional buffer should be added.

Shear DNA

12. Transfer the sample to a standard 1.5-ml microcentrifuge tube. Microcentrifuge 15 min at maximum speed, 4°C. Discard the supernatant and add 1 ml ice-cold FA lysis buffer to the pellet.

The pellet contains the cross-linked chromatin, cell debris, and unbroken cells. The purpose of this centrifugation step is to remove soluble protein, most of which is not cross-linked to DNA, as it might contribute to nonspecific background in the subsequent immunoprecipitations step. There is no need to resuspend the pellet at this point.

13. Holding the microtip probe near the bottom of the tube to prevent foaming, sonicate the sample 30 sec at 4°C using a continuous pulse at a power output of 20%. Cool in an ice-water bath > 1 min. Repeat two more times.

Take great care that the sample does not get too hot.

If a different sonication device is used, empirically determine the conditions necessary to achieve the desired level of DNA shearing. The shear size is determined as described below (see Critical Parameters and Troubleshooting).

14. Microcentrifuge 30 min at maximum speed, 4°C. Transfer the supernatant to a fresh 15-ml disposable conical tube, add 4 ml ice-cold FA lysis buffer, and gently mix by inversion. Remove 250 µl for checking DNA fragment size and freeze the remaining chromatin solution in 800-µl aliquots in liquid nitrogen.

Upon sonication, the cross-linked chromatin is solubilized and purified away from the pelleted material which contains cell debris and unbroken cells. The resulting chromatin solution constitutes the input sample for the subsequent immunoprecipitation. The frozen aliquots are stable for many months when stored at -70°C and are suitable for immunoprecipitations.

Check chromatin-fragment size

15. Add 250 µl ChIP elution buffer and 20 µl of 20 mg/ml Pronase in TBS to the 250-µl chromatin aliquot. Incubate 2 hr at 42°C, followed by 6 hr at 65°C. Phenol extract and ethanol precipitate sample (APPENDIX 3A).

While it is convenient to perform the reaction in a PCR machine overnight, it could just as easily be done in heat blocks or water baths. The same is true of the incubation described in step 26.

16. Resuspend in 30 µl TE buffer, pH 7.5, add 1 µl of 20 mg/ml DNase-free RNase A, and incubate 15 min at 37°C. Add 3 µl of 10× loading buffer and electrophoretically separate the material on a 1.5% agarose gel (APPENDIX 3A).

Fragments should be between 100 to 1000 bp, with an average length of 400 to 500 bp.

It is important to shear DNA fragments down to an average length of 400 to 500 bp. Longer fragments will increase the background and will decrease the resolution of the region to which the protein associates (see Commentary).

Immunoprecipitate

17. Incubate 800 µl chromatin solution with 10 µl primary antibody against the protein or epitope of interest and 20 µl of 50% (v/v) protein A–Sepharose beads in TBS on an end-over-end rotator 90 min at room temperature.

The actual amount of antibody needed has to be empirically determined and can vary considerably. The idea is to have an excess of antibody to efficiently precipitate at least 50% of the antigen in question. One way to assess the efficiency of antigen immunoprecipitation is to determine the amount of antigen present in the sample before and after the immunoprecipitation. An aliquot of 30 μ l chromatin solution, taken before and after immunoprecipitation, is usually sufficient to visualize the protein of interest via immunoblotting (UNIT 6.2) and standard chemiluminescent detection; however, the samples have to be boiled in SDS/PAGE sample buffer for 30 min prior to loading in order to reverse the formaldehyde cross-links. The immunoprecipitation conditions can be varied (e.g., time, temperature, salt concentration, presence of detergents) if necessary.

Protein A–Sepharose beads are used here because they work well with most monoclonal and polyclonal sera derived from mouse and rabbit, respectively. In some cases, the use of other beads (e.g., protein G–Sepharose) may improve binding of some antibodies, including rat IgG (see Table 7.2.1).

18. Microcentrifuge beads 1 min at 3000 rpm, room temperature. Transfer 300 μ l supernatant into a 0.5-ml PCR tube labeled “INPUT.” Discard the rest of the liquid.

Wash beads

19. Resuspend beads in 700 μ l FA lysis buffer, room temperature, and transfer mixture into a Spin-X centrifuge-tube filter.

The use of Spin-X filters aids in the recovery of the beads after washes and results in better uniformity between different samples. The procedure is also substantially faster with the filters, particularly when multiple samples are processed simultaneously. Alternatively, one could use conventional microcentrifuge tubes for the washes and aspirate the supernatant with a narrow-bore pipet tip after each spin.

20. Place the filter into a 1.5-ml microcentrifuge tube and mix sample 3 min on an end-over end rotator. Microcentrifuge 2 min at 3000 rpm, room temperature. Discard the flow-through liquid at the bottom of the tube.
21. Add 700 μ l FA lysis buffer, room temperature, to the beads and repeat step 20.

Elute protein from beads

22. Wash beads for 3 min each with 700 μ l FA lysis buffer/0.5 M NaCl, 700 μ l ChIP wash buffer, and finally 700 μ l TE.

For many polyclonal antibodies, the more stringent washes in this step result in a cleaner signal, while gentle washes frequently lead to an unacceptably high background. For some antibodies (e.g., monoclonal against peptide epitopes; see Alternate Protocol 1), repeated washes with FA lysis buffer, which are gentler, might be more appropriate.

23. Place filter unit containing the beads into a new 1.5-ml microcentrifuge tube and add 100 μ l of ChIP elution buffer. Gently pipet up and down two or three times in order to dislodge beads from the filter. Incubate 10 min in a 65°C water bath.

A water bath is used instead of other heating apparatuses in order to improve heat transfer.

24. Microcentrifuge beads 2 min at 3000 rpm, room temperature. Discard filter with beads. Transfer the eluate into a 0.5-ml PCR tube labeled “IP.”

Reverse cross-links and purify DNA

25. Add 80 μ l TE and 20 μ l Pronase in TBS to the IP tube. Combine 20 μ l INPUT material (step 18), 100 μ l ChIP elution buffer, 60 μ l TE, and 20 μ l TBS into a new 0.5-ml PCR tube.
26. To reverse cross-links, place tubes into a PCR machine. Incubate 2 hr at 42°C, followed by 6 hr at 65°C. Store samples at 4°C until use.

The incubation at 42°C allows for Pronase digestion of cross-linked polypeptides, while the 65°C incubation results in a reversal of the formaldehyde cross-links.

27. Purify DNA using a Qiagen PCR-purification spin column as per manufacturer's instructions.

This will require double loading of the spin column (i.e., 600 μ l spin through and then repeat).

Alternatively, add 20 μ l of 4 M LiCl and purify by extracting with 25:24:1 phenol/chloroform/isoamyl alcohol, followed by extraction with chloroform and ethanol precipitation (APPENDIX 3A). It is useful to add 2 μ l of Pellet Paint (Novagen) prior to the addition of ethanol, as this aids both the ethanol precipitation and visualization of the very small pellet.

28. Resuspend in 300 μ l TE and store at -20°C .

DNA pellets stored in this fashion should be stable for years.

Perform quantitative PCR

29. Design primer pairs for the desired genomic regions to be examined.

Success in obtaining high-quality data is critically dependent on good primer design (see Critical Parameters and Troubleshooting). In general, primers should be 20 to 30 bases long with a T_m of 55° to 60°C . The design of good primers is greatly facilitated by commercially available software packages such as Oligo 6.6 (see <http://www.oligo.net>) or Primer Express 1.5 (see <http://www.appliedbiosystems.com>). Most primers require no purification or special treatment prior to PCR. Amplification products should be 75 to 350 bp; longer products should be avoided, as the amplification efficiency is substantially lower. A final primer concentration of 1 μM works well for most primers, but in some instances, improved product specificity may be obtained by lowering the final primer concentration 5 to 10 fold. Refer to APPENDIX 3F for more information.

30. Dilute INPUT DNA (obtained from step 18) in three separate tubes by a factor of 5, 10, and 20. Set up standard PCR reactions (APPENDIX 3F) with 2 μ l DNA sample, primers at 1 pmol/ μ l, and total reaction volumes of 10 to 50 μ l. If PCR products will be detected by radioactivity, add 1 μCi of 3000 Ci/mmol [^{32}P]dATP.

For a typical measurement, the three dilutions of input DNA are tested along with duplicate immunoprecipitated samples (or undiluted and 5-fold diluted immunoprecipitated samples). This permits an assessment of whether the assay is being performed in the linear range as well as of the reproducibility of the PCR reaction. The immunoprecipitated DNA is typically used without dilution, although it is useful to analyze different amounts to ensure that it is also in the linear range.

There are several key parameters for achieving an optimum PCR reaction. For example, it is very important to have a quality repeat pipettor that can reproducibly dispense 2 μ l DNA. Pipetting inaccuracies at this stage will lead to greater well-to-well variability and poorer reproducibility among identical samples. Additionally, multiple primer pairs (up to 4 to 5) can be included in the same reaction, provided that the PCR products can be unambiguously resolved from each other by gel electrophoresis. This permits simultaneous and internally controlled analysis of multiple genomic regions in a single reaction. However, it is critical to ensure that there is no competition between the different primer pairs and PCR products. Also, comparable results are obtained when PCR reactions are performed in volumes between 10 to 50 μ l; using smaller volumes reduces the cost and facilitates loading of the reaction products on gels. See Critical Parameters and Troubleshooting for a discussion of primer choice.

Detection of PCR products by [^{32}P]label is recommended over detection by ethidium bromide or SYBR Green (see Alternate Protocol 2) staining as it improves the sensitivity and extends the linear range of detection; however, it necessitates using the usual precautions in working with radioactivity.

31. Carry out hot-start PCR using the following thermal cycling parameters.

Initial step:	10 min	95°C	(denaturation)
26 cycles:	30 sec	95°C	(denaturation)
	30 sec	55°C	(annealing)
	1 min	72°C	(extension)
Final step:	4 min	72°C	(final extension).

These conditions are generally appropriate for most situations. The annealing temperature may have to be adjusted if the melting temperatures of the primers is substantially above or below 55°C. The number of cycles might also have to be adjusted in some cases if reactions are not in the linear range. See Critical Parameters and Troubleshooting for more details.

Analyze PCR products

32. Add the appropriate loading buffer to the PCR products, and analyze by electrophoresis on nondenaturing polyacrylamide (UNIT 6.5) or agarose gels (APPENDIX 3A).

The gels should be stained either with ethidium bromide or SYBR Green dyes, or analyzed by autoradiography or PhosphorImager.

33. Quantitate the relative amount of PCR products using appropriate software for the accompanying instrument.

34. Calculate the apparent immunoprecipitation efficiency for a specific fragment by dividing the amount of PCR product obtained with the immunoprecipitated DNA by the amount obtained with the input DNA.

A volume of 2 μ l immunoprecipitated DNA sample (1/150 total immunoprecipitated material) contains ~200 times the number of cell equivalents as 2 μ l INPUT sample that has been diluted 5-fold (1/30,000 of the original aliquot that was immunoprecipitated). Thus, if the amount of PCR product in the immunoprecipitated sample is equal to the amount of PCR product in the 5-fold diluted INPUT sample, the apparent immunoprecipitation efficiency is 0.5%. The apparent immunoprecipitation efficiency for the background signal is typically ~0.025% to 0.05%, and it should not be higher than 0.1%.

SPECIFIC PEPTIDE ELUTION OF PROTEIN-DNA COMPLEXES IMMUNOPRECIPITATED FROM CROSS-LINKED CHROMATIN

Peptide elution represents an alternative method for removal of immunoprecipitated protein-DNA complexes from beads. In this procedure, beads containing the immunoprecipitated complexes are incubated with high concentrations of a peptide recognized by the antibody used in the immunoprecipitation. The added peptide competes with the protein antigen of interest for binding to the antibody, and specifically liberates the protein-DNA complexes from the beads. The high specificity of peptide elution reduces the nonspecific background (typically by a factor of 2 to 4), which makes it the method of choice, particularly for applications where the expected immunoprecipitation signal is low. Peptide elution is especially useful for chromatin immunoprecipitation experiments involving proteins that are tagged with the HA or myc epitopes (in single or multiple copies); however, it would also be appropriate in cases where the antibody used for the immunoprecipitation was generated against a defined peptide sequence. Peptide elution is slightly more expensive than conventional elution, due to the cost of the peptide. In general, peptide elution should be used in conjunction with gentle washes during the immunoprecipitation procedure described below, which minimizes antigen leaching. Stringent washes, such as those employed in the main method (see Basic Protocol), will often result in signals that are several-fold lower, with little or no improvement in background. Finally, peptide elution may vary in quality depending on factors such as the number of epitopes in the antigen and the relative stability of the antibody-antigen interaction.

ALTERNATE PROTOCOL 1

Additional Materials (also see *Basic Protocol*)

1 mg/ml peptide (e.g., myc, HA) in TBS (see *APPENDIX 2A* for buffer)

For this protocol, follow steps 1 to 21 of the main method (see *Basic Protocol*), replace steps 22 to 25 with the following, and continue with step 26 onwards.

22. Repeat FA lysis buffer wash (see *Basic Protocol*, steps 20 and 21) three additional times for a total of five washes.

Repeated washes with FA lysis buffer are much more gentle than the single washes with FA lysis buffer/0.5 M NaCl, ChIP wash buffer, and TE used in the Basic Protocol and result in higher signal-to-background ratios.

23. Place the Spin-X centrifuge-tube filter unit containing the beads into a new 1.5-ml microcentrifuge tube and add 100 μ l of 1 mg/ml peptide (typically myc or HA) in TBS. Gently pipet up and down two or three times in order to dislodge beads from the filter. Incubate 30 min at 30°C.
24. Microcentrifuge beads 2 min at 3000, room temperature. Discard filter with beads. Transfer the eluate into a 0.5-ml PCR tube suitable for PCR labeled "IP."
25. Add 150 μ l TE, pH 7.5, 250 μ l of ChIP elution buffer, and 20 μ l of 20 mg/ml Pronase in TBS.

**ALTERNATE
PROTOCOL 2**

**ANALYSIS OF CHROMATIN IMMUNOPRECIPITATION EXPERIMENTS BY
REAL-TIME QUANTITATIVE PCR WITH SYBR GREEN**

Quantitative PCR (QPCR) analysis in real time with SYBR Green has several advantages over the analysis of PCR reactions by gel electrophoresis (see *Basic Protocol*, step 32). First, the method saves considerable time because no gels are involved and because quantitative values are obtained directly from the data curves and do not require densitometry or phosphor imager analysis. As a consequence, this approach permits very rapid analysis of much larger numbers of chromatin immunoprecipitation samples than can be performed with the *Basic Protocol*. Using standard 96-well instruments, it is a straightforward procedure to analyze 100 to 200 samples/day (in replicates of three) with only 1 to 2 hr of total setup time. With newer 384-well instruments and automated robotics equipment, sample throughput can be further increased to thousands per week. Second, the data generated by this procedure are more accurate and reproducible, because quantitative values are determined from continuous sampling throughout the PCR reaction rather than a single end-point determination. Furthermore, the quality and "linear range" of every PCR reaction are directly visualized. Third, the procedure is significantly safer for the researcher, as no radioactive materials or toxic acrylamide are used. The major disadvantage of this procedure is that the measurements are performed individually and hence are not internally controlled, whereas the *Basic Protocol* permits the simultaneous analysis of multiple genomic regions in a single PCR reaction (provided the individual primer pairs function independently). As such, the *Basic Protocol* is more useful for analyzing the same small set of genomic regions under multiple experimental conditions and for simultaneous analysis of electrophoretically distinguishable alleles of a given genomic region.

SYBR Green is a sensitive and highly selective double-stranded DNA (dsDNA)-binding dye that remains associated even at the high temperatures normally used for PCR template extension. Real-time PCR reactions involving SYBR Green are performed with standard oligonucleotide primers, and hence are much less expensive than real-time PCR reactions using fluorophore-conjugated oligonucleotides (e.g., TaqMan or Lux probes). Measurements of SYBR Green fluorescence at the polymerase extension step of PCR, when

plotted against PCR cycle number, provide both a qualitative assessment of the progress of the PCR and a way to quantitate the relative amount of DNA template initially present in the reaction. Typical real-time QPCR graphs feature the plot of the \log_{10} (Net fluorescence) on the y axis versus the PCR cycle number on the x axis, and usually contain three well-defined stages: (1) baseline, (2) linear, and (3) plateau. In the baseline stage, the amount of DNA product formed is still below the sensitivity threshold of SYBR Green, so product formation is undetectable. This part of the curve is typically used as a baseline for SYBR Green signal drift. The linear part of the curve is the most important from the analytical standpoint, because it is at this stage that the rate of PCR product accumulation is both constant on a per-cycle basis and readily detectable by increased SYBR Green fluorescence. Finally, as all of the SYBR Green in the reaction becomes bound to the recently synthesized PCR products, the amount of fluorescence stays constant from cycle to cycle and the reaction reaches a plateau.

In the protocol described below, PCR is performed under special conditions that minimize the inhibitory effects of SYBR Green on *Taq* activity and maximize the linear range of product detection. After amplification is complete, raw data are stripped of outliers and exported in a format readable by a spreadsheet program such as Microsoft Excel. Finally, data points from replicate samples are averaged, and mean values are further manipulated and ultimately compared to some internal reference or control.

Additional Materials (also see *Basic Protocol*)

Input DNA (see Basic Protocol, step 28)
Immunoprecipitated fragments (“IP” sample; see Basic Protocol, step 23)
2× SYBR Green *Taq* mix (see recipe)

Real-time PCR machine and corresponding software (e.g., ABI)
96-well PCR plates (ABI, cat. no. 4306737) and optical adhesive covers
Centrifuge with swinging-bucket rotor and microtiter plate adapter
Spreadsheet program (e.g., Microsoft Excel)

Set up PCR reactions

1. Dilute input DNA to an approximate equivalent of 1×10^6 cells/ml in TE buffer, pH 7.5.

If immunoprecipitations were performed as described in the Basic Protocol, then a 1:25 dilution of the input sample from step 28 will result in 1:1000 overall dilution and will correspond to $\sim 5 \times 10^8$ to 1×10^9 cell equivalents.

2. If necessary, resuspend immunoprecipitated fragments in TE buffer, pH 7.5, so that the approximate cell equivalent is 1×10^9 cells/ml.

Immunoprecipitated DNA derived from the IP sample obtained by the Basic Protocol (step 23) is appropriately diluted and needs no further treatment.

3. Prepare PCR primer stocks by mixing each primer pair at a final concentration of 3.3 μ M in TE buffer, pH 7.5.

It is critical to test newly obtained primer pairs for amplification specificity and performance under conditions that will be used for real-time PCR with SYBR Green (see Critical Parameters and Troubleshooting). SYBR Green can inhibit PCR reactions, and primer pairs that are appropriate for quantitative PCR analysis in the absence of SYBR Green may not work well in the presence of SYBR Green. High-quality primer pairs should result in ~ 1.9 -fold amplification/cycle (this can be determined from quantitative analysis of raw fluorescence data for each cycle, which is generally available on commercial instruments). Amplified material at the completion of the PCR should contain only one band

(as assayed on high-percentage agarose or polyacrylamide gels). Specificity information may also be obtained by running dissociation curves on reactions following the conclusion of the PCR run. Typically, samples are melted for 15 min at 95°C, cooled to 60°C, and then slowly heated back up to 95°C over a period of 20 min. Plotting the first derivative of the fluorescence against the temperature allows for simple visual identification of sample heterogeneity. Some instrument-specific software packages have built-in modules for dissociation curve analysis.

4. Select and label the wells to be used in the run.

In general, individual samples should be run in triplicate. Obvious outliers occur with some frequency, generally at <5%. Triplicate analysis of samples permits removal of those outliers while still allowing for inclusion of two accurate measurements for each sample. While this reduces the number of different samples that can be run at any given time, the resulting data is much more reliable and accurate.

For each primer pair examined, the input DNA samples should be run alongside the immunoprecipitated samples. Amplification efficiencies among different primer pairs vary slightly on a per-cycle basis, but those slight variations in efficiency translate into substantially different amounts of amplified material in the cycle range used for analysis. Precise quantitation of relative binding cannot be accurately performed without primer pair-specific input signal.

Detailed instructions on the use of the real-time PCR machine and general issues, e.g., calibration and camera exposure settings, are addressed in the documentation that accompanies the instruments.

5. Program the real-time PCR machine as follows:

1 cycle:	10 min	95°C	(initial denaturation)
40 cycles:	30 sec	95°C	(denaturation)
	30 sec	53°C	(annealing)
	30 sec	72°C	(extension).

Collect the data only at 72°C.

The annealing temperature may have to be adjusted if the melting temperatures of the primers are substantially above or below 53°C. If the desired amplification product is >500 bp (this is not recommended), the extension time at 72°C should be increased to 1 min. See Critical Parameters and Troubleshooting for more details.

6. Using a small-volume automatic pipettor (20- μ l capacity), place a 2- μ l aliquot of each DNA template into the appropriate wells of a 96-well PCR plate. Gently tap the plate to allow the sample droplets to fall to the bottoms of the wells.

It is very important to have a quality repeating pipettor that can reproducibly dispense small volumes of sample into the wells. Pipetting inaccuracies at this stage will lead to greater well-to-well variability and poorer reproducibility among identical samples.

7. Using a small-volume automatic pipettor (20- μ l capacity), place a 3- μ l aliquot of primer mix (see step 3) into the relevant wells and tap the plate a few times to settle the contents.

On many real-time PCR machines, results from 10- μ l reactions are virtually indistinguishable from those of 25- and 50- μ l reactions in their accuracy and reproducibility. The use of 10- μ l reactions provides substantial savings in reagent costs. On some machines, the minimal reaction volume needed for accurate and reproducible results may be greater.

8. With a larger automatic pipettor (100- μ l capacity), add 5 μ l of 2 \times SYBR Green Taq Mix to every assayed well. Place microtiter plate into appropriate microtiter plate adapter and centrifuge 1 min at 200 \times g, room temperature, in a swinging-bucket rotor.

The 2× SYBR Green Taq Mix contains a variety of components that considerably reduce the inhibitory effect of SYBR Green, thereby resulting in more reproducible signals that require fewer amplification cycles. Comparable mixes containing proprietary buffers can be obtained commercially. It is critical that quantitative PCR reactions containing SYBR Green be performed under conditions of efficient amplification (e.g., 1.9-fold amplification/cycle).

9. Seal plate with clear optical adhesive covers, overlay foam compression pad with gold side facing up, and place into the real-time PCR machine. Secure lid.

The details of this step may differ, depending on the machine.

10. Start the PCR protocol (see step 5). After completion, save the run for future analysis.

DATA analysis

11. Open the file containing the real-time data according to the manufacturer's instructions for the instrument.

Although the specific protocol will depend on the software and instrument, the overall logic and approach to the analysis of real-time data is generally applicable.

12. Look at the different curves and set the baseline as needed.

Generally, the baseline should be set from cycle 3 to the cycle just prior to where the curves start increasing in a linear fashion. It is desirable to have at least 10 cycles for the calculation of the baseline, as this results in increased accuracy in the subsequent calculations of the threshold cycle.

13. Change the value in the Threshold box to be about halfway up in the linear range, and apply changes to the data set.

The threshold cycle is defined to be the PCR cycle at which the fluorescence is 10 times (10 is the default multiplier) the standard deviation obtained in the baseline calculation. When the multiplier is set to 10, the fluorescence at the threshold cycle is considered the lowest fluorescence value that is significantly above the background. In practice, this number frequently lies in the nonlinear range of many of the curves. For later calculations, it is easier to manually set the fluorescence value used to calculate the threshold cycles to 0.04. At this value, all the curves should be in the linear range and well above the baseline, allowing for far more accurate comparisons of the threshold cycles. On occasion, however, it will be necessary to adjust this value either up or down to better reflect the linear range of net fluorescence for most of the curves.

14. Manually select one group of triplicates and visually inspect their amplification plots. If curves are essentially superimposable and the threshold cycle (C_T) values are close to each other (maximal and minimal replicates within 1 cycle, preferably within 0.5 cycles), proceed to the next triplicate sample. Otherwise, remove the outlier and continue to the next triplicate.

Decisions regarding the removal of some outliers could either be straightforward or judgment calls, depending on the circumstances. In cases where two out of three curves are superimposable while the third is clearly off by more than a cycle, it is a fairly easy decision to consider the third replicate an outlier. If the curves are closer, the decision on which one to eliminate, if any, becomes much more difficult. As a general guide only, if the spread between the lowest and highest C_T values is less than 0.5, it is probably safe to average all the C_T values (see step 12). If the C_T range is <1 but >0.5 , the data are less reliable and the decision to remove any data points should probably be made on a case-by-case basis. It is highly recommended that the PCR be repeated for samples where the C_T ranges are >1 with no two curves superimposable.

15. Proceed to analyze the data for all triplicates in the manner described above. Save the results in a different file.

16. Export the data to a spreadsheet program such as Microsoft Excel by using built-in filters. The file should not contain omitted wells (see step 14) and should be in a column format containing well positions, descriptors, and C_T values for each selected well.

Final calculations are most easily handled in a spreadsheet but could also be performed with a scientific calculator.

17. Open the exported file. Proceed to average triplicate measurements for each sample in a new column ($AVERAGE C_T$).

For some samples, there may be only two measurements left as a result of the removal of outliers in step 14.

18. For each primer pair, subtract the $AVERAGE C_T$ (INPUT) from $AVERAGE C_T$ (IP) in a new column. This number is the $NET C_T$.

This value represents the difference in cycles between the immunoprecipitated sample and the input DNA.

19. Subtract $NET C_T$ for one primer pair (experimental; EXPT) from the $NET C_T$ of another primer pair that serves as a reference or a control (CTRL) in a new column. The resulting value is $NET C_T^{EXPT} - CTRL$. Repeat $NET C_T$ subtraction of control primer for all other experimental primers.

It is very desirable to have a control primer pair that can be used to assess the relative cross-linking efficiency at promoters of interest. Frequently, the control primer pair could be specific for a DNA region that does not bind to the immunoprecipitated protein of interest. The signal from the control primer pair could then be considered the background, and the binding efficiency of the protein to different promoter regions could be expressed as fold over background binding.

20. Evaluate the expression POWER ([mean primer slope], [$NET C_T^{EXPT} - CTRL$]), where the [mean primer slope] is the base and [$NET C_T^{EXPT} - CTRL$] is the exponent. Repeat the process with other primers by using the different $NET C_T^{EXPT} - CTRL$ values calculated in step 19.

The actual value calculated in the POWER expression above is the degree of occupancy of the immunoprecipitated protein at the sequence of interest relative to that of a control (or background) DNA region.

*Perfectly efficient PCR, in which the number of amplified molecules doubles every cycle, has a primer slope of 2. As defined, this value is independent of primer pair sequence, target sequence length, and other variables that under normal circumstances may adversely affect the efficiency of amplification. In practice, however, the mean primer slope is almost always <2 and is slightly variable from primer pair to primer pair; mostly due to differences in primer sequence and other parameters (e.g. GC content and length of amplified sequence). For the majority of primers designed to amplify *S. cerevisiae* promoter sequences, the mean slope is 1.9 ± 0.06 , and this value can be safely used in the calculations above. However, it is still good practice to calculate representative slopes for every newly synthesized primer pair; any substantial deviation from 1.9 (± 0.06), especially to the downside, should be viewed suspiciously. Frequently, a slope that substantially deviates from the normal range is indicative of problems in the amplification.*

The mean primer slope for a given primer pair is most easily calculated by performing linear regression on the linear portion of the amplification plot (log net fluorescence versus PCR cycle). In order to perform this calculation, it is first necessary (if possible) to export a file containing the fluorescence values for all the wells in use at every PCR cycle (see software manuals for more information). It is then possible to use linear regression to estimate the slope within the linear range (i.e., by using the MS Excel function LINEST). Since it is rather time-consuming to calculate slopes for an entire 96-well plate one-by-one, it may well be worthwhile to write a macro (or a stand-alone program) to automate this process.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

ChIP elution buffer

50 mM Tris·Cl, pH 7.5 (APPENDIX 2A)
10 mM EDTA (APPENDIX 2A)
1% (w/v) SDS (APPENDIX 2A)
Store up to 1 year at room temperature

ChIP wash buffer

10 mM Tris·Cl pH 8.0 (APPENDIX 2A)
0.25 M LiCl
1 mM EDTA (APPENDIX 2A)
0.5% (v/v) Nonidet P-40
0.5% (w/v) sodium deoxycholate
Store up to 1 year at room temperature

FA lysis buffer with and without 2 mM PMSF or 0.5 M NaCl

For FA lysis buffer:
50 mM HEPES: adjust pH to 7.5 with KOH
150 mM NaCl
1 mM EDTA (APPENDIX 2A)
1% (v/v) Triton X-100
0.1% (w/v) sodium deoxycholate
0.1% (w/v) SDS
Store up to 1 year at room temperature

For FA lysis buffer/2 mM PMSF add 100 mM phenylmethylsulfonyl fluoride (PMSF) in ethanol (store up to 1 year at -80°C) to a final concentration of 2 mM just before use. For FA lysis buffer/0.5 M NaCl, change the amount of NaCl added to 0.5 M. Store up to 1 year at room temperature

Loading buffer, 10 \times

20% (w/v) Ficoll 400
0.1 M disodium EDTA, pH 8 (APPENDIX 2A)
1.0% (w/v) sodium dodecyl sulfate
0.25% (w/v) bromphenol blue
0.25% (w/v) xylene cyanol (optional; runs $\sim 50\%$ as fast as bromphenol blue and can interfere with visualization of bands of moderate molecular weight, but can be helpful for monitoring very long runs)

RNase A stock solution (DNase-free), 2 mg/ml

Dissolve RNase A (e.g., Sigma) in DEPC-treated H_2O (APPENDIX 2A) to 2 mg/ml. Boil 10 min in a 100°C water bath. Store ≤ 1 year at 4°C .

SYBR Green Taq mix, 2 \times

12 mM Tris·Cl, pH 8.3 (APPENDIX 2A)
50 mM KCl
5 mM MgCl_2

continued

150 mM trehalose (Sigma)
100 mM betaine (Aldrich)
0.2% (v/v) Surfact-Amps 20 (Pierce; active ingredient, Tween 20)
0.2 mg/ml nonacetylated BSA (Sigma, B8667)
1 μ M 5(6)-carboxy-X-rhodamine (ROX; Helix Research)
0.133 \times SYBR Green (Molecular Probes; final dilution 1:75,000)
Store solution with above components indefinitely at -80°C or up to several months at 4°C in the dark
Add the following immediately prior to use
0.5 mM each dATP, dTTP, dCTP, dGTP
0.2 U/ μ l hot-start *Taq* DNA polymerase

The amount of Taq DNA polymerase may need to be slightly adjusted to account for batch/activity variations among different manufacturers.

COMMENTARY

Background Information

Direct protein-DNA contacts and indirect protein-DNA interactions regulate fundamental chromosomal functions such as DNA replication, gene expression, and chromosome segregation. Thus, knowledge about the distribution of particular proteins on specific chromosomal DNA sequences can provide important insights into the mechanisms that govern chromosomal functions, structure, and organization. In vivo footprinting methods provide high-resolution mapping of protein-DNA interactions but cannot directly identify the chromatin-associated protein(s) responsible for the footprint. Chromatin immunoprecipitation, by contrast, is ideally suited for determining the identity of proteins associated with specific DNA sequences in vivo, albeit with lower resolution (≤ 1 kbp).

Two groups, Gilmour and Lis and Solomon and Varshavsky, independently pioneered cross-linking and immunoprecipitation methods for in vivo chromatin analysis (Gilmour and Lis, 1984; Solomon and Varshavsky, 1985; Solomon et al., 1988; Gilmour et al., 1991). These methods exploited cross-linking to conserve in vivo chromatin structures and permit their isolation under the stringent conditions necessary to isolate soluble chromatin. Cross-linked protein-DNA complexes were purified by cesium chloride centrifugation (a time-consuming step) and subjected to immunoprecipitation. Their methods were distinguished primarily by the cross-linking agent: Gilmour and Lis employed UV irradiation, while Solomon and Varshavsky used formaldehyde. The biochemical characteristics of each method have been discussed extensively (Gilmour and Lis, 1984; Solomon and Varshavsky, 1985; Orlando et al., 1997,

and references therein). In short, UV irradiation cross-links only protein-DNA complexes in direct contact, which limits its use. Formaldehyde reacts with primary amines on amino acids and DNA and RNA bases, reversibly forming a covalent adduct between two primary amines in close proximity to each other (≤ 2 Å). Because protein-protein adducts are formed in addition to protein-DNA adducts, chromatin-associated proteins not directly bound to DNA can be cross-linked to DNA via other proteins such as histones, significantly broadening the applicability of this procedure. Cross-linking with formaldehyde is also more easily accomplished than UV irradiation, especially with larger culture volumes.

Formaldehyde-based chromatin immunoprecipitation was simplified and adapted for use in other experimental systems, including budding yeast, where it was first used to assess the association of differentially acetylated histones with the silent mating-type loci (Dedon et al., 1991; Braunstein et al., 1993). This method involved fractionation of cell extracts to isolate a chromatin fraction before immunoprecipitation. A closely related method was used to assess the composition of the budding yeast centromere (Meluh and Koshland, 1997). The protocol presented here (see Basic Protocol; Fig. 17.7.1) is for a simpler procedure derived by Hecht and Grunstein in which immunoprecipitations were performed with whole-cell extracts to assess the spatial distribution of SIR proteins on telomere-proximal DNA regions (Hecht et al., 1996; Strahl-Bolsinger et al., 1997). The Basic Protocol has also been applied to characterize the spatial and temporal associations of DNA replication proteins with chromatin at replication origins (Aparicio et al., 1997; Tanaka et al., 1997), the

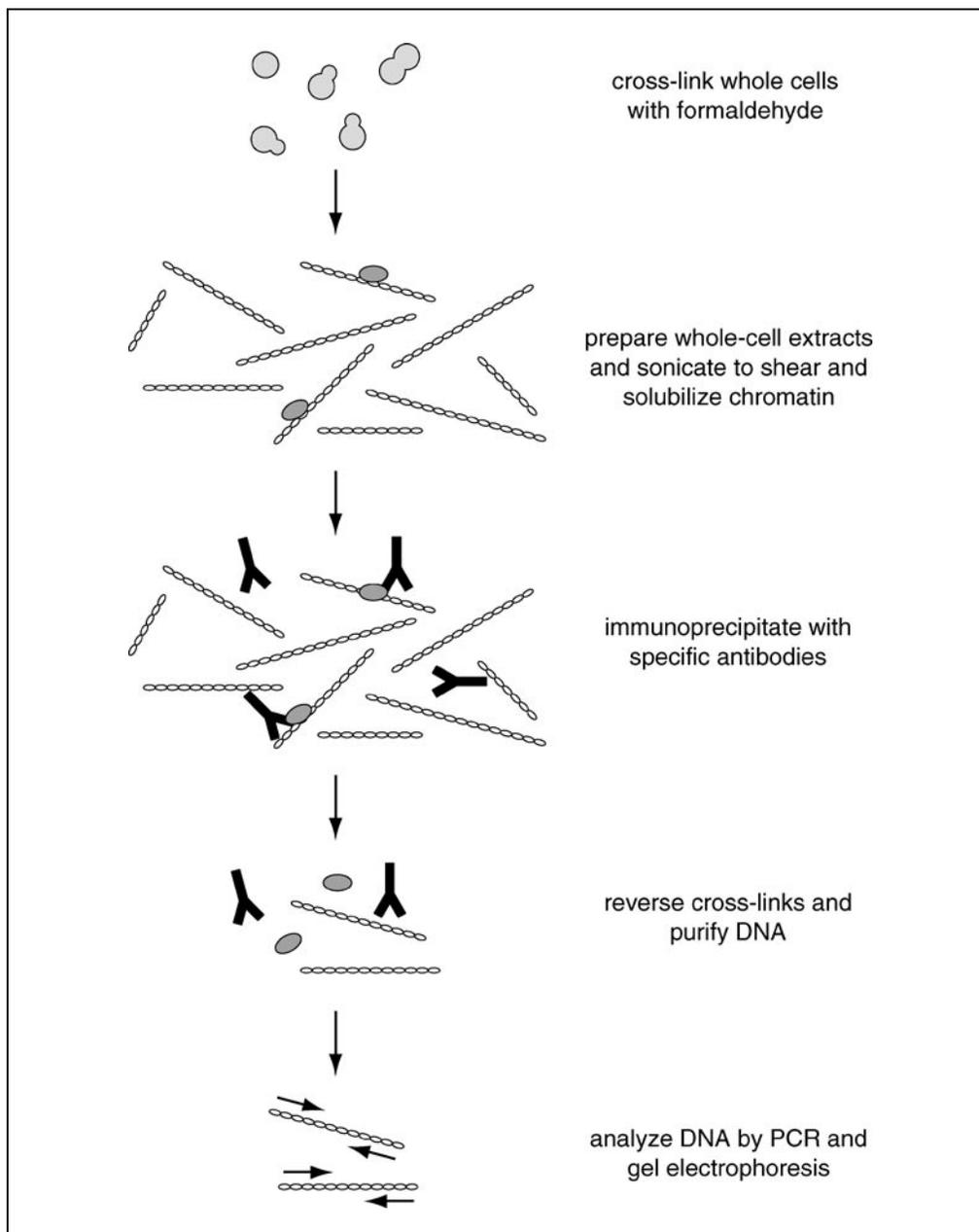


Figure 17.7.1 Scheme for chromatin immunoprecipitation from yeast whole-cell extracts.

association of general transcription factors at promoters (Kuras and Struhl, 1999; Li et al., 1999), and the dynamics of DNA-binding proteins and chromatin-modifying activities associated with a cell-cycle and developmentally regulated promoter (Cosma et al., 1999). In each of these latter studies, protein association with relevant DNA sequences was examined using PCR amplification.

ChIP can also be used to specifically follow the genomic association of mutant proteins that are unable to support cell growth (Mencia and Struhl, 2001). This involves a strain containing both an epitope-tagged version of the mutant protein and an untagged version of the

wild-type protein, which supports cell growth; ChIP is performed with an antibody against the epitope. Lastly, ChIP can be combined with microarray technology to identify the location of specific proteins on a genome-wide basis (Ren et al., 2000; Iyer et al., 2001). The immunoprecipitated DNA is PCR amplified with linkers, and the resulting material hybridized to microarrays containing the complete set of intergenic regions in *Saccharomyces cerevisiae*. Such “genome-wide location” or “ChIP-chip” is particularly powerful, because it allows one to identify novel regions of protein association, without any previous knowledge.

Critical Parameters and Troubleshooting

Controls

There are two basic types of controls for a standard ChIP experiment. One control is mock immunoprecipitation to determine the specificity of an observed signal. This is accomplished by performing parallel immunoprecipitations of a given cross-linked chromatin sample with the antibody of interest and with an irrelevant antibody (or simply not providing any antibody). Alternatively, when antibodies against epitope-tagged proteins are employed (e.g., anti-HA, anti-myc), a similar comparison can be made with parallel immunoprecipitations (primary antibody included) of chromatin samples from strains expressing epitope-tagged or untagged versions of the protein of interest. However, in such experiments, it is often observed that the apparent immunoprecipitation efficiency for any irrelevant genomic region is about 2- to 3-fold higher in the experimental sample than in the control sample. This probably reflects nonspecific, and perhaps artifactual, association of the protein of interest with chromatin; hence it is not indicative of specific protein association with the genomic region. For proteins that generally and strongly associate with the entire genome (e.g., histones), the apparent immunoprecipitation efficiency in the experimental sample will be much higher than the control sample.

The second control, which is usually more meaningful, is to examine a given pair of input and immunoprecipitated samples for multiple genomic regions. Control genomic regions (i.e., those not interacting with the protein) should all give the same background level of apparent immunoprecipitation efficiency (typically ~0.025% to 0.05%). Fragments bound by the protein of interest will give higher apparent immunoprecipitation efficiencies, and the difference can be expressed as fold enrichment over the background level. In the best cases, enrichments can be >100-fold, but even a factor of two can be meaningful if the experiment is repeated enough times and the experimentally determined error is sufficiently low. The advantage of this approach is that the identical samples are used to directly determine relative protein association to different genomic regions. Furthermore, differences in fold enrichments for different genomic regions represent relative quantitative measurements of protein association *in vivo*.

Additional controls may be used depending on the specific application. For example,

where binding to a putative binding site is being tested, a mutation in the binding site is a critical control (especially if such a mutation previously was shown to eliminate binding *in vitro*). In a related manner, it might be useful to determine the protein association in mutant strains or under particular environmental conditions that are suspected to be important for the protein of interest.

Cross-linking

The extent of formaldehyde cross-linking is an important variable that in principle may be modified by changing the duration of cross-linking, the concentration of formaldehyde, or the temperature at which the cross-linking is performed. The use of 1% (final concentration) formaldehyde for 15 min at temperatures ranging from 12°C to 37°C usually works well; however, at temperatures above 30°C, background sometimes increases. Therefore, when fixation at a higher temperature is required, reducing the duration of cross-linking or the formaldehyde concentration may be helpful. Excessive cross-linking can interfere with cell breakage by bead beating and effective fragmentation and solubilization of the DNA by sonication (see below). For some applications where protein cross-linking is particularly efficient (e.g., histones), it might be useful to decrease the cross-linking time or formaldehyde concentration. In particular, histone tails have a number of lysine residues that are likely to be modified by formaldehyde, and such modified lysines may interfere with the binding of antibodies against specific peptides corresponding to modified histones (e.g., by acetylation, phosphorylation, methylation).

Cell lysis

Although complete lysis of all cells is not absolutely necessary (and may be difficult to achieve), it is very important that lysis be as efficient as possible. Efficient lysis is important to obtain a reproducible degree of cell breakage among a group of samples to reliably compare results. Significant differences in cell lysis efficiency will result in immunoprecipitations with different ratios of antibody to chromatin, which will possibly alter immunoprecipitation efficiency. Cell breakage by a mini bead beater is generally more efficient than breakage by a multi-vortexing apparatus, although both methods work. In both cases, it is important to use flat-bottomed 2-ml microcentrifuge tubes. When using the mini bead beater, the sample and beads should nearly fill the tube, whereas for vortexing it is important

to maintain a small volume. The extent of cell breakage may be monitored microscopically by comparing the number of intact cells (determined by counting on a hemacytometer) in small, diluted aliquots of the sample taken before and after vortexing. In addition, the size of the remaining pellet (unbroken cells and debris) obtained in the first centrifugation following sonication (see Basic Protocol, step 14) is a good general indicator of the extent of lysis. The size of this pellet should be routinely checked (by rapid visual inspection) to compare the extent of lysis among samples.

The final yield of genomic DNA in the extract is also an important indicator of the extent of cell breakage, although the DNA yield is also dependent on the solubilization of chromatin by sonication (see below). Poor or variable cell breakage may result from excessive cross-linking that toughens the yeast cell wall and other structures. The procedure for lysis of *Saccharomyces cerevisiae* is appropriate for other yeast species. However, modified procedures are necessary for breaking mammalian cells.

Sonication

Shearing DNA to a small size (~500 bp average) by sonication is the critical factor in achieving resolution between a DNA sequence where a particular protein is bound and a nearby (*cis*-)DNA sequence that does not bind that protein. In addition, fragmentation of the chromatin is essential for its solubilization from the ruptured cells. As indicated above, the ability to fragment and solubilize the chromatin depends on the extent of chromatin cross-linking. In general, more cross-linking results in larger fragment size and lower solubility, resulting in lower yield (Orlando et al., 1997). Because of the importance of this variable, the shear size of the DNA should be assessed to confirm that the desired degree of fragmentation has been achieved, and it should be reassessed if fixation conditions are altered. The shear size is determined by electrophoresing DNA from step 16 of the Basic Protocol on a 1.5% to 2.0% agarose gel and visualizing with ethidium bromide. A smear of DNA should be apparent with an average size of 500 bp and most of the DNA (>90%) should be in the size range of 100 to 1000 bp.

As an alternative to sonication, DNA fragment size can be reduced by treatment of the cross-linked chromatin with micrococcal nuclease. Micrococcal nuclease preferentially cleaves DNA located in the linker re-

gions between nucleosomes. By varying the concentration of micrococcal nuclease, it is possible to generate samples in which average DNA size varies. The minimal useful size is about 150 bp, which corresponds to a mononucleosome. However, cleavage to mononucleosome-sized fragments may also result in a preferential loss of certain genomic regions due to the sequence-specificity of micrococcal nuclease.

Immunoprecipitation

The success of this procedure relies on the use of an antibody that will specifically and tightly bind its target protein in the buffer and wash conditions used. In addition, antibody should be present in excess with respect to its target protein so that differences in the amounts of the protein-DNA complexes of interest will be accurately measured. Perform preliminary experiments to confirm avid immunoprecipitation and determine an approximate amount of antibody to use. Chromatin extracts should be prepared without prior cross-linking of the cells and subjected to immunoprecipitation with varying concentrations of antibody (20 µg/ml antibody may be a good starting point). The amount of the protein of interest in the extracts before and after immunoprecipitation should be analyzed by immunoblotting (UNIT 6.2) to determine the lowest antibody concentration that depletes >90% of the protein of interest from the extract. This antibody concentration is a good starting point for the full protocol and may later be modified to maximize the signal-to-noise ratio (see Anticipated Results). With cross-linked chromatin, immunodepletion of the target protein is less efficient (~50%), presumably due to masking or modification of the epitopes, and a significant amount of the protein remains refractory to immunoprecipitation even with higher antibody concentrations. Thus, the ideal antibody concentration is ultimately determined empirically to maximize the yield of specific coprecipitated DNA while minimizing precipitation of nonspecific DNA.

Both monoclonal and polyclonal antibodies have been used in this procedure. The monoclonal antibodies 12CA5 (anti-HA), 17D09 (anti-HA), and 9E10 (anti-myc) have been used successfully in different laboratories. In general, triple-HA epitope tags work well (Hecht et al., 1996; Aparicio et al., 1997; Tanaka et al., 1997), and larger multi-myc epitope tags have also been successful (e.g., myc-9, myc-18; Tanaka et al., 1997). Protein G-Sepharose, Protein A-Sepharose, and

anti-mouse immunoglobulin-coupled magnetic beads have all been used to precipitate the immune complexes, although it should be noted that certain classes of mouse and rat immunoglobulins are not strongly bound by protein A (Harlow and Lane, 1988; see Table 7.2.1).

For optimal results, it is critical to minimize the background level of material that inevitably comes down during the immunoprecipitation. The procedures described here work well with a diverse set of antibodies, but it might be necessary to modify the binding and elution conditions in specific cases. Peptide elution is clearly preferred over heat elution, as it is more specific and results in lower experimental backgrounds and hence higher-fold inductions. However, peptide elution is only possible for experiments using antibodies against peptides (typically for analyzing epitope-tagged proteins, but analysis of native proteins should also be possible). In performing peptide elution, it is important to add enough peptide such that the protein-DNA complexes are efficiently eluted from the beads.

Another consideration is that the epitope of interest in the chromatin-bound protein might be inaccessible to the antibody due to associated proteins or DNA structures. In such a case, one might obtain a false-negative result. Whereas the majority of a given protein may be efficiently immunoprecipitated from the cross-linked cells, the fraction that is actually cross-linked might be undetectable. The

use of polyclonal antibodies (which often recognize multiple determinants within a protein) or epitope-tagged proteins (the epitope is unlikely to have a specific interaction with other proteins or DNA sequences, particularly if the epitope does not affect the biological function as determined by genetic complementation) minimizes, but does not eliminate this concern. Because of this caveat, negative results should be interpreted cautiously and alternative methods (e.g., *in vitro* DNA binding or association of the protein with bulk chromatin) should be tried. This concern is particularly relevant when a protein of interest does not appear to interact with any genomic sequence. However, if a protein selectively associates with some genomic sequences, this concern is significantly reduced—i.e., it is unlikely that epitope masking will occur at some loci, but not others.

PCR strategy

The choice of primers depends on the experimental goals. If binding to a specific site is being tested, a primer pair that flanks the site and at least one control primer pair recognizing a DNA sequence not expected to bind the protein of interest are the minimal requirements (see Fig. 17.7.2). When choosing primers, it is important to remember that resolution between adjacent sequences is limited by the shear size of the DNA. For an average DNA size of 500 bp, a significant fraction of the DNA molecules will be in the 500 to 1000 bp

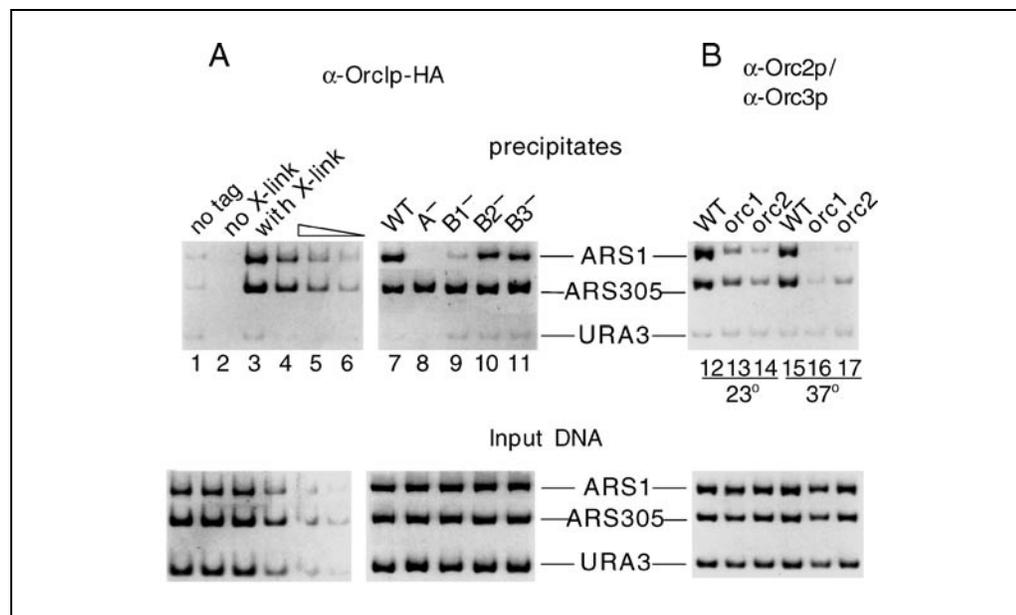


Figure 17.7.2 Anticipated results from chromatin immunoprecipitation analysis of origin recognition complex (ORC) with replication origin and nonorigin DNA sequences.

range, and hence DNA sequences 1000 bp distal from the actual protein binding site may be coprecipitated. Therefore, primer pairs used as controls should amplify a region of DNA that is far enough away from the expected binding site (e.g., >1 kbp) that coprecipitation of adjacent DNA is not detected. A good strategy is to design multiple sets of primers at increasing distances from a suspected binding site. Such a strategy has also been used to probe the “spreading” and “movement” of proteins on chromatin (Hecht et al., 1996; Aparicio et al., 1997; Strahl-Bolsinger et al., 1997).

Success in obtaining high-quality quantitative data is critically dependent on good primer design! In general, primers should be 20 to 30 bases long with a T_m of 55° to 60°C. Most primers require no purification or special treatment prior to PCR. Amplification products should be 75 to 300 bp. Longer PCR products should be avoided, because the amplification efficiency is substantially lower, and DNA fragments that do not bind to both primers will not be amplified (this can be a significant problem since the size of DNA fragments in the samples averages ~500 bp and ranges between 100 to 1000 bp). A final primer concentration of 1 μ M works well for most primers, but in some instances, improved product specificity may be obtained by lowering the final primer concentration 5 to 10 fold.

The design of good primers is greatly facilitated by commercially available software packages such as Oligo 6.6 or Primer Express 1.5. These packages allow for extensive customization of many different parameters, including T_m , oligonucleotide length, GC content, and more. While the success of each individual primer pair in the specific amplification of its target sequence is dependent on many variables, special care must be taken to minimize primer-dimers and hairpins. Finally, it is a good idea to check primers for hybridization to other genomic sequences through the use of a web-based program such as BLAST.

Newly obtained primer pairs must be tested for amplification specificity and performance under the conditions that will be used in quantitative PCR. Primer pairs that are suitable for reactions performed by the Basic Protocol might not be suitable for real-time PCR reactions using SYBR Green, because SYBR Green can inhibit *Taq* polymerase. It is particularly informative to analyze input DNA amplification by the primers in question on high-percentage agarose or polyacrylamide gels after completion of the PCR. The presence of multiple product bands indicates poor speci-

ficity and will invariably lead to unreliable results.

For the Basic Protocol, the best test for quality of a given primer pair is to carry out a standard curve using different dilutions of DNA. For a high-quality primer pair, the amount of PCR product should be directly proportional to the amount of DNA, with an error of less than $\pm 20\%$. The number of PCR cycles is determined empirically. Usually, 25 to 28 cycles is appropriate. More than 28 cycles can result in detection of nonspecifically precipitated sequences and/or lead to variable results due to inactivation of *Taq* polymerase. Multiple primer pairs can be used in combination if the PCR products are separable by gel electrophoresis (as many as five have been used), but some combinations interfere with efficient amplification of one or more products. It is essential to test primer pairs singly and in combination, with titrations of template DNA, to determine if this is a problem. The advantage of using multiple primer pairs is that individual reactions can generate data for multiple genomic regions in an internally controlled manner. In addition, the Basic Protocol can be used to simultaneously analyze two alleles of a given locus in an internally controlled manner, provided the individual alleles result in different-sized PCR products.

When quantitative PCR will be performed in real time using SYBR Green (see Alternate Protocol 2), high-quality primer pairs should result in ~1.9-fold amplification/cycle. Such amplification efficiency can be determined from quantitative analysis of raw fluorescence data for each cycle. Amplification efficiencies <1.8 are likely to cause problems, particularly if detection of the PCR product requires 30 cycles or more. Specificity information may also be obtained by running dissociation curves on reactions following the conclusion of the PCR run.

Quantitation

For the Basic Protocol, PCR products are analyzed by gel electrophoresis and detected by staining with ethidium bromide or SYBR Green or by radioactivity (typically by including a small amount of [32 P]dATP in the reactions). DNA staining has the advantage of not requiring radioactivity, but the linear detection range is relatively limited. In this regard, SYBR Green is more sensitive than ethidium bromide and is preferred. Radioactive detection is more sensitive and has a larger linear range than detection by DNA staining. Whatever detection method is employed, it is

essential to use a high-quality imager to accurately quantitate the amounts of PCR product in the various reactions.

When quantitative PCR is performed in real time using SYBR Green (Alternate Protocol 2), the linear range is directly visualized and the quality of the reactions can be directly assessed. For reactions involving a given primer, the curves should be superimposable with respect to shape, and they should differ only in the number of cycles needed to reach the threshold (C_T). Amplification efficiencies should be ~ 1.9 -fold/cycle. If the curves have different shapes and/or amplification efficiencies are < 1.8 , the reactions are not equivalent and accurate quantitation is impossible.

Data interpretation

In most experiments, it is presumed that the protein of interest associates specifically with certain genomic regions and associates only nonspecifically with other genomic regions. In general, it is very difficult to distinguish true nonspecific association from experimental background of the cross-linking procedure. In this regard, immunoprecipitations with the antibody of interest generally give 2- to 3-fold higher immunoprecipitation efficiencies than immunoprecipitations with control (or no) antibodies, but it is unclear whether this effect is physiologically meaningful or an experimental artifact.

For this reason, the best way to interpret the data for most experiments is to compare the immunoprecipitation efficiencies for different genomic regions from the same INPUT and IP samples. The immunoprecipitation efficiency is calculated by the amount of PCR product in the IP sample divided by the amount of PCR product in the INPUT sample. A typical background level for DNA fragments that do not associate with the protein of interest is 0.025% to 0.05%. However, background levels can vary, depending on the antibody used and the elution method. In general, monoclonal antibodies give lower background signals than polyclonal antibodies. Peptide elution is preferred over heat elution for the same reason, although this can only be employed for ChIP experiments involving antibodies against defined peptide epitopes. By definition, the background level should be the same for all DNA fragments that do not specifically associate with the protein of interest. In many cases, the choice of suitable negative control regions is based on expectation from other lines of evidence (e.g., the middle of protein-coding regions are unlikely to bind general transcrip-

tional initiation factors). In cases where there is no previous knowledge, the background level can only be based on multiple regions having similar immunoprecipitation efficiencies that are roughly at the level of a typical background immunoprecipitation efficiency. In this regard, it is particularly useful to use proteins tagged with a standard epitope (e.g., HA or myc), as there is considerable information on background levels in such cases.

DNA fragments that display immunoprecipitation efficiencies significantly above the background are indicative of protein association to those genomic regions *in vivo*. Moreover, for a given pair of INPUT and IP samples, the fold enrichment of a given genomic region over the background is directly related to the level of protein association *in vivo*. It is useful to define “relative protein occupancies” for different regions by subtracting the background from the observed immunoprecipitation efficiencies. For example, if the background level is arbitrarily defined as 1 occupancy unit, fragment A that shows 6-fold enrichment over background will have 5 occupancy units, whereas fragment B that shows 21-fold enrichment over background will have 20 occupancy units. Thus, one can conclude that the protein association with fragment B is 4-fold greater than with fragment A. Without further considerations (see below), this conclusion is only relevant for the particular pair of INPUT and IP samples because absolute immunoprecipitation efficiencies and fold enrichments can vary among repeated trials of the same experiment.

Absolute immunoprecipitation efficiencies and fold enrichments depend on multiple factors. First, the number and physical location of amino acid and nucleotide residues within the interacting protein surfaces that react with formaldehyde vary considerably among protein-protein and protein-DNA interactions. Second, proteins directly interacting with DNA can be cross-linked by a single event, whereas proteins that indirectly associate with DNA need multiple cross-linking events. In this regard, proteins directly binding DNA (e.g., specific DNA binding proteins and general transcription factors) typically give higher fold enrichments than other proteins (e.g., components of chromatin-modifying complexes). Third, some proteins might stably associate with genomic DNA sequences (maximally 100% occupancy), whereas association of other proteins might be transient. Fourth, the absolute immunoprecipitation efficiency depends on the quality of the specific

antibody-antigen interaction as well as the antibody concentrations, and the fold enrichment depends on both the absolute immunoprecipitation efficiency and on the background. Thus, absolute immunoprecipitation efficiencies and fold enrichments cannot be used to compare binding characteristics of different proteins. Furthermore, ChIP experiments do not provide absolute measurements of protein occupancy on specific genomic regions or relative stoichiometry of factors on a given sequence. Despite these limitations, ChIP experiments do provide direct quantitative information on the relative levels of protein association on different genomic sequences.

As mentioned above, absolute immunoprecipitation efficiencies and fold enrichments can vary among repeated trials of the same experiment due to potential differences in overall immunoprecipitation efficiency and experimental background. To account for these experimental variations, it is useful to arbitrarily define occupancy units for a specific protein-DNA association. In the example above, one might arbitrarily define fragment A to have 4 occupancy units. In independent experiments, association with fragment A will always be defined as having 4 occupancy units, and association with other fragments will be defined relative to that of fragment A in the same pair of INPUT and IP samples. In this manner, it is possible to accurately determine the relative level of protein association to multiple genomic regions even though the absolute immunoprecipitation efficiencies and fold enrichments might vary in different repeats of the same experiment. However, in actual practice, differences in absolute immunoprecipitation efficiencies and fold enrichments should not show significant sample-to-sample variation.

A related issue occurs when comparing the level of protein association under different physiological conditions (e.g., different growth conditions or strains). The ideal way to handle this situation is to analyze a “positive control” region that is predicted to be unaffected by the growth condition or genetic constitution. For example, in analyzing association of general transcription factors under a particular environmental condition, it would be useful to examine promoters that are regulated and promoters that are not. In some cases, such a control genomic region is not available, in which case one must rely on simple sample-to-sample reproducibility from independent trials of the same experiment.

ChIP can be used to determine the relative occupancy levels of different proteins at ge-

nomeric regions (Kuras et al., 2000). Ideally, this is accomplished by performing parallel immunoprecipitations with different antibodies on the same cross-linked chromatin sample. Alternatively, immunoprecipitations can be performed on different samples (this often occurs when using epitope-tagged strains or when multiple proteins are examined). To determine occupancy ratios for two different factors (e.g., X and Y), occupancy units for X and Y are calculated independently as described above. The X:Y occupancy ratios are then calculated for all genomic regions examined. The resulting occupancy ratios are defined in arbitrary units, but the relative occupancy ratios for the different genomic regions are valid. To account for potential sample-to-sample variations among repeats of the same experiment, a given X:Y occupancy ratio should be defined for a specific genomic region and ratios at all other genomic regions calculated in relative terms. Using this rationale, it has been shown that the relative associations of TBP and the general transcription factors TFIIA and TFIIB are essentially constant at all promoters, whereas the TAF:TBP occupancy ratios vary considerably (Kuras et al., 2000). Importantly, however, occupancy ratios determined from such experiments cannot address whether two proteins co-occupy a given genomic region or mutually compete for the same genomic region.

For some experiments, particularly those involving histone modifications, it is inappropriate to analyze the data in terms of occupancy units and specific versus nonspecific binding sites. Histones associate with essentially all genomic regions, and the level of a particular chromatin modification typically occurs in a continuum. Thus, it is very difficult to determine whether a given region is devoid of a particular modification, although information in this regard can be obtained in control immunoprecipitations using an irrelevant antibody. For these reasons, quantitative analysis of the relative level of a given histone modification is best presented using simple immunoprecipitation efficiencies. Again, to account for sample-to-sample variations, a specific genomic region should be given an arbitrarily defined value, which is used to determine the relative levels of all other genomic regions.

Anticipated Results

Figure 17.7.2 shows the results of chromatin immunoprecipitation of protein subunits of the origin recognition complex (ORC)

and relevant controls (Aparicio et al., 1997). In panel A, immunoprecipitation of Orc1p-HA was shown to specifically coprecipitate the replication origin sequences *ARS1* and *ARS305* but not the nonorigin DNA sequence *URA3*. Enrichment of *ARS1* and *ARS305* (~0.4% precipitated relative to total) compared to *URA3* (~0.01% precipitated) was ~40-fold and depended on formaldehyde cross-linking and on the epitope-tagged Orc1p (lanes 1 to 3). Mutation of DNA sequences (A and B1) in the *ARS1* replication origin (required in vivo for origin activity and in vitro for binding of ORC to origin DNA) greatly reduced or eliminated association of Orc1p-HA with *ARS1*. The continued association with the wild-type *ARS305* origin served as an additional control (lanes 7 to 9). Analysis of the totals demonstrated that the origin and nonorigin DNA sequences were equally represented in the whole-cell extract (lower panel, "input DNA"). In panel B, origin association of ORC was tested in strains with temperature-sensitive alleles of *ORC1* or *ORC2* demonstrating loss of ORC-origin DNA binding under nonpermissive conditions (lanes 15 to 17). Nevertheless, it should not be assumed that a mutation in a protein of interest would necessarily result in loss of its chromatin association.

Time Considerations

The Basic Protocol may be completed in a 2- or 3-day period. On the first day, cells are fixed with formaldehyde and harvested (1 hr). For convenience, or if preparation of the cells for cross-linking will require an extended period of time beforehand (e.g., induction of expression, cell cycle synchronization), the cells may be frozen and stored at -80°C as described (see Basic Protocol, step 6). Preparation of chromatin extracts (2 hr) and immunoprecipitations (primary antibody incubation, ~2 hr; incubation with secondary-coupled beads, ~1 hr; washing and elution, 2 hr) may be carried out in 1 day, after which the samples are placed at 65°C overnight to reverse the cross-links. If necessary, immunoprecipitation with the primary antibody or bead-coupled secondary antibody may be extended overnight; however, it is most efficient to perform the ≥ 6 hr cross-link reversal step overnight. On the final day, the DNA is purified (< 4 hr, including a 2-hr incubation with proteinase K), PCR amplified (< 3 hr, including 2 hr for the PCR program), and analyzed by gel electrophoresis (< 2 hr, including 1 hr of gel running time).

For Alternate Protocol 2, setup time is < 30 min, thermal cycling takes ~ 2 hr, and data analysis takes < 30 min.

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Key References

Hecht et al., 1996. See above.

Describes the technique from which the Basic Protocol was adapted.

Orlando et al., 1997. See above.

Describes formaldehyde cross-linking and immunoprecipitation for chromatin analysis in Drosophila, and discusses various parameters of the technique.

Solomon et al., 1988. See above.

Describes original formaldehyde cross-linking and immunoprecipitation technique for mapping protein-DNA interactions.

Solomon and Varshavsky, 1985. See above.

Characterizes formaldehyde cross-linking, cross-link reversal, and sensitivity of cross-linked protein-DNA complexes to proteases and endonucleases.

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Isothermal titration calorimetry (ITC) is the only technique that can directly measure the binding energetics of biological processes, including protein-ligand binding, protein-protein binding, DNA-protein binding, protein-carbohydrate binding, protein-lipid binding, and antigen-antibody binding. ITC has the ability to precisely determine the Gibbs energy, enthalpy, entropy, and heat capacity changes associated with binding.

In this unit several protocols are presented, from the basic ones that are aimed at characterizing binding of moderate affinity (see Basic Protocols 2 and 3) to the advanced ones that are for determining very high or very low affinity (see Alternate Protocols 1 and 2). Also, alternate protocols for special cases (homodimeric proteins and unstable proteins; see Alternate Protocols 3 and 4) and additional information obtainable by ITC (heat capacity and proton transfer coupled to binding; see Alternate Protocols 5 and 6) are presented.

PRINCIPLES OF THE TECHNIQUE

The ITC instrument is a heat-flux calorimeter operating according to the dynamic power compensation principle, i.e., it measures the amount of power ($\mu\text{cal}/\text{sec}$) required to maintain a constant temperature difference (close to zero) between the sample and the reference cell (see Basic Protocol 1). Initially, the feedback system continuously applies a small power to the sample cell, which determines the baseline level. Each injection of the syringe solution (usually termed as ligand) triggers the binding reaction and, depending on the binding affinity and the concentration of reactants (macromolecule, M, and ligand, L) in the cell, a certain amount of macromolecule/ligand (ML) complex is formed. The formation of complex is accompanied by the release (exothermic reaction) or the absorption (endothermic reaction) of heat that causes a difference in temperature between the two cells. Then, the feedback system either lowers or raises the thermal power applied to compensate such temperature unbalance. After each injection, the system reaches equilibrium and the temperature balance is restored. Therefore, the recorded signal shows a typical deflection pattern in the form of a peak (Fig. 17.8.1). Integrating the area under the peak, assuming the baseline as reference, provides the amount of heat associated with the injection. As the reactant in the cell becomes saturated, the heat signal diminishes until only the background heat, due to an unspecific phenomena (e.g., ligand dilution, liquid friction), is observed.

INSTRUMENTATION

Two identical coin-shaped cells, sample and reference, are enclosed in an adiabatic shield (jacket) as illustrated in Figure 17.8.1. The temperature difference between the reference cell and the jacket is continuously monitored to maintain a constant temperature. A feedback control system monitors the difference in temperature between the two cells through a semiconductor Peltier sensor device sandwiched between them. This temperature difference is kept constant and as close to zero as possible at any time. The feedback signal is the measured signal.

One of the reactants is placed in the sample cell and the other one in the injection syringe. During the course of an experiment, the syringe is used to add the titrant reactant to the sample cell in a stepwise fashion and, at the same time, stir the solution in the sample cell to achieve fast mixing. The reference cell serves only as a temperature reference.

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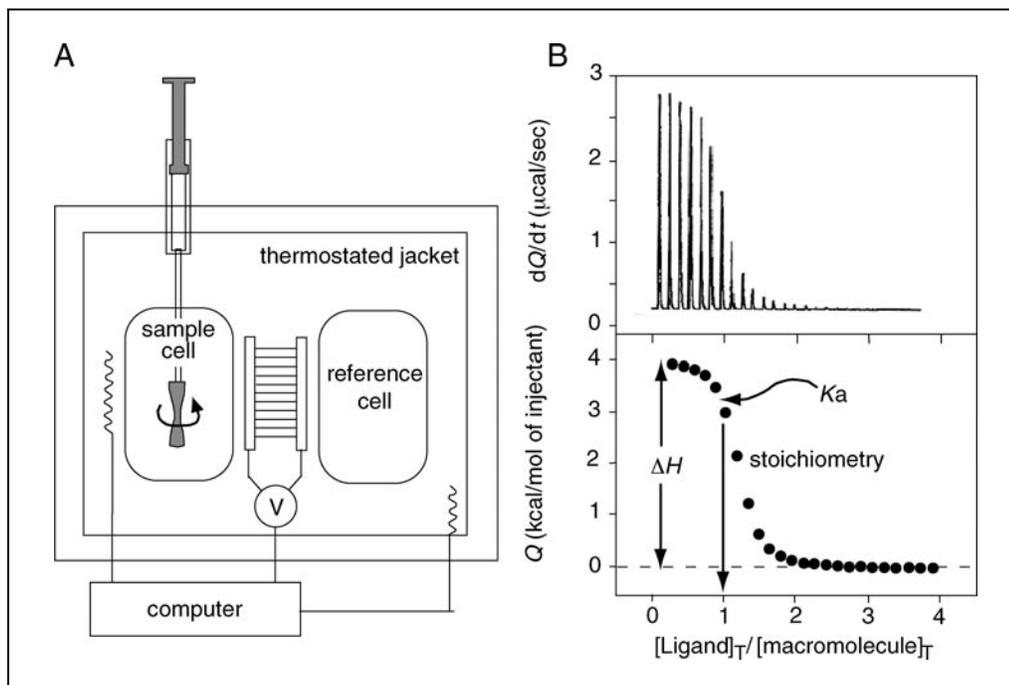


Figure 17.8.1 (A) Basic schematic illustration of the ITC instrument, showing the two cells (sample and reference) surrounded by the thermostated jacket, the injection syringe that also works as stirring device, and the computer-controlled thermostatic and feedback systems (using Peltier and resistor devices as sensor and actuator subsystems). (B) Example of a typical ITC experiment. The top panel shows the sequence of peaks, each one corresponding to each injection of the solution in the syringe. The monitored signal is the additional thermal power needed to be supplied or removed at anytime to keep a constant temperature in the sample cell and as close as possible to the reference cell temperature. This example corresponds to an endothermic binding. The bottom panel shows the integrated heat plot. The areas under each peak, calculated and normalized per mole of ligand injected in each injection, are plotted against the molar ratio (quotient of the total concentrations of ligand and macromolecule in the sample cell). From this plot, and applying the appropriate model, the thermodynamic parameters of the binding can be obtained: binding affinity, binding enthalpy, and stoichiometry.

BASIC PROTOCOL 1

ISOTHERMAL TITRATION CALORIMETRY

The protocols differ only in the way the experiment is planned and not in the procedure for using the instrument. This protocol consists of a detailed description of setting up and using the ITC instrument. The time required for performing a complete ITC experiment (setting up the instrument, running the experiment, and analyzing the results) is ~ 2 hr.

This protocol should not be considered a substitute for the instrument manual, where more detailed and specific explanations are provided. This protocol is written based on the VP-ITC MicroCalorimeter (Microcal LLC) and it can serve as a guide for other models.

Materials

Reactant solutions: macromolecule and ligand
Methanol

VP-ITC calorimeter (e.g., Microcal LLC or equivalent)
Vacuum pump
2.5-ml long-needle syringe (e.g., Hamilton)
12 \times 75-mm and 6 \times 50-mm glass tubes

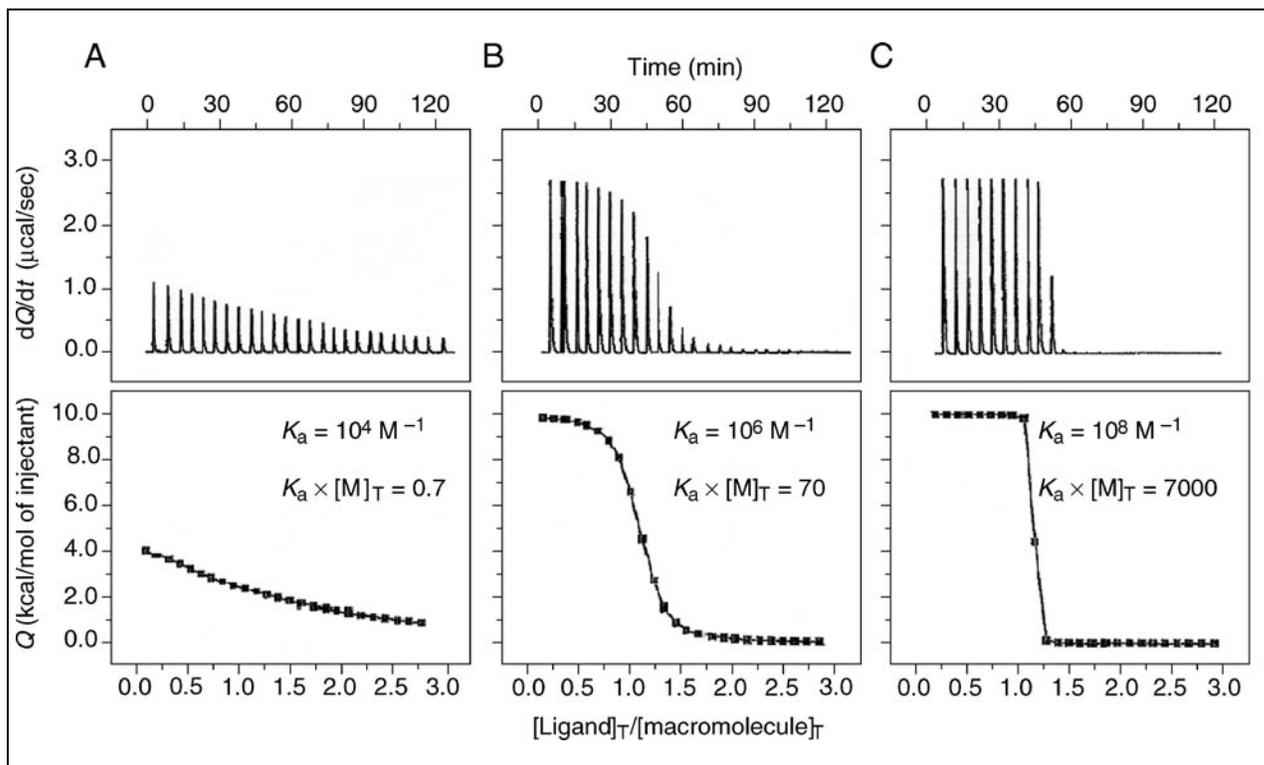


Figure 17.8.2 Illustration of the effect of the association constant value on the shape of a titration curve. The plots represent three titrations simulated using the same parameters (concentrations of reactants and binding enthalpy), but different binding affinities. Low (A), moderate (B), and high affinity (C) binding processes are shown. To obtain accurate estimates of the binding affinity an intermediate case is desirable (B, $1 < c = K_a \times [M]_T < 1000$). When the parameter c is large enough, a good estimate of the enthalpy can be determined from the y-axis intercept of the curve (if the heat effect associated with dilution is used as a reference). Such an intercept value is given by $(c/c+1) \times \Delta H_a$ (Indyk and Fisher, 1998).

Select concentration of reactants

1. Determine the appropriate concentrations of reactants.

Typically, the concentrations of reactants are in the micromolar range; if the heat associated with the reaction is significant, however, the concentrations can be lowered.

An important requirement for an ITC experiment is having appropriate concentrations of interacting molecules such that the heat associated with a given binding reaction is within the calorimetric determination range. Sensitivity of the instrument varies depending on brand and model. The Microcal VP-ITC instrument used in all the experiments described in this unit has a limiting sensitivity of 0.1 µcal. Therefore, to accurately determine the change of heat involved in the interaction between molecules, each injection should have a minimum of 1 µcal of associated heat.

The affinity of the interaction should also be considered in choosing the required concentrations of the interaction components. A dimensionless constant, c , describes the practical window of the instrument to accurately determine the binding constants (Wiseman et al., 1989) in Equation 17.8.1:

$$c = nK_a[M]_T$$

Equation 17.8.1

where, K_a is the binding constant, $[M]_T$ the total macromolecular concentration in the cell, and n is the stoichiometry of interaction. As illustrated in Figure 17.8.2, a large c value corresponds to very tight binding, resulting in an isotherm that is rectangular in shape and is devoid of data points in the transition region of binding, therefore, making the estimation of the binding affinity unreliable, although the binding enthalpy can still be obtained accurately. Conversely, a very low c value corresponds to weak binding and

the resultant isotherm is featureless, making the determination of both the binding affinity and enthalpy unreliable. Keeping other parameters constant (K_a , ΔH , etc), the c value could be reduced or increased by changing the $[M]_T$. ITC data with c values between 1 and 1000 can be used for determination of binding constants.

To obtain a complete binding isotherm within the specified number of injections, the ligand solution in the syringe should be more concentrated than the macromolecule in the cell, so that at the end of the experiment, the molar ratio of ligand to macromolecule inside the cell is 2 to 3 (for 1:1 stoichiometry). Considering the volume of the sample cell (1.4 ml), the typical injection volume (10 μ l), and the typical number of injections (\sim 30), it is advisable to use a concentration of ligand in the syringe 10 to 20 times higher than the solution in the cell. This will guarantee that the reaction reaches the neutralization point after 7 to 13 injections.

Prepare samples

2. Choose experimental conditions, taking into account the stability and the solubility of the reactants and the biological considerations of the system under study. Prepare reactant solutions for the cell and the syringe under identical conditions and with the same composition.

For the reactant solutions, buffer type, buffer concentration, pH, ionic strength, and co-solvents must be the same. In an ITC assay, consistency between the exact composition of the buffer in the cell and the syringe is of crucial importance to prevent dominance of unspecific heat effects. The composition, concentration, pH, and ionic strength of the buffer all affect the thermodynamic parameters, and the quality of the experiment depends on maintaining a perfect match of the buffer in the cell and syringe samples. One effective way of achieving this goal is to dialyze the macromolecule against the desired buffer and then using the filtered dialysis buffer to prepare the ligand solution. Certain additives such as DMSO, which increases the solubility of hydrophobic ligands, have an enormous effect on the ITC signal, therefore, utmost care should be taken to keep the concentration of DMSO in the cell and syringe nearly the same.

3. Prepare the reactant solutions in the appropriate volumes.

Although the apparent volume of the sample cell is 1.4 ml, the volume of solution required for proper filling of the cell is 2.2 ml. The volume required for the syringe is 0.5 ml.

4. Degas all solutions (reactants and buffer solution used for rinsing the cell) for 10 to 20 min (stirring and temperature control are optional) to avoid formation of bubbles in the sample cell during the experiment.
5. To prevent long equilibration delays, lower the calorimeter thermostat setting slightly below the running temperature (with a difference of 0.5° to 2°C).

Load solutions

6. Place degassed, distilled water or buffer solution in the reference cell using a 2.5-ml, long-needle syringe.
7. Rinse the sample cell several times with buffer solution, then remove all liquid.
8. Fill the cell with macromolecule solution, taking care to prevent the appearance of bubbles in the cell.

The most common configuration is one in which a macromolecule is placed in the reaction cell and a low-molecular-weight ligand is placed in the injecting syringe. For convenience, the injected reactant located in the syringe is referred to as “ligand” and the one in the reaction cell is referred to as “macromolecule,” throughout this unit. The reader should be aware that other configurations are possible.

9. Fill the calorimeter syringe with ligand solution according to the manufacturer's instructions. Rinse off excess ligand solution on the surface of the needle with water and then carefully blot the surface of the needle with a paper towel.

A special plastic syringe is used to fill the calorimeter injecting syringe. A purge-refill cycle may be performed to ensure the absence of air bubbles inside the syringe.

Set instrument settings

10. Set the total number of injections to 30.

11. Set measurement temperature to °C.

12. Set reference power to 10 µcal/sec.

The reference power can be modified if a large exothermic or endothermic reaction is expected.

13. Set the initial delay (time between the start and the first injection) to 200 sec.

14. Set the syringe ligand concentration to millimolar (mM).

15. Set the cell macromolecule concentration to millimolar (mM).

During the experiment and analysis, the concentrations are always expressed as millimolar.

16. Set the stirring speed ~500 rpm.

This speed is selected to ensure good mixing, but it can be modified depending on the sample (e.g., high viscosity, tendency to generate foam, etc.).

17. Set feedback mode/gain to high (high sensitivity and small response time).

18. Set injection volume to 10 µl.

A different injection volume can be set, for example, if the heat of reaction is expected to be very large or very small. However, if the ligand concentration can be varied, then it is preferable to adjust its concentration in the injecting syringe rather than the injection volume.

19. Set duration for injection to 20 sec.

This setting is automatically assigned according to the injection volume.

20. Set spacing between injections to 400 sec (usually).

The spacing might be increased depending on peak size or the reaction kinetics.

The user should allow enough time between peaks in order to keep the next injection from starting before the signal from the previous injection returns to baseline. Typically, there should be ~200 sec between the end of an injection and the beginning of the next one.

21. Set filter period (the signal-average time period) to 2 sec.

22. Set the volume of the first injection to ≤3 µl. Do not include the heat associated with the first injection in the data analysis.

Due to inter-diffusion of the solutions during the insertion of the syringe or the equilibration stage, the first injection is not useful in the analysis, therefore, the volume of the first injection should be set to ≤3 µl to minimize the amount of ligand wasted.

Start measurement and troubleshoot the baseline (noise, drift, level)

23. After gently setting the injection syringe in place, initiate the experiment. If fast equilibration is initially selected, both thermal and mechanical equilibration will proceed simultaneously.

The equilibration level will be slightly less than the reference power value entered in the parameter setting due to the power generated by stirring.

24. Check the quality of the baseline in a full scale of 1 µcal/sec. When the baseline becomes flat, with no significant drift or noise, start the injection sequence.

If the baseline is of poor quality, several possibilities should be considered. (1) Small irregular noise is probably due to air bubbles in reference or sample cell. In the case of bubbles in the sample cell, stop measurement, remove syringe, and check cell content. In the case of bubbles in the reference cell, replace the content of the cell. (2) Strong regular noise and/or drift indicates that the injection syringe is bent. Stop measurement and check the needle of the syringe by rolling it on a flat surface. (3) A high baseline level indicates low liquid level or air bubbles in the reference cell. (4) A low baseline level indicates that the viscosity of the cell solution is high or the cell is dirty.

25. Collect data.

Once the sequence of injections is initiated, the titration should be continued until saturation is observed.

Clean the ITC

26. Wash the injection syringe with ≥ 150 ml water. Frequently, and especially for low solubility reactants, wash the syringe with 50 ml methanol (or water-organic co-solvent) and 300 ml water. Dry syringe for ≥ 10 min.

27. Wash the plunger tip of the syringe injector with water and dry it.

28. Wash the sample cell with water (ten times or more) using the long-needle syringe.

For periodic deep cleansing or when using a different macromolecule or in cases where the sample precipitated or aggregated in the sample cell, the sample cell should be washed with a cleaning solution (compatible with the material the cells are made of) according to the manufacturer's instructions.

CAUTION: Do not leave any methanol or organic solvent in the injection syringe or the cells.

**SUPPORT
PROTOCOL**

DATA ANALYSIS FOR ITC EXPERIMENTS

The following section is an outline of the foundations for data analysis, including the basic equations describing the binding equilibrium and its implementation. Data analysis is usually performed by non-linear regression using fitting functions defined in the control and analysis software provided by the manufacturer.

Adjust baseline and integrate peaks

1. Carefully check the quality of the automatic baseline and, if applicable, manually modify the baseline and the integration interval.

The application automatically creates a baseline and integrates each peak when reading the data file. The baseline should follow a smooth path, clearly define a peak signal that relaxes and asymptotically merges with the baseline, and go through the noise when the reaction is finished. The appropriate integration interval should cover the entire peak, taking care to include all the area corresponding to the relaxation stage.

Enter concentration values

2. Enter the values of the ligand and macromolecule concentrations, as well as the sample cell volume to ensure the accuracy of the calculations.

Estimate heat of dilution

3. Note the heat observed after saturation; it reflects different unspecific phenomena, and it is referred to as "heat of dilution." If the fitting procedure does not account for the heat of dilution through an adjustable parameter, make an estimation of such value, e.g., by averaging the values of the last injections or by performing a blank experiment injecting reactant into a buffer solution (see below). If estimated, subtract the heat of dilution from the heat associated with each injection.

Select fitting function

- Determine the model for binding to be used.

Although traditional data analysis methods based on linearization of the binding equations have been commonly used, a non-linear regression is a more appropriate methodology for analyzing ITC data. A suitable model, according to the system under study, needs to be considered when selecting a fitting function to use in the non-linear least square regression analysis. The description for different models can be found in the literature (Cantor and Schimmel, 1980; Freire et al., 1990; Wyman and Gill, 1990; Van Holde et al., 1998; Jelesarov and Bosshard, 1999; Sigurskjold, 2000).

- Express the binding equations in terms of total concentrations. The total concentration of each reactant inside the calorimetric cell is known and after each consecutive injection, i , is given by Equation 17.8.2:

$$\begin{aligned} [M]_{T,i} &= [M]_0 \left(1 - \frac{v}{V}\right)^i \\ [L]_{T,i} &= [L]_0 \left(1 - \left[1 - \frac{v}{V}\right]^i\right) \end{aligned}$$

Equation 17.8.2

where $[M]_0$ is the initial concentration of macromolecule in the cell, $[L]_0$ is the concentration of ligand in the syringe, V is the cell volume, v is the injection volume and $1 - (v/V)$ is the factor that accounts for the change in the concentration of reactants due to the dilution that takes place upon each injection.

Since the concentration of each chemical species (free reactants and complex) is not known, the binding equations must be expressed in terms of total concentrations.

- Determine the heat released or absorbed due to each ligand injection, q_i , i.e., the heat associated with the formation of complex in injection i according to Equation 17.8.3:

$$q_i = V\Delta H_a \left([ML]_i - [ML]_{i-1} \left[1 - \frac{v}{V}\right] \right)$$

Equation 17.8.3

where ΔH_a is the binding or association enthalpy. Again, the dilution factor indicates that the heat change in each injection corresponds to different reactant concentrations.

- Using the mass action law and the conservation of mass for each species, the expression for the concentration of complex after each injection becomes Equation 17.8.4:

$$[ML]_i = \frac{1 + [M]_{T,i}K_a + K_a[L]_{T,i} - \sqrt{(1 + [M]_{T,i}K_a + K_a[L]_{T,i})^2 - 4[M]_{T,i}K_a^2[L]_{T,i}}}{2K_a}$$

Equation 17.8.4

- It is customary to include in this equation a factor n , the stoichiometry, which can be interpreted in two ways: (1) as the generalization for a macromolecule with n

equivalent and independent binding sites or (2) to account for the possibility of having a percentage of the macromolecule in a non-competent binding conformation:

$$[\text{ML}]_i = \frac{1 + n[\text{M}]_{\text{T},i} K_a + K_a [\text{L}]_{\text{T},i} - \sqrt{(1 + n[\text{M}]_{\text{T},i} K_a + K_a [\text{L}]_{\text{T},i})^2 - 4n[\text{M}]_{\text{T},i} K_a^2 [\text{L}]_{\text{T},i}}{2K_a}$$

Equation 17.8.5

Equations 17.8.4 and 17.8.5 constitute the formalism to analyze binding experiments in which a ligand binds a macromolecule with n equivalent and independent binding sites. Given the initial concentrations of reactants, $[\text{M}]_0$ and $[\text{L}]_0$, the binding affinity, K_a , the binding enthalpy, ΔH_a , and the stoichiometry, n , it is possible to estimate the heat involved in each injection, which will be the dependent variable used in the analysis, and incorporate that calculation in a fitting function. The thermodynamic parameters (K_a , ΔH_a , and n) will be estimated as adjustable parameters in the fitting procedure.

All examples presented in this unit can be analyzed with built-in functions embedded in Origin version 5.0 or 7.0.

The previous formalism can be applied to other cases (see Basic Protocol 2) by appropriately modifying Equations 17.8.3 and 17.8.4.

BASIC PROTOCOL 2

MACROMOLECULE/LIGAND INTERACTION WITH MODERATE AFFINITY

Ligands with moderate binding affinities to their target macromolecule constitute the majority of the cases in biological systems. However, ligands with very low or very high affinities are also encountered. Conducting ITC assays for a complete thermodynamic characterization of the binding of such ligands requires modified versions of the basic ITC protocol and will be explained later. When the affinity between the macromolecule and the ligand is moderate ($10^4 < K_a < 10^8 \text{ M}^{-1}$), it is possible to determine the affinity and the enthalpy of binding simultaneously in a single experiment.

This protocol uses commercially available ribonuclease A (RNase A), a well-studied endonuclease of 13.7 kDa, and its commercially available inhibitor 2'-cytidine-monophosphate (2'CMP) of 323 Da, to familiarize first-time users with the setup, procedure, and data analysis of isothermal titration calorimetry. The binding of 2'CMP to RNase A is an example of an exothermic binding reaction, and the binding constant for this reaction is within the range measurable by conventional ITC (Wiseman et al., 1989; Straume and Freire, 1992). It is highly recommended that every attempt at ITC begin with this simple protocol and that conducting experiments with other samples only take place after mastering the technique and analysis explained in this protocol. This protocol presents an efficient way of keeping the conditions in the ITC cell and syringe as close to each other as possible.

Materials

RNase A, lyophilized powder (Sigma)
15 mM potassium acetate buffer, pH 5.5
2'CMP, lyophilized powder (Sigma)

12 × 75-mm and 6 × 50-mm glass tubes
6-kDa MWCO dialysis membrane
0.22- μm filter

Prepare samples

1. Prepare RNase A stock solution in a 12 × 75-mm glass tube. Dissolve the protein in 15 mM potassium acetate buffer, pH 5.5, to a final concentration of 200 to 300 μM.

RNase A is commercially available as a lyophilized powder. A final volume of 5 ml of this stock solution should be sufficient to conduct several experiments.

2. Dialyze RNase A stock solution against ~1 liter 15 mM potassium acetate buffer, pH 5.5, overnight at 4°C using a dialysis membrane with a ~6.0-kDa MWCO.
3. After dialysis, filter the RNase A solution through a 0.22-μm filter. Determine the exact concentration of the stock solution by measuring its absorbance at 278 nm using an extinction coefficient of 9800 M⁻¹cm⁻¹. Store the dialyzed stock of RNase A up to several weeks at 4°C.
4. Prepare 2'CMP stock solution in a 12 × 75-mm glass tube. Filter 15 mM potassium acetate buffer, pH 5.5, used for dialysis using a filter with a 0.22-μm pore size. Dissolve the lyophilized 2'CMP (or 5'CMP) in the filtered buffer to a final concentration of 5 mM. Determine the exact concentration of the stock solution by measuring its

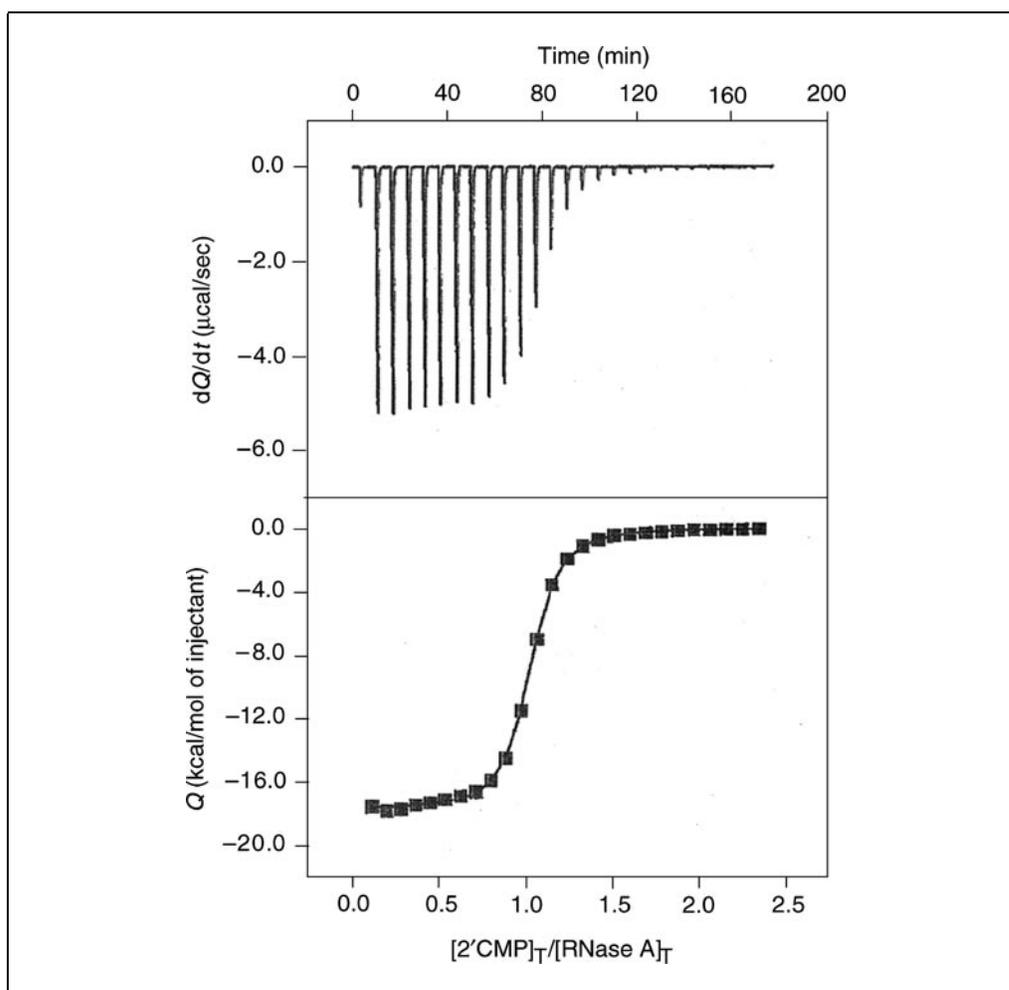


Figure 17.8.3 Titration of RNase A with 2'CMP. The experiment was performed in 15 mM potassium acetate, pH 5.5, at 25°C. The concentration of reactants are 76 μM RNase A and 1.13 mM 2'CMP. The solid line corresponds to the theoretical curve with $n = 1.02$, $K_a = 2.9 \times 10^6 \text{ M}^{-1}$ and $\Delta H = -19.3 \text{ kcal/mol}$.

absorbance at 260 nm using an extinction coefficient of $7400 \text{ M}^{-1}\text{cm}^{-1}$ in 100 mM phosphate buffer, pH 7.0.

A final volume of 1 ml should be sufficient to conduct several experiments.

5. Dilute the dialyzed RNase A stock to a final concentration of 50 to 100 μM in 15 mM potassium acetate buffer, pH 5.5.
6. Prepare cell sample (2.2 ml of diluted RNase A) to ensure proper loading of the cell.
7. Dilute the 2'CMP stock to a final concentration of 0.5 to 1.5 mM in 15 mM potassium acetate buffer, pH 5.5, depending on the concentration of RNase A.
8. Prepare syringe sample (0.5 ml of diluted 2'CMP) to ensure proper loading of the syringe.
9. Degas 10 ml of the same buffer employed, cell sample solution, and syringe sample solution 20 to 30 min.

This buffer solution will be used for rinsing the sample cell.

10. After preparation, transfer the syringe solution to a 6×50 -mm glass tube to fill the syringe.
11. Set the instrument settings, run the experiment, and analyze the data (see Basic Protocol 1 and Support Protocol).

Figure 17.8.3 shows the result from an experiment in which 2'CMP binds to the enzyme RNase A. The thermodynamic parameters obtained from this experiment are: $K_a = 2.0 \times 10^6 \text{ M}^{-1}$, $\Delta H_a = -17.8 \text{ kcal/mol}$, $n = 0.99$.

BASIC PROTOCOL 3

MACROMOLECULE/MACROMOLECULE INTERACTION WITH MODERATE AFFINITY

The same equations above (see Support Protocol) apply to macromolecule/macromolecule binding. The formalism can be applied to this case by considering the macromolecule in the syringe as the ligand. As in every situation, the less soluble reactant should be placed in the calorimetric cell.

This protocol uses commercially available porcine pancreatic trypsin (PPT), a well-studied serine protease of 23.8 kDa, and its commercially available inhibitor, soybean trypsin inhibitor (STI), of 20.0 kDa. The binding of STI to PPT is an example of an endothermic binding reaction, and the binding constant for this reaction is within the range measurable by conventional ITC (El Harrous and Parody-Morreale, 1997).

Materials

Porcine pancreatic trypsin (PPT), lyophilized powder (Sigma)
25 mM potassium acetate/10 mM calcium chloride, pH 4.5
Soybean trypsin inhibitor (STI), lyophilized powder (Sigma)

10-kDa MWCO dialysis tubing
0.22- μm filter

Prepare samples

1. Dissolve PPT in 25 mM potassium acetate/10 mM calcium chloride buffer, pH 4.5, to a final concentration of $\sim 400 \mu\text{M}$.

A final volume of 1 ml of this PPT stock solution should be sufficient.

- Dissolve STI in 25 mM potassium acetate/10 mM calcium chloride buffer, pH 4.5, to a final concentration of $\sim 30 \mu\text{M}$.

A final volume of 5 ml of this STI stock solution should be sufficient.

- Dialyze PPT and STI against ~ 1 liter of 25 mM potassium acetate/10 mM calcium chloride buffer, pH 4.5, overnight at 4°C using a dialysis membrane with a ~ 10 -kDa MWCO.
- After dialysis, filter the solutions through $0.22\text{-}\mu\text{m}$ pore filters.
- Determine the exact concentration of the PPT and STI stock solutions by measuring their absorbances at 280 nm using extinction coefficients of 35,700 and $18,200 \text{ M}^{-1}\text{cm}^{-1}$, respectively. Store the dialyzed stocks for several weeks at 4°C .
- Prepare cell sample. Prepare 2.2 ml of STI at a concentration of $\sim 30 \mu\text{M}$.

Since STI has very low solubility compared to PPT, the former will be placed in the calorimetric cell.

- Prepare syringe sample. Prepare 0.5 ml of PPT at a concentration of $\sim 400 \mu\text{M}$.

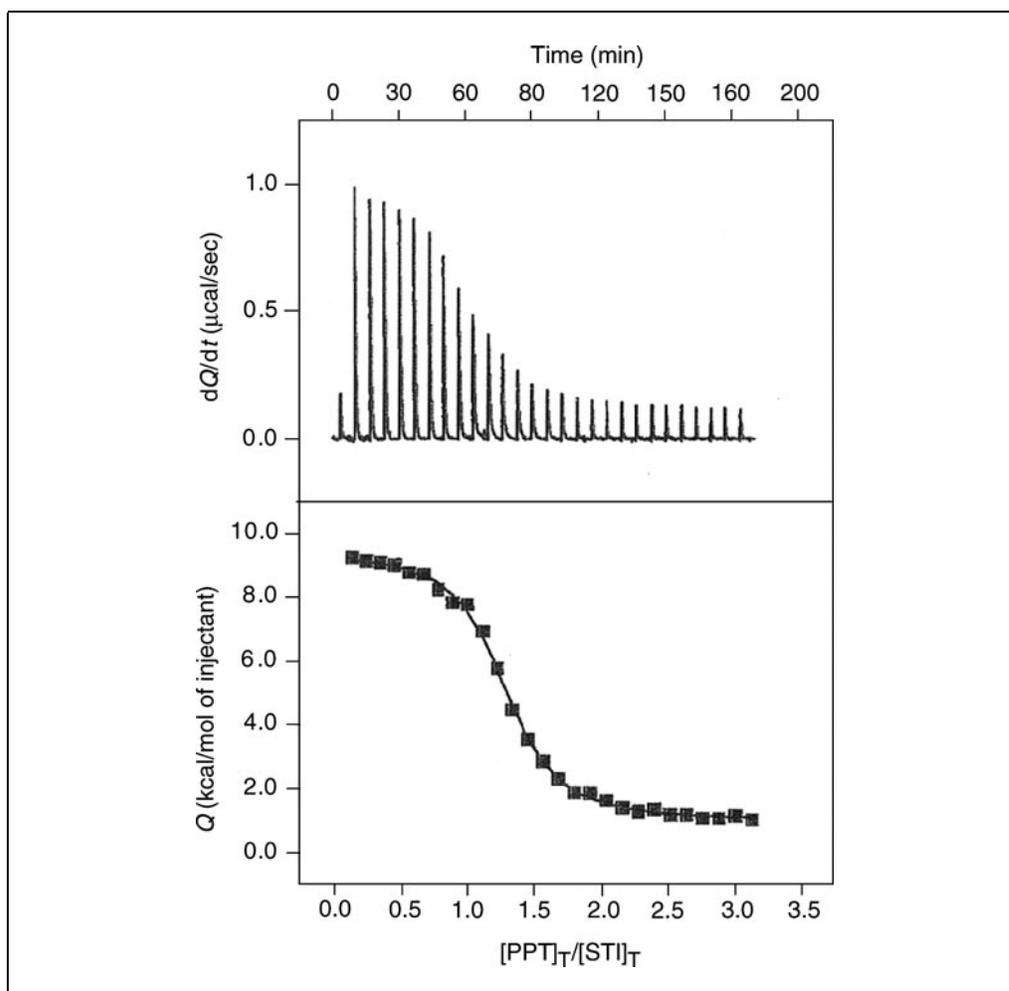


Figure 17.8.4 Titration of STI with PPT. The experiment was performed in 25 mM potassium acetate, pH 4.5/10 mM calcium chloride, at 25°C . The concentrations of reactants are $21 \mu\text{M}$ STI (in cell) and $312 \mu\text{M}$ PPT (in syringe). The inhibitor was placed in the calorimetric cell due to its low solubility. The solid line corresponds to theoretical curve with $n = 1.26$, $K_a = 1.5 \times 10^6 \text{ M}^{-1}$ and $\Delta H_a = 8.4 \text{ kcal/mol}$.

8. Set instrument settings, run the experiment, and analyze the data (see Basic Protocol 1 and Support Protocol).

Figure 17.8.4 shows the result from an experiment in which STI binds to PPT. The thermodynamic parameters obtained from this experiment are: $K_a = 1.5 \times 10^6 \text{ M}^{-1}$, $\Delta H_a = 8.4 \text{ kcal/mol}$, $n = 1.26$.

MACROMOLECULE/LIGAND BINDING WITH HIGH AFFINITY

If the affinity between the reactants is too high ($K_a > 10^8 \text{ M}^{-1}$), the affinity of binding cannot be reliably measured. To determine the affinity directly, the concentrations of the reactants need to be lowered to levels below the sensitivity limit of the calorimeter. However, in the case of tight binding ligands, the binding enthalpy can be measured very accurately.

Traditionally, several methods have been employed to determine high affinity. All of them rely on changing the experimental conditions (temperature, pH) to reduce the apparent affinity to a measurable value (Doyle et al., 1995; Doyle and Hensley, 1998). The disadvantages are important: (1) appropriate equations (e.g., van't Hoff relationship) have to be used in order to extrapolate (with cumulative errors) to the initially intended experimental conditions; and (2) changing experimental conditions can compromise the stability or the solubility of the reactants.

A method that does not require a change in the experimental conditions is the displacement method (Sigurskjold, 2000). In this method, the ligand solution is placed in the syringe and a solution of the macromolecule pre-bound to a weaker, competitive ligand is placed in the cell. In this way, the apparent affinity of the potent ligand is reduced because it has to displace the weak ligand from the binding site to bind to the macromolecule, therefore, incurring in an energy penalty. Thus, the thermodynamic parameters for the binding of the weak ligand and also the linkage equations for the binding of the potent ligand in the presence of the weak one should be well known.

The same reasoning as in the basic case is made: from the mass conservation equations and the mass action law, it is possible to reach a cubic equation from which the concentration of all species, free and bound, can be obtained and the analysis can be done in an exact manner. The details are explained elsewhere (Sigurskjold, 2000).

However, even if the exact analysis is always the one to be performed, an approximation can be considered when the weak ligand concentration is much higher than the macromolecule concentration, so that the concentration of free weak ligand is assumed to be equal to the concentration of total weak ligand (Zhang and Zhang, 1998). In this approximation, the apparent binding parameters in the displacement binding are related to the individual binding parameters as in Equations 17.8.6 and 17.8.7:

$$K_{a,A}^{\text{app}} = \frac{K_{a,A}}{1 + K_{a,B}[B]}$$

Equation 17.8.6

$$\Delta H_{a,A}^{\text{app}} = \Delta H_{a,A} - \Delta H_{a,B} \frac{K_{a,B}[B]}{1 + K_{a,B}[B]}$$

Equation 17.8.7

where A and B are the potent and the weak ligands, $K_{a,A}$ and $\Delta H_{a,A}$ are the affinity and enthalpy of binding of the potent ligand, and $K_{a,B}$ and $\Delta H_{a,B}$ are the affinity and enthalpy

of binding of the weak ligand, and the free concentration of ligand B can be approximated by its total concentration.

One of the most important applications of ITC is in the field of structure-based drug design. The goal in any drug design process is to obtain inhibitors with very tight binding affinities to the desired target, while minimizing the binding of the inhibitor to unwanted targets. Another goal is to design inhibitors that are able to adapt to genetic polymorphisms in their target. Genetic polymorphisms can arise as a result of natural population polymorphisms or mutations that cause drug resistance in viral, microbial, and parasitic targets.

Understanding the detailed mechanism and thermodynamic differences in the binding of various inhibitors allows the development of guidelines essential to the design of highly specific drugs that are able to adapt to genetic polymorphisms in their target. The first structure-based drugs were targeted against HIV-1 protease, an aspartic protease essential for the processing of the HIV virus. Measuring the thermodynamic parameters of the binding of HIV-1 protease inhibitors to their target is compounded by the very tight binding of these inhibitors ($K_a > 10^8 \text{ M}^{-1}$), which makes direct titration impossible. A displacement assay, in which a tight-binding inhibitor competes with a weaker binding inhibitor ($K_a = 10^6\text{--}10^7 \text{ M}^{-1}$), allows the measurement of all thermodynamic parameters in a single ITC experiment. This protocol uses HIV-1 protease, a well-studied aspartic protease of 21.6 kDa, and its commercially available inhibitors nelfinavir and acetyl-pepstatin. Unlike acetyl-pepstatin, the affinity of the binding of nelfinavir to HIV-1 protease is beyond the higher limit of measurable range by conventional ITC.

Three titrations should be made: two direct titrations with nelfinavir and acetyl-pepstatin binding to HIV-1 protease, respectively, and one displacement titration of nelfinavir binding to protease prebound to acetyl-pepstatin.

Materials

Acetyl pepstatin (Bachem)
9 mM NaOH
Nelfinavir (Viracept; or any other clinical or experimental inhibitor)
100% DMSO
HIV-1 protease (Todd et al., 1998 and Velazquez-Campoy et al., 2002)
10 mM sodium acetate, pH 5.0/2% (v/v) DMSO

Prepare samples

1. Prepare acetyl-pepstatin stock solution: dissolve the lyophilized acetyl-pepstatin in 9 mM NaOH (this compound is very insoluble in pure water) to a concentration of 8 to 9 mM.
2. Determine the concentration by analytical quantitative nitrogen content determination (Jaenicke, 1974; Pace et al., 1995).

Acetyl-pepstatin shows no absorbance at 280 nm.

3. Prepare nelfinavir stock solution at >15 mM in 100% DMSO.

Nelfinavir is not water soluble. The solution in pure DMSO should be very concentrated (15 to 40 mM). When DMSO is diluted in aqueous buffer, the concentration of nelfinavir is closer to 200 to 300 μM .

4. Prepare cell sample for first titration by purifying HIV-1 protease according to Todd et al. (1998) and Velázquez-Campoy et al. (2002) and storing at $\sim 20^\circ\text{C}$ at a

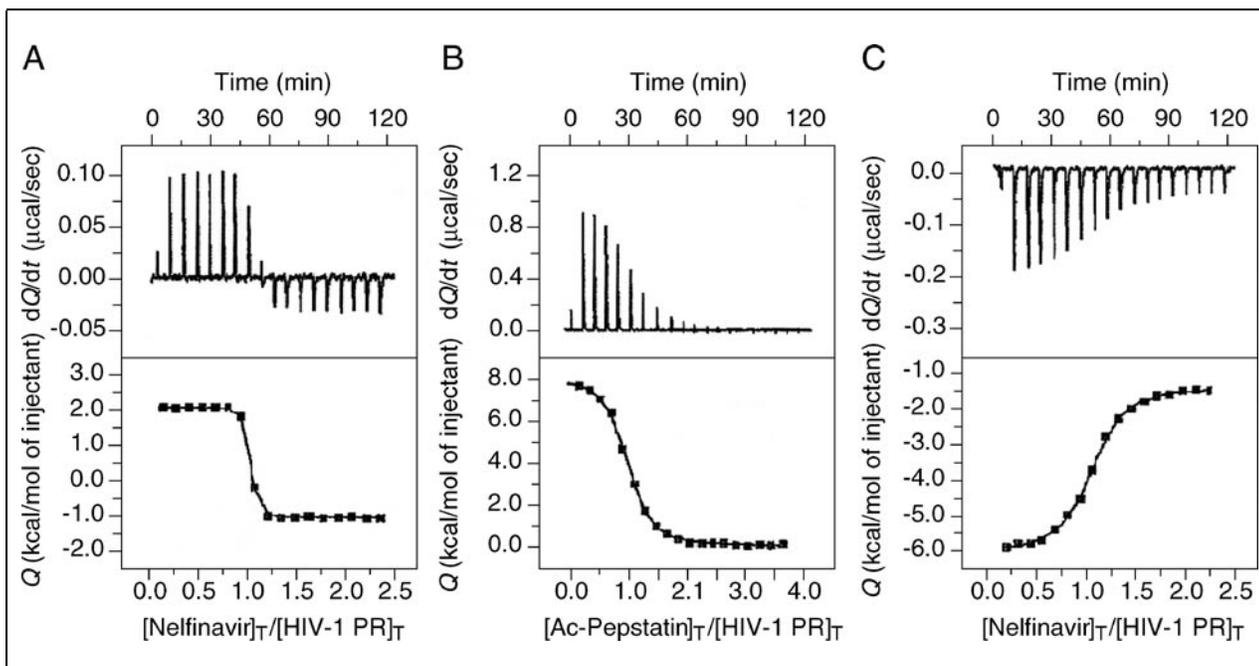


Figure 17.8.5 Set of calorimetric titrations corresponding to the implementation of the displacement protocol for the estimation of very high affinity. The concentrations of reactants are: **(A)** HIV-1 protease 11 μM in cell and nelfinavir 130 μM in syringe; **(B)** HIV-1 protease 19 μM in cell and acetyl-pepstatin 300 μM in syringe; and **(C)** HIV-1 protease 10 μM and acetyl-pepstatin 200 μM in cell, and nelfinavir 130 μM in syringe. The thermodynamic parameters obtained from these experiments are: nelfinavir binding to protease: $\Delta H_a = 3.1$ kcal/mol, $n = 1.02$, and K_a cannot be reliably obtained; acetyl-pepstatin binding to protease: $K_a = 2.3 \times 10^6$ M^{-1} , $\Delta H_a = 8.0$ kcal/mol, $n = 0.98$; nelfinavir binding to protease pre-bound to acetyl-pepstatin $K_a = 2.2 \times 10^9$ M^{-1} , $\Delta H_a = 3.3$ kcal/mol, $n = 0.99$.

concentration of >2.5 mg/ml. Dissolve the protein to a final concentration of 20 μM in 10 mM sodium acetate, pH 5.0/2% DMSO.

5. Determine the exact concentration of the cell sample by measuring its absorbance at 280 nm using an extinction coefficient of 25,500 $\text{M}^{-1} \text{cm}^{-1}$.
6. Prepare syringe sample for first titration: 2.2 ml of nelfinavir stock diluted to a concentration of ~ 300 μM in 10 mM sodium acetate, pH 5.0. If necessary, add DMSO to a final concentration of 2% to ensure that the inhibitor is dissolved in the aqueous buffer.
7. Perform the first titration with HIV-1 protease in the cell and nelfinavir in the syringe.
8. Prepare cell sample for second titration. Repeat step 4.
9. Prepare syringe sample for second titration: 0.5 ml of acetyl-pepstatin diluted to a concentration of ~ 300 μM in 10 mM sodium acetate, pH 5.0. Add DMSO to a final concentration of 2%.
10. Perform the second titration with HIV-1 protease in the cell and acetyl-pepstatin in the syringe.
11. Prepare cell sample for third titration. Repeat step 4 adding acetyl-pepstatin to the HIV-1 protease in the sample cell solution to a final concentration of 200 μM .
12. Prepare syringe sample for third titration. Repeat step 6.
13. Perform third titration.

Figure 17.8.5 shows the results from an experimental scheme aimed at determining the thermodynamic parameters for the binding of nelfinavir to HIV-1 protease. The thermodynamic

parameters obtained from these experiments are: nelfinavir binding to protease: $\Delta H_a = 3.1$ kcal/mol, $n = 1.02$; acetyl-pepstatin binding to protease: $K_a = 2.3 \times 10^6$ M⁻¹, $\Delta H_a = 8.0$ kcal/mol, $n = 0.98$; nelfinavir binding to protease prebound to acetyl-pepstatin: $K_a = 2.2 \times 10^9$ M⁻¹, $\Delta H_a = 3.3$ kcal/mol, $n = 0.99$ (applying the exact equations for the displacement experiment in the data analysis).

MACROMOLECULE/LIGAND BINDING WITH LOW AFFINITY

If the affinity between the reactants is too low ($K_a < 10^4$ M⁻¹), neither the affinity nor the enthalpy of binding can be reliably determined.

One way to determine both is again by using the displacement strategy (Zhang and Zhang, 1998). In this case, the binding parameters for the weak ligand are obtained from the change in the binding parameters of the potent ligand binding to the free macromolecule and its binding to the macromolecule pre-bound to the weak ligand. The same considerations and analysis procedure explained in the case of very high affinity ligands applies here.

Many natural ligands show very low affinity ($K_a < 10^4$ M⁻¹) for their biological targets. To measure such affinity, the displacement protocol can be implemented again.

In the case of the CMP inhibitors of RNase A, a change in the position of the hydroxyl group from 2' to 5' position causes a dramatic decrease in the strength of the interaction. This example illustrates the effect of minor modifications in the ligand on the thermodynamic parameters of binding.

To estimate the binding affinity for 5'-cytidine-monophosphate (5'CMP) binding to RNase A, a similar set of titrations as in the previous section should be done: two direct titrations with 2'CMP and 5'CMP binding to RNase A and one displacement titration of 2'CMP binding to RNase A prebound to 5'CMP.

For materials, see Basic Protocol 2.

Prepare samples

1. Prepare RNase A and inhibitors 2'CMP and 5'CMP stock solutions following instructions in the Basic Protocol 2.
2. Prepare cell sample for first titration: 2.2 ml of RNase A diluted to a concentration of 50 to 100 μ M in 15 mM potassium acetate buffer, pH 5.5.
3. Prepare syringe sample for first titration: 0.5 ml of 2'CMP diluted to a concentration of \sim 0.5 to 1.5 mM in 15 mM potassium acetate buffer, pH 5.5.
4. Prepare cell sample for second titration. Repeat step 2.
5. Prepare syringe sample for second titration: 0.5 ml of 5'CMP diluted to a concentration of \sim 0.5 to 1.5 mM in 15 mM potassium acetate buffer, pH 5.5.
6. Prepare cell sample for third titration. Repeat step 2 adding 5'CMP to the solution to a concentration of 0.6 mM.
7. Prepare syringe sample for third titration. Repeat step 3.

Figure 17.8.6 shows the results from an experimental scheme aimed at determining the thermodynamic parameters for the binding of 5'CMP to RNase A. Three titrations are shown: 2'CMP binding to RNase A, 5'CMP binding to RNase A, and 2'CMP binding to RNase A pre-bound to 5'CMP. In the first titration, the binding parameters are obtained as described earlier. It is not possible to obtain a reliable value for either the affinity or the enthalpy of binding from the second titration. On the other hand, applying the exact analysis

ALTERNATE PROTOCOL 2

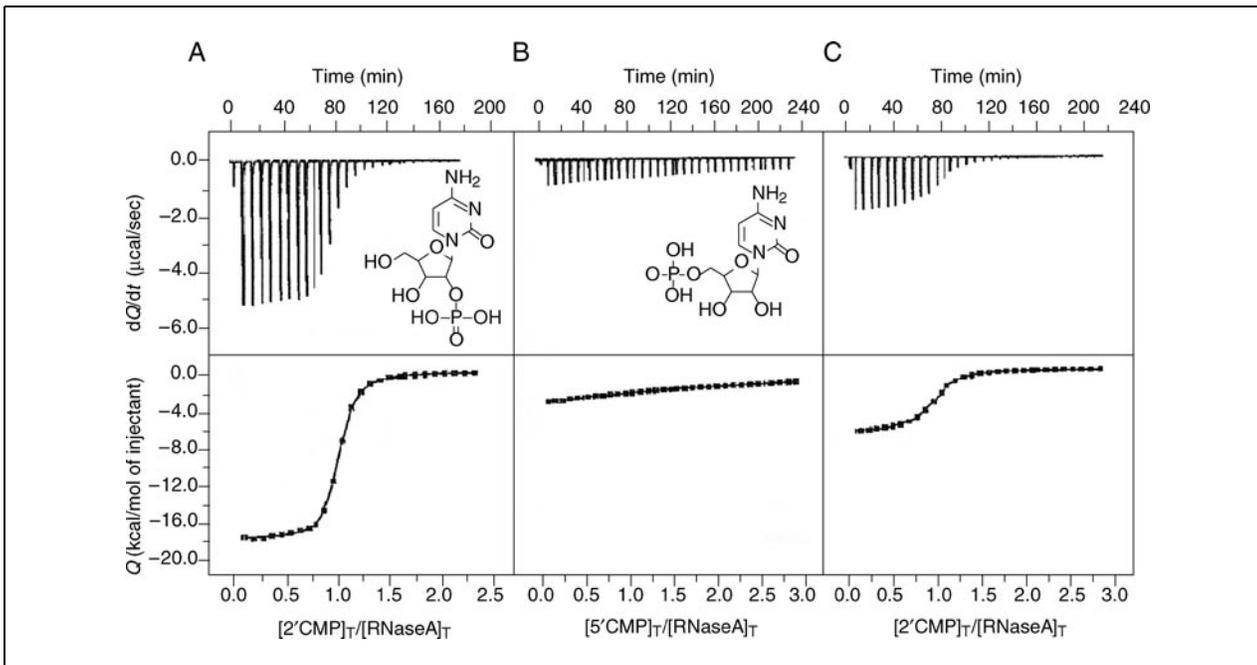


Figure 17.8.6 Set of calorimetric titrations corresponding to the implementation of the displacement protocol for the estimation of very low affinity. The concentrations of reactants are: **(A)** RNase A 76 μM in cell and 2'CMP 1.13 mM in syringe; **(B)** RNase A 76 μM in cell and 5'CMP 1.07 μM in syringe; and **(C)** RNase A 76 μM and 5'CMP 600 μM in cell, and 2'CMP 1.13 mM in syringe. The first titration corresponds to the one shown in Figure 17.8.1. In the second titration neither the binding affinity nor the binding enthalpy can be estimated. In the third titration, the thermodynamic parameters for the binding of 5'CMP obtained applying the exact analysis are: $K_a = 4250 \text{ M}^{-1}$, $\Delta H_a = -16.3 \text{ kcal/mol}$, $n = 0.99$. If using the approximation method, the values obtained for the binding of 5'CMP are: $K_a = 4300 \text{ M}^{-1}$, $\Delta H_a = -15.7 \text{ kcal/mol}$, $n = 0.98$, which are in agreement with the previous ones.

to the third titration yields the following thermodynamic parameters for the binding of 5'CMP: $K_a = 4250 \text{ M}^{-1}$, $\Delta H_a = -16.3 \text{ kcal/mol}$, $n = 0.99$. Using the approximation method, the values obtained for the binding of 5'CMP are: $K_a = 4300 \text{ M}^{-1}$, $\Delta H_a = -15.7 \text{ kcal/mol}$, $n = 0.98$, which are in reasonable agreement with the exact analysis.

ALTERNATE PROTOCOL 3

BINDING OF HOMODIMERIC PROTEINS

When the objective is to determine the thermodynamic parameters for the formation of homodimers, Basic Protocols 1 and 2 are not useful since they require the physical separation between reactants. However, it is still possible to modify the standard procedure towards that goal. In this case, the only reactant is placed in the syringe and the cell is filled with the appropriate buffer solution. The experiment consists of performing a series of injections of the reactant solution into the calorimetric cell. Since the concentration of reactant in the syringe is constant throughout the experiment, the fraction of monomers and dimers within the syringe remains constant. However, the dilution of the reactant in the cell upon injection promotes the dissociation of dimers into monomers. In these circumstances, the area under the peak associated with each injection is simply the heat released or absorbed during the dissociation reaction. Details and examples can be found in the literature (Burrows et al., 1994; Lovatt et al., 1996).

ALTERNATE PROTOCOL 4

BINDING OF UNSTABLE PROTEINS

Sometimes the unique nature of some macromolecules impedes the implementation of Basic Protocol 1 for ITC binding studies. Certain proteins tend to denature or aggregate under the vigorous stirring conditions of the calorimetric cell, which makes conducting standard ITC assays impossible.

Isothermal Titration Calorimetry

17.8.16

However, it is possible to modify the protocol to determine the enthalpy of binding of ligands to such proteins. A solution of the ligand is placed in the calorimetric cell and a solution of the macromolecule is placed in the syringe. Concentrations of reactants should be chosen so that, when injecting, all of the macromolecule binds to the ligand. Therefore, all the heat associated with the injection can be assigned to the binding of all injected macromolecules to the ligand. To obtain the enthalpy of binding, it is necessary to estimate how much heat is coming from binding and how much from unspecific processes (e.g., injection, friction, dilution). Blank experiments, in which buffer solution alone (without ligand) is placed in the cell, are needed to evaluate the non-binding heat. The binding enthalpy is obtained by subtracting such contribution from the observed heat (see Equation 17.8.8):

$$\Delta H_a = \frac{Q_T - Q_{\text{Blank}}}{v[S]_0}$$

Equation 17.8.8

where Q_T is the heat associated with the injection of macromolecule into the ligand solution, Q_{Blank} is the heat associated with the injection of macromolecule into the buffer solution, v is the injection volume, and $[S]_0$ is the concentration of reactant in the syringe.

Similar experiments can also be used in Basic Protocol 1 to estimate the heat produced by ligand dilution and to determine if the ligand self-associates or aggregates at high concentration in the syringe. Details and examples can be found in the literature (Nezami et al., 2002, 2003).

MEASURING THE HEAT CAPACITY CHANGE ASSOCIATED WITH BINDING

ALTERNATE PROTOCOL 5

A single ITC assay can provide information regarding the binding constant, the Gibbs free energy of binding, the binding enthalpy, the binding entropy, and the stoichiometry of the binding reaction. In addition to this information, the change in heat capacity upon binding and the change in ionization state upon binding can be obtained by repeating the experiment with varying temperature or buffer conditions.

The heat capacity change, at constant pressure, is simply the temperature derivative of the enthalpy change (see Equation 17.8.9):

$$\Delta C_p = \left(\frac{\partial \Delta H}{\partial T} \right)_p$$

Equation 17.8.9

Therefore, one can determine its value by measuring the enthalpy change of a binding reaction at different temperatures. Plotting ΔH versus temperature would yield ΔC_p as its slope. The heat capacity of binding reflects the burial of polar and non-polar surfaces as a consequence of the binding reaction (Privalov and Makhatadze, 1992; Murphy and Freire, 1992; Gómez et al., 1995).

ΔC_p is usually determined by measuring the enthalpy of binding from 15° to 35°C at 5° intervals under identical buffer and pH conditions. Figure 17.8.7 shows the temperature dependence of the binding enthalpy of amprenavir and TMC-126, a clinical and an experimental inhibitor of HIV-1 protease, respectively (Ohtaka et al., 2002).

Macromolecular Interactions in Cells

17.8.17

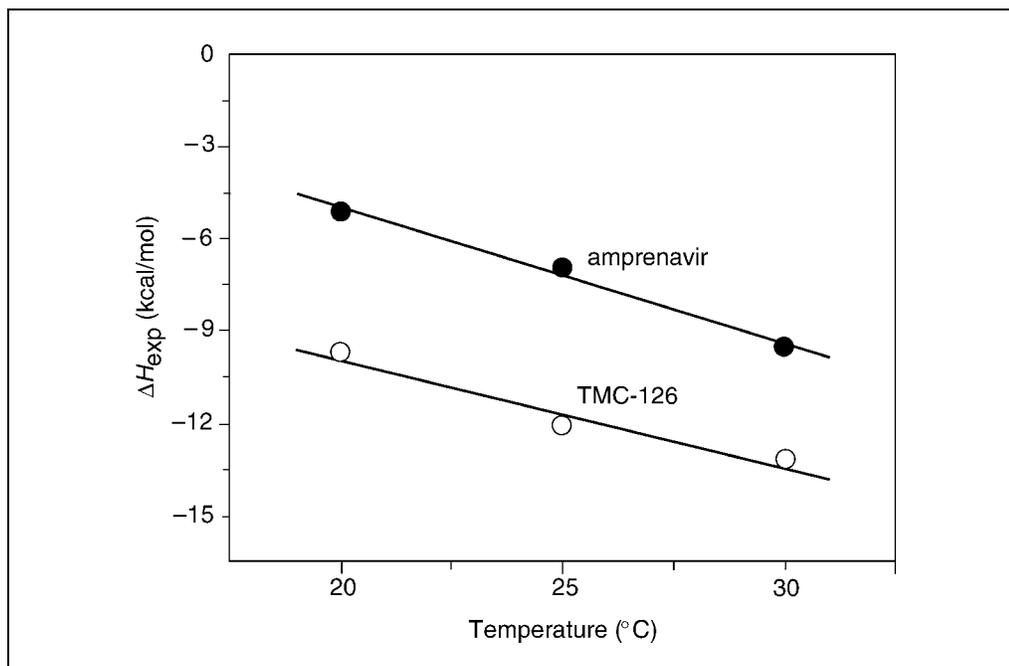


Figure 17.8.7 Temperature dependence of the binding enthalpy for amprenavir and TMC-126 binding to HIV-1 protease (Ohtaka et al., 2002). The experiments were done in 10 mM sodium acetate, pH 5.0/2%DMSO. The slope of the plot is equal to the heat capacity change upon binding and it is related to the burial of accessible surface areas from both the protein and the ligand. The values of the binding heat capacity for amprenavir and TMC-126 are -440 cal/K·mol and -350 cal/K·mol, respectively.

The heat capacity change of binding processes between a protein and a ligand is usually negative and <1 kcal/K·mol in absolute value. However, the binding of two macromolecules can be associated with a higher heat capacity change, which is indicative of a larger burial of solvent-accessible surface area upon binding or the structuring of some regions of the macromolecule.

**ALTERNATE
PROTOCOL 6**

**MEASURING PROTONATION/DEPROTONATION PROCESSES
COUPLED TO BINDING**

The change in the protonation state of certain residues involved in the binding process produces a proton transfer between the complex and the bulk solution. This phenomenon can be studied by detecting the heat effect produced by the protons exchanged between the protein and the buffer as a consequence of the inhibitor binding (Gómez and Freire, 1995; Baker and Murphy, 1996, 1997). In this case, the experimental enthalpy of binding, ΔH_{exp} , is the sum of the intrinsic enthalpy of the binding reaction, $\Delta H_{\text{binding}}$, independent of the buffer used and a term proportional to the enthalpy of ionization of the buffer (see Equation 17.8.10):

$$\Delta H_{\text{exp}} = \Delta H_{\text{binding}} + N_{\text{H}^+} \Delta H_{\text{ion}}$$

Equation 17.8.10

where the proportionality constant, N_{H^+} , is the number of protons that are exchanged between the complex and the bulk solution, and ΔH_{ion} is the ionization enthalpy of the buffer. Experiments are done under the same pH and temperature, using buffers with different ionization enthalpies. Figure 17.8.8 shows the protonation/deprotonation effect associated with the binding of amprenavir and TMC-126 to HIV-1 protease (Ohtaka et al., 2002). The coupling of protonation/deprotonation processes to the binding of a

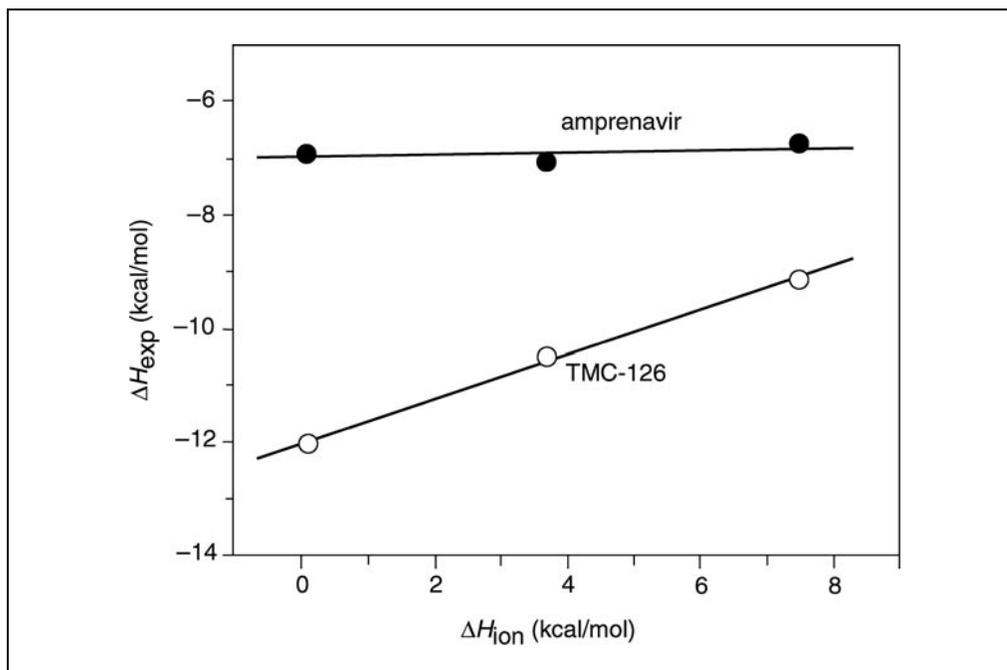


Figure 17.8.8 Dependence of the binding enthalpy for amprenavir and TMC-126 binding to HIV-1 protease on the ionization enthalpy of the buffer used in the experiment (Ohtaka et al., 2002). The experiments were done at pH 5.0 in 2% DMSO and 10 mM buffer concentration with buffers of different ionization enthalpy (acetate 0.12 kcal/mol, MES 3.72 kcal/mol, ACES 7.51 kcal/mol). The number of protons exchanged (slope) for amprenavir and TMC-126 is 0.02 and 0.39, respectively. The buffer-independent binding enthalpy (intercept with y-axis) for amprenavir and TMC-126 is -6.9 kcal/mol and -12.0 kcal/mol, respectively. Unlike in the case of amprenavir, where there is no net proton exchange between the ML complex and bulk solution upon binding, for TMC-126 there is a significant proton transfer.

ligand could make the experimental enthalpy measured directly, ΔH_{ion} , very different (even opposite) from the actual binding enthalpy, $\Delta H_{\text{binding}}$, if either N_{H^+} or ΔH_{ion} is large enough.

COMMENTARY

Background Information

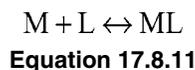
Isothermal titration calorimetry (ITC) is a technique that directly measures the energetics associated with the binding of two components. ITC is the only biophysical method that directly determines the enthalpy (ΔH) associated with binding and provides a complete thermodynamic profile of the interaction, including binding affinity (K_a), number of binding sites (n), entropy change (ΔS), and Gibbs energy change (ΔG). ITC can be utilized for determining very low (10^3 M^{-1}) to very high binding affinities (10^{12} M^{-1}). Besides providing fundamental information about binding, ITC does not require immobilization of the binding components and takes very little time, unlike other techniques [e.g., surface plasmon resonance (UNIT 17.6) and ultra-centrifugation]. Moreover, since heat is universally generated or absorbed during any

molecular interaction, ITC has become a more common and general detection method without the need of reporter labels. An ITC instrument has a simple setup consisting of two identical cells, a sample cell that contains a solution of one of the reactants and a reference cell that serves as a temperature reference. The instrument is equipped with a syringe that contains the second binding component and adds aliquots stepwise to the sample cell. In the last two decades, ITC has become an important technique in determining the binding energetics of various biochemical processes (Wiseman et al., 1989; Freire et al., 1990; Doyle, 1997; El Harrou and Parody-Morreale, 1997; Jelesarov and Bosshard, 1999; Leavitt and Freire, 2001; Ward and Holdgate, 2001; Ladbury, 2001), including protein-ligand binding, protein-protein binding, DNA-protein binding, protein-carbohydrate binding,

protein-lipid binding, and antigen-antibody binding. Moreover, with the ability to precisely determine the enthalpic and entropic components of binding, ITC has become an important component of the drug discovery process.

Thermodynamics of binding

Consider a binding reaction at equilibrium where a biological macromolecule *M* binds another molecule *L* (ligand; see Equation 17.8.11):



Assuming that there is a single binding site, the binding affinity or association constant, K_a , which is the inverse of the dissociation constant, K_d , determines the partition of the reactants into free and bound species, is given by (see Equation 17.8.12):

$$K_a = [ML]/[M][L]$$

Equation 17.8.12

The binding affinity is related to the free energy of binding, ΔG (see Equation 17.8.13):

$$\Delta G = -RT \ln K_a$$

Equation 17.8.13

where R is the gas constant and T the absolute temperature. The free energy of binding can be expressed in terms of the enthalpy (ΔH) and entropy (ΔS) of binding (see Equation 17.8.14):

$$\Delta G = \Delta H - T\Delta S$$

Equation 17.8.14

and therefore (Equation 17.8.15):

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S$$

Equation 17.8.15

As it can be seen from Equation 17.8.15, enthalpy (ΔH) and entropy (ΔS) are the two determinants of the binding affinity. The binding enthalpy primarily reflects the strength of interactions between the ligand and the target (non-covalent interactions, e.g., van der Waals, hydrogen bonds, electrostatics) relative to those existing with the solvent. On the other hand, the entropy change mainly reflects two contributions, changes in solvation entropy, ΔS_{solv} , and changes in conformational entropy, ΔS_{conf} (Lee et al., 1994; D'Aquino et al., 1996; D'Aquino et al., 2000). Other energetic contributions, such as those due to the

loss of translational degrees of freedom are similar for all bimolecular associations and amount to 8 to 10 cal/K-mol (Murphy et al., 1994; Amzel, 1997, 2000). As the ligand binds to the macromolecule, desolvation occurs, and water molecules are released from the binding site and the ligand, producing a significant increase in degrees of freedom. This desolvation process is favorable to binding as it increases the entropy of the system. At the same time, however, the ligand and certain groups in the protein lose conformational freedom as they bind to each other (relative to their conformational degree of freedom in water). Therefore, this loss in conformational entropy, ΔS_{conf} , is usually unfavorable for the binding process, unless the binding reaction is coupled to the loss in structure of distal regions from the binding site. Since the enthalpy and entropy changes are the manifestation of different types of inter- and intra-molecular interactions, two ligands that exhibit different enthalpic and entropic contributions to the Gibbs energy of binding reflect different binding mechanisms, even if their binding affinities might be the same (Myzka et al., 1997; Parker et al., 1999; Velázquez-Campoy et al., 2000a,b; Velázquez-Campoy et al., 2001). Additional information and more advanced formalism and developments about binding thermodynamics can be found in the literature (Cantor and Schimmel, 1980; Wyman and Gill, 1990; Luque and Freire, 1998; Van Holde et al., 1998).

Applications

ITC has evolved to become a very useful technique in determining the energetics of a wide variety of chemical and biochemical interactions. Furthermore, ITC can be utilized as a standard technique in determining the precise binding affinities of components from nanomolar range to micromolar range and coupling of the binding reaction to protonation/deprotonation processes. The implementation of displacement titration experiments has extended range for affinity determination, as explained below.

Ligand binding energetics

The useful range for an accurate binding affinity measurement by standard ITC is between 10^4 and 10^8 M^{-1} . However, the practical range of binding affinities experimentally accessible can be expanded to 10^3 to 10^{12} M^{-1} by using an appropriate well-characterized secondary ligand (ITC displacement technique). This is particularly useful in the drug design

process, where the ability to accurately measure binding affinities in the sub-nanomolar range is crucial during the lead optimization stage. ITC displacement experiments will be discussed in detail in later sections.

Drug development

ITC has been utilized in the characterization and optimization of lead compounds as viable drug candidates as it provides complete information of the binding affinity of a ligand to a target in terms of its thermodynamic components ΔG , ΔH , ΔS , and ΔC_p . Current strategies for lead identification and optimization depend almost exclusively on binding affinities or Gibbs energy of binding. The Gibbs energy of binding is a result of a delicate balance between the enthalpic and the entropic contributions, and different combinations of ΔS and ΔH can give rise to the same ΔG values and, therefore, elicit the same binding affinity. A higher affinity can be achieved by two means: (1) obtaining a more favorable (negative) enthalpy change, i.e., improving ligand-protein interactions over those with the solvent, and (2) obtaining a more favorable (positive) entropy change, which in turn can be achieved by making a ligand more hydrophobic in order to maximize the solvation entropy, and/or pre-shaping the ligand in order to minimize the loss of conformational entropy. Traditional drug-design methods rely on hydrophobicity and pre-shaping compounds through the use of conformational constraints, therefore, giving rise to entropically optimized compounds (Todd et al., 2000; Velázquez-Campoy et al., 2002; Ohtaka et al., 2002). Such an approach exhibits several pitfalls, the most important ones being the lack of specificity, the susceptibility to mutations in the target, and the impossibility to achieve extremely high affinity. For example, in the case of HIV-1 protease inhibitors, there is not a single reported case of an entropically driven inhibitor with an affinity stronger than 0.3 nM (Todd et al., 2000; Ohtaka et al., 2002). Therefore, ITC can be utilized not only to characterize existing ligands but also to provide a step-wise guide in the design process by permitting a direct evaluation of the energetic effects of specific chemical modifications.

Evaluation of mutations effects

Genetic diversity and mutations associated with drug resistance are the two major causes of failure of drug therapy in various bacterial and viral diseases, e.g., HIV infection. These two phenomena involve changes in the drug

binding sites of the target molecules. Since drug molecules developed under the traditional drug-design paradigm, based on the lock-and-key concept, are conformationally constrained and pre-shaped to the geometry of the target molecule, they cannot effectively adapt to changes in binding site geometry due to naturally occurring polymorphisms or drug resistance mutations (Velázquez-Campoy et al., 2002; Ohtaka et al., 2002). As a result, they lose significant binding affinity. Therefore, a major challenge in drug design is the integration of binding site heterogeneity in the design process. An ideal molecule would be one that is able to adapt to the variability in the target while simultaneously displaying high binding affinity and specificity. ITC being the only technique to provide the individual components of the Gibbs free energy of binding (ΔH , ΔS , and ΔC_p), is able to determine subtle changes in the binding process arising from polymorphisms or mutations in the target. Therefore, ITC can be utilized in the design process of an adaptive ligand that will overcome the current deficiency in the drug design process against variable targets (Velázquez-Campoy et al., 2001; Velázquez-Campoy and Freire, 2001; Freire, 2002; Nezami et al., 2003).

Critical Parameters and Troubleshooting

For a titration experiment to be successful and well designed, the following critical points should be considered.

1. ITC measures the global heat effect associated with the binding of two molecules. This includes the actual heat of binding (binding enthalpy) and all other heat effects originated from non-specific events (dilution of reactants, friction of injected liquid, etc.). Therefore, it is important to ensure that these contributions are minimized or considered in the analysis. For example, in the case of the dilution of reactants, the last peaks in the experiment after saturation can be an estimate of the heat effect due to such unspecific phenomena. Also, a titration of the reactant in the syringe into the buffer solution gives an estimation of the dilution effect of the titrant. In the case of friction of the injected liquid, small injection volumes (between 3 and 10 μl) and the recommended rate of injection (0.5 $\mu\text{l}/\text{sec}$) should be employed.

2. Regarding the possibility of aggregation or association of the reactants, the reactant with the lower solubility should be placed in the calorimetric cell.

3. As explained above, the appropriate range of concentrations and the proper ratio of ligand/macromolecule concentrations needs to be employed to guarantee completion of titration, reaching the saturation point in a reasonable number of injections (<30). If saturation is achieved in fewer injections (<5), then, the ligand/macromolecule concentration ratio should be lowered. On the contrary, if saturation is hardly reached, then the ratio should be increased.

4. A perfect match between the buffers has to be achieved to avoid spurious heat effects due to protonation of different species and mixing of different components that could be larger than the actual heat effect associated with the binding reaction. Accordingly, extreme care should be taken when using organic co-solvents (e.g., DMSO), because a mismatch in the concentration of these components between the solutions in the cell and the syringe will cause large heat effects.

5. From a practical point of view, it should always be emphasized that a clean cell and a perfectly straight syringe are decisive to avoid spurious results and to give excellent baselines with high signal-to-noise ratio.

Anticipated Results

Different titration experiments and representative examples are shown in each protocol.

Typical titration experiments in the range of moderate affinity ($K_a \sim 10^6 \text{ M}^{-1}$) corresponding to exothermic and endothermic binding processes are shown in Figures 17.8.3 and 17.8.4, respectively. The following are several features that will indicate the quality of the experiment:

1. The curvature of the titration curve is appropriate to obtain a reliable estimation of the binding affinity.

2. The saturation point has been achieved and the final molar ratio is ~ 2.5 to 3.

3. There is a good signal-to-noise ratio. The data in Figure 17.8.3 is better than that of Figure 17.8.4 because the concentrations of reactants used in the titration are ten times higher. A good signal-to-noise ratio will guarantee small errors in the integration stage in data analysis.

4. In terms of thermal power between the peaks before and after the titration, there is a 50-fold decrease ($5 \mu\text{cal/sec}$ compared to $0.1 \mu\text{cal/sec}$, in absolute value) and a five-fold decrease ($1 \mu\text{cal/sec}$ compared to $0.2 \mu\text{cal/sec}$, in absolute value) in Figure 17.8.3 and 17.8.4, respectively. The standard deviation of the noise is $0.002 \mu\text{cal/sec}$ and $0.004 \mu\text{cal/sec}$ in

Figure 17.8.3 and 17.8.4, respectively, much lower than the height of the peaks before and after saturation.

Time Considerations

The time required for a complete ITC experiment can be divided into different steps. Preparing sample solutions requires ~ 30 min. Running the ITC experiment takes ~ 2 to 3 hr. Cleaning the ITC instrument requires ~ 20 min. Analyzing the results takes ~ 30 min.

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CHAPTER 18

Cellular Aging and Death

INTRODUCTION

This chapter covers two distinct processes, cellular aging and cellular death. It is essential for multicellular organisms that the attainment of a permanent non-dividing state (replicative senescence) and patterned cell death (apoptosis) occur in a highly regulated manner. While the aging and death of cells are not strictly linked mechanistically, they are juxtaposed within this chapter since both processes occur at the end of the cellular life span and since the failure of both processes is intimately linked to tumor formation and cancer.

Somatic cells do not divide indefinitely under normal circumstances, although they may acquire the ability to do so if they, for instance, express viral gene products that allow them to escape the controls of senescence. Senescence is marked by phenotypic changes such as irreversible growth arrest and altered differentiated functions, as well as an increased resistance to apoptotic stimuli. For primary fibroblasts in culture, it is possible to follow the entry of cells into senescence through both cellular morphology and gene expression. Furthermore, a great deal has been learned within the past few years about how the proliferative lifetime of cells is determined. There is now good evidence that cells sense the number of cell divisions they have undergone through changes in their telomeres. Telomeres are structures at the ends of linear chromosomes that are comprised of short, repeated DNA sequences and specialized proteins associated with these repeats. This structure is essential to the stability of linear chromosomes, but each round of DNA replication results in shortening of the telomeric repeats because the replication machinery inevitably leaves an unreplicated 3' overhang. It has been proposed that senescence is triggered when telomeres shorten below some critical length, and considerable evidence is consistent with this hypothesis. Experimentally, telomere length can be measured by a number of different methods, including Southern blotting of chromosome terminal restriction fragments (*UNIT 18.6*), Q-FISH (*UNIT 18.4*) or Flow FISH. The elegant work of Blackburn and colleagues has demonstrated that the enzyme telomerase is capable of de novo addition of telomeric repeats to the ends of chromosomes. However, most somatic cells do not express telomerase, although immortal cells do. Some tumor cell lines express telomerase, but it does not appear that telomerase expression is strictly necessary or sufficient to escape senescence. Experimentally, telomerase activity can be measured by a sensitive PCR-based protocol known as telomere repeat amplification or TRAP (*UNIT 18.6*).

As discussed in *UNIT 18.1*, cell death can occur in either a programmed (apoptotic) or more accidental (necrotic) manner. Apoptosis and necrosis can be distinguished by a number of criteria including cellular morphology and the patterns of DNA fragmentation (*UNIT 18.3*). Apoptosis is generally a preferred mode of cell death in multicellular organisms, since it is a more orderly dismantling of cellular structures and degradation of the cell's macromolecular contents and is therefore less likely to cause damage to surrounding cells. Apoptosis occurs in response to a number of stimuli, including developmental signals, the engagement of receptors for cell death stimuli, DNA damage, or large amounts of unfolded protein within the endoplasmic reticulum. These pathways eventually lead to the activation of cysteinyl aspartases (caspases; *UNIT 18.2*), a family of related proteases that act in a proteolytic cascade to bring about the fragmentation of the cellular contents and cell death.

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Cellular Aging
and Death

18.0.1

Supplement 22

Activation of poly(ADP-ribose)polymerase 1 (PARP1) is an early event after DNA damage (*UNIT 18.7*). Once active, poly(ADP-ribosyl)ation by this enzyme is likely to promote chromatin remodeling in order to allow DNA repair and changes in gene expression. Caspase-7 and Caspase-3 efficiently cleave PARP1 after the activation of apoptotic pathways, allowing the use of its breakdown intermediates and enzymatic products to be used as a sensitive indicator of cell physiology during apoptosis.

During the last decade, it has become clear that mitochondria play a surprisingly central role in the regulation and execution of apoptosis. *UNIT 18.5* describes protocols for the assessment of mitochondrial status during apoptosis. This unit includes protocols for the preparation of mitochondria, for the detection of mitochondrial release of cytochrome c and other intermembrane-space proteins, and for assaying apoptosis-associated transitions in mitochondrial membrane permeability and calcium release. A variety of flow cytometric assays (*UNIT 18.8*) utilize caspase activation, PARP1 cleavage, collapse of the mitochondrial transmembrane potential, and other changes in cell physiology to monitor apoptosis in cell populations. Finally, it should also be noted that many of the processes of apoptosis have been reconstituted within cell-free mammalian and *Xenopus* systems (*UNIT 11.12*), and these systems should be increasingly helpful in understanding the molecular events surrounding programmed cell death.

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This unit attempts to define cell toxicity that leads to cellular demise, with a strong emphasis on cell death via both apoptosis and necrosis, by summarizing some of the more recent developments in cellular, molecular, and biochemical studies of the events that govern the induction and execution of cell death.

CELL DEATH

Cell death can occur via two processes, which are fundamentally different in their nature and biological significance (Kerr et al., 1972). These are termed necrosis and apoptosis and their features are summarized in Table 18.1.1.

Necrosis, or “accidental” cell death, was the classic model and was thought to be the universal mode of cell death until apoptosis was identified in 1972 as a separate mode of cell death. In general, necrosis is considered to be a passive process (Trump and Ginn, 1969) that is usually caused by extreme trauma or injury to the cell (Kerr et al., 1972). In tissues, necrosis is often seen as a lesion or patch consisting of many disintegrated cells. Typically, necrosis involves irreversible changes within the nuclei (such as karyolysis), loss of cytoplasmic structure, dysfunction in various organelles (especially mitochondria), and finally, cytolysis as a result of high-amplitude swelling. The release of the dying cell’s contents into the extracellular space can cause further injury or even death of neighboring cells, and may result in inflammation or infiltration of proinflammatory cells into the lesion, leading to further tissue damage (Haslett, 1992).

The first references to a different type of cell death came from developmental studies. Glucksmann (1951) emphasized the existence of this phenomenon for the first time. This was followed by a surge of interest in the 1960s (for review see Lockshin, 1981). In the 1970s, histochemical studies of lysosomal changes in hepatic ischemia provided direct evidence for the existence of two distinct types of cell death (Kerr, 1971; Kerr et al., 1972). It was observed that rounded masses that developed in dead hepatic tissue contained cells that were morphologically different from those found in necrosis. These differences initially caused some confusion—i.e., the process was mistaken for a variation of necrosis and was called “shrinkage necrosis” (Kerr, 1971). The following year,

Kerr, Wyllie, and Currie proposed the term “apoptosis” to describe the distinct morphological changes associated with this form of cell death (Kerr et al., 1972).

Apoptosis has been described as a form of cellular suicide, since death appears to result from induction of active processes within the cell itself. Typically, apoptosis involves margination and condensation of nuclear chromatin (pyknosis), cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation, and finally, formation of apoptotic bodies.

A cell that is dying, whether by apoptosis or necrosis, undergoes rapid changes, which are reflected in its structure, morphology, and biochemistry. These are generally a result of various enzymes activated through elaborate signaling pathways. Since the first description of apoptosis in 1972, there has been a surge of interest in the study of apoptosis, and very little attention has been given to necrosis. This lack of parallel study of the two processes is also reflected in the degree of detail provided here describing apoptosis and necrosis.

STRUCTURAL CHANGES DURING CELL DEATH

Considerable biochemical changes occur within the apoptotic cell, which facilitate neat packaging and removal of apoptotic bodies by neighboring cells; however, as described above, necrosis is accompanied by high-amplitude cell swelling. Modifications in the cytoskeleton and cytoplasmic membranes are required for both shrinkage and swelling to occur. During apoptosis, this results in the loss of cell-cell contact, untethering of the plasma membrane, and rapid blebbing or zeiosis (Saunderson, 1982). Similarly, necrotic cells undergo membrane blebbing, but in contrast to apoptosis, the tracts of contiguous dying necrotic cells collapse without loss of contact with neighboring cells. This results in clumps of necrotic cells rather than single cells.

Tissue transglutaminase, a Ca^{2+} -dependent protein-glutamine γ -glutamyltransferase, is induced and activated in liver hyperplasia and in glucocorticoid-treated thymocytes (Fesus et al., 1987). This enzyme cross-links cytokeratin, a component of the cytoskeleton, through ϵ -(γ -glutamyl) lysine bonds. During apoptosis there is a significant increase in transglutaminase mRNA, as well as in the protein itself, its

Table 18.1.1 Features of Apoptosis and Necrosis

Characteristics	Apoptosis	Necrosis
Stimuli	Physiological or pathological	Pathological (injury)
Occurrence	Single cells	Groups of cells
Reversibility	Limited	Limited
<i>Cellular level</i>		
Cell shape	Shrinkage and formation of apoptotic bodies	Swelling and later disintegration
Adhesion between cells	Lost (early)	Lost (late)
Phagocytosis by other cells	Present	Absent
Exudative inflammation	Absent	Present
<i>Cellular organelles</i>		
Membranes	Blebbing	Blebbing prior to lysis
Cytoplasm	Late-stage swelling	Very early swelling
Mitochondrial permeability transition	Present	Present
Nucleus	Convolution of nuclear outline and breakdown (karyorrhexis)	Disappearance (karyolysis)
<i>Biochemical level</i>		
Gene activation	Present ^a	Absent ^a
Requirement for protein synthesis	Present ^a	Absent ^a
Lysosomal enzyme release	Present	Present
Activation of nonlysosomal enzymes	Present	Present
Activation of caspases	Present	Absent
Cleavage of specific proteins	Present	— ^a
Changes in cytoskeleton	Present	Present
Level of ATP required	High	Low
Bcl-2 protection	Present	Present
Nuclear chromatin	Compaction in uniformly dense masses	Clumping not sharply defined
DNA breakdown	HMW and internucleosomal	Randomized
RNA degradation	Present	— ^a
Phosphatidylserine exposure	Present	Absent

^aThis feature is not a universal event or there are conflicting reports.

enzyme activity, and protein-bound (γ -glutamyl) lysine (Fesus et al., 1987). It is thought that transglutaminase activity may stabilize apoptotic cells and prevent membrane leakage during the early stages of the process by forming a shell around the cell. In contrast, transglutaminase activity does not appear to change during necrosis. For example, it was recently reported that ethanol-induced cytotoxicity in astroglial cells was due to necrotic cell death, and that it occurred in the absence of any change in transglutaminase activity (Holownia et al., 1997).

A critical part of apoptosis is the efficient recognition and removal of the apoptotic cells by phagocytes (Savill and Fadok, 2000). This involves the rearrangement and biochemical alteration of the plasma membrane in the dying cell. There are a number of changes that occur in the plasma membrane of apoptotic cells. One such change results in the alteration of carbohydrates on the plasma membrane, which helps preferential binding of macrophages to apoptotic cells (Duvall et al., 1985). Recognition of apoptotic cells by macrophages can also be

mediated via the vitronectin receptor (CD36; Savill and Fadok, 2000). The loss of phospholipid asymmetry in the plasma membrane of cells undergoing apoptosis is yet another significant change leading to the externalization of phosphatidylserine at the surface of the cell, thereby enabling cell recognition by macrophages (Fadok et al., 2000). Development of fluorescently labeled annexin V, which binds specifically to phosphatidylserine residues, enables detection of this externalization in apoptotic cells (Koopman et al., 1994); however, under *in vitro* culture conditions, where phagocytic cells are absent, apoptotic cells and their fragments lyse in a process very similar to that observed in necrosis. This phenomenon is termed secondary necrosis or post-apoptotic necrosis. Necrotic cells do not undergo phagocytosis to a similar extent, and the spillage of their cellular contents through plasma membrane disruption into the extracellular milieu induces cellular inflammatory responses.

Cell-volume changes are another feature of cell death. Interestingly, the cell volume changes during apoptosis and necrosis go in different directions. Apoptotic cell shrinkage, which leads to a loss of 30% to 50% of the cell volume (Saunderson, 1982), has been proposed to be due to budding of the endoplasmic reticulum. Vesicles thus generated migrate and fuse to the plasma membrane, releasing their contents into the extracellular region. This process requires energy (ATP), since water is moved against the osmotic gradient (Saunderson, 1982). Mitochondria, which are postulated to remain structurally and functionally intact during the early phase of apoptosis, provide the necessary energy. In contrast, during necrosis the cell loses control of ion flux, resulting in a reduction in concentration gradients of Na^+ , K^+ , Ca^{2+} , and Mg^{2+} , as well as changes in osmotic pressure. This, in turn, leads to uptake of water, giving rise to high-amplitude swelling of the cell and its organelles. This process is energy-independent and does not require a large amount of ATP.

MACROMOLECULAR DEGRADATION DURING CELL DEATH

Cell death is associated with activation of lipases, nucleases, and different classes of proteases. In apoptosis there is an efficient and neat packaging of the cell contents into apoptotic bodies. This requires the rearrangement and breakdown of lipids, proteins, and nucleic acids. Another possible reason for efficient deg-

radation of macromolecules in apoptotic cells prior to their phagocytosis is that contents from dead cells are thus prevented from entering the phagocyte or neighboring cells to cause injury. In necrosis, the enzymes activated appear to be different, with very little overlap with the apoptotic enzymes. The necrotic enzymes are largely lysosomal acidic hydrolases with less substrate specificity than the apoptotic enzymes. These enzymes are thought to be activated also in late apoptosis and in the absence of phagocytosis, when the cells undergo secondary necrosis. Thus, there is a similarity between the features of necrotic cells and those of apoptotic cells undergoing secondary necrosis. The major macromolecules and the mode of their degradation during apoptosis and necrosis are discussed below.

DNA Degradation

Three patterns of DNA degradation are already known to occur during apoptosis. One or more of these may occur during the progression of apoptosis in a single cell (for review see Robertson et al., 2000). These are: single-strand nicks (Gorczyca et al., 1992), chromatin cleavage with the formation of large (i.e., 50- to 300-kbp) fragments (Brown et al., 1993; Zhivotovsky et al., 1994), and finally formation of nucleosome-size fragments of 180 to 200 bp, which produce the ladder pattern that has long been accepted as a biochemical hallmark of apoptosis (Wyllie, 1980).

One or more nuclear endonucleases have been suggested as being responsible for these patterns of DNA fragmentation, since isolated nuclei can be induced to produce the same pattern of fragmentation. A number of endonucleases have been identified in different cell systems, with different ion requirements for their activity (for review see Robertson et al., 2000). For example, the endonuclease in thymocytes is $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent, whereas the endonuclease operating in HL-60 cells appears to function independently of these ions.

Among the different $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonucleases, DNase I is of particular interest. Several years ago, it was suggested that DNase I is involved in radiation-induced lymphoid cell death (for review see Robertson et al., 2000). DNase I is a secreted digestive enzyme that produces DNA strand breaks containing 3'-OH end groups—the same end groups found in DNA fragmentation products during apoptosis. This enzyme is localized within the rough endoplasmic reticulum, the Golgi complex, and in small (secretory) vesicles. In apoptotic cells,

DNase I is also found within the perinuclear space, which led to the speculation that during apoptosis damage to endoplasmic reticular and nuclear membranes permits entry of this enzyme into the nucleus where it is involved in DNA fragmentation. It is important to note that DNase I activity is inhibited by G actin gelsolin segment 1, suggesting that cytoskeletal interaction might play a role in the control of this enzyme. A potential problem in linking DNase I activity with apoptosis is the fact that this enzyme is believed to produce mainly single-strand breaks in DNA with 10-bp periodicity and only in the presence of Mn^{2+} can it introduce double-strand breaks; therefore, additional experiments are required to further clarify the role of DNase I in effecting DNA fragmentation during apoptotic cell death.

Endonuclease activities that are completely or partially independent of Ca^{2+} and Mg^{2+} have also been described. Specifically, endonuclease activity that is independent of Ca^{2+} and dependent on Mg^{2+} was implicated in the formation of high-molecular-weight DNA fragments, whereas Ca^{2+}/Mg^{2+} -independent and Mn^{2+} -dependent endonuclease activity was linked to the formation of DNA ladders in several experimental models (for review see Robertson et al., 2000). Moreover, some cell types appear to lack any constitutive Ca^{2+} -dependent endonuclease activity.

Little is known about the ionic requirement for the DNA fragmentation factor-40/caspase-activated DNase (DFF-40/CAD) and, hence, it is difficult to associate this enzyme with any of the nuclease classifications described above. Under normal or unstressed conditions, DFF-40/CAD is bound in an inactive form to DNA fragmentation factor-45/inhibitor of CAD (DFF-45/ICAD), which is an inhibitory component of DFF-40/CAD (for review see Nagata, 2000). In the presence of proapoptotic stimuli, DFF-45/ICAD is cleaved by specific proteases (caspases, see below) and liberates DFF-40/CAD resulting in the activation of nuclease activity, nuclear condensation, and DNA fragmentation. ICAD knock-out mice are refractory to DNA fragmentation and nuclear condensation in response to different apoptotic stimuli, indicating that the inhibitory molecule to CAD is required for normal function of this enzyme (for review see Nagata, 2000). Lastly, a recent study by Liu et al. (1999) indicated that DFF activation is dependent upon the association of DFF-40 with histone H1 and high-mobility group 2 protein, which grants DNA-binding ability and nuclease activity to this mole-

cule. DFF-40/CAD cleaves DNA at internucleosomal spaces.

Endonuclease activity in cells undergoing apoptosis can be regulated by protooncogenes and tumor-suppressor genes, such as *c-myc*, *Ha-ras*, *bcl-2*, and *p53*, as shown by analysis of genetic control of susceptibility to apoptosis (Arends et al., 1993). The apoptotic endonuclease or endonucleases cleave DNA at the exposed linker regions, and it has been suggested that these nucleases are topologically constrained rather than sequence constrained. Results obtained from cloning and sequencing DNA fragments from apoptotic cells show that the nuclease has no preference for specific DNA sequences or for the type of DNA to cleave. This randomness may be functionally important, and reflects the known properties of the endonucleases. The finding that apoptotic endonucleases are constitutive enzymes and that their activity can be modulated by different signals in cells undergoing apoptosis underlines the important role of nucleases in both physiological and pathophysiological processes. Although the role of intracellular endonucleases is not yet understood, one may speculate that a major function is to maintain genomic stability. Unfortunately, both purpose and mechanisms of DNA fragmentation in apoptotic cells are still unclear. Such fragmentation could serve to destroy the genetic information of unwanted cells and thus act as an irreversible step in the process. On the other hand, it may be simply a mid-to-late event that reflects ion redistribution and subsequent chromatin hypersensitivity to endonuclease(s) present in apoptotic cells. Alternatively, it may facilitate cleavage of DNA before uptake of apoptotic bodies during phagocytosis. Whatever the reason, it is still important to understand the mechanisms of this intriguing step of apoptosis.

As compared to apoptosis, at least two patterns of DNA degradation are recognized as occurring during necrosis. The first is the formation of both single- and double-strand DNA breaks. The second is randomized chromatin fragmentation. It is unclear if there is a link between these two steps of chromatin disintegration, although the probability for that is rather high. DNA fragments isolated from necrotic cells contain 5'-OH and 3'- PO_4 end groups. Among different endonucleases isolated and characterized up to now, only one is able to produce such DNA fragments—i.e., DNase II, which is localized in lysosomes. This makes sense, since the activation of lysosomal enzymes has been observed in necrotic cells.

The search for enzymes responsible for cleavage of chromatin during necrosis is still in progress in many laboratories.

Several publications have implicated the involvement of DNase II in apoptosis as well, although its precise role in the apoptotic process remains unclear given the following considerations: (1) DNase II is a lysosomal enzyme and in order to cleave nuclear DNA in intact cells, this enzyme would need to be released from lysosomes and transported into the nucleus. While this scenario is possible, it seems unlikely since it has been demonstrated that lysosomes maintain membrane integrity during apoptosis. (2) DNase II-mediated cleavage of DNA produces 3'-phosphates, which have not been linked to cells undergoing apoptosis. Nevertheless, it cannot be ruled out that DNase II may contribute to DNA fragmentation during apoptosis when the intracellular pH may be more acidic. Recently, it was reported that intracellular acidification during apoptosis can occur even in the absence of a nucleus, suggesting that intracellular acidification and DNA fragmentation are independent consequences of caspase action during apoptosis (Wolf and Eastman, 1999). In this respect, it is likely that DNase II does not induce DNA fragmentation in whole cells, but digests chromatin within phagocytes, where pH is low and apoptotic remnants must be processed. Indeed, two recent publications have suggested that the DNA fragmentation that occurs during apoptosis not only can result from DFF-40/CAD or any other endonuclease activities within the apoptotic cell but can also be attributed to a lysosomal acid DNase after the apoptotic cells are engulfed (McIlroy et al., 2000; Wu et al., 2000).

In conclusion, it is still unclear how all of the nuclease activities described in this section correlate with each other and are involved in apoptosis. It may be that there are cell type and/or insult-dependent specificities for the activation of different enzymes, or it could be that different enzymes involved in DNA fragmentation are active at different stages in the overall process.

RNA Degradation

Changes in ribosomal counts occurring in response to cell injury have been known for a number of years. This phenomenon may be a result of the release of ribosomes from the endoplasmic reticulum at particular stages leading to necrosis (for review see Bowen, 1981). A detailed analysis of the role of ribosomes in the pathogenesis of liver-cell necrosis was conducted by Bernelli-Zazzera (1975). He

showed that ischemia resulted in a decrease in the number of ribosomes, and that stripped rough endoplasmic reticulum from ischemic cells bound fewer added ribosomes than did the reticulum from normal cells. It was concluded that there is a "loosened relationship" between endoplasmic reticulum membranes and ribosomes in ischemic livers; however, it is unknown how specific these changes are for necrosis and what relationship they bear to this type of cell death.

There are also a number of reports that RNA, as well as DNA, is susceptible to cleavage during apoptosis. The changes in ribosomal RNA (rRNA) during apoptosis are best studied. Ribosomal RNA consists of conserved and divergent (or variable) domains numbered from D1 to D12 (for review see Houge and Doskeland, 1996). The cleavage of rRNA during apoptosis selectively affects the two largest divergent domains, D2 and D8, in the large 28S rRNA molecule of the 60S ribosomal subunit, while the 18S rRNA molecule in the 40S ribosomal subunit remains unaffected (Houge et al., 1993); however, the pattern of rRNA cleavage occurring during apoptosis cannot be reproduced in necrotic cells or when exposing cell lysates to random RNase activity, suggesting that it is specific to apoptotic cells.

Recently it was demonstrated that rRNA and DNA cleavages can occur independently in apoptotic cells (Samali et al., 1997). In other words, the previously observed correlation is likely to be coincidental. The absence of apoptotic rRNA cleavage in some cell types suggests that this phenomenon is tightly regulated and unrelated to DNA fragmentation; therefore it appears that the pattern of rRNA fragmentation seen during apoptosis is not part of a scheme for general macromolecular disintegration and degradation, but rather a trait present in only some cell types.

Protein Degradation

The prerequisite for proteolysis in apoptosis is well documented. Several proteolytic activities were implicated in both the induction and execution steps of cell killing (for review see Zhivotovsky et al., 1997). Early evidence of a role for proteases in apoptosis came from studies on granule proteases (granzymes/fragmentins). These investigations identified granzymes involved in the exocytosis pathway of lymphocyte-mediated cytotoxicity, which are responsible for the lethal damage inflicted by these cells upon target cells (Shi et al., 1992). More recently, a family of aspartic acid-spe-

cific cysteine proteases have been discovered in mammalian cells, which share homology with the *Caenorhabditis elegans* death gene, *ced-3* (Yuan et al., 1993). The newly adopted nomenclature for these enzymes, caspases, refers to the aspartic acid-specific cysteine protease activity specific to this family (Alnemri et al., 1996). At least fourteen of these proteases have now been purified and their genes cloned (for review see Budihardjo et al., 1999; Fadeel et al., 2000). The activation of the caspase family of proteases has been detected in numerous tissues and cell types and may function as a common pathway through which apoptotic mechanisms operate. Caspases are synthesized as zymogens, and an apoptotic signal converts the precursors into mature enzymes (Stennicke and Salvesen, 2000). Caspases differ in the length and sequence of their N-terminal prodomain and can be divided into two subgroups: procaspases-1, -2, -4, -5, -8, -9, and -10 with long prodomains, and procaspases-3, -6, -7, -11, and -13 with short prodomains. In the long prodomain-containing group of caspases, two distinct modules essential for protein-protein interaction have been identified. The first is referred to as the death effector domain (DED) and is present in both procaspases-8 and -10. The second is termed caspase recruitment domain (CARD) and is found in procaspases-1, -2, -4, and -9. The CARD domain is also present in Apaf-1, the mammalian homologue of the CED-4 protein. It seems that most, if not all, of the long prodomain-containing caspases are activated via oligomerization-induced autoproteolysis. For example, the oligomerization of procaspases-2 and -8 at the plasma membrane is sufficient for their autoactivation. The activation of procaspase-9 requires binding to Apaf-1 and the presence of at least two other components—i.e., dATP and cytochrome *c*. Apaf-1 first forms oligomers and then facilitates procaspase-9 autoactivation by oligomerizing its precursor molecules. On the other hand, the activation of short prodomain-containing caspases requires an initial first step cleavage (e.g., by active caspases or granzyme B) followed by a second autoproteolytic step. For full activation short prodomain-containing caspases also require cleavage in the interdomain linker. Thus, activated caspase-9 can initiate a caspase cascade involving the downstream executioners, caspases-3, -6, and -7.

All members of the caspase family share a number of amino acid residues crucial for substrate binding and catalysis. Despite their uniform requirement for an aspartate residue in the

P₁ position of the substrate, caspases can be divided into three different groups according to their substrate preferences (Nicholson and Thornberry, 1997). The predicted caspase specificities correspond to the cleavage sites in the different intracellular target proteins. The effects of caspases in apoptosis appear to be accomplished by the cleavage of a large number of proteins located in the cell membranes, cytoplasm, and nucleus, although the significance of the particular cleavages for the cell death process is still unclear.

There have been a large number of reports on caspase involvement in apoptosis; however, there is very little evidence for the involvement of this family of proteases in necrosis. Recent work from our own group has shown that menadione-induced necrosis of hepatoma cells is independent of caspase activation (Samali et al., 1999a). Furthermore, Tomaselli and co-workers (Armstrong et al., 1997) observed the activation of caspase-3 in cerebellar granule neurons undergoing apoptosis but not necrosis. These results appear to be in line with the work carried out in our laboratory which has shown that after the acute, necrotic death of cerebellar granule neurons exposed to glutamate, the remaining neurons undergo a delayed, apoptotic death (Ankarcrona et al., 1995); however, we did not detect any caspase activation during the “necrotic” step of our experimental model (M. Ankarcrona, B. Zhivotovsky, S. Orrenius, and P. Nicotera, unpub. observ.).

Apart from the caspase family of proteases, a number of other proteolytic enzymes are also implicated in cell death. Studies from our laboratory have suggested the involvement of Ca²⁺-dependent proteolytic activity in oxidant injury in the liver (Nicotera et al., 1986). Although the substrates for this protease activity during cell injury remain largely unidentified, it appears that cytoskeletal and membrane-integral proteins may be a major target for this proteolytic event during chemical toxicity (Mirabelli et al., 1989). Calpain, another Ca²⁺-dependent neutral protease and member of the papain family of cysteine proteases, degrades a number of key cellular proteins including protooncogenes, steroid-hormone receptors, protein kinases, and cytoskeletal proteins (Croal and DeMartino, 1991; Vanags et al., 1996). Calpain, one of the degradative nonlysosomal proteases, is implicated in both apoptosis and necrosis (Arai et al., 1990).

In apoptotic cells calpain cleaves a number of substrates, including fodrin, which can also be cleaved by caspase-3. The cleavage sites for

both proteases are different, and it is still unclear why several enzymes should cleave the same protein during cell death. *In vitro*, calpain can cleave procaspase-3, although it is unclear if this cleavage activates or inactivates this very important effector caspase (for review see Johnson, 2000). Calpain also cleaves several apoptosis regulatory proteins, such as p53, Bax and procaspase-9; however, the impact of calpain-mediated cleavage of these substrates *in vivo* remains to be elucidated. Enhanced calpain activity has been observed in anoxic hepatocytes and neurons. Moreover, inhibition of calpain by acidosis, calpain protease inhibitors, and glycine delays anoxic injury. Precise mechanisms responsible for the stimulation of calpain activity during either necrosis or apoptosis are still unclear. More recently, it has been shown that hepatocellular carcinoma cells resist necrosis during anoxia by preventing phospholipase-mediated calpain activation (Arora et al., 1996); however, it is unknown whether sustained calpain activity promotes necrosis as a “deathase” by degrading key cytoskeletal proteins such as spectrin or by acting as a “signaling protease.” Thus a better understanding of the protein substrates cleaved in necrosis will be required before the role of calpains in necrosis can be elucidated.

Another example of noncaspase proteases that may be involved in apoptosis is seen in the plasminogen activator system, which is a major proteolytic complex responsible for the breakdown of the extracellular matrix. Expression of the plasminogen activator inhibitor type 2 (PAI-2) in HeLa cells, which do not synthesize PAI-2, protects them from tumor necrosis factor- (TNF-) induced apoptosis (Dickinson et al., 1995). Interestingly PAI-2 has a high degree of structural similarity to crmA, which inhibits caspase-1-induced apoptosis (Dickinson et al., 1995). PAI-2 itself is not spared during apoptosis, and it loses its activity after cleavage by other proteases (Jensen et al., 1994). There are no reports about the involvement of this protease in necrosis.

An alternative proteolytic system, which is thought to play a role in cell death, is that of proteasomes. The contribution of proteasomal activity during apoptosis has primarily been evaluated by using inhibitors. There are several observations that show that inhibition of proteasomal activity by “specific” peptide inhibitors either fails to prevent receptor-mediated apoptosis (B. Zhivotovsky, D.H. Burgess, I. Ares-Pörn, and S. Orrenius, unpub. observ.) or drives cells into apoptosis (Shino-

hara et al., 1996); however, there are a few experimental models in which proteasome inhibitors antagonized apoptosis (for review see Orłowski, 1999). Currently there is no evidence that these enzymes directly activate caspases. Thus, a conclusive role for this enzymatic system in apoptosis remains to be elucidated.

The lytic nature of lysosomal enzymes and their release by various experimental procedures suggested long ago that they might be responsible for cell necrosis in damaged cells and tissues (for review see Bowen, 1981). The concept of the lysosome as a “suicide bag” was developed in the 1960s by de Duve (1963). This hypothesis states that particle-bound acid hydrolase can be released into the surrounding cytoplasm under appropriate conditions and bring about the cell’s ultimate destruction. Such a response might occur during cellular injury. Much attention was paid to acid hydrolases in earlier publications. Hydrolases, however, were later shown to occur in association with the Golgi and/or Golgi–endoplasmic reticulum lysosomes, endoplasmic reticulum extracisternal spaces, and plasma membranes (for review see Bowen, 1981). In reference to inflammatory cells, it has been shown that acid hydrolases are directly transferred from the endoplasmic reticulum into foci of cytoplasmic degeneration (Van Lancker, 1975). It was later concluded that when cells die, areas of the cytoplasm become segregated for autolysis, and that these areas are supplied with hydrolases, either from primary lysosomes or directly from the endoplasmic reticulum. Furthermore, an accumulation of free acid phosphatases around ribosomes in the extracisternal space of the endoplasmic reticulum has been linked to this type of cell death (for review see Bowen, 1981). This early extracisternal buildup appears as a prelude to cell autolysis. On the other hand, histochemical studies showed that the liberation of acid phosphatases and esterases was not an early change (Kerr et al., 1972), and the authors of that study suggested that the release of lysosomal enzymes is not responsible for the initiation of necrotic cell death, but rather accompanies this type of death. There is no evidence for preferable degradation of any proteins by lysosomal proteases during necrosis. Thus, although the involvement of both lysosomal and nonlysosomal protease activities in necrosis was documented, the precise role of these protease activities in either induction or execution of necrosis, as well as the specific cleavage of cellular proteins, remain to be elucidated.

Recently some lysosomal proteases have entered the “apoptotic death scene” (Salvesen, 2001). It has been shown that *in vitro* cathepsins can readily cleave several caspases; however, it is unclear whether cathepsin-mediated cleavage of procaspases results in the activation of these enzymes. Moreover, it is unknown whether this cleavage occurs in whole cells (for review see Johnson, 2000). Given the lack of evidence for a direct role in caspase activation by lysosomal proteases *in vivo*, the authors hypothesized that an indirect mode of caspase activation may involve the Bcl-2 family member Bid (Stoka et al., 2001). Indeed, Bid is cleaved by lysosomal protease(s) and this cleavage may represent a mechanism by which proteases that leak from the lysosomes can participate in perpetuation of the apoptotic pathway. It seems that lysosomal rupture can trigger apoptosis in certain pathological conditions as well as during normal aging. Nevertheless additional work is required to understand the precise role of lysosomal enzymes in induction and execution of apoptosis.

CELLULAR SIGNALING DURING CELL DEATH

Signal transduction is thought to play a key role in the onset of both apoptosis and necrosis, and this may be mediated by an increase in intracellular Ca^{2+} levels, protein kinase C (PKC), cyclic adenosine monophosphate/protein kinase A (cAMP/PKA), or phosphatases. It has been demonstrated that glucocorticoid-induced cell death involves Ca^{2+} influx in lymphocytes and that this type of cell death can be mimicked using calcium ionophores (Kaiser and Edelman, 1978). Based on these observations, it appears that intracellular Ca^{2+} levels may initiate a death signal, probably through the activation of Ca^{2+} -dependent enzymes (proteases, phospholipases, and endonucleases) in this system. The evidence for a role of Ca^{2+} in necrotic cell killing is particularly strong in the central nervous system. Ca^{2+} appears to mediate the neurotoxicity of cyanide, heavy metals like lead and mercury, and organotin compounds (for review see Nicotera and Orrenius, 1998). Furthermore, intracellular Ca^{2+} overload, resulting from excessive stimulation of excitatory amino acid receptors and enhanced Ca^{2+} influx through membrane channels, appears to play an important role in ischemic brain damage. Much recent research has focused on glutamate-induced excitotoxicity and its contribution to brain damage in various diseases (Ankarcrona et al., 1995; Sims and Robinson,

1999 and references therein). The calcium ion plays a critical role in this process, and intracellular Ca^{2+} overload appears to mediate the lethal effect of *N*-methyl-D-aspartate (NMDA) receptor overactivation. This mechanism is responsible not only for the brain damage induced by certain neurotoxins, but also for excitotoxicity, which is strongly implicated in neuronal death following insults such as ischemia and trauma. Thus, NMDA receptor antagonists not only block Ca^{2+} influx and neuronal death elicited by glutamate or NMDA *in vitro*, but can also reduce the volume of infarction produced by focal ischemia *in vivo*. As discussed above, glutamate can trigger the onset of both apoptosis and necrosis in cerebellar granule cells (Ankarcrona et al., 1995). Radical scavengers and agents that inhibit the generation of nitric oxide by nitric oxide synthase were ineffective in preventing cell death in this system, whereas NMDA receptor/channel blockers prevented both necrosis and apoptosis in glutamate-treated cerebellar granule cells. These data suggest that Ca^{2+} overload, mediated by NMDA-receptor-operated channels, is sufficient to induce either necrosis or apoptosis in cerebellar granule neurons *in vivo*. It is clear that intracellular Ca^{2+} overload is an important factor in various *in vitro* models, and results from clinical studies appear to support this hypothesis. The Ca^{2+} overload seems to result from increased Ca^{2+} influx through both receptor-operated and L-type Ca^{2+} channels, as indicated by the neuroprotective effects of glutamate-receptor antagonists and L-type channel blockers. Various Ca^{2+} -dependent degradative processes have been found to contribute to Ca^{2+} -mediated necrotic cell killing in *in vitro* studies, and although their relative contribution is unclear, it appears that perturbation of cytoskeletal organization, impairment of mitochondrial function, and/or activation of certain proteases may be of particular importance. For example, it has been proposed that activation of phospholipase A2 by a sustained increase in cytosolic calcium plays an important role in necrotic cell killing. Support for this hypothesis comes from several observations that necrosis in the liver and heart caused by ischemia, as well as in hepatocellular carcinoma cells in anoxic condition, is prevented by inhibitors of this enzyme or by the inhibition of calpain activation (for review see Nicotera and Orrenius, 1998). Thus, it is likely that the calcium ion may play a determinant role in the necrotic process.

One of the classic signaling pathways is that of PKC, which is a multifunctional ser-

ine/threonine kinase that utilizes diacylglycerol as a second messenger (Nishizuka, 1984). Twelve different isoforms of PKC have been identified to date and are classified according to their calcium dependence and phorbol ester binding activity (for review see Lavin et al., 1996). There are some reports as to the involvement of PKC in apoptosis. For example, it has been reported that treatment of mouse thymocytes with phorbol esters, which activate PKC, induces apoptosis (Kizaki et al., 1989). Similarly, apoptosis induced by the calcium ionophore A23187 can be inhibited by the PKC inhibitor H7; however, most of the evidence to date indicates that PKC activation inhibits apoptosis (McConkey et al., 1996). This paradox may be explained by the large number of isoforms, which may be differentially regulated during apoptosis (Forbes et al., 1992).

Similarly, the role of protein phosphatases during apoptosis is not clear. There are some reports that a number of cell lines react to okadaic acid by undergoing apoptosis (Boe et al., 1991), while there are other reports that the inhibition of protein phosphatase activity blocks apoptosis in some other cell lines (Baxter and Lavin, 1992). From these reports it appears that protein activation/modification, through reversible protein phosphorylation by kinases and protein phosphatases rather than by de novo protein synthesis, may play a more central role in the regulation of apoptosis in some cells.

The anatomy of receptor-mediated death signaling machinery is well characterized. This pathway involves initiation of a cascade of signaling events leading to the activation of caspases. Members of this family, such as CD95 (Apo1/Fas), contain a death domain (DD) in their cytoplasmic tail, which is essential for transducing the apoptotic signal. Upon binding with its natural ligand, CD95-L, receptors undergo trimerization followed by very fast formation of death-inducing signaling complex (DISC). The first step in DISC formation involves binding of DD in CD95 with DD present in the adaptor protein Fas-associated death domain (FADD), which also carries a so-called death effector domain (DED). Next, DED in FADD by homologous interaction recruits the DED in another protein, FLICE, which contains on C-termini procaspase-8. During CD95-initiated cell death, small-accelerator for death signaling (SADS) enhances interaction of FADD and FLICE, and formation of DISC, thereby accelerating processing/activation of procaspase-8 (for review see Kramer, 2000). Active caspase-8 is either directly

involved in cleavage and activation of procaspase-3, which results in completion of the cell death process, or it cleaves Bid, a Bcl-2 family member, which in turn activates the so-called, mitochondria-mediated pathway.

ROLE OF MITOCHONDRIA DURING CELL DEATH

Work from several laboratories has indicated that mitochondrial damage may be a common event in the development of cell injury caused by various agents (Nicotera and Orrenius, 1998). It was postulated that mitochondrial damage is initially manifested by a decrease in the mitochondrial membrane potential followed by ATP depletion (Zamzami et al., 1995). During necrosis, these changes occur irreversibly and usually lead to disruption of the mitochondrial structure. These changes set necrosis aside from apoptosis, during which mitochondrial structure remains morphologically intact. Mitochondria have nevertheless been implicated in apoptosis ever since the discovery that the Bcl-2 protein localizes to the outer mitochondrial membrane (Hockenbery et al., 1990). Moreover, by using different cell-free systems, it has been shown that nuclear apoptosis depends on the presence of ATP (Lazebnik et al., 1993; Newmeyer et al., 1994; Kass et al., 1996). It has been demonstrated that cytochrome *c* can induce cleavage and activation of procaspase-3 in cytochrome *c*-minus cytoplasmic extracts (Liu et al., 1996). It has also been shown that cytochrome *c* is released from the mitochondrial intermembrane space into the cytosol by cells undergoing apoptosis. In this case cytochrome *c* release is not preceded by changes in mitochondrial membrane potential (for review see Hengartner, 2000).

During apoptosis a number of proteins that are normally confined to the intermembrane space of the mitochondria are translocated to the cytosol, where they trigger reactions that give rise to the apoptotic process. The role of cytochrome *c* in activation of procaspases has already been described. Recently mitochondrial localization of at least three procaspases (2, 3, and 9) was described. It is still unclear whether any of these procaspases become active within the mitochondria or after their release into the cytosol. Mitochondrial procaspase-3 is present in a complex with HSP10 and 60, and upon induction of apoptosis, procaspase-3 is dissociated from the HSP complex simultaneously with the release of HSP from mitochondria and activation of caspases (Samali et al., 1999b). Another important regu-

lator of apoptosis, second mitochondria-derived activator of caspases (Smac) or direct IAP binding protein with low pH (DIABLO), is released from mitochondria into the cytosol, where it eliminates the inhibitory effect of many inhibitors of apoptosis proteins (IAPs) on the activation of several caspases (for review see Hengartner, 2000). Apoptosis-inducing factor (AIF) is also located in the intermembrane space of mitochondria and upon apoptosis induction redistributes to the nucleus where it is involved in nuclear chromatin condensation and chromatin fragmentation with formation of high-molecular-weight DNA fragments (Daugas et al., 2000). The role of other proteins released from mitochondria (i.e., adenylate kinase-2 and sulfite oxidase) in the apoptotic process is unknown.

Two groups have presented evidence that a high ATP level is required during the apoptotic process and suggested that the level of intracellular ATP determines whether a cell will die by apoptosis or necrosis (Nicotera and Leist, 1997; Tsujimoto, 1997). During exposure to glutamate, both mitochondrial membrane potential and ATP levels decline in many neurons (Ankarcrona et al., 1995). In neurons with irreversibly dissipated mitochondrial potentials, necrosis rapidly ensued. The surviving population recovered both mitochondrial membrane potential and energy levels and subsequently underwent delayed apoptosis. It was postulated that the loss of energy and the onset of rapid necrosis simply prevents the activation of the default apoptotic program. This postulate was supported by the observation that treatment of cerebellar granule cells with a combination of glutamate and the irreversible mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) resulted in necrosis for most of the neuronal population, rather than in the delayed apoptosis observed with glutamate alone.

There is abundant evidence for an ATP requirement in several apoptosis models, although the critical ATP-dependent steps in the process have not been characterized in detail. They are likely to be linked to both the signaling and the execution phases of apoptosis. It was suggested (Tsujimoto, 1997) that the caspase protease cascade is regulated by ATP-dependent reactions, and it has been shown (Yasuhara et al., 1997) that the active nuclear transport mechanism, which requires ATP hydrolysis, is involved in the apoptotic changes of the nuclei. It is likely that another ATP-dependent step or steps downstream from caspase activation is important for perpetuation of the apoptotic

process. This fact could explain the previously published data on the ATP requirement for induction of nuclear apoptosis induced by different cytoplasmic extracts (Lazebnik et al., 1993; Newmeyer et al., 1994; Kass et al., 1996). Thus, it is not surprising that the depletion of cellular ATP blocks various events in apoptosis. The question that remains to be answered is whether and how ATP depletion redirects the death process towards necrosis when cells have been triggered with apoptotic stimuli, such as anti-CD95 antibodies. In addition to the possible requirement of ATP for caspase activation, redirection could also be explained by an unknown mechanism, which shortens the survival time of cells that would eventually die by necrosis resulting from energy deprivation.

The next important question that remains to be resolved is an apparent contradiction between, on the one hand, the induction of the mitochondrial permeability transition associated with a cessation of mitochondrial ATP synthesis, and on the other, a need for maintenance of the intracellular ATP level during development of apoptosis, which may take many hours or days. In addition, hyperproduction of reactive oxygen species (ROS) results from the loss of cytochrome *c* from the respiratory chain; yet the cell needs to maintain a reducing environment for optimal caspase activity. These controversies could be resolved if the proposed apoptosis-induced changes were restricted to a subset of mitochondria, or that the maintenance of the energy level required by cells to undergo apoptosis could occur by glycolysis. This would be consistent with the observation by Jacobson et al. (1993) that cells lacking mitochondrial DNA and a functional respiratory chain can still undergo apoptosis. It is very likely that the mitochondrial permeability transition associated with a cessation of mitochondrial ATP generation plays an important role in necrotic cell death, whereas leakage of cytochrome *c* from morphologically intact mitochondria via a yet unknown and more selective pathway than outer-membrane rupture following permeability transition-induced mitochondrial swelling plays an important role in apoptotic cell death.

GENETIC MODULATION OF APOPTOTIC CELL DEATH

In recent years, enough evidence has been gathered to suggest a role for the protein products of a number of oncogenes and tumor-suppressor genes, as well as stress proteins and acute phase proteins, in the modulation of cell

death. These can be grouped into genes whose products are positive or negative regulators of cell death. The genes with a key role in the modulation of cell death and their effects on both apoptosis and necrosis are reviewed below.

c-myc

The *c-myc* proto-oncogene is a short-lived nuclear protein and a member of a family of closely related genes that also include *v-myc*, *n-myc*, and *b-myc*. The *Myc* family plays a key role in the regulation of cell proliferation (for review see Ryan and Birnie, 1996). It has been demonstrated that *c-myc* is capable of driving apoptosis (Evan et al., 1992). For instance, when T cell hybridomas were stimulated through their CD3/T cell receptor, they rapidly underwent apoptosis that could be blocked using an antisense oligonucleotide to *c-myc* mRNA. Furthermore, in fibroblasts, *c-myc* overexpression can induce apoptosis. The decision to proliferate or die in cells overexpressing *c-myc* seems to depend on the presence or absence of extracellular survival factors (Evan and Littlewood, 1998).

p53

The p53 protein is the product of a tumor-suppressor gene, which is mutated in over half of all human tumors, thus implicating p53 inactivation in tumorigenesis. Nevertheless, the main functions of p53 appear to be in mediating the cellular response to DNA damage and maintaining genomic stability (Kastan et al., 1991). Many forms of genotoxic stress induce a rapid increase in p53 protein levels, through both stabilization of the protein (Kastan et al., 1991) and an increase in p53 mRNA levels (Sun et al., 1995). This protein induces growth suppression by regulating cell-cycle arrest and/or inducing apoptosis. Reintroduction and overexpression of wild-type p53 in cells that have lost endogenous p53 function may induce apoptosis (Yonish-Rouach et al., 1991). In this regard, p53 is shown to be required for both irradiation-induced G1 arrest and apoptosis in several cell systems (Lowe et al., 1993). Induction of growth arrest by p53 has been shown to be transcription activation-dependent and appears to involve induction of *p21* (*Waf1/Cip/sdi1*) and growth arrest and DNA damage-inducible gene 45 (*Gadd45*; for review see Somasundaram and El-Deiry, 2000).

Although transcriptional activation appears to be involved in the induction of growth arrest, little is known about the possible downstream elements in this apoptotic pathway. p53 has

been shown to induce apoptosis in the presence of transcriptional activation, in which case p53 may induce expression of CD95. Moreover, p53 transcription activates several genes whose products can translocate to the mitochondrial outer membrane (e.g., the two proapoptotic Bcl-2 family members Bax and Noxa) or to an unknown submitochondrial compartment, presumably to the mitochondrial matrix (p53AIP1). In addition, direct death signal-induced translocation of p53 to mitochondria has been documented (for review see Ferri and Kroemer, 2001).

Recent identification of two homologues, p63 and p73, revealed that p53 is a member of a family of related transcription factors. Given that they share amino acid sequence identity, all three proteins should have redundant functions in the regulation of gene expression. Indeed, p73 can activate p53-regulated genes and suppress growth or induce apoptosis. Moreover, p73 and p53 are both induced by DNA damage, albeit through distinct mechanisms. Other evidence, however, suggests that p63 and p73 are important for regulation of normal development and in many p53-independent pathways (for review see Levrero et al., 2000; Yang and McKeon, 2000).

***Bcl-2* family**

This is a family of genes in which many members hold key positions in the apoptotic machinery. The proteins in this family share highly conserved regions, which have been referred to as *Bcl-2* homology domains (i.e., BH1 to BH4; for review see Gross et al., 1999). Family members that act as inhibitors of cell death harbor at least BH1, BH2, and BH4 domains, which are required for protein-protein interaction and the suppression of apoptosis, whereas BH3 serves as the minimal “death domain” in the proapoptotic members studied so far (for review see Fadeel et al., 1999).

Bcl-2 was the first member of this family to be identified in follicular B cell lymphomas (Bakhshi et al., 1985). Several other family members, which were discovered later, may function to inhibit apoptosis, including *Bcl-2*, *Bcl-X_L*, *Bcl-W*, *Bfl1/A1*, *Mcl-1*, *Bag-1*, *BRAG-1*, *NR-13*, *Boo/Diva*, and *BHRF-1*, while others function to accelerate the rate of apoptosis once it has been induced, including *Bax*, *Bcl-X_s*, *Bad*, *Bak*, *Bar*, *Bid*, *Bik/Nbk*, *Bok/Mtd*, *Bim/Bod*, *Blk*, *Hrk/DP5*, *Noxa*, *Bnip3*, *Nix*, *Bcl-G*, and *MAP-1* (for review see Fadeel et al., 1999).

Localization is an important theme in signal transduction and increased evidence has been

provided that the regulation of apoptotic signals by Bcl-2-like proteins is dependent on their specific intracellular movement.

Bcl-2 protein is localized to the mitochondria, endoplasmic reticulum, and nuclear membrane (Hockenbery et al., 1990). Bcl-2 appears to have antioxidant properties, as it can inhibit apoptosis induced by reactive oxygen species (Hockenbery et al., 1993); however, it has also been demonstrated that Bcl-2 can inhibit cell death under conditions that preclude the formation of reactive oxygen species (Jacobson et al., 1993). Thus, the ability of Bcl-2 to protect against the damaging effects of free radicals does not appear to account for the suppression of cell death by Bcl-2.

Bax is more ubiquitously expressed than Bcl-2 (Krajewski et al., 1994). The *bax* gene promoter contains four p53-binding sites, and it has been shown that the levels of its protein can be up-regulated by p53. The countervailing roles of pro- and antiapoptotic Bcl-2 family members can be regulated through protein-protein interaction and formation of homo- and/or heterodimers. Thus, Bcl-2 can heteromerize with Bax, Bid might interact with Bax and Bak, and Bax can also interact with Bcl-X_L, Mcl-1, and A1. Function of Bcl-2 like proteins in addition to homo- or heteromerization can be regulated via phosphorylation (i.e., Bcl-2, Bcl-X_L, Bim, and Bad), *N*-myristoylation (Bid), translocation (Bid, Bax, Bcl-X_L, and Bad), insertion (Bcl-2, Bcl-X_L, Bax, and Bid), conformational changes (Bak and Bax), and cleavage (Bcl-2, Bcl-X_L, and Bax). Many protein-protein interactions take place either at the level of mitochondria or induce the movement of proteins to mitochondria. Thus, after cleavage by caspase-8, the cleaved Bid (tBid) interacts in the cytosol with Bax. This interaction leads to conformational changes of Bax, its insertion into mitochondrial membrane and oligomerization. All these events result in cytochrome *c* release. Cleaved Bid can also translocate and insert into the outer mitochondrial membrane, where it might induce conformational changes of Bak constitutively anchored to this membrane. This again leads to oligomerization of Bak, its insertion into the membrane, and release of cytochrome *c*. The antiapoptotic proteins Bcl-2 and Bcl-X_L inhibit the conformational changes and/or oligomerization and insertion of Bax and Bak. Antiapoptotic proteins from the Bcl-2 family may counteract the effect of both caspase-dependent and caspase-independent models of cell death through manifold independent functions. Although much pro-

gress has been made toward understanding the mechanisms of pro- and antiapoptotic functions of Bcl-2 family proteins, numerous issues remain unresolved.

It has been shown that overexpression of Bcl-2 can also inhibit necrosis, at least in neuronal cells, when induced by such diverse stimuli as viruses, hypoxia, oxidative stress, or exposure to toxicants (Lezoulac'h et al., 1996; Shimizu et al., 1996). Whether this effect is cell-specific remains unclear. The mechanism of action of Bcl-2 in cells undergoing necrosis may simply involve antioxidant activity. The second possibility is that Bcl-2 prevents the consequences of mitochondrial dysfunction (i.e., collapse of the mitochondrial transmembrane potential, uncoupling of the respiratory chain, hyperproduction of superoxide anions, disruption of mitochondrial biogenesis, outflow of matrix calcium and glutathione, and release of soluble intermembrane proteins), all of which can lead to a bioenergetic catastrophe culminating in the disruption of plasma membrane integrity, which is the ultimate result of necrosis (Kroemer, 1997); however, more work should be done to justify this hypothesis and to understand the mechanism of Bcl-2-induced protection from necrosis.

Ras

Members of the Ras family of proteins play an essential role in the control of normal cell growth and may induce transformation. Certain evidence has led a number of groups to advocate that Ras, in addition to inducing transformation, can also prevent cell death as overexpression of the *ras* gene was shown to inhibit apoptosis in a number of cases (Wyllie et al., 1987; Arends et al., 1993). Ras appears to be a focal point for the convergence of many signaling pathways, especially during receptor/ligand interaction, such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and nerve growth factor (NGF), T cell receptor-CD3 (TCR-CD3), and CD95. The question as to which protein downstream of the pathway is the key effector of the antiapoptotic signal through Ras is unclear, but it may involve the activation of MAP kinase or phosphatidylinositol 3 kinase or both.

Stress Proteins

The stress proteins are a set of evolutionarily conserved proteins that are synthesized or activated in response to stress (for review see Lindquist and Craig, 1988). The main function of these proteins is to afford protection to cells

in times of stress. When the stress element is removed, these cells continue to function normally and the levels drop back to normal. Heat-shock proteins (HSPs), a subgroup of stress proteins, were first demonstrated to be induced as a response to elevated temperatures.

There is strong evidence suggesting that the induction of HSPs coincides with the acquisition of tolerance to higher doses of stress, which may otherwise be lethal to the cell. In this regard, it was demonstrated that a mild heat shock induces the rapid synthesis of a number of HSPs (Jaattela et al., 1992; Mosser and Martin, 1992) and that heat-shocked or thermotolerant cells demonstrated a greater degree of resistance to environmental stress (Jaattela et al., 1992), probably by resisting apoptosis (Samali and Cotter, 1996). Indeed, several recent publications reveal an important role for heat-shock proteins in apoptosis (for review see Xanthoudakis and Nicholson, 2000). These proteins can positively or negatively regulate the apoptotic process. Thus, a direct interaction of caspase-3 with HSP60 ultimately results in stimulation of proenzyme maturation. Direct binding of HSP70 or HSP90 to Apaf-1 precludes the eventual recruitment of procaspase-9 to the apoptosome complex. HSP70 can inhibit apoptotic processes downstream of the release of cytochrome *c* and upstream of the activation of caspase-3 as well as exert a protective effect against apoptosis by targeting one or more steps downstream of caspase-3. Elevated levels of HSP70 prevent the morphological changes that are characteristic of dying cells as well as the death of tumor cells despite the presence of cytochrome *c* and active caspase-3 in the cytosol. In addition, HSP27 was shown to bind to cytochrome *c* upon its release from mitochondria, thus preventing its interaction with Apaf-1. HSP27 might also directly bind and inhibit processing of procaspase-3. Thus, it remains to be determined whether these observations reflect a difference in signaling between different cell types or whether the results can be reconciled on the basis of some experimental variable. It was demonstrated that thermotolerant or HSP-overexpressing cells are more resistant to cell death induced by hyperthermia or growth-factor withdrawal (Mailhos et al., 1993). This may suggest a possible role for HSPs in the resistance mechanism. Furthermore, HSPs are also implicated in drug resistance (McClellan and Hill, 1992) and heat-shocked or HSP-overexpressing cells become more resistant to the cytotoxic effects of some anticancer drugs (Oesterreich et al., 1993) that

are otherwise capable of inducing both apoptosis and necrosis (Martin et al., 1990).

CONCLUSIONS

Cell damage leading to cell death may occur via apoptosis or necrosis. The mode of cell death depends on the nature of the stimuli but in extreme situations the final outcome is cell disruption and disintegration. In other words, necrosis is the acute type of cell death or the cellular response to extreme injury. During necrosis, chromatin adopts a highly flocculated form, and the DNA from these cells is digested randomly to give a smear when analyzed by agarose gel electrophoresis. The main feature of necrosis is an increase in cell volume. The rapid increase in cell volume results in membrane rupture and cell lysis (for review see Trump and Berezsky, 1995).

After two decades of research and development in the field of cell death, it is now universally accepted that apoptosis is an essential strategy for maintaining the dynamic balance and equilibrium of living systems, and is observed to occur as a normal mechanism in development and homeostasis, as well as an altruistic mode of inducible cell death. Classification of cell death is now based on morphological and biochemical criteria, their circumstantial occurrence, or a combination of both. Although morphological characterization of apoptosis and the features distinguishing it from necrosis have been well documented, progress in our understanding of the mechanisms underlying the process has been quite slow.

There is now much evidence that classic apoptosis and necrosis represent only definitive endpoints for these two processes; however, both types of cell death can occur either simultaneously or sequentially in the same cell population or tissue depending on type and/or dose of toxic insult. This suggests that some early biochemical and morphological events might be similar or even identical in both types of cell death. If this is the case, different downstream events might be important in determining whether the cell will undergo apoptosis or necrosis. It could also be that after intensive treatment, the cellular apoptotic machinery becomes suppressed, after which necrosis would predominate. The events summarized in Table 18.1.1 can be observed at different levels of cellular organization. Thus, both physiological or pathological stimuli can induce apoptosis, but only pathological injury leads to necrosis. Apoptosis, in contrast to necrosis, occurs in individual cells in an asynchronous fashion

without damaging neighboring cells. Both processes can be protected by certain inhibitors before a point of no return is reached. Once the cascade of caspase proteolytic activities becomes active, apoptosis cannot be completely reversed. The point of no return during necrosis remains to be elucidated. Nevertheless, cells can be rescued from necrosis as well as from apoptosis.

Cellular shrinkage is a hallmark of apoptosis and is seen in many if not all cell types that undergo this type of death. Cell-volume regulation is a complex phenomenon, involving the homeostasis of intracellular ions such as chloride, sodium, and potassium. As the most abundant intracellular ion, K^+ has been implicated in several of the regulatory mechanisms governing cell volume, including regulatory volume decrease. It has been shown that intracellular K^+ content decreases in cells undergoing apoptosis induced by diverse stimuli (for review see Yu and Choi, 2000). Work in the authors' laboratory has shown a potential link between glutathione (GSH) efflux and apoptotic cell shrinkage (Nobel, 1997). Inhibitors of apoptotic GSH efflux were able to inhibit cell shrinkage in thymocytes induced to undergo apoptosis by etoposide without affecting DNA fragmentation. Moreover, the GSH efflux was accompanied by K^+ efflux in Jurkat cells treated with anti-CD95 antibody. The K^+ efflux was not blocked either by the inhibitor of GSH efflux or by any known inhibitors of K^+ channels and cotransporters tested. Concomitant with apoptotic K^+ efflux, significant inhibition of Na^+/K^+ -ATPase activity was observed. Inhibition of this enzyme by ouabain caused a K^+ efflux of similar magnitude to that induced by anti-CD95 antibody, and it potentiated CD95-mediated cell shrinkage. This suggested that with Na^+/K^+ -ATPase activation, K^+ and GSH effluxes facilitated apoptotic cell shrinkage, which was followed by the formation of apoptotic bodies. In contrast, necrotic cells showed extensive swelling, which resulted in subsequent disintegration of cells.

Apoptotic cells lose adhesion at the very early stages, while necrotic cells stay adherent until very late stages. As mentioned before, individual apoptotic cells can be efficiently recognized either by neighboring cells or professional macrophages and can be removed by phagocytosis. This step of the apoptotic process involves rearrangement of the plasma membrane. Removal of the dead-cell fragments before their plasma membranes are lysed has important implications for other cells within the tissues. It is assumed that apoptosis occurs

without inflammation. Lysis of plasma membranes in necrotic cells seems to precede the removal of these remnants, and exudative inflammation is a common event during necrosis.

The significance of apoptosis is based on the fact that apoptotic cells tend to be "environmentally friendly" and package their contents into membrane-bound vesicles ready for ingestion by phagocytic cells, without releasing their contents into the intercellular matrix. Hence there is no inflammatory response. Apoptosis is also an altruistic mode of cell death, in that damaged or injured cells commit suicide to allow the neighboring cells to continue to proliferate without being affected by the death of the neighbor. In addition, sacrifice of individual abnormal, or potentially malignant, cells benefits the whole organism.

Most if not all mechanisms leading to cell death involve molecular damage that is potentially reversible by cellular repair mechanisms; therefore, if the repair mechanisms operate effectively, they may prevent cell death. It could be that in cells undergoing apoptosis, the cell-repair machinery is repressed by specific signals, which program cells to die. On the other hand, in cells undergoing necrosis this machinery might be directly suppressed by extremely strong insult (high doses of drugs or irradiation). Since apoptosis is a gene-directed process and many genes are involved in the regulation of this multistage action, the repression of repair processes can also be regulated on this level. There is no evidence for expression of any specific genes during necrosis, which is why it is difficult to discuss the possible requirement of gene regulation for necrotic cell death. Thus, it seems that classic necrosis is a passive process. An inhibition of the activity of a number of gene products involved in apoptosis can switch the death pathway from apoptosis to necrosis. Also rapid changes in the cellular ATP level, discussed above, and an imbalance in the ratio of nitric oxide and superoxide can switch the form of cell death. Recently, an additional form of cell death was described and named paraptosis by the authors (Sperandio et al., 2000). This internally programmed cell death is related to pathogenesis in the central nervous system and to development of some primitive eukaryotes. Although experiments indicate that this type of death depends on RNA and protein synthesis, the machinery required for apoptosis is inactive in paraptotic cells. Thus, morphologically these cells are characterized by swelling of endoplasmic reticulum and cytoplasmic vacuolization,

and do not display nuclear fragmentation and formation of apoptotic bodies. Paraptotic cells are TUNEL-negative and their DNA does not fragment. Moreover, paraptotic death is independent of well known sequential caspase activation, although expression of caspase-9 in the presence of pan-caspase inhibitors induced nonapoptotic cell death; therefore it is clear that there is "more than one way to go" (Wyllie and Golstein, 2001). The search at both molecular and biochemical levels for mechanisms of apoptosis, necrosis, and paraptosis, along with attempts to understand the differences/similarities between these types of cellular response to treatment, are currently subject to intense biological research.

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Analysis of Caspase Activation During Apoptosis

This unit describes methods for the detection of caspase activation as cells undergo apoptosis. Caspases are intracellular cysteine proteases that are synthesized as zymogens and then proteolytically activated early in the course of apoptosis. This activation process can be detected by assays for cleavage of suitable fluorogenic or chromogenic substrates, by immunoblotting with monospecific antibodies, or by affinity labeling with reactive substrate analogs. Because the caspases are organized into distinct proteolytic cascades, identifying the caspases that are activated and their order of activation can provide insight into the biochemical pathways involved in that activation.

Three different approaches for assessing caspase activation are described. Enzymatic assays using suitable substrates are simple and relatively quantitative (see Basic Protocol 1). Because the various low-molecular-weight substrates available for these assays are not selective, however, the assays do not accurately distinguish between various caspases. Immunoblotting can be utilized to follow the activation of specific caspases (see Basic Protocol 2 and Alternate Protocol 1). When coupled with subcellular fractionation (Graham and Rickwood, 1997), this method can provide large amounts of temporal and spatial information about caspase activation. Like all immunological methods, however, immunoblotting is limited by the quality and availability of immunological reagents. Finally, affinity labeling can be utilized to detect active caspases in whole-cell lysates (see Basic Protocol 3 and Alternate Protocol 2) or subcellular fractions. With suitably designed affinity labels, this method can detect active caspases even when their identity and substrate specificity are unknown (e.g., when caspases are being explored in a new species before immunological reagents are available). Affinity labeling, however, is the most labor intensive of these techniques. In addition, because some procaspases label poorly with commercially available affinity labels, the lack of affinity labeling does not absolutely rule out the presence of active caspases.

ENZYMATIC ASSAYS FOR CASPASE ACTIVITY

In principle, the easiest method for determining whether caspases have been activated in apoptotic cells is to assay for their activity. This approach became feasible after the identification of sequences that are cleaved in various caspase substrates during apoptosis (reviewed in Nicholson and Thornberry, 1997; Earnshaw et al., 1999) and the subsequent synthesis of low-molecular-weight substrates containing the appropriate peptides coupled to fluorogenic or chromogenic leaving groups (Thornberry et al., 1992; Nicholson et al., 1995; Martins et al., 1997). It must be kept in mind, however, that the apoptotic caspases show varying degrees of preference rather than absolute specificity for the currently available caspase substrates. Accordingly, these enzymatic assays can indicate that caspases have been activated but will not necessarily provide an accurate indication of the individual caspases that have been activated.

The following protocol describes the preparation of cytosol and an assay for cleavage of the substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (acetyl-DEVD-AFC). For this procedure, cells are initially subjected to conditions that are thought to induce apoptosis. These apoptosis-inducing treatments must be optimized for each cell line and inducing reagent before the protocol can be carried out. Because caspases are intracellular proteases, cells must then be lysed or fractionated. After the protein content in the lysates or subcellular fractions is measured, samples containing equal amounts of protein are incubated with the fluorogenic substrate. The change in

BASIC PROTOCOL 1

Cellular Aging and Death

18.2.1

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fluorescence is measured and compared to a standard curve constructed using known amounts of the fluorescent product.

Materials

Cells of interest and appropriate medium
Apoptosis-inducing stimulus
CMF-DPBS (*APPENDIX 2A*), ice cold
Lysis buffer (see recipe), 4°C
0.5 M EDTA, pH 7.4 (see recipe)
1 M dithiothreitol (DTT; *APPENDIX 2A*)
5 mM EDTA (pH 7.4)/1 mM DTT in lysis buffer, ice cold
20 mM (14.6 mg/ml) acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (acetyl-DEVD-AFC; Biomol), or other fluorogenic or chromogenic caspase substrate, in dimethyl sulfoxide (DMSO)
HEPES/CHAPS buffer (see recipe), room temperature and ice cold
10 mM (4.6 mg/ml) 7-amino-4-trifluoromethylcoumarin (free AFC; Sigma), or appropriate standard for other substrate, in DMSO

Cell scraper (optional)
2-ml (total volume) tight-fitting Dounce homogenizers
Beckman TL100 ultracentrifuge and TL100.2 rotor, or equivalent, 4°C, and appropriate ultracentrifuge tubes
Fluorometer

Additional reagents and equipment for Ficoll-Hypaque density sedimentation (*UNIT 2.2*), cell trypsinization (for adherent cell lines only; *UNIT 1.1*), trypan blue staining to detect lysed cells (*UNIT 1.1*), and determination of protein concentration (*APPENDIX 3B*)

NOTE: Fluorogenic and chromogenic caspase substrates are available from a number of suppliers, including Bachem Bioscience, Biomol Research Laboratories, Calbiochem-Novabiochem, Molecular Probes, and Osaka Peptide Institute. Stock solutions for substrates and standards are prepared as 20 mM solutions in DMSO and stored in aliquots for up to 1 year at -20°C.

Induce apoptosis

1. Remove cells that have spontaneously become apoptotic before starting assay. For adherent cell lines, tap the flask containing cells of interest and change the medium to remove the dead cells. For nonadherent cell lines, remove dead cells by Ficoll-Hypaque density sedimentation (*UNIT 2.2*).

This step reduces the background activity in this assay. For most adherent cell lines, apoptotic cells become nonadherent and can be simply washed away. Nonadherent cell lines such as leukemia and lymphoma cells require Ficoll-Hypaque density sedimentation to remove dead cells (Martins et al., 1997).

2. Treat cells with a suitable apoptosis-inducing stimulus or mock treatment. Use 1–3 × 10⁸ cells per time point for treatment and controls.

The optimal treatment regimen (e.g., Kaufmann and Earnshaw, 2000) must be determined separately for each cell type and inducing agent. For reasons that are unclear, some cell lines (particularly leukemia cell lines) undergo apoptosis within 3 to 6 hr, whereas other cell lines (fibroblasts and solid tumor cell lines) take several days to die even though the same fraction of cells will ultimately die. These differences reflect varied lengths in the latent phase of apoptosis.

Harvest cells

3. Harvest cells for each time point. For adherent cells, use a cell scraper or trypsinization (UNIT 1.1) to release cells from their substratum.

When working with an adherent cell line, cells that have become detached during the course of the experiment should also be collected. For many cell lines, one of the changes that occurs during apoptosis is detachment from the substratum. Discarding detached cells will cause an underestimation of the amount of apoptosis.

4. Transfer cells to a 50-ml conical centrifuge tube and centrifuge 10 min at $200 \times g$, room temperature.
5. Remove supernatant, resuspend cells in 10 ml ice-cold CMF-DPBS, and centrifuge 10 min at $200 \times g$, room temperature. Repeat once.

All subsequent steps should be performed at 4°C unless otherwise indicated.

Isolate cell extracts

6. Remove supernatant and resuspend pellet in 1 ml ice-cold lysis buffer.

This yields a cytosolic extract with a protein concentration ranging from 4 to 8 mg/ml.

7. Incubate sample 20 min on ice, then lyse cells in a 2-ml tight-fitting Dounce homogenizer.

Glass-glass homogenizers work well for homogenizing cells under the conditions specified. Note that the power stroke with the homogenizer is the upstroke. Excessive force on the downstroke will cause a fountain effect. For leukemia cell lines, which have a sparse cytoskeleton, 20 to 30 strokes in a tight-fitting homogenizer are generally adequate. For epithelial cell lines, which have a dense cytoskeleton, many more strokes may be required. For some cell lines, alternative procedures such as freeze-thawing (see Basic Protocol 3) may also be employed. However, freeze-thawing disrupts membrane-enclosed compartments, making it difficult to assess subcellular distribution of activities.

8. Confirm cell lysis by mixing 3 to 5 μl homogenate with an equal volume of 0.4% (w/v) trypan blue in Hanks' balanced salt solution and examining with a hemacytometer (UNIT 1.1).

If plasma membranes have been successfully disrupted, nuclei will stain blue. Continue homogenization until $>95\%$ of the nuclei stain blue.

9. Transfer homogenate to a suitable tube and centrifuge 10 min at $800 \times g$ or 3 min at $16,000 \times g$, 4°C , to remove the nuclei.
10. Transfer the postnuclear supernatant to an ultracentrifuge tube. Add 0.01 vol of 0.5 M EDTA, pH 7.4.

EDTA is added only after removal of nuclei to avoid the high viscosity that accompanies extraction of chromatin after chelation of divalent cations.

11. Centrifuge the postnuclear supernatant 60 min at $280,000 \times g_{\text{max}}$, 4°C , in a Beckman TL100 ultracentrifuge with a TL100.2 rotor.
12. Collect the supernatant (cytosol) and use an aliquot to determine its protein concentration (APPENDIX 3B).

Caspase activity can also be examined in other fractions after suitable purification procedures (Martins et al., 1997).

The authors generally use the bicinchoninic acid method (Smith et al., 1985) for protein concentration determination. Alternatives include the Lowry method (Cadman et al., 1979), the Coomassie blue method (Bradford, 1976), and the nitric acid method (Bible et al., 1999). No matter which method is utilized, protein measurements are required to adjust various samples to the same concentration.

- To the remaining cytosol, add 1 M DTT to a final concentration of 1 mM.

DTT is added to maintain sulfhydryl groups at caspase active sites in a reduced state. It could be added at earlier steps but would interfere with some protein assay methods.

- Freeze the cytosol in 50- μ l aliquots at -70°C .

The authors generally avoid repeated freezing and thawing steps by freezing small aliquots. Control experiments from the authors' laboratory have indicated that activity capable of cleaving acetyl-DEVD-AFC is stable for ≥ 3 months at -70°C .

Measure enzymatic activity

- Thaw a sample aliquot at 4°C and transfer a volume containing 50 μg cytosolic protein to a microcentrifuge tube. Dilute to 50 μl with ice-cold 5 mM EDTA (pH 7.4)/1 mM DTT in lysis buffer.

- Add 50 μl ice-cold 5 mM EDTA (pH 7.4)/1 mM DTT in lysis buffer to a second microcentrifuge tube and treat in parallel as a negative control reaction.

Alternatively, a reagent blank containing 50 μg boiled cytosolic protein or bovine serum albumin would also be acceptable.

- Prepare 100 μM acetyl-DEVD-AFC in HEPES/CHAPS buffer and add 225 μl to each tube. Incubate 2 hr in a 37°C water bath.

The 100 μM working substrate solution should be prepared immediately before use.

- To stop the reaction, add 1.225 ml ice-cold HEPES/CHAPS buffer (final 1.5 ml) and place samples on ice.

As indicated below, the authors have found the release of product to be a linear function of time for up to 4 hr when saturating substrate concentrations (five times the K_M) are utilized. If the reaction is run as an endpoint assay as described above, it is important to verify its linearity over time. Alternatively, continuous monitoring of fluorochrome release can be utilized to examine the kinetics of product release and/or the kinetics of enzyme inhibition if suitable equipment is available (Thornberry et al., 1992).

- Use 10 mM free AFC in DMSO to set up a series of tubes containing a range of concentrations for a standard curve. Use 0 to 1500 pmol free AFC in 50 μl of 5 mM EDTA (pH 7.4)/1 mM DTT in lysis buffer. Add 1.45 ml HEPES/CHAPS buffer (final 1.5 ml at 0 to 1 μM AFC).

- Measure the fluorescence of all samples in a fluorometer using an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Under the conditions specified, the reaction is fairly robust and can be monitored in a relatively inexpensive filter fluorometer. Alternatively, for continuous monitoring, fluorometric plate readers and fluorometers with thermostatically controlled cuvette holders are available.

Some filter fluorometers do not have excitation and emission filters of the correct wavelength for AFC fluorescence. The authors have successfully used an excitation wavelength of 360 nm and an emission wavelength of 475 nm to measure AFC.

- Prepare a standard curve from the fluorescence intensity of the known amounts of free AFC (step 19). Compare the fluorescence values in the cell extracts to determine the absolute amount of AFC liberated in each sample.

- Subtract the amount of AFC liberated in the reagent blank (step 16) from each value to determine the amount of enzyme-mediated release.

- Convert this to an activity measurement by expressing the data as product released per unit protein per unit time.

DETECTION OF CASPASE ACTIVATION BY IMMUNOBLOTTING

BASIC PROTOCOL 2

Immunoblotting provides an alternative method for detecting active caspases within cells. Caspases are synthesized as zymogens that undergo proteolytic cleavage during their activation (see Background Information, discussion of immunoblotting). Accordingly, the precursors and active caspase species can be distinguished by size differences on immunoblots using anti-caspase antibodies. The usual practice is to look for disappearance of zymogen and corresponding appearance of processed caspase subunit(s). Alternatively, it is possible to use antibodies that recognize neoepitopes (i.e., epitopes generated during caspase maturation, such as epitopes that contain the carboxylate moiety of aspartate at the C terminus of the large subunit or the free α -amino group at the N terminus of the small subunit). These anti-neoepitope antibodies have the advantage that they recognize species present only in cells containing active caspases. In using either type of antibody, the overall approach for detecting caspase activation is the same (also see *UNIT 6.2*). A second method for cell lysis is described (see Alternate Protocol 1).

Table 18.2.1 Selected Properties of Human Caspases^a

New name	Old name(s)	Molecular weight (kDa) ^b			Preferred small substrates ^c	Antibody suppliers ^d
		Pr	Lg	Sm		
Caspase-1	ICE	45	24 20	14 10	YEVD/X WEHD/X	BP, ORP, UB
Caspase-2	Ich-1 _L , NEDD2	48	32 18	14 12	VDVAD/X DEHD/X	BP, BTL, ORP, UB
Caspase-3	CPP32, YAMA, Apopain	32	20 17	12	DMQD/X DEVD/X	BP, BTL, CI, ORP, UB
Caspase-4	Tx, Ich-2, ICE _{rel} II				LEVD/X (W/L)EHD/X	BP, ORP
Caspase-5	ICE _{rel} III, Ty				Unknown (W/L)EHD/X	CN
Caspase-6	Mch2	34	21 18	13 11	VEID/X VEHD/X	BP, CI, CN, NEB, UB
Caspase-7	Mch3, CMH-1, ICE-LAP3	34	20	12	DEVD/X DEVD/X	BP, BTL, NEB, ORP
Caspase-8	Mch5, FLICE, MACH	53 55	43 18	12 11	IETD/X LETD/X	BP, CI, CN, NEB, ORP
Caspase-9	ICE-LAP6, Mch6	50	37	12	Unknown LEDH/X	BP, CI, CN, NEB, UB
Caspase-10	Mch4, FLICE-2	55	43 17	12	IEAD/X Unknown	CI, CN, NEB, UB
Caspase-13	ERICE				Unknown Unknown	Unknown
Caspase-14	MICE	29	18	10	Unknown Unknown	BTL, ORP

^aModified from Earnshaw et al. (1999).

^bThe appearance of multiple entries indicates partially processed and fully processed large (Lg) and small (Sm) subunits that result from sequential cleavage at the C-terminal end of the large subunit followed by removal of the linker peptide from the small subunit and the prodomain (Pr) from the large subunit. A blank in this column indicates that the molecular weight of the processed forms has not been reported.

^cThe left and right columns indicate the preferred low-molecular-weight substrate specificity reported by two groups: Talanian et al. (1997) and Thornberry et al. (1997), respectively. It is important to note, however, that additional factors also affect caspase cleavage of full-length polypeptides. Not all sites conforming to the indicated sequences are cleaved, perhaps due to limited accessibility. Conversely, polypeptides are sometimes cleaved at sites that would not be predicted based on analysis of the tetrapeptide preferences indicated in this table (Samejima et al., 1999).

^dAbbreviations: BP, BD Pharmingen; BTL, BD Transduction Laboratories; CI, Chemicon International; CN, Calbiochem-Novabiochem; NEB, New England Biolabs; ORP, Oncogene Research Products; UB, Upstate Biotechnology.

18.2.5

Materials

CMF-DPBS (APPENDIX 2A), ice cold
Serum-free tissue culture medium (appropriate for cells of interest), ice cold, optional
SDS sample buffer (see recipe)
Fast green dye solution: 0.1% (w/v) fast green FCF/20% (v/v) methanol/5% (v/v) acetic acid
Fast green destain: 20% (v/v) methanol/5% (v/v) acetic acid
Blocking buffer (see recipe)
Anti-caspase primary antibody (Table 18.2.1)
PBS-T (see recipe)
Appropriate secondary antibody conjugated to horseradish peroxidase (HRP), alkaline phosphatase (AP), or a radiolabel
3% (w/v) nonfat dry milk in CMF-DPBS
Enhanced chemiluminescence reagents (e.g., ECL from Amersham Pharmacia Biotech), for HRP-conjugated secondary antibodies
X-ray film
Cell scraper (optional)
Sonicator equipped with microprobe (e.g., Branson)
70° or 100°C water bath or heating block
Additional reagents and equipment for inducing apoptosis (see Basic Protocol 1), trypsinizing and counting cells (optional; UNIT 1.1), SDS-polyacrylamide gel electrophoresis (UNIT 6.1), and electrophoretic transfer of polypeptides to a solid support (UNIT 6.2)

Induce apoptosis

1. Subject the desired number of cells to an apoptotic stimulus or mock treatment (see Basic Protocol 1, steps 1 and 2).

It is convenient to load $2\text{--}5 \times 10^5$ cells per well for immunoblotting. To permit the running of multiple duplicate gels, each processed sample should contain $1\text{--}10 \times 10^6$ cells.

Harvest cells

2. Harvest cells at an appropriate time point. For adherent cells, use a cell scraper or trypsinization (UNIT 1.1) to release cells from their substratum.

When working with an adherent cell line, cells that have become detached during the course of the experiment should also be collected. For many cell lines, one of the changes that occurs during apoptosis is detachment from the substratum. Discarding detached cells will cause an underestimation of the amount of apoptosis.

3. Transfer cells to a 15-ml conical centrifuge tube. Centrifuge 10 min at 200 to 400 × g, room temperature.
4. Remove supernatant and resuspend cells in 10 to 15 ml ice-cold CMF-DPBS or serum-free tissue culture medium.

The purpose of this step is to remove serum proteins so that they do not interfere with subsequent protein determination, electrophoresis, or immunoblotting.

5. Count the cells in the sample (UNIT 1.1).

If samples will be loaded based on protein concentration, cells do not need to be counted.

6. Centrifuge 10 min at 200 to 400 × g, room temperature. Remove supernatant without disturbing cell pellet.

Lyse cells

7. Add 20 μ l SDS sample buffer per $2\text{--}5 \times 10^5$ cells to lyse cells. Vortex sample vigorously.

If a cell count was not performed, a suitable sample volume is determined empirically by the inability to sonicate volumes that are too small and the inability to load volumes that are too large onto SDS-polyacrylamide gels. An alternative procedure for lysing cells in larger volumes and then concentrating them is given (see Alternate Protocol 1).

8. Use a sonicator equipped with a microprobe to deliver 40 pulses (0.33 sec each at 40% of the maximal output of the power supply) to the sample to shear the DNA.
9. Freeze sample indefinitely at -80°C or proceed to step 10 immediately.

If samples are going to be loaded based on protein content, it is convenient to remove an aliquot for protein assays prior to freezing. Protein should be estimated using an assay that is not subject to interference by SDS, urea, or reducing agents, such as the nitric acid method (Bible et al., 1999) or the Lowry method after protein precipitation (APPENDIX 3B).

Electrophoresis sample

10. Heat sample 20 min to 70°C or 3 min to 100°C in a water bath or heating block.

This heat treatment is required to denature the protein and ensure that polypeptides are uniformly bound to SDS. If frozen samples are not heated prior to electrophoresis, there is a risk that SDS will not be uniformly bound to polypeptides and migration will be aberrant.

11. Immediately load a volume of sample containing $2\text{--}5 \times 10^5$ cells in the lane of an SDS-polyacrylamide gel with suitable separating capabilities.

Alternatively, samples containing equal amounts of protein can be loaded.

The authors prefer $20 \times 20\text{-cm}$ gels for better resolution. Adequate results, however, can be obtained with minigels ($6 \times 8\text{-cm}$).

Because the subunits of active caspases are 10 to 12 kDa and 16 to 35 kDa, a gel that separates in this molecular-weight range is required. If the standard 30:0.8 acrylamide/bisacrylamide ratio (Laemmli, 1970) is utilized, a gel containing somewhere between 12% and 16% acrylamide is required to resolve in this molecular-weight range. Alternatively, a 5% to 15% or 5% to 20% gradient gel gives adequate separation of caspases and also permits analysis of their substrates by immunoblotting.

12. Electrophoresis gel (UNIT 6.1) and electrophoretically transfer (UNIT 6.2) sample to a nitrocellulose or PVDF membrane.

Because of their small sizes, caspases should readily transfer even from gels containing a high acrylamide content. If desired, transfer of higher-molecular-weight caspase substrates from these gels can be facilitated by including 0.02% to 0.1% (w/v) SDS in the transfer buffer. If this approach is taken, it is important to cool the transfer apparatus, as the SDS increases the current flow during transfer.

13. Stain the membrane for 1 min using fast green dye solution to confirm equal transfer of sample proteins. Remove unbound stain by rinsing briefly in fast green destain.

Alternatively, blots can be stained in 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid (UNIT 6.2).

14. Rinse membrane several times in CMF-DPBS to remove residual acetic acid.

15. Incubate membrane on an orbital platform shaker ≥ 1 hr in blocking buffer at room temperature to block unoccupied protein-binding sites.

This blocking step should be performed immediately after the transfer if PVDF membranes are used. Nitrocellulose membranes can be dried and blocked later.

Perform immunoblotting

16. Dilute an anti-caspase primary antibody in blocking buffer according to supplier's instructions.

Most antisera or antibodies give good results when diluted in milk-containing buffer. Some, however, yield cleaner results when a different carrier protein, such as 1% to 3% (w/v) bovine serum albumin, is used.

17. Incubate membrane with diluted primary antibody on an orbital platform shaker overnight at room temperature.

Some investigators prefer to incubate filters with antibody for a shorter period of time. The authors have obtained good signals after a 4 to 6 hr incubation with primary antibody. Others have reported good signals with incubation times as short as 1 hr, although the authors' experience has been that higher concentrations of antibody are required to detect a signal after a 1-hr incubation.

18. Remove the primary antibody and store it at 4°C for subsequent use.

Aliquots of primary antibody can be used three to four times without detectable loss of signal. Additional penicillin, streptomycin, and sodium azide can cut down on microbial growth if the antibody is to be stored for several weeks.

19. Wash the membrane three times in 100 ml PBS-T (for a 20 × 20-cm membrane) for 15 min each.

If there is extensive background on the film after an initial immunoblotting experiment, it may be helpful to use a wash buffer of increased stringency (e.g., PBS-T supplemented with 2 M urea or additional NaCl).

20. Wash the membrane twice with 100 ml CMF-DPBS for 5 min each.

21. Dilute an appropriate secondary antibody in 3% (w/v) nonfat dry milk in CMF-DPBS according to the supplier's instructions.

If HRP-coupled secondary antibodies are used, it is important to avoid sodium azide from this point onward because azide inhibits peroxidase.

22. Add diluted secondary antibody to the membrane and incubate on an orbital platform shaker 1 hr at room temperature.

23. Discard the secondary antibody and wash filter with 100 ml PBS-T as follows:

twice for 5 min each
twice for 15 min each
twice for 5 min each.

24. Follow manufacturer's instructions for enhanced chemiluminescence reagents for HRP- or AP-conjugated secondary antibodies.

This step is omitted if radioconjugated secondary antibodies are used.

25. Expose the membrane to X-ray film for an appropriate length of time.

When using ECL enhanced chemiluminescence reagents, the authors start with an initial 2-min exposure and then adjust the exposure time empirically.

Primary and secondary antibodies can be removed from the membrane for reprobing (see Support Protocol 1).

CELL LYSIS WITH GUANIDINE HYDROCHLORIDE FOR IMMUNOBLOTTING

ALTERNATE PROTOCOL 1

It is sometimes inconvenient to lyse and sonicate cells in a small enough volume to permit loading on a gel. In addition, high concentrations of SDS and 2-mercaptoethanol (2-ME) interfere with many protein estimation methods. Finally, some of the authors' observations have raised the possibility that active caspase species bound to cleaved intermediate filaments (MacFarlane et al., 2000) might not be solubilized efficiently by SDS sample buffer (T.J.K. and S.H.K., unpubl. observ.). The following protocol provides an alternative that allows cells to be rapidly lysed in the strong denaturing agent guanidine hydrochloride, dialyzed into dilute SDS, and then concentrated to the desired volume prior to electrophoresis. This protocol is suitable for detecting a variety of polypeptides in addition to caspases (Kaufmann et al., 1997).

Additional Materials (also see Basic Protocol 2)

- Guanidine hydrochloride lysis buffer (see recipe)
- 100 mM PMSF (*APPENDIX 2A*)
- 2-Mercaptoethanol (2-ME)
- 1.54 M (285 mg/ml) iodoacetamide in guanidine hydrochloride lysis buffer, prepared fresh
- 4 M urea (see recipe)/50 mM Tris-Cl, pH 7.4 at 4°C (*APPENDIX 2A*)
- 0.1% (w/v) SDS
- 1-cm dialysis tubing (MWCO 8000 to 10,000), double knotted at one end, and dialysis clips
- Additional reagents and equipment for determining protein concentration (*APPENDIX 3B*)

CAUTION: 2-ME has a strong odor and its use is confined to the hood in some laboratories.

NOTE: All steps involving iodoacetamide should be performed under subdued light because of the light sensitivity of the carbon-iodine bond.

Prepare cell lysate

1. Subject cells of interest to apoptosis-inducing or mock treatment, harvest, and wash as described (see Basic Protocol 2, steps 1 to 6). Remove as much supernatant as possible after the second centrifugation.
2. To 1 ml guanidine hydrochloride lysis buffer add 10 μ l of 100 mM PMSF and 10 μ l of 2-ME. Immediately add this mixture to cell pellet and vortex vigorously.
3. Use a sonicator equipped with a microprobe to deliver 40 pulses (0.33 sec each at 40% of the maximal output of the power supply) to the sample to shear the DNA.

At this point samples can be stored ≤ 10 weeks at room temperature without loss of reactivity as detected by a variety of antibodies.

4. Add 0.1 vol of 1.54 M iodoacetamide in guanidine hydrochloride lysis buffer to each sample. Incubate 1 hr at room temperature.

This reaction blocks free sulfhydryl groups, which can otherwise reoxidize to form large disulfide-cross-linked polypeptide oligomers in the wells of an SDS-polyacrylamide gel.

5. Add 0.01 vol of 2-ME.

This reducing agent reacts with any remaining iodoacetamide.

Dialyze lysate

6. Transfer sample to a length of knotted 1-cm dialysis tubing and close tubing with a dialysis clip.
7. Dialyze sample at 4°C against four or five changes (≥90 min each) of 10 to 100 vol ice-cold 4 M urea/50 mM Tris·Cl, pH 7.4.

The purpose of dialyzing into urea initially is to avoid the simultaneous presence of guanidine and SDS, which form a poorly soluble salt. Because each change of dialysis buffer decreases the concentration of solutes by a multiplicative factor, the key to effective dialysis is multiple changes with moderate volumes rather than a few changes with large volumes. Sufficient time should be permitted for equilibration to occur before each buffer change.

8. Dialyze sample at 4°C against three changes (≥90 min each) of 10 to 100 vol of 0.1% SDS.
9. Transfer sample to a 2-ml microcentrifuge tube. Remove a small aliquot and determine its protein concentration (APPENDIX 3B).

The authors prefer the bicinchoninic acid BCA method (Smith et al., 1985) or the folin (Lowry) method suitably modified for samples containing SDS (Cadman et al., 1979).

10. Measure and record the volume of the remaining sample and then lyophilize it until dry.

Lyophilized sample can be stored up to 5 years at -20°C.

Electrophoresis and immunoblot caspases

11. Reconstitute the lyophilized sample in SDS sample buffer at 5 mg protein/ml, transfer 10 to 15 µl to a microcentrifuge tube, and continue with analysis as described (see Basic Protocol 2, steps 10 to 25).

The authors find it convenient to reconstitute samples at 5 mg protein/ml SDS sample buffer and then load 50 to 75 µg protein per well, which is about the loading limit of a 2-mm-wide × 0.75-mm-thick lane.

SUPPORT PROTOCOL 1

REMOVING (STRIPPING) PRIMARY AND SECONDARY ANTIBODIES FROM BLOTS

It is often useful to probe filters with more than one antibody solution. If the antigens of interest have sufficiently different molecular weights, it is possible to reprobe a blot without stripping it. Likewise, if the primary antisera were raised in different species and can therefore be distinguished by different secondary antibodies, it is not necessary to strip blots. However, sometimes it is useful to remove antibodies without removing the original proteins transferred to the blot. The following protocol serves this purpose.

Materials

Nitrocellulose or PVDF membrane with bound antibodies (see Basic Protocol 2 or Alternate Protocol 1)

Blot erasure buffer (see recipe)

CMF-DPBS (APPENDIX 2A)

Resealable plastic bags

65°C water bath

1. Place a nitrocellulose or PVDF membrane with bound antibodies in a resealable plastic bag.

2. Add 50 ml blot erasure buffer (for a 20 × 20–cm membrane). Seal the bag.
3. Incubate 20 to 30 min in a 65°C water bath with gentle agitation every 5 to 10 min.

When blotting is performed after transfer of polypeptides to nitrocellulose, complete removal of some antibodies requires heating of blot erasure buffer to ≥50°C for 20 to 30 min (Kaufmann et al., 1987). On the other hand, after immobilization of polypeptides on PVDF, antibodies are efficiently dissociated by erasure buffer at room temperature.

4. Discard the erasure buffer. Wash the blot twice for 5 min each with 50 to 100 ml CMF-DPBS.
5. Reprobe the membrane as described (see Basic Protocol 2, steps 15 to 25).

Although some antigens are destroyed by the stripping process, most can be readily detected even after blots have been stripped multiple times (Kaufmann et al., 1987).

Be sure to incubate the membrane in blocking buffer after each stripping to ensure that all nonspecific binding sites are coated.

LABELING AND DETECTING ACTIVE CASPASES USING BIOTINYLATED SUBSTRATE ANALOGS

BASIC PROTOCOL 3

A protocol for detection of active caspases in cytosol preparations using *N*-(*N*^α-benzyloxycarbonylglutamyl-*N*^ε-biotinyllysyl)aspartic acid ([2,6-dimethylbenzoyl]oxy)methylketone, or zEK(bio)D-aomk, is described below. Although not as sensitive as assays based on cleavage of substrate polypeptides in intact cells or fluorogenic substrates under cell-free conditions, this assay can nonetheless detect picogram levels of active caspases when combined with very sensitive chemiluminescent substrates currently available. This affinity-labeling approach is particularly useful in at least three situations. First, this assay can be utilized to unequivocally identify active caspase species in apoptotic cell extracts by comparing migration of species present in the extracts with migration of similarly labeled recombinant caspases (Martins et al., 1997). Second, by taking advantage of the affinity of streptavidin for the biotin moiety, it is possible to purify the affinity-labeled caspases for further study (Nicholson et al., 1995; Faleiro et al., 1997). Third, because only two inhibitor molecules bind to each caspase tetramer, the labeling procedure can be used to determine the molar concentration of labeled active caspases.

Materials

- Incomplete KPM buffer (see recipe), 4°C
- Complete KPM buffer (see recipe), 4°C
- 100 μM *N*-(*N*^α-benzyloxycarbonylglutamyl-*N*^ε-biotinyllysyl)aspartic acid ([2,6-dimethylbenzoyl]oxy)methylketone [zEK(bio)D-aomk; Osaka Peptide Institute] in dimethyl sulfoxide (DMSO), stored in aliquots up to 2 years at –80°C
- 3× SDS sample buffer (see recipe)
- 5% (w/v) nonfat dry milk in PBS-T
- PBS-T (see recipe)
- Peroxidase-coupled streptavidin (e.g., Amersham Pharmacia Biotech)
- Enhanced chemiluminescence reagents (e.g., ECL; Amersham Pharmacia Biotech)
- Cell scraper (optional)
- 8 × 34–mm polycarbonate ultracentrifuge tubes (e.g., Beckman)
- Beckman Optima TLX tabletop ultracentrifuge and TL100.1 rotor, or equivalent, 4°C
- Additional reagents and equipment for inducing apoptosis (see Basic Protocol 1), cell trypsinization (optional; UNIT 1.1), protein determination (APPENDIX 3B), SDS-polyacrylamide gel electrophoresis (UNIT 6.1), and electrophoretic transfer of polypeptides to a solid support (UNIT 6.2)

Cellular Aging and Death

18.2.11

Induce apoptosis

1. Subject $\sim 8 \times 10^6$ cells of interest at the desired density to suitable apoptosis-inducing conditions or mock treatment (see Basic Protocol 1, steps 1 and 2).

It is important to include a sample that contains control (nonapoptotic cells) so that endogenous biotinylated proteins can be distinguished from species that label with zEK(bio)D-aomk. A second control for labeling specificity (see Support Protocol 2) can also be included.

The authors estimate that a 10-cm tissue culture plate will yield $\sim 8 \times 10^6$ cells or 400 μg cellular protein when prepared as described.

Harvest cells

2. At an appropriate time point, collect cells in a 15-ml tube, releasing cells from their substratum by trypsinization (UNIT 1.1) or scraping if they are adherent.

All subsequent steps prior to incubation with the affinity labeling agent should be performed at 4°C.

3. Centrifuge cells 5 min at $300 \times g$, 4°C. Gently resuspend them in 5 to 10 ml incomplete KPM buffer and centrifuge again.

In this and all following steps, incomplete and complete KHM buffer (see Alternate Protocol 2) can be substituted for incomplete and complete KPM buffer, respectively.

4. Gently resuspend cells in 1 ml complete KPM buffer and transfer them to a 1.5-ml microcentrifuge tube.
5. Centrifuge cells 3 min at $800 \times g$, 4°C. Remove supernatant and resuspend cell pellet in a volume of complete KPM buffer approximately equal to that of the pellet.

Prepare cytosolic lysates

6. Lyse cells using three cycles of freezing in liquid nitrogen (or on dry ice) and incubating in a 37°C water bath until sample thaws.

It is not necessary for the cells to reach 37°C. The freeze-thaw lysis procedure ruptures mitochondria and lysosomes, providing cell lysates that contain soluble polypeptides from multiple subcellular organelles. As an alternative, it is possible to prepare various subcellular fractions and then affinity label them with zEK(bio)D-aomk (Martins et al., 1997).

7. Transfer lysed cells to an 8×34 -mm polycarbonate ultracentrifuge tube and centrifuge 1 hr at $157,000 \times g_{\text{max}}$, 4°C, in a Beckman Optima TLX tabletop ultracentrifuge with a TL100.1 rotor.
8. Transfer cleared lysate to a 1.5-ml microcentrifuge tube.
9. Measure protein concentration (APPENDIX 3B).

The authors prefer the folin (Cadman et al., 1979), BCA (Smith et al., 1985), or Coomassie blue methods (Bradford, 1976).

10. Dilute lysates to a final protein concentration of 5 mg/ml in complete KPM buffer. Freeze 200- μg aliquots up to 2 months at -80°C .

Affinity label lysate

11. Thaw an aliquot of lysate on ice.
12. To 40 μl cell lysate (200 μg) add 0.4 μl of 100 μM zEK(bio)D-aomk. Mix the sample thoroughly without forming bubbles and incubate 5 min at 37°C.

When mixing the sample, it is important to avoid forming bubbles (e.g., by vortexing), as enzymes can be denatured at the resulting air-liquid interface.

13. Add 25 μl of $3 \times$ SDS sample buffer. Boil the sample 5 min.

Electrophoresis labeled lysate

14. Apply samples (10 to 40 µg protein) to a 16% SDS-polyacrylamide gel (UNIT 6.1).

Because the large subunits of active caspases have molecular weights of 17 to 35 kDa, a 16% gel gives good separation of various caspase species. Optimal resolution of the caspases is obtained using 0.75-mm-thick, 6 × 8-cm gels with separation at 200 V constant voltage for 1 hr. Alternatively, separation of charge variants of caspase species can be accomplished by two-dimensional isoelectric focusing/SDS-polyacrylamide gel electrophoresis (Martins et al., 1997; also see UNIT 6.4). With this technique, 16% gels also give good separation in the second dimension.

As an alternative to SDS-polyacrylamide gel electrophoresis and ligand blotting, the derivatized caspases can be affinity purified using immobilized avidin (Nicholson et al., 1995; Faleiro et al., 1997).

15. Transfer polypeptides electrophoretically to a nitrocellulose membrane (UNIT 6.2).

For example, polypeptides can be transferred 30 min at 12 V, current limited to 5.5 mA/cm², in a Bio-Rad TransBlot SD SemiDry Transfer Cell.

Visualize labeled caspases

16. Incubate membranes in 5% (w/v) nonfat dry milk in PBS-T for 1 hr at room temperature to block unoccupied binding sites.

17. Wash membrane once for 15 min and two times for 5 min each with PBS-T.

18. Dilute peroxidase-coupled streptavidin in PBS-T and incubate blot 3.5 hr at room temperature in this solution.

The authors use a 1:3000 dilution of peroxidase-coupled streptavidin supplied by Amersham Pharmacia Biotech. Suitable dilutions of similar reagents from other suppliers will need to be determined empirically.

19. Wash blot once for 15 min and four times for 5 min each with PBS-T to remove unbound peroxidase-coupled streptavidin.

20. Visualize the bound streptavidin using enhanced chemiluminescence reagents as described by the supplier.

The membrane can be stripped and probed with a suitable antibody to confirm the activation of specific caspases (see Support Protocol 3).

IN VITRO ACTIVATION OF CASPASES IN NAIVE LYSATES FOLLOWED BY AFFINITY LABELING

ALTERNATE PROTOCOL 2

It is sometimes useful to assess the competence of lysates from nonapoptotic cells to activate caspase pathways. This can be accomplished by incubating lysates with reagents that are known to activate caspase cascades, such as active caspase-8 (Muzio et al., 1997) or cytochrome *c* plus dATP (Liu et al., 1996; Mesner et al., 1999). Detailed below is a protocol for the detection of active caspases following cytochrome *c* and dATP-dependent apoptosome formation or caspase-8 activation. For such experiments, lysates are prepared from nonapoptotic cells essentially as described (see Basic Protocol 3).

Additional Materials (also see Basic Protocol 3)

Incomplete KHM buffer (see recipe), 4°C

Complete KHM buffer (see recipe), 4°C

5 µg/ml active caspase-8 (BD PharMingen) in complete KHM buffer *or* 5 mg/ml cytochrome *c* (Sigma) in complete KHM buffer

10 mM dATP (Sigma) in complete KHM, pH 7.4, optional

Cellular Aging and Death

18.2.13

100 μM *N*-(*N* ^{α} -benzyloxycarbonylglutamyl-*N* ^{ϵ} -biotinyllysyl)aspartic acid ([2,6-dimethylbenzoyl]oxy)methylketone [zEK(bio)D-aomk; Osaka Peptide Institute] in dimethyl sulfoxide (DMSO), stored in aliquots up to 2 years at -80°C

1. Prepare cell lysates from nonapoptotic cells (i.e., untreated cells; see Basic Protocol 3, steps 1 to 11), using incomplete and complete KHM buffer instead of incomplete and complete KPM buffer, respectively, throughout.
2. To 8 μl (40 μg) cell lysate add 10 μl of 5 $\mu\text{g}/\text{ml}$ active caspase-8 in complete KHM buffer or 10 μl of 5 mg/ml cytochrome *c* in complete KHM buffer and 2 μl of 10 mM dATP (optional).

*Active caspase-8 can be utilized to trigger the caspase activation process without interfering with affinity labeling of endogenous caspases because caspase-8 does not label with zEK(bio)D-aomk. In contrast, caspase-9 does label with zEK(bio)D-aomk. To trigger caspase activation using endogenous caspase-9, cytochrome *c* can be added to cytosol (Li et al., 1997). It is important to keep in mind, however, that extracts from some cells contain sufficiently high levels of ATP to support this reaction, whereas other cell lysates might require addition of dATP or ATP (Liu et al., 1996; Mesner et al., 1999).*

3. Mix the sample thoroughly without forming bubbles and incubate 1 hr at 37°C .

It is important that mixing be performed without creating froth in the sample. Caspases, like other enzymes, can be denatured at air-water interfaces.

4. Add 0.2 μl of 100 μM zEK(bio)D-aomk in DMSO. Mix the sample and incubate 5 min at 37°C .
5. Add 10 μl of $3\times$ SDS sample buffer. Boil the sample 5 min.
6. Electrophorese and detect the biotinylated species as described (see Basic Protocol 3, steps 14 to 20).

SUPPORT PROTOCOL 2

CONTROLS FOR SPECIFICITY OF AFFINITY-LABELED ACTIVE CASPASES

It is possible to competitively inhibit binding of the affinity label with caspase inhibitors. For example, preincubation of lysates with certain peptide chloromethyl ketones or fluoromethyl ketones completely abolishes subsequent labeling with zEK(bio)D-aomk (Martins et al., 1997). This type of competition experiment serves two purposes. First, cell lysates contain many polypeptides that are intrinsically biotinylated. These are unaffected by preincubation of the lysates with 100 μM acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone (acetyl-YVAD-CMK), whereas labeling of caspases can be completely suppressed under the same conditions (Martins et al., 1997). Second, the same approach can sometimes be utilized to suggest the identity of the labeled caspase responsible for a particular biological function. For example, the SPI-2 serpin from rabbitpox virus and underivatized peptides corresponding to the lamin A cleavage site—two different types of reversible caspase inhibitors—were shown to inhibit lamin cleavage in vitro and simultaneously inhibit affinity labeling of one particular caspase (Takahashi et al., 1996a). In performing this latter type of experiment, it is important to optimize the labeling conditions so that the length of exposure to the affinity labeling reagent is minimized (e.g., 1 min). Because reversible inhibitors continually dissociate and rebind to the caspase active site (thereby providing an opportunity for binding to the affinity label), longer labeling times allow covalent modification of caspases even in the presence of a potent reversible inhibitor.

**STRIPPING MEMBRANE IN THE PRESENCE OF d-BIOTIN FOR
REPROBING WITH ANTIBODY**

It is sometimes useful to confirm the activation of specific caspases detected by affinity labeling using suitable antibodies. In order to do so, it is necessary to dissociate the peroxidase-coupled streptavidin without removing the biotinylated caspases. To minimize removal of the membrane-bound caspases, the authors have devised a way to compete the peroxidase-coupled streptavidin from the membrane using biotin.

Additional Materials (also see *Basic Protocol 3*)

Membrane containing affinity-labeled caspases (see *Basic Protocol 3*)
2 mM d-biotin (Sigma) in PBS-T

1. Wash a membrane containing affinity-labeled caspases two times for 15 min each with PBS-T.

It is convenient to use 50-ml of buffer for a 6 × 8-cm blot.

2. Incubate membrane 1 hr with 2 mM d-biotin in PBS-T at room temperature.
3. Wash membrane once for 15 min and two times for 5 min each with PBS-T.

The membrane can be stored dry up to 6 months at room temperature.

4. Perform immunoblot procedure with an appropriate antibody as described (see *Basic Protocol 2*, steps 16 to 25).

REAGENTS AND SOLUTIONS

Use deionized or distilled water and ACS reagent-grade chemicals (or better) in all recipes and protocol steps. Filter sterilize buffers that are to be stored for long periods. For common stock solutions, see APPENDIX 2; for suppliers, see SUPPLIERS APPENDIX.

Blocking buffer

10 mM Tris-Cl, pH 7.4 (APPENDIX 2A)
150 mM NaCl
10% (w/v) nonfat dry milk
100 U/ml penicillin G
100 µg/ml streptomycin
1 mM sodium azide
Store up to 4 weeks at 4°C

CAUTION: *Sodium azide is poisonous. Follow appropriate precautions for handling, storage, and disposal.*

A 100× penicillin G/streptomycin solution is commercially available from Life Technologies.

Blot erasure buffer

62.5 mM Tris-Cl, pH 6.8 at 21°C
2% (w/v) SDS
Store up to 6 months at 4°C
Add 100 mM 2-mercaptoethanol (2-ME) immediately before use

CAUTION: *2-ME has a strong odor and its use is confined to the hood in some laboratories.*

CLAP, 1000×

Dissolve 5 mg chymostatin, 5 mg leupeptin, 5 mg antipain, and 5 mg pepstatin A (all from Sigma) in a total volume of 5 ml dimethyl sulfoxide (DMSO), for a final concentration of 1 mg/ml each. Store 50-µl aliquots up to 1 year at -20°C.

CMF-DPBS containing 0.05% Tween 20 (PBS-T)

To 900 ml H₂O add:

11.5 g Na₂HPO₄

2.96 g NaH₂PO₄

5.84 g NaCl

Add H₂O to 1 liter

Add 2.5 ml 20% (w/v) Tween 20 (e.g., Bio-Rad)

The pH of the CMF-DPBS should be 7.5 (before addition of Tween 20).

Complete KHM buffer

50 ml incomplete KHM buffer (see recipe)

50 µl 100 mM PMSF (*APPENDIX 2A*; 0.1 mM final)

50 µl 20 mM cytochalasin B (see recipe; 20 µM final)

500 µl 100 mM Na₃VO₄ (see recipe; 1 mM final)

50 µl 1000× CLAP (see recipe; 1× final)

Prepare on ice immediately before use

Complete KPM buffer

50 ml incomplete KPM buffer (see recipe)

50 µl 100 mM PMSF (*APPENDIX 2A*; 0.1 mM final)

50 µl 20 mM cytochalasin B (see recipe; 20 µM final)

500 µl 100 mM Na₃VO₄ (see recipe; 1 mM final)

50 µl 1000× CLAP (see recipe; 1× final)

Prepare on ice immediately before use

Cytochalasin B, 20 mM

Dissolve 10 mg cytochalasin B (Sigma) in 1 ml dimethyl sulfoxide (DMSO).

Store 50-µl aliquots up to 1 year at -20°C.

EDTA, 0.5 M, pH 7.4

Add 93.1 g Na₂EDTA·2H₂O to 400 ml H₂O. While stirring and continuously monitoring pH at room temperature, add two pellets solid NaOH every 5 to 10 min. Adjust pH to 7.4. Add H₂O to 500 ml. Store indefinitely at 4°C.

The white crystalline EDTA will go into solution as the pH rises toward 7.0.

EGTA, 0.5 M, pH 7.0 and pH 7.4

Add 19.02 g EGTA to 75 ml H₂O. Adjust pH to 7.0 with 10 M NaOH or to 7.4 with 5 M KOH, stirring vigorously. Add H₂O to 100 ml. Store indefinitely at 4°C.

The EGTA will not go into solution until the pH approaches 7.0. As the solution starts to clear, be careful not to overshoot the desired pH.

Guanidine hydrochloride lysis buffer

114.64 g guanidine hydrochloride (Sigma; 6 M final)

50 ml 1 M Tris-Cl, pH 8.5 at 21°C (*APPENDIX 2A*; 250 mM final)

4 ml 0.5 M EDTA, pH 7.4 (see recipe; 10 mM final)

Bring to 200 ml final volume

Store indefinitely at room temperature

HEPES, 1 M, pH 7.4

Dissolve 23.8 g HEPES in 50 ml H₂O. Adjust pH to 7.4 with 5 M KOH. Add H₂O to 100 ml. Store indefinitely at 4°C

HEPES/CHAPS buffer

To 450 ml H₂O add:

2.98 g HEPES (25 mM final)

Adjust pH to 7.5 with 10 M NaOH

Add 0.77 g dithiothreitol (DTT)

500 mg 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; 0.1% final)

H₂O to 500 ml

Store up to 2 months at 4°C

Immediately before use, for each 1 ml buffer add:

100 U aprotinin (Bayer)

10 µl 100 mM PMSF (*APPENDIX 2A*)

Incomplete KHM buffer

5 ml 1 M HEPES, pH 7.4 (see recipe; 50 mM final)

2.5 ml 2 M KCl (50 mM final)

2 ml 0.5 M EGTA, pH 7.4 (see recipe; 10 mM final)

100 µl 2 M MgCl₂ (2 mM final)

H₂O to 100 ml

Store indefinitely at 4°C

Incomplete KPM buffer

5 ml 1 M PIPES, pH 7.0 (see recipe; 50 mM final)

2.5 ml 2 M KCl (50 mM final)

2 ml 0.5 M EGTA, pH 7.0 (see recipe; 10 mM final)

100 µl 2 M MgCl₂ (2 mM final)

H₂O to 100 ml

Store indefinitely at 4°C

K-EDTA, 0.1 M, pH 7.4

3.72 g Na₂EDTA·2H₂O

50 ml H₂O

Adjust pH to 7.4 with 1 M KOH

Add H₂O to 100 ml

Store indefinitely at room temperature

Lysis buffer

To 450 ml H₂O add:

2.98 g HEPES (25 mM final)

0.238 g MgCl₂ (5 mM final)

1 ml 0.5 M EGTA, pH 7.0 (see recipe; 1 mM final)

Adjust pH to 7.5 at 4°C with 10 M NaOH

Add H₂O to 500 ml

Store indefinitely at 4°C

Immediately before use, for each 1 ml buffer add:

10 µl 100 mM PMSF (*APPENDIX 2A*)

1 µl 10 µg/ml pepstatin A in ethanol

1 µl 10 µg/ml leupeptin in ethanol

Pepstatin A and leupeptin in ethanol can be stored indefinitely at -20°C.

Na₃VO₄, 100 mM

Add 1.84 g Na₃VO₄ (Sigma) to ~75 ml H₂O (pH ~14). Add concentrated HCl until the pH is 10. The solution will turn yellow at this point. Boil 3 min. The pH will then be ~9 and the solution will be almost white. Add 2.5 ml of 1 M NaOH and boil 3 min. Add H₂O to 100 ml. Store 1-ml aliquots indefinitely at -20°C.

PIPES, 1 M, pH 7.0

Dissolve 30.24 g PIPES in 50 ml H₂O. Adjust pH to 7.0 with 5 M KOH. Add H₂O to 100 ml. Store indefinitely at room temperature.

PIPES powder will not dissolve until the pH is adjusted to ≥ 6.7 .

SDS sample buffer

To 200 ml H₂O add:

31.25 ml Tris base (62.5 mM final)

1 ml 0.5 M EDTA, pH 7.4 (1 mM final)

Adjust to pH 6.8 with concentrated HCl

Add 10 g SDS (2% final)

250 ml 8 M urea (see recipe; 4 M final)

Add H₂O to 500 ml

Store up to 3 months at 4°C

Immediately before use, add 50 μ l of 2-mercaptoethanol (2-ME) per 950- μ l aliquot

CAUTION: SDS is a severe irritant. Use a dust mask to prevent inhaling SDS powder. 2-ME has a strong odor and its use is confined to the hood in some laboratories.

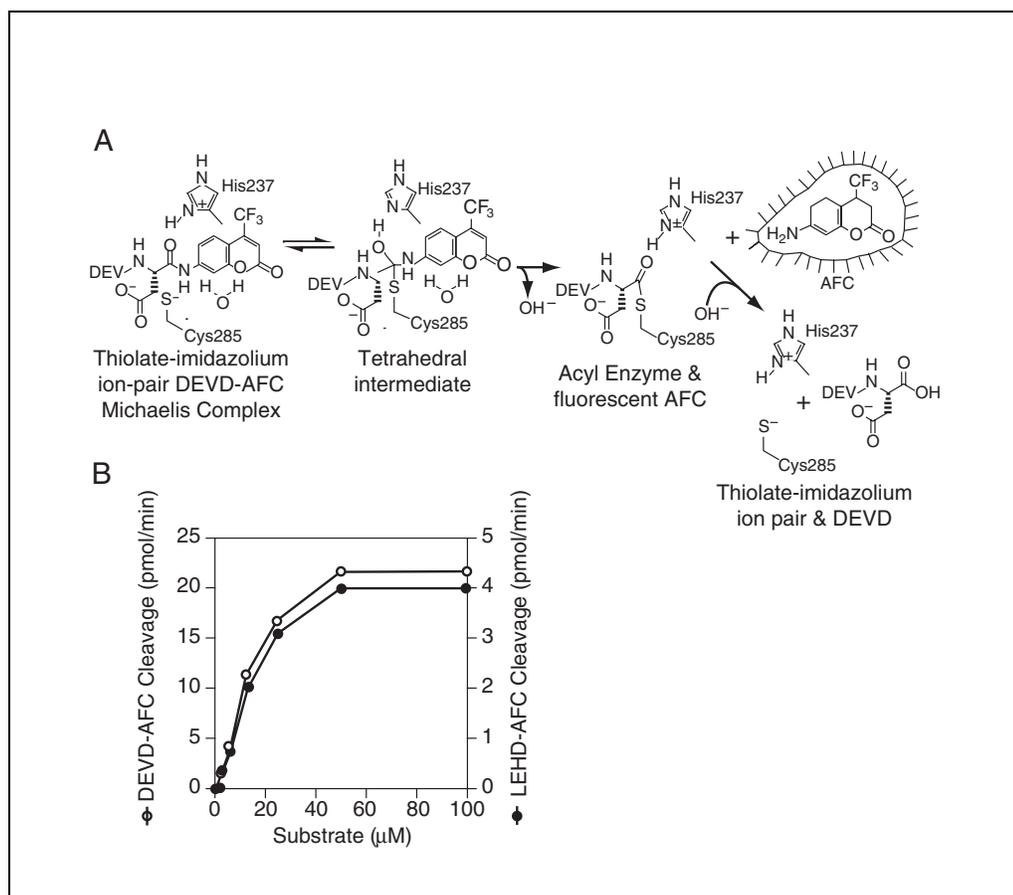


Figure 18.2.1 Measurement of caspase activity using a fluorogenic substrate. **(A)** Conversion of the substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (acetyl-DEVD-AFC) to tetrapeptide and free 7-amino-4-trifluoromethylcoumarin (AFC). **(B)** Velocity versus substrate concentration curves for caspase-3. Purified recombinant human caspase-3 was incubated for 2 hr with increasing concentrations of acetyl-DEVD-AFC or acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (acetyl-LEHD-AFC). From these data it is possible to determine that the maximal velocity is 5-fold lower for acetyl-LEHD-AFC than for acetyl-DEVD-AFC, whereas the concentration that results in half-maximal velocity (K_M) is the same (~ 12.5 μ M) for each substrate.

SDS sample buffer, 3×

30 ml 0.5 M Tris·Cl, pH 6.8 at 20°C (150 mM final)
45 g sucrose (45% final)
6 ml 0.1 M K-EDTA, pH 7.4 (see recipe; 6 mM final)
30 ml 20% (w/v) SDS (9% final)
6 ml of 0.5% (w/v) bromphenol blue (0.03% final)
H₂O to 100 ml
Store 3-ml aliquots at −20°C
Just before use add 0.1 vol of 2-mercaptoethanol (2-ME; 10% final)

CAUTION: *SDS is a severe irritant. Use a dust mask to prevent inhaling SDS powder. 2-ME has a strong odor and its use is confined to the hood in some laboratories.*

Urea, 8 M

Gradually add 1920 g urea to 2 liters H₂O while stirring. Add H₂O to 4 liters. Filter through four layers of cheesecloth. Store 500-ml aliquots up to 2 months at room temperature. Prior to use, deionize aliquot with AG 501X-8 D mixed-bed resin (Bio-Rad).

Deionization removes cyanate breakdown products that can carbamylate polypeptides.

COMMENTARY

Background Information

Enzyme activity assays

Measurements of caspase enzymatic activity are performed to determine whether caspases have been activated after various treatments and to assess the timing of caspase activation relative to other events occurring as cells undergo programmed cell death. Similar assays can also be utilized to study the biochemistry of the caspase activation process under cell-free conditions and to purify active caspases.

The chemistry of the basic enzyme assay is illustrated in Figure 18.2.1A. Cell extracts (or recombinant proteases) are generally incubated with a substrate that generates a highly fluorescent or colored product when the amide bond on the C-terminal side of aspartate is cleaved. The substrates utilized in these reactions have been patterned after the cleavage sites in known caspase substrates (Sleath et al., 1990; Lazebnik et al., 1994; Takahashi et al., 1996b) or identified by studying relative rates of cleavage of libraries of derivatized peptides (Thornberry et al., 1992, 1997). The assays based on these substrates are not absolutely specific for caspases but are relatively specific because most mammalian proteases do not cleave very efficiently on the carboxyl terminal side of aspartate. Indeed, at the present time, the only other mammalian protease known to prefer aspartate in the P₁ position (i.e., immediately to the amino side of the cleavage site) is granzyme B, a serine protease that appears to be

expressed exclusively in the granules of cytotoxic lymphocytes (Pham and Ley, 1997).

The principal advantages of assessing caspase activation using an enzymatic assay are its sensitivity and reliance on commonly available reagents and equipment. Because each caspase molecule can cleave multiple substrate molecules, there is an inherent amplification compared to techniques like affinity labeling (see Basic Protocol 3). Previous studies from the authors' laboratory have demonstrated low but detectable amounts of caspase activity in subcellular fractions whose content of active caspases is below the limit of detection by affinity labeling (Samejima et al., 1998). Moreover, the assay described above can also be performed with chromogenic substrates (e.g., commercially available peptidyl-*p*-nitroanilides), making it possible to assay for active caspases in any laboratory that has a spectrophotometer.

The principal disadvantage of this approach is its lack of specificity for any particular caspase. Currently available fluorogenic and chromogenic substrates were designed to mimic the sites that are cleaved in various substrate polypeptides (Earnshaw et al., 1999). Because most caspases have similar cleavage-site specificities (Table 18.2.1), these assays are not specific for individual caspases. Several examples illustrate this problem.

Analysis of tetrapeptide preferences has demonstrated that caspase-9 prefers acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl

coumarin (acetyl-LEHD-AFC) to other peptides tested (Thornberry et al., 1997). Contrary to the common interpretation of these results, this does not mean that acetyl-LEHD-AFC is a specific substrate for caspase-9. When caspase-3 is incubated with increasing concentrations of acetyl-DEVD-AFC or acetyl-LEHD-AFC, the enzyme displays the same K_M for these two substrates (Figure 18.2.1B). Although caspase-3 does display a lower turnover rate or maximum initial velocity (V_{max}) for acetyl-LEHD-AFC than acetyl-DEVD-AFC, it nonetheless cleaves both substrates quite well. Accordingly, cleavage of acetyl-LEHD-AFC in a cell lysate cannot be taken as evidence that caspase-9 has

been activated. Indeed, if acetyl-LEHD-AFC is cleaved in a cellular extract, it is not immediately obvious how to determine the amount that has been cleaved by caspase-9 as opposed to the more abundant caspase-3.

This problem is not limited to acetyl-LEHD-AFC. Acetyl-DEVD-AFC, a substrate modeled after the cleavage site in poly(ADP-ribose) polymerase (PARP; Lazebnik et al., 1994), can be cleaved by caspases-1, -2, -3, -4, -6, -7, -8, -10, and -14 (Fernandes-Alnemri et al., 1995, 1996; Boldin et al., 1996; Srinivasula et al., 1996; Talanian et al., 1997; Hu et al., 1998; Faubion et al., 1999). Thus, when acetyl-DEVD-AFC cleavage activity is detected in a cell lysate, it

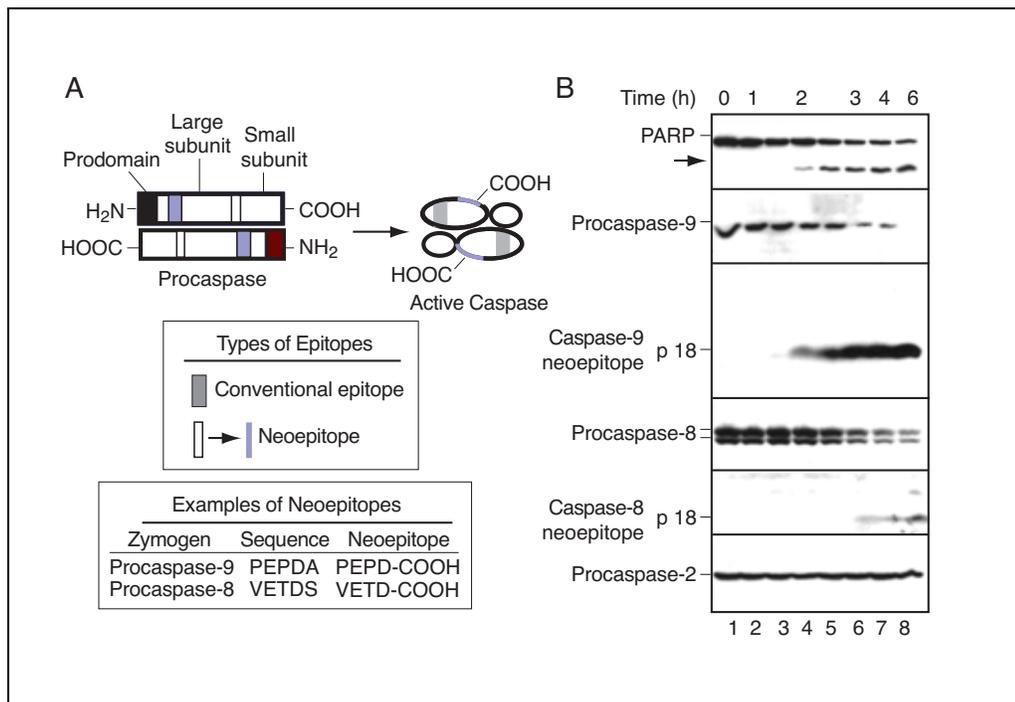


Figure 18.2.2 Assessment of caspase activation by immunoblotting. **(A)** Caspase activation. The prodomain, large subunit, and small subunit are indicated. Two types of epitopes, a conventional linear epitope in the large subunit and a neopeptide at the C terminus of the large subunit, are indicated. The lower box indicates the peptide sequences of procaspase-9 and procaspase-8 at the junction between the large and small subunits. When cleavage occurs on the C-terminal side of aspartate, new C-terminal epitopes (neopeptides) are formed. **(B)** Results obtained when HL-60 human leukemia cells are treated with 68 μ M etoposide, a topoisomerase II-directed agent that causes DNA damage, for 0, 1, 1.5, 2, 2.5, 3, and 4 or 6 hr. Adjacent lanes were loaded with 50 μ g of total cellular protein harvested according to the guanidine hydrochloride cell lysis procedure (see Alternate Protocol 1). Blots were probed with monoclonal anti-poly(ADP-ribose) polymerase (PARP), a sensitive marker of caspase activation (Lazebnik et al., 1994); reagents that detect procaspases-2, -8, and -9; or anti-neopeptide sera that react with active caspase-9 (Mesner et al., 1999) or active caspase-8 species. Two major splice variants of procaspase-8 are detected in these cells. The arrow indicates the product resulting from PARP cleavage by caspases. The anti-neopeptide antisera were raised by immunizing rabbits with the neopeptide tetrapeptides indicated in (A). Note that the increased signal for active caspase-9 is at least as readily detected with the anti-caspase-9 neopeptide serum as it is with the anti-procaspase-9 serum. Also note that the signal for procaspase-2 does not change during the course of apoptosis in this cell line, illustrating the selective cleavage of some procaspases and not others during the course of apoptosis in situ (Martins et al., 1997).

is unclear whether the activity reflects caspase-3 activity or not. A more formal analysis of the K_M and k_{cat} (forward rate constant) of purified caspases acting on a variety of low-molecular-weight substrates (Talanian et al., 1997) revealed that caspases-3 and -7 preferred the *p*-nitroaniline (pNA)-coupled peptide acetyl-DEVD-pNA ($K_M = 11$ and $12 \mu\text{M}$, respectively), caspase-1 preferred acetyl-YEVD-pNA ($K_M = 7.3 \mu\text{M}$), and caspase-6 preferred acetyl-VEID-pNA ($K_M = 30 \mu\text{M}$). Further analysis, however, revealed that these preferences were not absolute. Caspase-3 displayed a k_{cat}/K_M (a measure of catalytic efficiency) of $21.8 \times 10^4 \text{ sec}^{-1}$ for acetyl-DEVD-pNA, $6.1 \times 10^4 \text{ sec}^{-1}$ for acetyl-VEID-pNA, and $3.9 \times 10^4 \text{ sec}^{-1}$ for acetyl-YEVD-pNA, indicating that the cleavage efficiencies for the various substrates differed by only a factor of five. Aside from the inefficient cleavage of acetyl-DEVD-pNA by caspase-6, other caspases showed similar promiscuity. This lack of specificity of currently available substrates must be taken into account in interpreting results of these assays.

Given the ability of multiple caspases to cleave the same substrate, it is logical to ask whether other classes of proteases can also cleave these compounds. It has been reported that the proteasome can cleave acetyl-YVAD-AFC, albeit relatively inefficiently (Kobayashi et al., 1996). The possibility that other proteases might also cleave these substrates cannot absolutely be ruled out. Two approaches can be utilized to help ensure that cleavage of caspase substrates by other proteases is not misinterpreted as caspase activity. First, it is possible to perform the assay in the presence of a battery of protease inhibitors that inhibit many potentially confounding protease activities. The protease inhibitors used in Basic Protocol 1 will inhibit a wide range of serine, cysteine, and acid proteases and metalloproteinases. Second, it is usually important to bolster the conclusion derived from activity assays with some other measure of caspase activation (e.g., immunoblots showing cleavage of caspase zymogens or caspase substrates).

As an alternative to tetrapeptide or pentapeptide substrates, *in vitro* translated proteins have also been used as substrates to assay caspase activity *in vitro* (Nicholson et al., 1995; Liu et al., 1997; Margolin et al., 1997). Work of Andrade et al. (1998) has demonstrated the feasibility of measuring the K_M and k_{cat} using these more relevant macromolecular substrates. Recent data suggest that caspase cleavage preferences derived solely from the study

of tetrapeptide-based substrates might not accurately predict cleavage sites in substrate polypeptides (Samejima et al., 1999). Additional experiments have shown that phosphatase treatment of extracts has no effect on cleavage of acetyl-DEVD-AFC but causes a significant increase in cleavage of PARP (Martins et al., 1998). Collectively, these studies suggest that macromolecular substrates might still have an important role in the study of caspases. On the other hand, disadvantages of this alternative approach include the need to perform both *in vitro* transcription and translation reactions to generate substrates and SDS-PAGE followed by fluorography to analyze results. In short, this labor-intensive approach is not suitable for casual inquiry.

A second disadvantage of using enzymatic assays to assess caspase activation is the potential to underestimate caspase activation in certain cell types. Previous studies have demonstrated that a number of caspase substrates are cleaved in MCF-7 human breast cancer cells. Nonetheless, activity that cleaves acetyl-DEVD-AFC is undetectable in cytosol prepared from this cell line (Janicke et al., 1998). Further analysis has revealed that caspase-3 and other active caspases can form tight complexes with caspase-generated fragments of intermediate filaments (MacFarlane et al., 2000) and are not solubilized under the conditions described. Whether this is a problem unique to the MCF-7 line, or whether substantial amounts of active caspases are also sequestered in an insoluble fraction in other cell types, remains to be established.

Immunoblotting

The ability to detect caspase activation by immunoblotting reflects the fact that these proteases are synthesized as zymogens that undergo proteolytic processing during the course of activation. The zymogens have the general organization shown in Figure 18.2.2A. An N-terminal prodomain is followed by sequences encoding a large subunit and a small subunit. In some procaspases, these two subunits are separated by a small spacer. Maturation of each zymogen appears to proceed by an ordered series of proteolytic cuts (Earnshaw et al., 1999). The first incision, which appears to increase the catalytic activity of the protease, occurs at the C terminus of the large subunit. If a spacer is present between the subunits, it is usually excised next. The final cut, which appears to be catalyzed by the caspase molecule

that is being matured, severs the prodomain from the rest of the molecule.

Three further aspects of caspase maturation require additional comment. First, mature caspases are $\alpha_2\beta_2$ tetramers consisting of two large subunits and two small subunits. Because the large subunit derived from one zymogen molecule associates with the small subunit derived from a second zymogen molecule in the dimeric precursor, caspase maturation actually involves cleavage of two zymogen molecules. Of course, the quaternary structure of the precursor and the mature caspase is lost under denaturing conditions. Second, the maturation of caspases appears to involve conformational changes as well as proteolytic cleavages. These conformational changes can be observed by immunofluorescence using certain conformation-sensitive anti-caspase antibodies (Srinivasan et al., 1998). Again, because SDS-PAGE and immunoblotting are performed after protein denaturation, these conformational changes are not detected by the present protocols. Finally, it is not clear that all caspases require cleavage for activation. In particular, recent results suggest that apoptotic protease activating factor-1 (Apaf-1) is capable of acting as an allosteric activator of procaspase-9 (Rodriguez and Lazebnik, 1999), even under conditions where procaspase-9 cannot be cleaved (Stennicke et al., 1999). Thus, when procaspase-9 is cleaved to its signature fragments (Table 18.2.1), this can be taken as presumptive evidence of procaspase-9 activation; but the lack of procaspase-9 cleavage cannot be taken as proof for a lack of caspase-9 activation.

Two types of reagents are available for detecting caspase cleavage by immunoblotting. First, antibodies raised against epitopes present in either the large or small subunits of the active enzymes are commercially available from a number of sources (Table 18.2.1). These antibodies should theoretically recognize the corresponding zymogen and active caspase with equal affinities. More recently, antibodies generated against neoepitopes (i.e., epitopes that are generated during the proteolytic activation of caspases) have been developed (Fig. 18.2.2). Such an antibody might recognize the C terminus of the large subunit, including the COOH group that is only present after zymogen cleavage at the proper site (i.e., activation). These reagents, which selectively detect active caspases on immunoblots, are becoming increasingly available.

The advantage of antibodies raised against epitopes present in the large or small subunit is

the possibility that decreased zymogen levels can be directly correlated with increased amounts of processed caspases. Interestingly, a stoichiometric relationship between loss of caspase zymogen and appearance of processed caspase is rarely observed. Usually a decreased signal for caspase zymogen is accompanied by a much weaker increase in signal for the active caspase large or small subunit. One possible explanation for this phenomenon is a rapid degradation of caspase species once they are activated. Another possibility is that active caspases might leak from cells as they undergo secondary rupture late in the process of apoptosis. Whatever the explanation, the lack of a 1:1 relationship between loss of zymogen and appearance of active caspase species in cells undergoing apoptosis has practical implications: It makes it difficult to detect the decrease in zymogen levels and the appearance of active caspase species on the same blot with a single exposure of X-ray film. In many studies, a decrease of a procaspase signal is presented as the sole evidence of caspase activation. Whereas a decrease in zymogen level is consistent with caspase activation, it is also consistent with proteolytic degradation to fragments that lack caspase activity. Ideally, X-ray films exposed for different lengths of time are presented, one showing decreased zymogen levels and another showing the appearance of active subunits.

Anti-neoepitope antisera provide an alternative approach for detecting caspase activation (Fig. 18.2.2B). These sera are raised against epitopes that are present at the sites where caspases are cleaved during maturation. Accordingly, if these reagents are of high quality, they recognize only caspases that have been cleaved at the proper site to allow their activation to mature species. Cleavages at other sites will not liberate the epitopes and will not yield a signal. In other words, these sera provide information not only about the size of the caspase fragments that are generated during apoptosis, but also about the precise location of one of the cleavages giving rise to those fragments. The disadvantages of these anti-neoepitope antisera, however, are their current lack of widespread availability and the possibility that they might recognize the same two- to four-amino-acid epitope in other proteins that are cleaved during apoptosis. Nonetheless, these are potentially quite useful reagents.

Affinity labeling

The mapping of caspase cleavage sites in PARP, the lamins, and other substrates (Lazebnik et al., 1994; Takahashi et al., 1996a,b; Nicholson and Thornberry, 1997; Earnshaw et al., 1999) enabled the design of caspase-specific affinity labeling reagents. Each of these reagents consists of a four- or five-amino-acid peptide linked to a detector group (e.g., biotin) and a reactive group that irreversibly binds the caspase active site (e.g., chloromethyl, fluoromethyl, or acyloxymethyl

ketone). In principle, the peptide binds to the substrate binding pocket of the caspase, the reactive group forms a covalent bond with the active site cysteine (Thornberry et al., 1994; Wilson et al., 1994), and the covalently modified subunit of the enzyme is then detected or purified using a reagent that reacts with the detector group (e.g., avidin, streptavidin).

This approach is feasible because caspases can tolerate the insertion of detector groups on substrate-like peptides. As was first shown for caspase-1 (Sleath et al., 1990), these enzymes

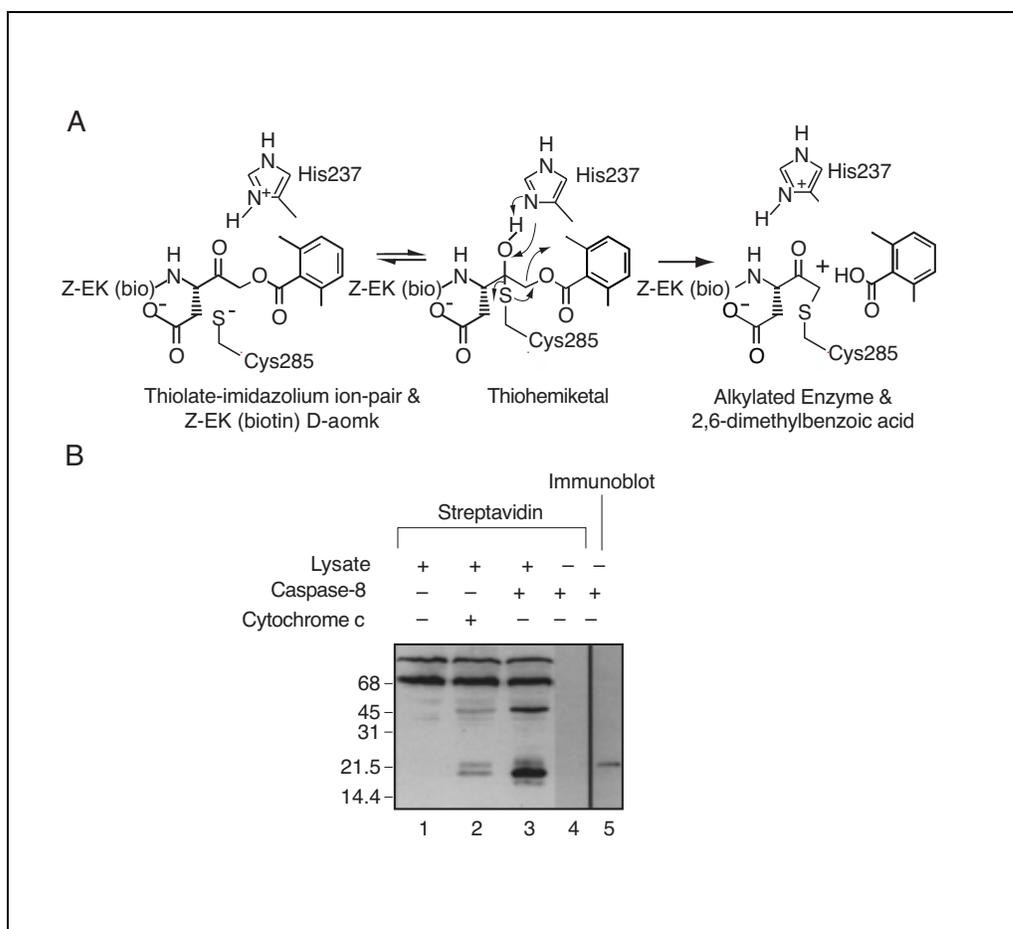


Figure 18.2.3 Labeling of active caspases by acyloxymethylketones. **(A)** The reaction mechanism of zEK(bio)D-aomk (Martins et al., 1997). The active site cysteine initially forms a reversible thiohemiketal. In a slower reaction, the methyl group of the substituted methylketone undergoes S_N2 attack by the same active site thiolate to form the thioether (alkylated enzyme) and liberate the leaving group 2,6-dimethylbenzoic acid. **(B)** Results obtained when *N*-(*N*^α-benzyloxycarbonylglutamyl-*N*^ε-biotinyllysyl)aspartic acid ([2,6-dimethylbenzoyl]oxy)methylketone (zEK[bio]D-aomk) is used to detect caspase activation. Cytosol from 1×10^6 cells (40 μ g) was incubated for 1 hr at 37°C with diluent (lane 1), 100 ng cytochrome *c* (lane 2), or 50 ng active caspase-8 (lane 3). Samples were then covalently modified with zEK(bio)D-aomk as described in Basic Protocol 3. Lanes 4 and 5: 50 ng of active caspase-8 without cell lysate. Samples in lanes 1 to 4 were visualized using peroxidase-coupled streptavidin and enhanced chemiluminescence reagents. Lane 5 was probed with anti-caspase-8 antiserum followed by peroxidase-coupled secondary antibody. Collectively, these results demonstrate that endogenous biotinylated species are visualized by this technique (lane 1), that multiple zEK(bio)D-aomk-modified bands between 15 and 50 kDa are detected after caspase activation (lanes 2 and 3), and that active caspase-8 does not label with this reagent (lanes 4 and 5). Numbers at left, molecular weights of marker proteins in kilodaltons.

exhibit an absolute requirement for aspartate in the P₁ position. In contrast, because the sidechain of the P₂ residue is pointed away from the enzyme (Wilson et al., 1994), a wide range of unmodified or modified amino acids is permitted at this position (Thornberry et al., 1992). The observation that lysine is tolerated in the P₂ position (Thornberry et al., 1992) opened the door to a new family of labeling reagents that contain biotin on the ε-amino group of lysine. The first member of this family, acetyl-Tyr-Val-(ε-biotin)Lys-Asp-([2,6-dimethylbenzoyl]oxy)methylketone (YVK[*bio*]D-aomk), was shown to be a potent inhibitor of caspase-1 that could easily be detected with avidin-based reagents (Thornberry et al., 1994). The reagent utilized in Basic Protocol 3, *N*-(*N*^α-benzyloxy-carbonylglutamyl-*N*^ε-biotinyllysyl)aspartic acid ([2,6-dimethylbenzoyl]oxy)methylketone (zEK[*bio*]D-aomk; Martins et al., 1997), was synthesized based on the sequence of the caspase cleavage site in PARP (EVD↓G; Lazebnik et al., 1994) and lamin A (EID↓N; Takahashi et al., 1996a). This reagent has recently become commercially available.

The reaction leading to caspase derivatization by zEK(*bio*)D-aomk is illustrated in Figure 18.2.3A. Evaluation of the reaction of caspase-1 with a large number of aspartic ketones (Brady et al., 1999) has revealed that the inhibitory effects of these compounds could be classified as reversible, inactivating, or bimodal (i.e., reversible inhibition followed by slow inactivation). The acyloxymethyl ketone inhibitor zEK(*bio*)D-aomk can be categorized as a bimodal inhibitor, with a first-order rate of conversion of the reversible thiohemiketal complex to the inactive thioether. The thiolate-imidazolium ion pair of the active site Cys²⁸⁵ and His²³⁷ reversibly binds to the zEK(*bio*)D-aomk inhibitor to form a thiohemiketal complex. Here the imidazolium functions to polarize the carbonyl, enabling subsequent attack by the thiolate anion. The S-C-C_α-LG dihedral angle thereby adopts a 180° conformation necessary for SN₂ displacement. This conformation places the His²³⁷ in a position that allows it to stabilize the charge that develops on the leaving group during the displacement reaction. In an irreversible SN₂ displacement reaction the thiolate attacks the C_α carbon, thereby displacing the 2,6-dimethylbenzoic acid leaving group and simultaneously forming a thioether bond (and an alkylated enzyme). It is this alkylated enzyme that is detected upon subsequent blotting with streptavidin.

Critical Parameters

Enzyme activity assays

When setting up assays to measure caspase activity, there are a number of critical parameters to consider. Some of these relate to the experimental design, whereas others relate to the reagents utilized.

In terms of experimental design, it is important to remove cells that spontaneously become apoptotic before starting an experiment. For adherent cell lines, spontaneously apoptotic cells can be removed simply by changing the tissue culture medium at the start of the experiment. For nonadherent cells, these spontaneously apoptotic cells can be removed by Ficoll-Hypaque density gradient sedimentation (Martins et al., 1997). If this is not done, the spontaneously apoptotic cells contribute to a high level of caspase activity in the control sample.

At the time of the enzyme assay, it is important to use an appropriate substrate concentration. If the assay is being performed to quantify relative amounts of enzyme present, as is usually the case, it is important to use saturating substrate concentrations (e.g., four to five times the *K*_M of the enzyme). The *K*_M can be estimated from data such as those presented in Figure 18.2.1B. If the substrate concentration is not saturating, changes in the amount of product released might be due to changes in either the number of active enzyme molecules (altered *V*_{max}) or the affinity for the substrate (altered *K*_M). Importantly, the common interpretation that changes in the amount of product released correspond to changes in the number of active enzyme molecules is only valid when the reaction is run under conditions where the substrate is saturating.

When the assay is run as an endpoint assay (see Basic Protocol 1), it is important to confirm that product release is a linear function of the length of incubation. If extracts are prepared from a variety of human cell lines after induction of apoptosis, the assay described above is linear for up to 4 hr under the specified conditions. If the assay is not linear, the most common causes of nonlinearity are substrate exhaustion and enzyme instability. Substrate exhaustion occurs when the enzyme uses most of the substrate, causing the actual substrate concentration to drop below saturating levels. This potential explanation for nonlinearity is suggested by the presence of a high concentration of product (i.e., approaching the nominal concentration of the substrate added). This problem

can be solved by decreasing the amount of enzyme (extract) present or by adding more substrate and determining whether linearity is restored.

If nonlinearity occurs in the absence of substrate exhaustion, the possibility of caspase instability needs to be considered. Based on the authors' experience, it does not appear that the caspases themselves are intrinsically labile at 37°C. However, nonlinearity can also arise if caspases are degraded by other components in the cellular extracts (e.g., proteases that are resistant to the cocktail of protease inhibitors). For certain tissues or cell types, it might be necessary to add additional protease inhibitors in order to preserve caspase stability in the apoptotic extracts.

Another critical parameter for these assays is the quality of the substrate utilized. The authors have found that acetyl-DEVD-AFC from some suppliers (not listed above) yields high levels of fluorescence when diluted in buffer without caspases, apparently as a consequence of contamination by free AFC.

Immunoblotting

When attempting to assess caspase activation by immunoblotting, there are at least two critical parameters. The first involves the method for preparing cell lysates. It has been reported that lysis of lymphoid cells in neutral detergents such as Triton X-100 can lead to artifactual caspase activation by granzyme B present in cytotoxic granules (Zapata et al., 1998). Other serine proteases can also activate certain caspases under cell-free conditions (Zhou and Salvesen, 1997), again raising the potential for artifactual caspase activation during preparation of cell lysates. To avoid this potential pitfall, it is important to solubilize cells under conditions that prevent procaspase cleavage and activation after cell lysis. The protocols described above involve lysis of cells under strongly denaturing conditions, such as in 2% (w/v) SDS containing 4 M urea and reducing agent (Martins et al., 1997; see Basic Protocol 2) or in 6 M guanidine hydrochloride containing reducing agent (Kaufmann et al., 1997; see Alternate Protocol 1). These conditions should avoid spurious caspase activation.

A second critical parameter is the quality of the anti-caspase antibody. Some currently available antibodies exhibit such a weak signal that the instructions accompanying them indicate that they can be used to detect caspase zymogens only in cells that have been transfected with cDNA encoding the procaspase

under the control of a strong promoter. Other commercially available anti-caspase antibodies recognize purified recombinant protein but exhibit extensive cross-reactivity when applied to whole-cell lysates. Clearly, these reagents are not useful for the studies envisioned by most cell biologists. Because of the cost of obtaining commercially available antibodies from multiple sources (to say nothing of the frustration of failed experiments), it is important to assess the sensitivity and cross-reactivity of reagents whenever possible before purchasing them.

Affinity labeling

Several parameters are critical for successful detection of active caspases using the affinity labeling approach. First, it is essential to optimize detection conditions in order to achieve maximum signal and minimum background. High backgrounds can be a serious problem with this assay, particularly when caspase concentrations in the cell lysates are low. The most common cause for high backgrounds in the authors' experience is the choice of blocking agent. Although milk is traditionally discouraged when using avidin-biotin detection systems, the authors have found nonfat dry milk to be far superior to biotin-free blocking agents. However, they have also found that nonfat dry milk from different sources results in different levels of background. Accordingly, nonfat dry milk from several different sources might have to be compared to arrive at an optimal signal-to-noise ratio.

A second critical parameter is the chemistry of the reactive group on the affinity labeling reagent. The authors have found that affinity labeling reagents from some commercial sources give unacceptably high background labeling of polypeptides in nonapoptotic cells. Part of this problem might relate to the choice of reactive group. Chloromethyl ketones are highly reactive but relatively nonspecific reagents that covalently modify a wide range of sulfhydryl-containing enzymes (Shaw, 1990). Fluoromethyl ketones are also widely reactive with sulfhydryl-containing enzymes, as illustrated by the demonstration that zVAD-fmk and acetyl-DEVD-fmk can irreversibly inhibit cathepsins B and H (Faubion et al., 1999; Schotte et al., 1999) and by the observation that biotinylated zVAD-fmk can affinity label cathepsin B (Schotte et al., 1999). Accordingly, the authors recommend using peptidyl acylloxymethyl ketones in preference to the more readily available chloromethyl or fluoromethyl ketones.

A third critical factor is the quality of the affinity labeling reagent. The authors have found that the quality of commercially available acyloxymethyl ketones varies widely. These compounds are extremely difficult to synthesize cleanly. When the authors analyzed some commercially available reagents by high performance liquid chromatography, mass spectroscopy, and nuclear magnetic resonance, they noticed several problems, most notably cyclization and racemization of the aspartate. These alterations might reflect the reaction conditions utilized to synthesize these molecules, the quality of the purification step after the multistep synthesis, or the subsequent conditions used to store or ship these somewhat labile molecules. In any case, these impurities can contribute to lack of ability of the reagents to react with caspases and/or spurious reactions with other proteins.

Unfortunately, cost and quality of available reagents remains a consideration in deciding whether to apply this approach. Hopefully the presence of a potentially lucrative market will stimulate suppliers to improve the quality and availability of these reagents.

Anticipated Results

Enzyme activity assays

Despite all the caveats and limitations listed above, activity assays can be extremely useful when applied appropriately. They have been utilized as one means of successfully confirming that caspases are activated under a wide range of conditions. In addition, they have proven particularly useful in assessing the timing of caspase activation relative to other apoptotic events, in comparing the ability of cells to undergo apoptosis after different treatments, and in assessing the mechanism by which various antiapoptotic agents exert their effects. Compared to the immunoblotting and affinity labeling procedures, activity assays are easier and more quantitative. They must, however, be interpreted cautiously because they do not yield a definitive identification of the caspases that are activated.

Immunoblotting

Shown in Figure 18.2.2B are results obtained when HL-60 human leukemia cells were treated with the topoisomerase II poison etoposide, lysed in guanidine hydrochloride lysis buffer, and subjected to immunoblotting using several immunological reagents. Caspase activation, as reflected by the cleavage of the

caspase substrate PARP (Lazebnik et al., 1994), is first detectable at 2 hr. Loss of caspase-9 zymogen, reflecting presumed proteolytic activation, and appearance of a species corresponding to the large subunit of active caspase-9 is also evident at 2 hr. Thus, immunoblotting with antibodies against either caspases or their substrates can be utilized to assess the timing of caspase activation. In addition, examination of other immunoblots in Figure 18.2.2B shows that some caspase zymogens are degraded to active species after caspase-9 activation (e.g., caspase-8) or are not activated at all (e.g., procaspase-2). Thus, by employing a series of reagents that recognize zymogens and active caspases, it is possible to confirm that specific caspases have been activated and to assess the relative timing of their activation in a population of cells undergoing somewhat synchronous apoptosis.

Affinity labeling

Results obtained in a typical experiment are shown in Figure 18.2.3B. In this example, cell lysate prepared from nonapoptotic cells has been treated with cytochrome *c* or active caspase-8 to activate endogenous caspases. After treatment with zEK(bio)D-aomk, the samples have been subjected to SDS-PAGE followed by detection with peroxidase-coupled streptavidin. As illustrated in lane 1, this technique detects several endogenous biotinylated polypeptides. Most of these have molecular weights above $M_r \sim 50,000$ and do not interfere with the detection of active caspase species. As shown in lanes 2 and 3, the most prominent polypeptides that label after caspase activation are species of $M_r \sim 15,000$ to 22,000. Two-dimensional gel electrophoresis has demonstrated that multiple discrete charged species are hidden in each band (Martins et al., 1997). Two different approaches have revealed that the most abundant species detected in apoptotic cell lysates are active forms of caspase-3 and caspase-6 (Faleiro et al., 1997; Martins et al., 1997). Further analysis has suggested that caspase-3 and caspase-6 each give rise to at least two size variants, a mature variant lacking the prodomain and a partially processed intermediate containing the prodomain. The charge variants appear to reflect, at least in part, differences in caspase phosphorylation (Martins et al., 1998).

As also illustrated in Figure 18.2.3B, zEK(bio)D-aomk does not react with active caspase-8. In contrast, biotinyl-DEVD-fmk has been reported to derivatize this enzyme (Muzio

et al., 1997). These observations illustrate the potential importance of testing several different affinity labeling reagents before concluding that active caspases cannot be detected in a particular biological system by affinity labeling.

Time Considerations

Enzyme activity assays

The entire enzyme activity assay procedure can be completed in 1 day. After the induction of apoptosis, the time required for preparing cell extracts is ~3 hr. These extracts can be stored for several months at -70°C without appreciable loss of caspase activity. Protein assays take 1 to 2 hr to complete. If the solutions are premixed, the assay itself can be set up in 30 min to 1 hr and then read 2 to 4 hr later.

Immunoblotting

It takes several days from start to finish to treat cells, prepare cell lysates, perform SDS-polyacrylamide gel electrophoresis, transfer polypeptides to nitrocellulose, and probe immunoblots. It should be obvious, however, that there are many stopping points in the procedure. The process can be speeded up by using minigels and a minitransfer apparatus, but the resolution of the smaller blots might be somewhat limited.

Affinity labeling

The entire affinity labeling procedure outlined above can be completed in 2 days. After the induction of apoptosis, the time required for preparing cell extracts is ~3 hr. These extracts can be stored for several months at -80°C without appreciable loss of reactivity. Protein assays take 1 to 2 hr to complete. The samples can then be subjected to electrophoresis (1 hr), transferred to nitrocellulose (30 min), blocked (overnight at 4°C), and incubated with streptavidin and visualized (4 to 5 hr).

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This paper illustrates one of the first uses of immunoblotting to demonstrate caspase activation during apoptosis.

Takahashi et al., 1996b. See above.

This paper describes the first use of affinity labeling to detect active caspases in extracts from apoptotic cells.

Internet Resource

www.peptide.co.jp

The Web site of the Osaka Peptide Institute contains a variety of caspase substrates as well as acyloxymethyl ketones that can be used as affinity labels.

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Assessment of Apoptosis and Necrosis by DNA Fragmentation and Morphological Criteria

Cell death may occur by two mechanisms: apoptosis, or programmed cell death, and necrosis, or cell death due to injury or trauma (see UNIT 18.1). Both types of cell death have their own specific and distinct morphological and biochemical hallmarks. Apoptotic cells share a number of common features, such as phosphatidylserine (PS) exposure, cell shrinkage, chromatin cleavage, nuclear condensation, and formation of pyknotic bodies of condensed chromatin. Necrotic cells exhibit nuclear swelling, chromatin flocculation, loss of nuclear basophilia, breakdown of cytoplasmic structure and organelle function, and cytolysis by swelling. Cell death can be induced by a wide variety of stimuli, such as growth factor withdrawal, heat shock, cold shock, radiation, heavy metals, genotoxic drugs, and a number of biological ligands such as Fas-L and tumor necrosis factor (TNF). Most if not all of these can induce both apoptosis and necrosis in a time- and dose-dependent manner.

A variety of techniques have been developed to assess cytotoxicity in untreated and/or treated cells, most of which are based on the loss of plasma membrane integrity. Membrane disruption can be detected based on the uptake of vital dyes (see Basic Protocol 1). However, during apoptosis, the cell membrane remains intact for a relatively long time, and therefore some of these assays cannot detect the early stages of apoptosis. Alternatively, assays that detect all types of cell death do not discriminate between apoptosis and necrosis. Therefore, more specific techniques have been developed to determine cell death, and the combination of several methods is required to distinguish between the two separate types. These techniques rely on specific morphological and molecular or biochemical changes associated with these two processes.

This unit describes some of the techniques most commonly used to detect cell death. Morphological assays include trypan blue exclusion (see Basic Protocol 1), differential staining (see Basic Protocol 2), and Hoechst staining (see Basic Protocol 3). Methods to detect chromatin cleavage include TUNEL assays for whole cells (see Basic Protocol 4) and paraffin sections (see Alternate Protocol 1), DNA fragmentation assays using whole cells (see Basic Protocol 5), assays of total genomic DNA (see Alternate Protocol 2), analysis of DNA fragmentation by agarose gel electrophoresis (see Alternate Protocol 3), phenol extraction of DNA for analysis of fragmentation (see Alternate Protocol 4), a quantitative assay for DNA fragmentation (see Basic Protocol 6), and detection of DNA fragmentation by pulsed-field gel electrophoresis (see Basic Protocol 7). A protocol is also provided for Cytospin preparations from cell suspensions (see Support Protocol 1). Table 18.3.1 describes how these assays are used to distinguish apoptotic cells from necrotic cells.

MORPHOLOGY ASSAYS

Loss of membrane integrity, which occurs late in apoptosis and relatively early in necrosis, can be detected by cellular uptake of the vital dye trypan blue (see Basic Protocol 1). Apoptosis and necrosis also can be identified morphologically based on the criteria described elsewhere (Bowen, 1980). These include cell shrinkage, nuclear condensation, and cleavage for apoptotic cells; and nuclear swelling, chromatin flocculation, and loss of nuclear basophilia for necrotic cells. The levels of both apoptosis and necrosis in a particular cell population can be estimated from Cytospin preparations (see Support Protocol 1) stained with RAPI-DIFF (see Basic Protocol 2; see Figure 18.3.1), Hoechst stain (see Basic Protocol 3), or propidium iodide.

Table 18.3.1 Methods for Determination of Apoptosis and Necrosis

Method	Protocol	Result for	
		Apoptosis	Necrosis
<i>Morphological/cytological analysis</i>			
Trypan blue staining	Basic Protocol 1	No staining (early apoptosis) Blue staining (late apoptosis)	Blue cells
Differential staining	Basic Protocol 2	Membrane blebbing, chromosome condensation and nuclear shrinkage, cytoplasmic constriction and loss of cell volume	Nuclear swelling, chromatin flocculation, membrane blebbing and disruption, appearance of cell “ghosts”
Hoechst staining	Basic Protocol 3	Increased blue fluorescence, fragmented or condensed nuclei	Blue nucleus with diffuse staining
Flow cytometry	—	Reduced forward and side scatter	Increased forward and side scatter
Annexin V binding and PI staining	—	Positive fluorescence response for annexin V binding; exclusion of PI	Positive fluorescence; uptake of PI
<i>Assays for chromatin cleavage</i>			
TUNEL assay	Basic Protocol 4 and Alternate Protocol 1	Detection of strand breaks	Detection of strand breaks
Whole-cell DNA fragmentation	Basic Protocol 5	DNA ladder	DNA smear
Total genomic DNA fragmentation	Alternate Protocol 2	DNA ladder	DNA smear
Simple protocol for DNA fragmentation	Alternate Protocol 3	DNA ladder	DNA smear
Phenol extraction for DNA fragmentation	Alternate Protocol 4	DNA ladder	DNA smear
Quantitative assay of DNA fragmentation	Basic Protocol 6	Increase in the percentage of fragmented DNA	Increase in the percentage of fragmented DNA
Pulsed-field detection of high-molecular-weight DNA	Basic Protocol 7	High-molecular-weight bands	DNA smear
<i>Assays for caspase activation</i>			
Specific substrate cleavage assay	UNIT 18.2	Positive detection of cleaved fluorescent substrate	No changes in fluorescence detected
Immunodetection of caspase activation	UNIT 18.2	Positive detection of cleavage products of procaspase	No cleavage products detected

**BASIC
PROTOCOL 1****Measurement of Cell Death by Trypan Blue Exclusion**

This common cell viability assay is based on the ability of a cell with an intact membrane to exclude the dye trypan blue. Therefore, this assay allows one to distinguish between cells with intact and disrupted membranes. Since this method does not give an indication of the mode of cell death, it should be used only in conjunction with a more informative morphological method.

Materials

Cell suspension to be assessed
 2× PBS tablets, pH 7.2 to 7.4 (Sigma) or 2× PBS (APPENDIX 2A)
 0.2% (w/v) trypan blue (Sigma) in 2× PBS (store up to 1 to 2 months at 4°C)

**Determination of
Apoptosis and
Necrosis****18.3.2**

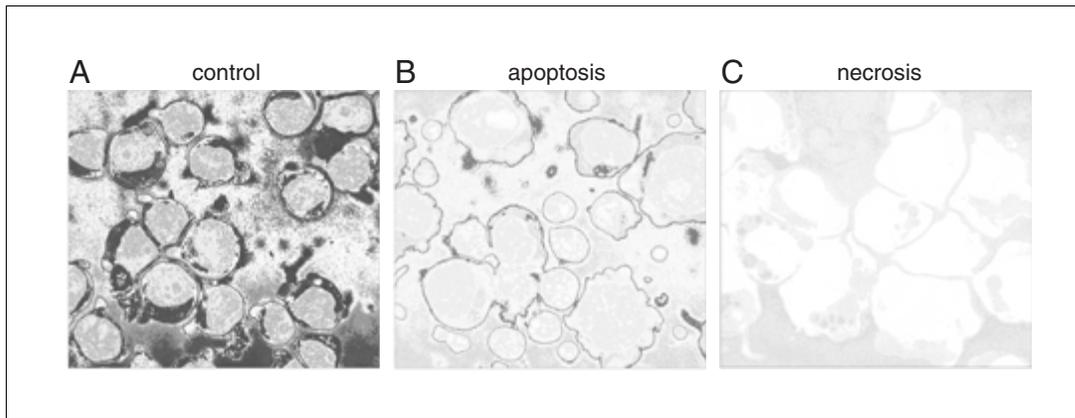


Figure 18.3.1 RAPI-DIFF staining of Cytospin preparation of HL-60 cells. Cells were treated with different concentrations of a cytotoxic drug to induce apoptosis or necrosis. **(A)** Untreated cells, **(B)** apoptotic cells, and **(C)** necrotic cells.

Hemocytometer: improved Neubauer type (Karl Hecht; Baxter) or equivalent
 Coverslips (e.g., Chance Propper)
 Light microscope

1. Remove from the cell suspension a sample containing $\sim 5 \times 10^4$ cells.
2. Add an equal volume of 0.2% trypan blue, mix, and incubate 1 to 2 min at room temperature to permit dye uptake.
3. Load samples onto the hemacytometer.
4. Count the total number of cells and the number of unstained cells in five of the major sections of the hemacytometer (see Fig. 1.1.1). Calculate the average number of cells per section.
5. Calculate the number of cells/ml culture medium by multiplying the average number of cells per section by the dilution factor (2 in this case) and hemacytometer index (10^4). Determine the percentage viability according to the following formula:

$$\% \text{ viability} = \frac{\text{number of unstained cells}}{\text{total number of cells}} \times 100$$

Control cells and cells in early stages of apoptosis exclude trypan blue; cells in the late stages of apoptosis and necrotic cells take up the dye and appear as blue cells.

Differential Staining of Cells

This staining procedure involves three steps: first cells are fixed in 100% methanol, then the nuclei are stained with an acid dye, and finally the cytoplasm is stained using a basic dye. Such three-step procedure allows differential staining and contrast between the cytoplasm and the nucleus. Apoptotic cells exhibit membrane blebbing, chromatin condensation and nuclear shrinkage, cytoplasmic constriction and loss of cell volume, and formation of apoptotic bodies. Necrotic cells undergo nuclear swelling, chromatin flocculation, cell membrane blebbing and disruption, and finally cell lysis resulting in the appearance of “ghost cells” (see Figure 18.3.1). This method can also be applied to cells growing attached to coverslips or chambered culture slides.

**BASIC
 PROTOCOL 2**

**Cellular Aging
 and Death**

18.3.3

Materials

Cytospin preparations of cells (see Support Protocol 1)
100% methanol
Acid dye: 0.1% (w/v) eosin Y/0.1% (w/v) formaldehyde/0.4% (w/v) sodium phosphate dibasic/0.5% potassium phosphate monobasic
Basic dye: 0.4% (w/v) methylene blue–polychromed/0.4% (w/v) azure/0.4% (w/v) sodium phosphate dibasic/0.5% (w/v) monobasic potassium phosphate
DPX mountant (in solution; BDH)
Coverslips (e.g., Chance Propper)
Microscope slides (e.g., Menzel-Glaser)
Light microscope

1. Fix cells after Cytospin preparation (see Support Protocol 1) by immersing the slide ten times in 100% methanol.

The three solutions required for this procedure are available as a kit, called RAPI-DIFF Stain kit, from Diagnostic Developments. The solutions can be stored 1 to 2 months at room temperature.

2. Stain the cell nuclei by dipping the slide in the acid dye ten times.
3. Stain the cell cytoplasm by dipping the slide in basic dye ten times.
4. Rinse the slide by dipping in distilled water, let it air dry, and mount it using DPX mountant.
5. Score the cells as normal, apoptotic, condensed and/or fragmented nuclei, or necrotic swollen cells, based on their morphological appearance under a light microscope (see *UNIT 18.1* and Fig. 18.3.1).

Hoechst Staining of Cells

Hoechst 33342, a bisbenzimidazole dye, is a cell-permeant, minor group-binding DNA stain that fluoresces bright blue upon binding to DNA. It is water soluble and relatively nontoxic. Hoechst 33342 can be excited with the UV lines of the argon-ion laser and most conventional fluorescence excitation sources, and it exhibits relatively large Stokes shifts (excitation and emission maxima ~350 and 460 nm), making it suitable for multicolor labeling experiments (Pollak and Ciancio, 1990). Cells are scored as apoptotic if they have fragmented nuclei. This method can also be applied to cells growing attached to coverslips or chambered culture slides.

Materials

Cell suspension
1× and 2× PBS, pH 7.2 to 7.4 (from Sigma 2× PBS tablets, or see *APPENDIX 2A*)
4% (w/v) paraformaldehyde (see recipe)
10 µg/ml Hoechst 33342 dye (Molecular Probes) in PBS (*APPENDIX 2A*)
50/50 (v/v) PBS/glycerol
Microscope slides (e.g., Menzel-Glaser)
Coverslips (e.g., Chance Propper)
Fluorescent microscope
Additional reagents and equipment for Cytospin preparations (see Support Protocol 1)

1. Place an aliquot of cell suspension containing $0.3\text{--}0.5 \times 10^6$ cells in a microcentrifuge tube, and centrifuge 5 min at $1000 \times g$, 4°C.

2. Remove and discard supernatant. Resuspend the dry pellet in 30 to 50 μl of 4% paraformaldehyde.
3. Spin the fixed cells onto a glass slide using a Shandon/Lipshaw Cytospin centrifuge (see Support Protocol 1). Alternatively, spread the cells onto a slide with a pipet tip, and air dry slide.
4. Stain slide with 10 $\mu\text{g}/\text{ml}$ Hoechst dye.

Hoechst 33342 dye can be diluted from 100 \times stock kept in the dark at 4°C.

5. Leave slide in the dark for 10 min at room temperature.
6. Rinse by dipping slide five times in distilled water, and let it air dry in the dark.
7. Mount slide with coverslip using PBS/glycerol. Monitor fluorescence with a blue filter in the fluorescence microscope, and score cells with fragmented nuclei as apoptotic.

This method is useful for discrimination of apoptotic from nonapoptotic (untreated or necrotic) cells.

Cytospin Preparation of Cells for Analysis

Cytospin preparations can be used in many protocols for cell biology. Once slides are prepared they can be stained with variety of dyes and analyzed using light, fluorescent, and confocal microscopy, depending on the dye type. Before staining, slides can be stored up to 1 year at -20°C covered with aluminum foil. Fixed and stained slides can be stored indefinitely once mounted permanently.

Materials

Cell suspension
 PBS (APPENDIX 2A)
 Cytospin centrifuge and cups (Shandon/Lipshaw)

1. Place a sample of cell suspension containing $\sim 0.5\text{--}1 \times 10^5$ cells in a microcentrifuge tube, and centrifuge 5 min at $1000 \times g$, 4°C .
2. Remove and discard supernatant. Resuspend pelleted cells in 100 μl PBS.
3. Add suspension to the Cytospin cup and slide setup, and centrifuge 2 min at $500 \times g$, 4°C .
4. Air dry the slide.

ASSAYS FOR CHROMATIN CLEAVAGE

Chromatin cleavage is a hallmark of apoptosis and involves the formation of high-molecular-weight ($>50\text{-kbp}$) and nucleosome-sized (200-bp) DNA fragments. The high-molecular-weight fragments can be separated by pulsed-field gel electrophoresis, and the nucleosome-sized fragments, when separated on a conventional agarose gel, demonstrate a ladder pattern. The DNA from necrotic cells, on the other hand, has a random and general cleavage pattern and produces a smear when electrophoresed on either a pulsed-field or conventional gel (see Fig. 18.3.2). Prior to the formation of the cleavage products, endogenous nucleases must generate a large number of DNA strand breaks. These strand breaks in DNA can be detected by attaching biotin-conjugated nucleotides to the 3' hydroxyl termini in a reaction catalyzed by exogenous terminal deoxynucleotidyl transferase (TdT). A number of methods can be used to identify apoptotic or necrotic DNA. All of these methods are equally successful and require the same number of cells.

SUPPORT PROTOCOL 1

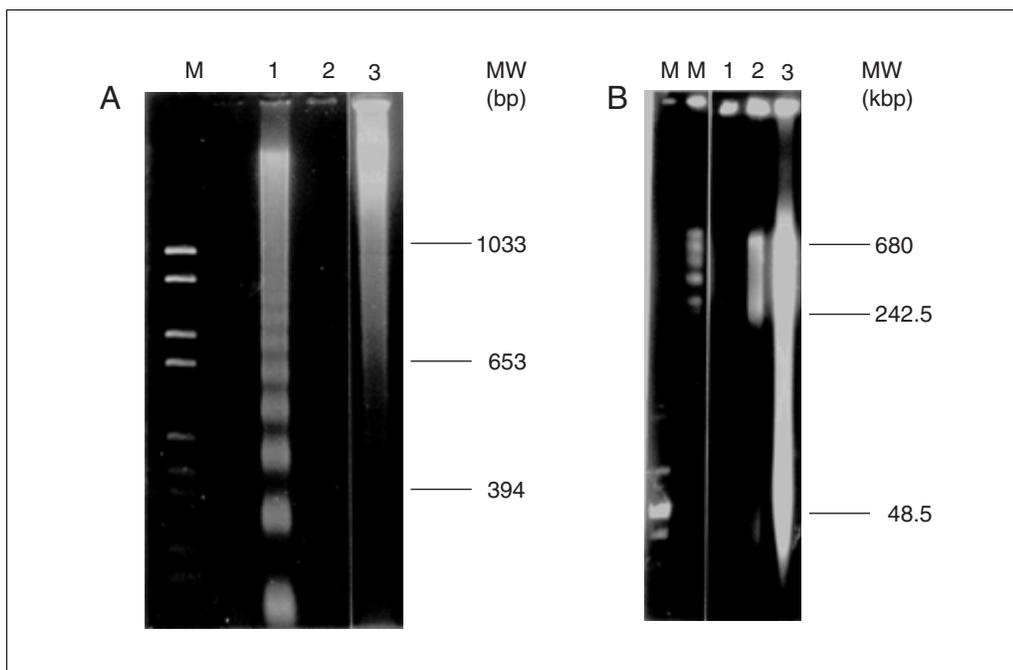


Figure 18.3.2 Gel electrophoresis of DNA from Jurkat cells treated with different concentrations of a cytotoxic drug. **(A)** Conventional gel: M, marker; lanes 1 to 3, DNA from apoptotic, untreated, and necrotic cells, respectively. The DNA ladder, when present, is a strong indicator of apoptosis. **(B)** Pulsed-field gel: MM, markers; lanes 1 to 3, DNA from untreated, apoptotic, and necrotic cells, respectively.

BASIC PROTOCOL 4

TUNEL Assay for DNA Fragmentation in Cells

Induction of apoptosis results in the generation of single-strand DNA breaks. These can be detected using the TdT-mediated dUTP-biotin nick end-labeling (TUNEL) method (Gavrieli et al., 1992). This method requires cell fixation with cross-linking agents such as formaldehyde, which, unlike some of the alcohols (such as ethanol), prevents the extraction of degraded DNA. In other words, this fixation step prevents the loss or reduction of the cellular DNA content after the extensive washings and staining involved in this protocol. Once cells are fixed with formaldehyde, treatment with alcohols does not affect the DNA content of cells.

Materials

- Cell suspension
- 100% methanol, -20°C
- PBS, pH 7.2 to 7.4 (APPENDIX 2A)
- 1% (v/v) formaldehyde
- 70% ethanol, ice cold
- Terminal deoxyribonucleotidyltransferase (TdT; 25 U/ml) and 10 \times buffer (0.3 M Tris base/1.4 M sodium cacodylate, pH 7.2/1 mM DTT; e.g., Boehringer Mannheim)
- 25 mM CoCl_2
- 1 mM Bio-16-dUTP (e.g., Boehringer Mannheim)
- Termination buffer: 300 mM NaCl/30 mM sodium citrate (e.g., Sigma or equivalent)
- Staining buffer (e.g., Sigma; see recipe)
- FACS fluid (e.g., Becton Dickinson; optional; for flow cytometry)
- DPX mountant (BDH)
- 5 $\mu\text{g}/\text{ml}$ propidium iodide in PBS (APPENDIX 2A)
- Flow cytometer (e.g., Becton Dickinson) and tubes; *or* fluorescent microscope
- Coverslips (e.g., Chance Propper)
- Microscope slide (e.g., Objektträger, Menzel-Glaser)

1. Wash a sample of cell suspension containing $\sim 10^6$ cells by centrifuging 5 min at $1000 \times g$, 4°C , discarding supernatant, and resuspending in PBS. Transfer to microcentrifuge tube and centrifuge 5 min at $1000 \times g$, 4°C .
2. Remove and discard supernatant. Resuspend cells in 1% formaldehyde and fix for 15 min on ice.
3. Centrifuge 5 min at $1000 \times g$, 4°C . Remove and discard supernatant. Resuspend cell pellet in PBS, and repeat centrifugation. Remove and discard supernatant.
4. Resuspend cell pellet in 0.5 ml PBS and add to 5 ml ice-cold 70% ethanol.

Cells can be stored in ethanol for several weeks at -20°C .

5. Centrifuge 5 min at $1000 \times g$, 4°C . Remove and discard ethanol. Resuspend cells in PBS, and repeat centrifugation.
6. Prepare elongation buffer (50 μl per sample):
 - 41.5 μl ultrapure water
 - 5 μl $10\times$ TdT buffer
 - 2 μl 25 mM CoCl_2
 - 1 μl 1 mM Bio-16-dUTP
 - 0.5 μl 25 U/ μl TdT enzyme.

Add 50 μl elongation buffer to each cell pellet and resuspend. Incubate 30 min at 37°C .

It is recommended that more than the required amount of elongation buffer be prepared; that is, multiply the total number of samples + 1 by 50 μl (the volume of the elongation buffer per sample) when calculating how much buffer to prepare. This buffer needs to be made fresh.

7. Add 5 ml PBS and centrifuge 5 min at $1000 \times g$, 4°C . Remove and discard supernatant.

The reaction may first be stopped by incubating cells with termination buffer for 15 min at room temperature, though omission of this step does not appear to affect the results.
8. Resuspend cell pellet in 100 μl staining buffer, and incubate 30 min at room temperature.

Again, it is recommended that extra buffer be prepared.
9. Add 2 ml PBS, resuspend cells, and centrifuge 5 min at $1000 \times g$, 4°C . Remove and discard supernatant. Repeat.
10. Add 1 ml of 5 $\mu\text{g}/\text{ml}$ propidium iodide and incubate 30 min at room temperature in the dark.
11. Centrifuge 5 min at $1000 \times g$, 4°C , and remove supernatant.

12a. *For flow cytometry:* Resuspend cells in sufficient amount of PBS or FACS fluid and measure fluorescence (for FITC, 488 nm excitation, 520 ± 20 nm emission, FL-1 channel; for PI, 560 nm excitation, 640 nm emission, FL-2 or FL-3 channels).

12b. *For fluorescence microscopy:* Resuspend cells in 50 to 100 μl of PBS and spin them onto a slide as described under Cytospin preparations (see Support Protocol 1). Mount slides with DPX mountant and observe fluorescence with green and red filters under microscope.

Cells for fluorescence microscopy can be counterstained with hematoxylin for ≤ 30 sec, and then rinsed with water, before microscopy.

The number of single-strand breaks is increased during the early stages of apoptosis (increase in green fluorescence) without any change in the DNA content (measured by PI staining). With time the DNA content is decreased (red fluorescence) due to formation of apoptotic bodies, which also results in the reduction of TUNEL signal.

TUNEL Assay in Paraffin-Embedded Sections

The TUNEL assay can also be performed on paraffin-embedded tissue sections.

Additional Materials (also see Basic Protocol 4)

Paraffin-embedded tissue sections on slides
4% (w/v) paraformaldehyde (see recipe) *or* 4% formaldehyde, in PBS (*APPENDIX 2A*)
96%, 90%, and 80% ethanol
Xylene
BSA (e.g., Sigma)
10 mM Tris·Cl, pH 8 (*APPENDIX 2A*)
20 µg/ml proteinase K in 10 mM Tris·Cl (*APPENDIX 2A* for Tris·Cl)
3% (v/v) methanol
2% (w/v) BSA in PBS (*APPENDIX 2A* for PBS)
ExtrAvidin-peroxidase (Sigma) diluted 1:50 in PBS/1% BSA/0.5% Tween 20
3-Amino-9-ethylcarbazole (AEC)

Fluorescent microscope
Coverslips (e.g., Chance Propper)
Microscope slide (e.g., Objektträger, Menzel-Glaser)

1. Fix section in 4% paraformaldehyde or formaldehyde, then immerse in PBS.
2. Remove paraffin from sections by incubating slide 10 min at 70°C or 30 min at 58° to 60°C.
3. Rehydrate sections by sequential incubation in xylene (twice, 5 min each), 96% ethanol (twice, 3 min each), 90% ethanol (3 min), and 80% ethanol (3 min), followed by 3 min in water.

Because paraffin traces might interfere with the enzymatic reaction in tissue sections, use of fresh solvents is recommended.

4. Treat sections with 10 mM Tris·Cl, pH 8, for 5 min, then incubate 15 min in 20 µg/ml proteinase K at room temperature. Wash slide four times in ultrapure water, 2 min each time.
5. Incubate sections in 3% methanol for 30 min at room temperature, and rinse by dipping five times in ultrapure water.
6. Prepare 100 µl per slide of elongation buffer (see Basic Protocol 4, step 6; the buffer needs to be fresh). Incubate the sections 60 min at 37°C in humid atmosphere.
7. Rinse the sections by dipping three times in ultrapure water, and incubate in termination buffer for 15 min at room temperature.
8. Place sections sequentially in PBS (5 min), 2% BSA (10 min), and PBS (5 min), all at room temperature.

Addition of BSA prevents nonspecific binding of ExtrAvidin-peroxidase.

9. Incubate the sections in ExtrAvidin-peroxidase for 15 min at room temperature.
10. Incubate the sections in 2% BSA, and wash four times in PBS.
11. Stain the sections with AEC for 30 min at 37°C, then rinse by dipping three times in ultrapure water.
12. Mount slides and observe for fluorescence.

After mounting the covered slides can stored for many months at 4°C.

Tissue sections, like cells, can be counterstained with hematoxylin for ≤30 sec, followed by rinsing in water.

Detection of DNA Fragmentation in Whole Cells

In 1976, Skalka et al. reported that the DNA in chromatin of irradiated lymphoid tissues degrades *in vivo* into oligonucleosome-length fragments. This observation was first linked to endonuclease activation in 1980 (Wyllie, 1980). Since then several methods have been developed to measure internucleosomal DNA fragmentation. This method was adapted from the protocol first described by Sorenson et al. (1990) and does not require DNA purification. Appearance of a DNA ladder will correspond to apoptosis, whereas a DNA smear will indicate necrosis. This also applies to all the alternate protocols in this section.

Materials

1× and 5× TBE buffer (see recipe), pH ~8.0 at room temperature (do not adjust pH)
SeaKem GTG agarose (FMC Bioproducts)

Cell suspension

50 mg/ml RNase A (see recipe)

4× DNA loading buffer: 4× TBE buffer (see recipe) containing 40% (w/v) sucrose and 0.25% (w/v) bromphenol blue (e.g., Sigma; store up to 2 to 3 weeks at 4°C)

Ultrapure agarose (Life Technologies)

10% (w/v) SDS (see recipe)

20 mg/ml proteinase K in water (store in aliquots up to 1 year at -20°C)

1× DNA loading buffer: 1× TBE buffer (see recipe) containing 10% (w/v) sucrose and 0.25% (w/v) bromphenol blue (e.g., Sigma)

DNA marker VI (pBR328 DNA cleaved with *Bgl*I and *Hin*FI; Boehringer Mannheim), 1 µl in 20 µl of 1× DNA loading buffer

TE buffer, pH 8.0: 10 mM Tris·Cl/1 mM EDTA

10 mg/ml ethidium bromide (see recipe)

Boiling water bath or microwave oven

Gel electrophoresis apparatus: GNA-100 (Amersham Pharmacia Biotech), Buffer Puffer (Owl Scientific), or equivalent

Power supply (Power-Pac 300, Bio-Rad, or equivalent)

Shaker at 4°C

MacroVue UV Transilluminator (Hoefer Scientific Instruments) or equivalent

Photoman Polaroid gel documentation system (Hoefer Scientific Instruments) or equivalent

Prepare agarose gel for electrophoresis

1. Prepare sufficient volume of 1× TBE to cast the gel and to fill the electrophoresis tank.

It is important to use the same batch of electrophoresis buffer in both the electrophoresis tank and in the gel. Differences in pH or ionic strength can greatly affect the DNA mobility.

2. Weigh 0.9 g SeaKem agarose in a flask, and add 50 ml of 1× TBE (for GNA-100 gel electrophoresis apparatus). Heat in a boiling water bath or microwave oven until dissolved, then allow the agarose to cool to 60°C for 10 to 15 min.

This is a standard 1.8% agarose gel. The volume should not occupy >40% of the volume of the flask.

For the Buffer Puffer gel electrophoresis apparatus, use 2.7 g agarose and 150 ml TBE.

3. Pour gel into mold. Remove unwanted air bubbles. Place comb in the gel and allow gel to set 1 hr.

Prepare sample

4. Place a sample of cell suspension containing $4\text{--}5 \times 10^5$ cells in a microcentrifuge tube, and centrifuge 3 min at $1000 \times g$, 4°C .

If necessary, the pellet may be stored up to 2 to 3 weeks in microcentrifuge tubes at -20°C before proceeding.

5. Carefully remove supernatant and resuspend pellet in $16 \mu\text{l}$ ultrapure water.
6. Add $4 \mu\text{l}$ of 50 mg/ml RNase A (final concentration 10 mg/ml), mix, and leave 20 min at room temperature.
7. Add $5 \mu\text{l}$ of $4\times$ DNA loading buffer.
8. Using a scalpel, cut out a piece of gel between the comb and the upper edge of the gel (do not remove the comb), leaving the gel above the first one or two wells intact.

Prepare digestion gel

9. Prepare 5 ml of digestion gel by weighing 40 mg Ultrapure agarose, adding 2.75 ml ultrapure water, and stirring on a hot plate to dissolve.

Do not use the SeaKem agarose in this step because it causes SDS to come out of solution.

For the Buffer Puffer gel electrophoresis apparatus, prepare 10 ml of digestion gel.

10. When steam starts to rise from the flask, add 1 ml of $5\times$ TBE and 1 ml of 10% SDS. When agarose has boiled and dissolved, take flask off the heat and allow gel to cool to 50°C .
11. Add $250 \mu\text{l}$ of 20 mg/ml proteinase K, mix gently, and pour into the gap above the main gel. Allow to cool, and remove the comb.

Set up for electrophoresis

12. Mount the gel in the electrophoresis tank, and add just enough $1\times$ TBE buffer to cover it to a depth of 1 mm.
13. Add $1 \mu\text{l}$ DNA marker VI to $30 \mu\text{l}$ of $1\times$ DNA loading buffer and load the marker into the well that is separated from the digestion gel. Load all other samples into the wells connected with the digestion gel.

For the Buffer Puffer apparatus, use $2 \mu\text{l}$ DNA marker VI dissolved in $30 \mu\text{l}$ of $1\times$ DNA loading buffer.

14. Run the gel overnight (~ 14 hr) at 20 V to facilitate digestion of the sample.
15. Turn the voltage up to 90 V and run for an additional 1.5 hr.

For the Buffer Puffer apparatus, run gel 2 hr at 100 V after the initial 20-V overnight run.

Develop gel

16. Remove the gel from the tank and rinse with water.
17. Add 100 ml TE buffer and $40 \mu\text{l}$ of 50 mg/ml RNase A. Place gel on shaker for 3 to 4 hr.
18. Rinse the gel with distilled water. Add 100 ml TE buffer and $5 \mu\text{l}$ of 10 mg/ml ethidium bromide. Place gel on the shaker for 40 min.
19. Wash several times with fresh TE to remove ethidium bromide, and photograph under UV light.

Detection of DNA Fragmentation in Total Genomic DNA

This method is a modified version of the protocol described by McGahon et al. (1995). The presence of ethidium bromide in the gel avoids later staining and destaining of the gel. Although Basic Protocol 5 and this alternate protocol are equally sensitive, the latter is less time consuming. In addition, dry loading prevents the loss of material.

Additional Materials (also see Basic Protocol 5)

Lysis buffer: 2 mM EDTA/100 mM Tris·Cl, pH 8.0/0.8% (w/v) SDS (store at room temperature)

1. Place sample of cell suspension containing $4\text{--}6 \times 10^5$ cells in a 1.5-ml microcentrifuge tube. Centrifuge 5 min at $2000 \times g$, 4°C .
2. Remove and discard supernatant. Add 20 μl lysis buffer.
3. Add 2 μl of 50 mg/ml RNase A. Mix well by flicking the tip of the tube. Do not apply vigorous vortexing. Incubate at least 30 min at 37°C .
4. Add 10 μl of 20 mg/ml proteinase K, and incubate at least 1.5 hr at 50°C .
5. Add 8 μl of $4\times$ DNA loading buffer.

If necessary, after this step the samples can be stored for at least 1 week at 4°C .

6. Place 0.9 g SeaKem agarose (1.8%) in 50 ml of $1\times$ TBE buffer in a flask (for GNA-100 gel electrophoresis apparatus). Heat in a boiling water bath or microwave oven until dissolved. Allow the agarose to cool to 60°C for 10 to 15 min. Add 3 μl of 10 mg/ml ethidium bromide.
7. Dry load the samples. Dissolve 1 μl DNA marker VI in 30 μl of $1\times$ DNA loading buffer and load into the gel. Apply low current (~ 35 mA) for ≥ 7 hr or higher current (~ 60 mA) for 4 hr.

Samples are dry loaded and enough TBE buffer is added to the apparatus so it touches both sides of the gel but does not cover it. This is to ensure that the sample is not lost. After 10 min, when the samples have entered the gel, the current is stopped and more TBE is added so it covers the whole gel, and the current is then reapplied.

8. Photograph the gel under UV light.

Simple Protocol for Detection of DNA Fragments

This simple protocol was developed in the authors' laboratory. It is particularly useful for detection of chromatin cleavage in lymphoid cells (Zhivotovsky et al., 1995) but less so for hepatocytes and epithelial cells. For these types of cells, see Alternate Protocol 4.

Additional Materials (also see Basic Protocol 5)

Lysis buffer, 4°C : 5 mM Tris·Cl, pH 8.0/20 mM EDTA/0.5% (v/v) Triton X-100
100% ethanol, -20°C

5 M NaCl (APPENDIX 2A)

RNase T1/A stock (see recipe)

Vacuum lyophilizer (e.g., Hetovac, Heto-Holten)

Prepare DNA sample

1. Place sample containing $1\text{--}2 \times 10^6$ cells in a microcentrifuge tube, and centrifuge 5 min at $2000 \times g$, 4°C . Resuspend pellet in 1 ml PBS and repeat centrifugation.
2. Resuspend pellet in 250 μl TE buffer, add 250 μl of 4°C lysis buffer, and vortex. Refrigerate 30 min.
3. Centrifuge sample 15 min at $15,000 \times g$, 4°C .

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4. Transfer supernatant to a fresh microcentrifuge tube. Add 1 ml -20°C ethanol and 30 μl of 5 M NaCl. Mix and place in -20°C freezer overnight.
5. Centrifuge sample for 15 min at $15,000 \times g$, 4°C . Remove and discard supernatant.
6. Place precipitate in vacuum lyophilizer for 20 to 25 min.
7. Add 20 to 30 μl TE buffer and 1 μl RNase T1/A stock. Incubate 1 hr at 37°C .
8. Add 1 μl of 20 mg/ml proteinase K, and incubate an additional 1 hr at 37°C .
9. Add 8 μl of $4\times$ DNA loading buffer.

Cast and run the gel

10. Dissolve 0.9 g SeaKem agarose in 50 ml TBE buffer (for GNA-100 gel electrophoresis apparatus) or 2.7 g SeaKem agarose in 150 ml TBE buffer (for Buffer Puffer gel electrophoresis apparatus). Heat in a boiling water bath or microwave oven until dissolved, then cool gel and pour into mold.
11. Mount the gel in the electrophoresis tank, and add just enough $1\times$ TBE buffer to cover the gel to a depth of 1 mm.
12. Load DNA marker VI in the first well and samples in the rest of wells (see Basic Protocol 5, step 13).
13. Run the gel at 60 mA (for GNA-100) or 70 mA (for Buffer Puffer) until the bromphenol blue front is ~ 1 to 2 cm from the end of the gel.
14. Stain gel with ethidium bromide and photograph under UV light (see Basic Protocol 5, steps 18 to 19).

**ALTERNATE
PROTOCOL 4**

Phenol Extraction of DNA Fragments for Agarose Gel Electrophoresis

This protocol is a modification of the classical method described by Wyllie (1980). This method requires DNA purification. Although it takes much more time than other techniques and needs many more cells for preparation, it yields very clean, protein-free DNA fragments.

Additional Materials (also see Basic Protocol 5)

- Lysis buffer (see Alternate Protocol 3), 4°C
- 100% ethanol, -20°C
- 5 M NaCl (APPENDIX 2A)
- RNase T1/A stock (see recipe)
- Phenol, TE-saturated (see recipe)
- 24:1 (v/v) chloroform/isoamyl alcohol (store mixture at room temperature in a fume hood)
- 0.5% (w/v) SDS

Prepare DNA sample

1. Place sample containing $2-5 \times 10^6$ cells in microcentrifuge tube, and centrifuge 5 min at $2000 \times g$, 4°C .
2. Resuspend pellet in 250 μl TE buffer, add 250 μl of 4°C lysis buffer, vortex, and refrigerate 30 min at 4°C .
3. Centrifuge sample 15 min at $15,000 \times g$, 4°C .
4. Transfer supernatant to fresh microcentrifuge tube. Add 1 ml -20°C 100% ethanol and 30 μl of 5 M NaCl. Mix and place in -20°C freezer overnight.
5. Centrifuge sample 15 min at $15,000 \times g$, 4°C . Remove and discard supernatant.

6. Add 500 μ l TE buffer and 5 μ l RNase T1/A stock to pellet. Incubate 30 min at 37°C.
7. Add 250 μ l TE-saturated phenol and 250 μ l of 24:1 chloroform/isoamyl alcohol. Vortex, and centrifuge for 2 to 3 min at 5000 \times g, 4°C. Transfer upper layer to fresh 2-ml microcentrifuge tube.
8. Add 500 μ l of TE buffer to lower layer; repeat step 7. Transfer upper layer to 2-ml microcentrifuge tube and mix with upper layer from step 7.
9. Extract the mixed samples from steps 7 and 8 with 1000 μ l of 24:1 chloroform/isoamyl alcohol and centrifuge again.
10. Transfer the upper layer to 1.5-ml microcentrifuge tubes, 500 μ l per tube. Precipitate overnight with ethanol and NaCl as in step 4.
11. Repeat step 5.
12. Place precipitate in vacuum lyophilizer for 20 to 25 min.
13. Dissolve and mix precipitates from both microcentrifuge tubes in a total of 20 to 30 μ l TE buffer/0.5% SDS. Add 5 μ l of 4 \times DNA loading buffer.

Cast and run the gel

14. Mix a 1.8% agarose gel by dissolving 0.9 g agarose in 50 ml TBE buffer (for GNA-100 gel electrophoresis apparatus) or 2.7 g agarose in 150 ml TBE buffer (for Buffer Puffer). Cool gel and pour into mold.
15. Mount the gel in the electrophoresis tank, and add just enough 1 \times TBE buffer to cover the gel to a depth of 1 mm.
16. Load DNA marker VI in the first well (see Basic Protocol 5, step 13) and samples in the rest of the wells.
17. Run the gel at 60 mA (for GNA-100) or 70 mA (for Buffer Puffer) until the bromophenol blue front is ~1 to 2 cm from the end of the gel.
18. Stain gel with ethidium bromide (see Basic Protocol 5, steps 18 to 19) and photograph under UV light.

Quantitative Assay of DNA Fragmentation

In addition to qualitative analysis by gel electrophoresis, DNA fragmentation can be quantitatively determined by using the diphenylamine reagent. This method, introduced by Burton (1956), cannot, however, discriminate between apoptotic and necrotic chromatin cleavage.

Materials

Cell suspension to be assessed
 Lysis buffer (see Alternate Protocol 3), ice cold
 10% (w/v) and 5% (w/v) trichloroacetic acid (TCA; keep at room temperature in dark flasks)
 Diphenylamine reagent (see recipe)
 10-ml conical glass tubes
 Round-bottom glass tubes
 Water bath, 100°C

1. Prepare cell suspension containing 1–10 \times 10⁶ cells in a 1-ml volume.
2. Transfer 0.8 ml of cell suspension to microcentrifuge tube. Add 0.7 ml ice-cold lysis buffer. Vortex, and allow lysis to proceed 15 to 30 min at 4°C.

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3. Centrifuge 15 min at $15,000 \times g$, 4°C . Transfer supernatant to labeled conical glass tube.
4. Add 0.65 ml of 5% TCA to the pellet in microcentrifuge tube, and add 1.5 ml of 10% TCA to the sample in labeled glass tube.
5. Precipitate both samples overnight (≥ 4 hr) at 4°C .
6. Centrifuge conical tube 10 min at $2500 \times g$, room temperature.
7. Remove supernatant after centrifugation and add 0.65 ml of 5% TCA to the pellet. Prepare two blank tubes with 0.65 ml of 5% TCA to be treated the same for the remaining steps.
8. Make hole on the top of each microcentrifuge tube from step 5, and cover each conical tube with a marble.
9. Boil both sets of tubes for 15 min in 100°C water bath.
10. Cool to room temperature. Centrifuge samples 5 min at $2500 \times g$, room temperature.
11. Transfer 0.5 ml of each supernatant (from both glass and microcentrifuge tubes) to labeled, round-bottom glass tubes.
12. Add 1 ml diphenylamine reagent to each tube. Incubate ≥ 4 hr at 37°C with a marble covering each tube.
13. Read absorbance at 600 nm in spectrophotometer. Set zero with blanks from step 7. Express results as the percentage of DNA fragmented:

$$\% \text{ fragmented DNA} = \frac{\text{absorbance of the supernatant}}{\text{absorbance of supernatant} + \text{pellet}} \times 100$$

**BASIC
PROTOCOL 7**

Detection of High-Molecular-Weight Chromatin Fragments by Pulsed-Field Agarose Gel Electrophoresis

Not all forms of apoptosis are accompanied by internucleosomal DNA fragmentation (Oberhammer et al., 1993a). However, the formation of high-molecular-weight apoptotic DNA fragments of 50 to 700 kbp (see Figure 18.3.2B), which was first observed by Walker et al. (1991), has been reported to occur in all the cell types studied to date (see Background Information in Commentary).

One of the forms of pulsed-field gel electrophoresis, field-inversion gel electrophoresis (FIGE), has been successfully used in several laboratories to identify high-molecular-weight DNA fragments in apoptotic cells and a smear in the same range of DNA size in necrotic cells. The authors have used both vertical and horizontal gel chambers; resolution of DNA fragments is the same. This protocol is an adaptation of an earlier method described by Anand and Southern (1990).

Materials

- Cell suspension
- Agarose buffer (for molds; see recipe)
- SeaPlaque GTG low-melting-point agarose (FMC Bioproducts)
- 20 mg/ml proteinase K in water (store in aliquots up to 1 year at -20°C)
- Proteinase buffer (for plugs; see recipe)
- TE buffer, pH 8.0:10 mM Tris-Cl/1 mM EDTA
- 50 mM EDTA, pH 8.0 (APPENDIX 2A)
- SeaKem GTG agarose (FMC Bioproducts)
- 5 \times TBE buffer (see recipe)

DNA size pulse markers: chromosomes from *Saccharomyces cerevisiae* (225 to 2200 kbp) and a mixture of λ DNA *Hind*III fragments, λ DNA, and λ DNA concatemers (0.1 to 200 kbp; Sigma; supplied premade in syringe)

Gel leveling table

100- μ l insert molds (Amersham Pharmacia Biotech), stored in 0.1 M HCl

12- or 24-well tissue culture plates

50°C incubator

100°C water bath or microwave oven

Pulsed-field gel electrophoresis system: vertical gel chamber with cooling elements (Protean II, Bio-Rad); horizontal gel chamber (HE 100B); power supply (PS 500 XT); and Switchback pulse controller (PC 500, Hoefer Scientific Instruments)

Thermostatic circulator Multi Temp III (Amersham Pharmacia Biotech)

Prepare sample plugs of agarose

1. Rinse insert molds extensively in distilled water and dry. Wrap Parafilm around the bottom of the molds and place them on ice to chill at least 10 min before pouring in the gel.

Make sure to mark the sample wells with pencil or pen.

2. Place sample of cell suspension containing 1×10^6 cells in a microcentrifuge tube, and centrifuge 3 min at $2000 \times g$, 4°C. Remove and discard supernatant. Resuspend pellet in 100 μ l of agarose buffer.
3. Prepare 1% low-melting-point agarose in agarose buffer, and place in a 60°C water bath until melted.
4. Add 100 μ l of molten 1% agarose to the cell suspension, and mix with pipet. Immediately pipet mixture into two prechilled insert molds, 100 μ l per mold.
5. Place the filled insert molds on ice for 10 to 15 min.
6. Remove Parafilm from the bottom of the insert molds, and dislodge each plug into separate well of a tissue culture plate.

It is convenient to use 12- or 24-well tissue culture plates for this purpose.

7. Add 1 ml of proteinase buffer and 10 μ l proteinase K (final concentration 0.2 mg/ml plug) to each well. Wrap Parafilm or clear tape around the plate, and incubate ≥ 24 hr on a shaker at 50°C.
8. Remove wrapping from plate, and remove buffer with pipet.

If it is necessary to simultaneously analyze the low-molecular-weight DNA, it is possible to remove the DNA fragments that have leached out from the plugs during incubation with proteinase K by precipitating them from this buffer over 48 hr at -20°C . Add 0.2 volume of 10.5 M ammonium acetate and 2 volumes of cold absolute ethanol. This DNA can then be lyophilized, dissolved in 1 \times DNA loading buffer, and analyzed by conventional gel electrophoresis.

9. Wash each plug three times with 1 ml TE buffer, 2 hr per wash, on a shaker at 4°C.
10. Remove TE buffer, and add 1 ml of 50 mM EDTA.

Plugs are now ready to put on a gel. To save samples for a long time, store the plugs in EDTA, in which DNA is stable for several months, at 4°C.

Prepare separating gel

11. Prepare 1% agarose (SeaKem GTG) gel in 0.5 \times TBE.

For horizontal gels

- 12a. Dissolve 2.5 g agarose in 250 ml 0.5× TBE in a boiling water bath or microwave oven. Allow the agarose to cool to 60°C for 10 to 15 min.
- 13a. Seal the edges of the gel platform with tape, and place platform on leveling table.
- 14a. Pour the gel into the mold, and remove unwanted air bubbles. Place the comb into the gel, and allow to set for 1 hr.
- 15a. Remove the comb very carefully so as not to break the gel. Remove tape.

For vertical gels

- 12b. Clamp two frosted glass plates together with 3-mm spacers in between. Seal the bottom with Parafilm to prevent leakage of warm agarose.
- 13b. Warm glass plates for 30 min in 50°C incubator.
- 14b. Melt agarose as in step 12a (1 g agarose in 100 ml 0.5× TBE), and pour into the prewarmed gel assembly to ~5 mm below top of plates. Insert comb into the gel, and allow to set for 1 hr.
- 15b. Remove the comb very carefully so as not to break the gel, and wash the wells with 0.5× TBE to remove partially polymerized agarose.

Load and run gel

16. Using a scalpel, cut out a 2-mm slab from one marker, and insert into first well of the gel with a spatula.
17. Repeat this procedure with the second marker.

Make sure that the slab is flat on the bottom of the well. This applies to loading of samples as well.

18. To load sample, cut off 3 mm from sample plug, and insert it into individual well with a spatula. Repeat same procedure for all samples. Avoid introducing any air bubbles into the wells.

Note and record the order of the samples!

19. Cement the slabs into wells with the remaining molten 1% agarose.
20. Transfer the loaded gel (either horizontal or vertical) into a precooled tank filled with 0.5× TBE.

The buffer temperature should be 11° to 12°C at the beginning of the experiment.

21. Run the gel at a constant voltage with a suitable switcher program to achieve the desired resolution. The programs listed in Table 18.3.2 give good resolution of 50-, 300-, and 700-kbp fragments.
22. Remove gel carefully, and stain it in ethidium bromide for 1 hr (see Basic Protocol 5, step 18). Destain it in 0.5× TBE for 2 to 3 hr.
23. Visualize and photograph bands under UV light.

METHODS FOR ANALYSIS OF CASPASE PROTEOLYTIC ACTIVITY

Several proteolytic activities are implicated in apoptosis and necrosis (see *UNIT 18.1*). The activation of caspases, a family of apoptotic proteases, plays a key role in apoptosis induced by diverse stimuli. Caspases, which have been detected in numerous tissues and cell types, are synthesized as a precursor form, or proenzyme, and an apoptotic signal converts the precursor into mature enzyme. Moreover, until now there has been no

Table 18.3.2 Parameters for Running Pulsed-Field Agarose Gels

	Horizontal gel	Vertical gel
Running voltage ^a	170 V	200 V
Procedure	10 min: run the DNA into gel with continuous forward pulse 6 hr: apply 20 sec forward time, 3:1 forward/back ratio, ramp factor 1.5 ^b 6 hr: apply 10 sec forward time, 3:1 forward/back ratio, ramp factor 2 12 hr: apply 0.8 sec forward time, 3:1 forward/back ratio, ramp factor 12.5	10 min: run the DNA into gel with continuous forward pulse 2 hr: apply 12 sec forward time, 3:1 forward/back ratio, ramp factor 2 ^c 3 hr: apply 2.4 sec forward time, 3:1 forward/back ratio, ramp factor 5.0 2.5 hr: apply 8.0 sec forward time, 3:1 forward/back ratio, ramp factor 3.0
Total running time	24 hr, 10 min	7 hr, 40 min

^a Constant voltage

^b This means that at the end of these 6 hr, the forward time should be 30 sec.

^c This means that at the end of these 2 hr, the forward time should be 24 sec.

evidence for the involvement of caspase activities in necrosis. Therefore the detection of caspase activity in cells can be used as a discriminating criterion to distinguish apoptosis from necrosis.

All members of the caspase family of proteases share a number of amino acid residues crucial for substrate binding and catalysis. Despite their uniform requirement for an aspartate residue at the P₁ position of the substrate site, caspases can be divided into three different groups according to their substrate preferences. The first group (caspases 1, 4, and 5) has the optimal substrate cleavage sequence WEXD; the second (caspases 2, 3, and 7) preferentially cleaves targets at the DEXD motif; and the third (caspases 6, 8, and 9) has the optimal substrate cleavage sequence (L/V)EXD (Thornberry et al., 1997). The predicted caspase specificities correspond to the cleavage sites in the different intracellular caspase targets. Analysis of caspase activation is also based on the substrate specificity of the enzymes (see *UNIT 18.2*).

REAGENTS AND SOLUTIONS

Use Milli-Q-purified or other ultrapure water for all recipes and protocol steps. For common stock solutions, see *APPENDIX 2*; for suppliers, see *SUPPLIERS APPENDIX*.

Agarose buffer (for molds; used in *Basic Protocol 7*)

Prepare 500 ml in H₂O:

0.15 M NaCl

2 mM KH₂PO₄/KOH, pH 6.8

1 mM EGTA

5 mM MgCl₂

Filter sterilize through 0.2- μ m filter

Store at 4°C

Diphenylamine reagent

100 ml glacial acetic acid

1.5 g diphenylamine

1.5 ml concentrated sulfuric acid

0.5 ml 16 mg/ml acetaldehyde stock

Prepare just before use

Prepare acetaldehyde stock in ultrapure water; store in a dark flask up to 1 month at 4°C.

Ethidium bromide, 10 mg/ml

Stir 10 mg/ml ethidium bromide in water on magnetic stirrer for several hours. Store up to 2 months in a dark bottle at 4°C.

Paraformaldehyde, 4% (w/v)

Add 8 g paraformaldehyde powder (e.g., Sigma) to 100 ml water and heat to 60°C in a fume hood. If necessary, add NaOH dropwise (one or two drops each time) to help dissolve the powder. When the solids have completely dissolved, let the solution cool to room temperature. Then add 100 ml of 2× PBS (APPENDIX 2A). Store at 4°C.

Because this solution cannot be kept indefinitely, it is better to prepare small volumes every week.

Phenol, TE saturated

Allow phenol to warm to room temperature, and then melt it at 68°C. Add hydroxyquinoline to a final concentration of 0.1%. Add a volume of TE buffer, pH 8.0 (10 mM Tris·Cl/1 mM EDTA), equal to the volume of phenol, and vortex extensively. Allow the two phases to separate, and aspirate the upper aqueous phase. Repeat this saturation with TE buffer two to three times until volume of TE remains unchanged and the pH of the phenol phase has reached 8.0 (as measured with pH paper). Remove the aqueous phase, and add 0.1 vol fresh TE. Store up to 1 month in the dark at 4°C.

Hydroxyquinoline, a yellow antioxidant, provides a convenient way to identify the organic phase during extraction.

Proteinase buffer (for treatment of plugs)

Prepare 500 ml of buffer consisting of:

10 mM NaCl

10 mM Tris·Cl, pH 9.5

25 mM EDTA

Add *N*-lauroylsarcosine (Sarkosyl) to 1% final concentration and dissolve using magnetic stirrer. Check pH, and if necessary adjust to 9.5 with NaOH. Filter through 0.2-µm filter and store at 4°C.

RNase A, 50 mg/ml

Dissolve RNase A (e.g., Sigma) at a concentration of 50 mg/ml in 10 mM Tris·Cl, pH 7.5/15 mM NaCl. Heat 15 min at 50°C. Allow to cool at room temperature. Divide into aliquots and store up to several months at -20°C.

RNase T1/A stock

Mix 50 µl of 500,000 U/ml RNase T1 and 50 mg of RNase A in 5 ml of 10 mM Tris·Cl, pH 7.5/15 mM NaCl. Heat for 15 min at 50°C. Allow to cool at room temperature. Divide into aliquots and store up to several months at -20°C.

SDS, 10% (w/v)

Dissolve 10 g of sodium dodecyl sulfate (SDS) in 90 ml of water by mixing on magnetic stirrer for several hours or by heating to 65°C to assist dissolution. Adjust the volume to 100 ml with water. Store at room temperature for up to 1 month.

Staining buffer

54.2 μ l water
25 μ l 20 \times sodium cacodylate (SSC) stock solution
20 μ l blotto (25% low-fat dried milk in PBS)
0.7 μ l avidin-FITC (160 \times)
0.1 μ l Triton X-100
Store up to several weeks at 4°C

TBE (Tris/borate/EDTA) buffer, 5 \times

445 mM Tris base
445 mM boric acid
10 mM trisodium EDTA
Store at room temperature

All components may be obtained from Sigma. Do not adjust pH, which should be about 8.0 at room temperature. When this solution is stored for a long time, a precipitate forms. To avoid problems, discard batches that develop a precipitate.

COMMENTARY

Background Information

A number of techniques have been developed to detect cytotoxicity and cell death in various cell types. The methods developed in the last two decades have been aimed at distinguishing between cell toxicity leading to apoptosis and that leading to necrosis. The choice of a particular method for analysis of cellular toxicity depends on the cell system; the toxin or toxicant; the expected mode of cell death; the type of information being sought; and, finally, technical limitations. There is no one single method that is satisfactory for obtaining all the information required. This is largely a result of technical and practical limitations. Therefore, to make the right interpretation of data and to draw the correct conclusions regarding the mode of cell death, a combination of some of the methods mentioned above should be used. It is recommended that a combination of at least three criteria of cell death be evaluated: cell morphology, DNA fragmentation (see Assays for Chromatin Cleavage), and/or caspase activation (see UNIT 18.2).

Although morphological changes are obviously secondary to biochemical alterations, many of the published reports on cellular toxicity are based mainly on histological, cytological, and electron microscopy studies (Kerr et al., 1972; Bowen, 1980). The interpretation of histological data is based on the changes in membrane permeability. However, as mentioned in the introduction to this unit, analysis of plasma membrane integrity fails to identify cells in the early stages of apoptosis, although it can be used to identify necrotic cells in a population of interest. Alternative morphologi-

cal methods draw on a mixture of optical and electron microscopy techniques. Combination of these methods allows one to distinguish between apoptotic changes—cell shrinkage, high cytoplasm density, membrane blebbing, compaction of chromatin, and formation of apoptotic bodies—and those indicative of necrosis—chromatin clumping, gross swelling of organelles, early membrane breakdown, and cell disintegration. In addition, confocal microscopy allows the combination of morphological analysis with subcellular localization of biochemical changes within damaged cells.

Other changes that accompany cell death can be used as indicators of the mode of cell death. It has been shown that phagocytosis is a response to cell death and is particularly efficient in apoptosis. Apoptotic cells can be recognized by macrophages through a number of cell surface markers. The presence of PS on the outer plasma membrane is one such marker, and it can be detected efficiently by phagocytic cells, as blockage of PS renders phagocytosis less efficient. Therefore, PS exposure can be considered a biochemical feature of apoptosis with physiological importance (Koopman et al., 1994). Annexin V has recently been utilized as a probe to monitor changes in phospholipid distribution in the plasma membrane of cells undergoing apoptosis. In combination with propidium iodine, annexin V staining can reliably discriminate apoptosis from necrosis. Flow cytometry is a useful technique to examine not only changes in the plasma membrane, but also changes in cell size and granularity (Darzynkiewicz et al., 1994; Robinson et al., 1999). Apoptosis is accompanied by a dramatic

loss of water and consequent cell shrinkage, which can be distinguished from necrosis by a difference in light-scatter pattern. This is a rapid technique to identify apoptotic versus necrotic cells and is very widely used nowadays.

Several biochemical methods based on the detection of changes in chromatin structure have been developed. These vary from methods for detecting single-strand DNA nicks or breaks, to those for demonstrating high-molecular-weight DNA fragments, to those for showing the subsequent internucleosomal cleavage. DNA nicks can be detected using TUNEL (see Basic Protocol 4; Gavrieli et al., 1992). However, this method apparently recognizes not only nicks formed during apoptosis, but also those formed during necrosis. Thus this method should always be used in combination with other more discriminating methods.

Some other methods used for the analysis of DNA or chromatin damage appear to be better suited to discriminate between the two types of cell death. The different DNA gel electrophoresis techniques that are described here (see Basic Protocol 5; see Alternate Protocols 2, 3, and 4) can be utilized for a qualitative analysis (Wyllie, 1980; Sorenson, 1990). Although the quantitative analysis (see Basic Protocol 6) can provide limited information concerning the general cleavage of chromatin (Burton, 1956), the combination of different methods of gel electrophoresis provides more specific information concerning the type of chromatin cleavage (high-molecular-weight DNA fragments, DNA ladder, or smear). It has been shown that DNA laddering is not apparent in all apoptotic systems (Oberhammer et al., 1993a). In many types of cells, chromatin cleavage is restricted to the formation of high-molecular-weight DNA fragments (Walker et al., 1991; Oberhammer et al., 1993b). In such cases, again, the combination of two independent methods assists in drawing the correct conclusions in the study of cell death or toxicity. However, when DNA laddering occurs, it is highly indicative of apoptosis.

Activation of the caspase family of proteases has been detected in numerous cell systems and appears to function as a pathway through which apoptotic mechanisms operate (for review see Zhivotovsky et al., 1997). Upon apoptotic triggering, a hierarchy of caspases is believed to become activated in a process where more proximal caspases cleave and activate downstream caspases, giving rise to a proteolytic cascade that serves to amplify the death signal. However, not all of these caspases

normally function in cell death. A recent classification divided caspases into two groups, one involved in cytokine processing (caspase 1–like proteases) and the other playing a role in cell death (Alnemri et al., 1996; Cryns and Yuan, 1998). This latter group can be further subdivided according to function as activators or executioners of cell death. Up to now there has been no evidence concerning the involvement of caspase activities in necrosis. Therefore the detection of caspase activity in cells can be used as a discriminating criterion to distinguish apoptosis from necrosis. Caspase activity is assessed by proteolytic cleavage of fluorogenic substrates (see UNIT 18.2) or by immunodetection of protein bands corresponding to active caspases (see UNIT 18.2).

In summary, a combination of morphological and biochemical methods is recommended to discriminate between the mode of cell death, especially if the manifestations of cell death or the stimuli used have not been previously characterized in the cell type of interest.

Critical Parameters and Troubleshooting

Morphological assays

A characteristic feature of apoptosis is that membrane integrity of the dying cell is maintained long after the process has begun. However, a reduction in membrane integrity usually occurs during the late stages of apoptosis when the cells undergo a process commonly called “secondary necrosis” or “postapoptotic necrosis.” Therefore, a disadvantage of the trypan blue exclusion assay (see Basic Protocol 1) is underestimation of the level of apoptosis in a cell population.

If the cytopsin preparations are understained after a full cycle of fixation and differential staining (see Basic Protocol 2), then the procedure can be repeated a second time until the desired staining is achieved. If overstaining is the problem, preparations can be destained by immersing them in 100% methanol for up to 5 sec, followed by rinsing with water. Because membrane blebbing is a feature common to both apoptosis and necrosis, one should take care not to use this as the sole criterion for determination of the mode of cell death.

If assessing the nuclear changes in apoptotic cells is problematic, then use other conventional methods of inducing apoptosis in cells as a positive control, stain cells treated both ways with Hoechst dye (see Basic Protocol 3), and observe nuclear changes under the UV micro-

scope. If no apoptotic changes are evident in cells, try to increase the time of incubation with apoptosis-inducing agents.

Chromatin cleavage assays

The TUNEL technique (see Basic Protocol 4) is used to detect DNA strand breaks. There are rare situations when apoptosis proceeds without DNA degradation. Conversely, extensive DNA degradation may accompany necrosis. Thus, one should always use another independent assay, along with the TUNEL method, to confirm whether cells have undergone apoptosis. Costaining cells with propidium iodide or Hoechst is a very useful method to discriminate between apoptosis and necrosis in samples that have stained positively with the TUNEL technique. TUNEL-positive cells that express condensed, fragmented nuclei after staining with PI or Hoechst are apoptotic, whereas those with diffuse staining of the nuclei with PI or Hoechst are necrotic.

If a high background is present in TUNEL-treated samples, make sure that there is no DNase contamination in the test tubes or buffers. It is also possible that the number of DNA strand breaks is so large that one cannot quantify the degree of cell labeling. To eliminate this problem, use fewer cells or decrease the incubation time.

For exogenous enzymes, such as the TdT enzyme used in TUNEL, to enter the cell, the plasma membrane has to be permeable. In many cases, the fixation procedure is sufficient to permeabilize cells; however, sometimes an additional short incubation on ice or short (1- to 2-min) treatment with proteinase is required. To avoid loss of low-molecular-weight DNA, the permeabilized cells have to be fixed with formaldehyde before permeabilization. This fixation cross-links DNA fragments to other cellular constituents and prevents their leakage during the permeabilization step.

The procedures for assessment of DNA fragmentation in whole cells (see Basic Protocol 5) may cause the samples to become viscous and difficult to load into gel sample wells. If this is the case, dilute samples with 1× DNA loading buffer. If no detectable DNA laddering is observed in a population of cells that are known to be apoptotic, increase the number of cells used for the assay. If it is difficult to observe distinctive bands because of high background, wash gel extensively with water. If an unusual band appears in the middle of the gel in all lanes, incubate gel longer with RNase (step 17).

For analysis of fragmentation in total genomic DNA (see Alternate Protocol 2), the

reagents are optimized for the stated number of cells. If larger numbers of cells are used, RNA and protein digestion will not be complete, which may interfere with electrophoresis. In addition, the procedures cause the samples to be viscous. Therefore, for minimum sheering of DNA, it is better to use pipet tips that have the ends (3 to 4 mm) cut off with a razor blade to make wide-bore tips.

If no laddering is observed in DNA phenol extracted from a population of apoptotic cells (see Alternate Protocol 4), the DNA may have been lost in the organic phase during extraction. The aqueous phase should be removed carefully during extraction to prevent DNA loss. Another possible reason for failure to detect laddering may be degradation of DNA by exogenous nucleases. All solutions must be free of nuclease contamination. If, instead of a DNA ladder, a smear appears on the gel, this indicates random and general cleavage of chromatin, indicative of necrosis (see Figure 18.3.2A, lane 3).

For quantitative assays of DNA fragmentation (see Basic Protocol 6), if absorbance of DNA in supernatant samples is below the sensitivity of detection, increase the number of cells. If all absorbance is present in the supernatant and absent from the pellet, then the pellet was mistakenly transferred together with supernatant to the labeled conical glass tubes (step 4).

There could be several reasons for large fragments not to appear on the pulsed-field electrophoresis gel (see Basic Protocol 7). If the material is still in the wells, the pulse generator power supply may not have been working properly. If some of the material is in the wells, but no sharp bands are visible and the material moved in the wrong direction, the current may not have been applied in the correct direction. If there is no material in the wells, the chromatin may have been digested by exogenous exonucleases during the preparation. If very little material is in the well, a bigger slab may have to be cut from the sample plugs. If too much material is in the lane, a smaller slab may have to be cut from the sample plugs. If thin stripes appear in any lanes in the gel, it means that either a bubble was in the well or the slab was not placed flat. Sometimes there is a leakage of buffer from the top reservoir, which may also influence the results.

Anticipated Results

Morphological assays

In the trypan blue exclusion assay (see Basic Protocol 1), several thousand cells are sufficient

to quantify the number of trypan blue–positive cells and to count the percentage of cells with an intact plasma membrane. Necrotic cells with rapidly disrupted membrane take up trypan blue as well.

Differential staining (see Basic Protocol 2) is one of the fastest and easiest methods for identifying dead cells. Cells are viewed under a light microscope (40× magnification), and ~300 cells are counted from separate fields of view and scored as normal, apoptotic, or necrotic based on the following morphological characteristics: membrane blebbing, chromatin condensation and nuclear shrinkage, cytoplasmic constriction and loss of cell volume, and formation of apoptotic bodies for apoptotic cells; nuclear swelling, chromatin flocculation, cell membrane blebbing and disruption, and finally cell lysis resulting in the appearance of “ghost cells” for necrotic cells (see Figure 18.3.1).

Because chromatin condensation is one of the early events of apoptosis, apoptotic cells stained with Hoechst 33342 have increased blue fluorescence compared with live cells similarly stained (see Basic Protocol 3). The intensity of the blue fluorescence changes with the incubation time, so that an optimal incubation time should be found for each cell system for discrimination of apoptotic cells (usually 5 to 10 min). For quantification of apoptosis, a minimum of 150 cells, from 10 to 15 fields, should be evaluated for the changes described above.

Chromatin cleavage assays

In both TUNEL protocols (see Basic Protocol 4 and Alternate Protocol 1), very little material is required for quantitative measurement of TUNEL-positive cells. Proportions of apoptotic cells as low as 10% to 15% can be detected by this method. Although the enzymatic labeling methods are time consuming (due to multiple incubation and washing steps), they are very sensitive. The method can also be applied to cryostat sections. In this case, the proteolytic pretreatment (see Alternate Protocol 1, step 4) can be shorter or even be omitted.

The whole-cell DNA fragmentation method (see Basic Protocol 5) requires only $4\text{--}5 \times 10^5$ cells. It can be used for cells growing in suspension as well as attached cells. Usually, the resolution of DNA fragmentation using this protocol is distinct and high. This method does not require DNA purification and is relatively simple and fast. Although Basic Protocol 5 and Alternate Protocol 2 are equally sensitive, the latter is less time consuming. In addition, the dry loading prevents the loss of material. Alter-

nate Protocol 3 is particularly useful for detecting chromatin cleavage in different lymphoid cells.

Phenol extraction (see Alternate Protocol 4) is the most commonly used technique for investigating internucleosomal chromatin cleavage. Although it takes much more time than other techniques (almost 2.5 working days) and needs many more cells for preparation ($2\text{--}5 \times 10^6$ cells), it yields very clean, protein-free DNA fragments.

The quantitative assay of DNA fragmentation described (see Basic Protocol 6) is only one quantitative method for measurement of DNA fragmentation. This method is very simple, and very reproducible results can usually be obtained.

Pulsed-field gel electrophoresis can be used to detect the presence of high-molecular-weight DNA fragments in apoptotic cells (see Basic Protocol 7). The method is very sensitive and requires 5×10^5 cells. Depending on which system is available, one can use either horizontal or vertical gel systems. If necessary, plugs can be kept for several months and run on another gel.

Time Considerations

Morphology assays

The time required for preparing solutions for trypan blue exclusion is 20 to 30 min; the solutions can then be kept up to 1 to 2 months at 4°C. Trypan blue staining takes 5 min, and calculation takes 10 min.

It takes 30 min to prepare the solutions for differential staining (which can be stored 1 to 2 months at room temperature); this time is not required if the commercial kit is used. 1 hr is required to prepare and analyze samples, and 30 min to evaluate results.

It takes about 30 to 40 min to prepare solutions for Hoechst staining and 1 hr to run the test. No more than 30 min are required to evaluate results.

Chromatin cleavage assays

Approximately 3 to 4 hr are required to run TUNEL assays. The evaluation of results requires an additional 1 to 2 hr, depending on the investigator's experience.

About 1 to 2 hr are required to prepare the buffers for assessment of fragmentation in whole cells, which then can be stored for at least 1 month. Usually, gel and sample preparation, which can be done simultaneously, take 2 hr, and gel electrophoresis takes 15 to 16 hr (over-

night is better). Incubation with RNase, staining, and destaining take another 6 to 7 hr. Thus, the whole experiment takes almost 1.5 days.

Analysis of fragmentation in total genomic DNA takes 1 working day.

Preparing solutions for quantitative analysis of DNA fragmentation takes 1.5 to 2 hr; however, the solutions can be stored for up to several weeks. The procedure takes 1.5 working days and 1 hr to evaluate results.

It takes about 1 week to perform the whole pulsed-field gel electrophoresis procedure. Vertical gels require a relatively shorter time (1 day less) compared with horizontal gels, with no difference in resolution.

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Quantitative Fluorescence In Situ Hybridization (Q-FISH)

This unit describes a quantitative technique for measuring the lengths of telomere repeat sequences in individual chromosomes from single metaphase cells (see Basic Protocol). The technique is based on fluorescence in situ hybridization (FISH) adapted for use with peptide nucleic acid (PNA) probes. PNA is an example of novel synthetic oligonucleotide “mimetic” which has a higher affinity than regular oligonucleotide (RNA or DNA) probes for complementary single-strand (ss) DNA sequences. PNA oligonucleotides have excellent penetration properties due to their small size (typically 15 to 18-mers) and can be directly labeled with fluorochromes. These properties have been exploited to develop quantitative fluorescence in situ hybridization (Q-FISH) onto denatured single-stranded chromosomal DNA target sequences. The latter can be present in preparations of fixed metaphase cells on slides (Q-FISH) or in heat-treated (interphase) cells in suspension (flow-FISH).

Q-FISH requires fluorescence microscopy in combination with digital image acquisition to measure the length of telomere repeats in preparations of metaphase chromosomes. The basic Q-FISH techniques are also very suitable to obtain qualitative information about the presence of specific repeat sequences in the nuclei of cells. The bright fluorescence signals combined with the low background of directly labeled PNA probes offer unique possibilities for a variety of studies including studies of nuclear organization. Such studies are not discussed here but can be explored using the Basic Protocol provided.

The Q-FISH technique described here consists of four distinct steps: (1) sample preparation; (2) image capture; (3) image analysis; and (4) telomere length normalization and calibration. First, slides suitable for Q-FISH analysis are prepared. For this purpose, fixed metaphase cells are dropped onto slides and hybridized with directly labeled PNA probes using an optimized FISH technique (see Basic Protocol). Second, images of telomeres and chromosomes are captured using digital fluorescence microscopy (see Support Protocol 1). Third, the captured images are analyzed to extract telomere fluorescence and chromosome identification (karyotype) information (see Support Protocol 2). Finally, similar procedures incorporating all the three components are used to calibrate and validate Q-FISH for quantitative purposes and determine calibration values for telomere length measurements (see Support Protocol 3).

USING PNA PROBES TO VISUALIZE TELOMERES IN METAPHASE SPREADS

*BASIC
PROTOCOL*

The following is a basic “generic” method to visualize telomeres and chromosomes in metaphase cells using fluorescence hybridization with directly labeled PNA probes. The slides that result from application will typically be of high quality and can be used for any type of qualitative analysis. However, in order to extract the quantitative information that is contained within these preparations, close attention to all steps described in subsequent protocols (see Support Protocols 1, 2, and 3) is essential.

The aim is to first prepare and deposit metaphase cells onto microscope slides. Standard cytogenetic techniques are used to prepare metaphase chromosomes from dividing cells. After fixation, metaphase cells are dropped onto slides and air-dried. A small volume of hybridization mixture containing Cy3- or FITC-labeled telomere (PNA) probe is placed onto coverslips and added to the chromosome preparations on the slides. The slides are subsequently heated at 80°C for 3 min using a thick metal (aluminum) plate preequili-

**Cell Aging and
Death**

brated to 80°C in an oven. The heat and high (70%) formamide content of the hybridization mixture will result in complete denaturation of chromosomal DNA. Removal of the slides from the oven and subsequent incubation of the slides at room temperature result in conditions that allow hybridization of the PNA probes to chromosomal target sequences but not the renaturation of complementary DNA. Conventional FISH procedures (using RNA or DNA probes) must use hybridization conditions that allow renaturation of complementary DNA strands as well. In Q-FISH only the PNA probes can hybridize to the denatured telomere repeat target sequences in chromosomal DNA. As a result, the number of probes attached to target sites will correlate to the number of repeat sequences at that site. In this way, telomere probes provide information on the length of telomere repeats at individual telomeres. After hybridization, the slides are washed to remove unbound PNA, dried, and sealed using a coverslip and a small volume of an antifade solution containing DAPI (4',6-diamidino-2-phenylindole,fs15 dihydrochloride) as a DNA counterstain. DAPI allows the visualization of cells and individual chromosomes and facilitates subsequent image acquisition, karyotyping, and telomere length analysis steps described in Support Protocols 1, 2, and 3.

Materials

Cells of interest
Cell-specific tissue culture medium supplemented with growth factors
10 µg/ml Colcemid (Life Technologies)
75 mM hypotonic KCl buffer (12.3 mM HEPES/0.53 mM EGTA/64.4 mM KCl;
or commercially available from Stem Cell Technologies or Life Technologies),
37°C
3:1 (v/v) methanol/glacial acetic acid fixative (Fisher; BDH)
70%, 90%, 95%, and 100% ethanol
Phosphate buffered saline, pH 7.4 (PBS; without Ca²⁺ and Mg²⁺, Stem Cell
Technologies; also see recipe)
37% formaldehyde, reagent-grade (BDH)
Pepsin (Sigma-Aldrich)
1 N HCl, pH 2.0
Hybridization mixture (see recipe)
Wash solution I (see recipe)
Wash solution II (see recipe)
Mounting medium containing antifade and DAPI (see recipe)
25-cm² tissue culture flasks with vented blue plug seal caps (Becton Dickinson)
15- and 50-ml polypropylene conical tube (Becton Dickinson)
Precleaned microscope slides for hematology and histology (e.g., Micro World;
<http://www.mwrn.com/>)
Lint-free paper towels
Phase-contrast microscope
100- and 150-ml glass slide staining jars and corresponding slide rack
Coverslips (22 × 60–mm; VWR)
Thick metal plate (~1-cm aluminum), e.g., 6 × 10–in.
80°C oven
Molded plastic slide box for 25 slides (Fisher)
Parafilm
Platform shaker (Barnstead/Thermolyne)
Light-protected slide storage box

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and aseptic techniques should be used accordingly. Furthermore, it is prudent to consider cultured and primary human cells as biohazardous and adhere to appropriate biohazard

containment and disposal guidelines. Consult your institutional safety officer for specific instructions.

NOTE: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain pH 7.4.

CAUTION: To avoid inhalation of fumes from glacial acetic acid, methanol, formaldehyde, and formamide solutions, it is recommended that cell fixation and formamide washes be performed in a fume hood. Formamide is a harmful and irritant organic solution. Wear suitable protective clothing and gloves when handling; avoid inhalation of fumes and skin contact. Use an excess of water in case of skin contact. Refer to Material Safety Data Sheet and consult your institutional guidelines for appropriate disposal of chemical waste.

Prepare metaphase-arrested cells

1. Culture cells for several days in tissue culture medium supplemented with growth factors in 25-cm² tissue culture flasks.

It is important to maximize the fraction of dividing cells in order to obtain sufficient mitotic cells and metaphase cells (chromosomes) in subsequent steps.

2. Add 10 µg/ml Colcemid to the culture medium (final 0.05 µg/ml) prior to harvesting mitotic cells.

The duration of the Colcemid treatment varies with cell type (e.g., for lymphocytes, from 30 min to 2 hr; for fibroblasts, from 6 to 24 hr), the proportion of dividing cells, and the required quality of the metaphase chromosomes (the longer the Colcemid treatment, the more condense the chromosomes).

3. Harvest the cells by trypsinization and/or pipetting as required and transfer to 15-ml tubes.
4. Centrifuge cell suspension 8 to 10 min at 200 to 300 ×g, room temperature.
5. Remove supernatant, resuspend pellet in remaining supernatant (<0.5 ml) by vortexing the tube and adding 2 to 10 ml of prewarmed (37°C) 75 mM hypotonic KCl buffer depending on the size of the pellet.
6. Incubate 20 to 30 min in a 37°C water bath.

Fix cells

7. Centrifuge cells 8 to 10 min at 200 to 300 ×g, room temperature, remove all but 0.5 ml of the hypotonic KCl solution, resuspend the cell pellet by hand tapping the tube, and add 1 to 5 ml of fresh 3:1 methanol/glacial acetic acid fixative with a Pasteur pipet. Let stand 15 min at room temperature.
8. Centrifuge the tubes again as in step 7, discard supernatant, and suspend the cells in 1 to 5 ml fresh 3:1 methanol/glacial acetic acid fixative. Repeat this step one or two times until a suitable cell pellet is obtained.

A suitable preparation will show nuclei devoid of cytoplasm as judged by microscopy after dropping the cells onto slides.

After the last fixation, cells can be stored in fixative in a closed tube for at least 3 months at -20°C prior to the preparation of slides. However, typically, slides are prepared within 7 days.

Prepare slides

9. Clean glass microscope slides with 95% ethanol. Wipe slides with dry lint-free paper towels.

Alternatively, precleaned slides can be used directly.

10. Resuspend the cells in a small amount of fresh 3:1 methanol/glacial acetic acid fixative until the suspension looks slightly turbid ($\sim 5 \times 10^6$ cells/ml). Drop 3 to 4 drops evenly on a slide covered with a thin film of distilled water. After the slide is completely dry, examine with a phase-contrast microscope to check the cell density and spread of metaphase chromosomes.

If the cell density is too high, add a few more drops of fixative to the cell suspension. If the cell density is low, centrifuge the suspension and resuspend the pellet in a smaller amount of fixative.

11. With a Pasteur pipet, let two drops of cell suspension fall onto each cleaned, wet slide. Mark the slide with a diamond pen or a pencil. Air dry slide(s) overnight in the fume hood.
12. Pour 150 ml PBS without Ca^{2+} and Mg^{2+} in a 150-ml staining jar with a removable slide rack. Place slides in glass slide rack and immerse in PBS to rehydrate for 15 min at room temperature.

Fix slides in formaldehyde

13. In a fume hood, prepare 4% formaldehyde in PBS by adding 21.6 ml of 37% formaldehyde stock solution to 200 ml PBS.
14. In the fume hood, transfer the 4% formaldehyde solution into a staining jar. Transfer the slides in a slide rack into the fixative solution and fix for 2 min. Save fixative so it can be reused in step 18.
15. Wash slide(s) in three changes of fresh PBS for 5 min each change.

Treat slides with pepsin

16. Prepare pepsin solution by dissolving 200 mg pepsin in 200 ml of 0.01N HCl, pH 2.0. Pour the pepsin solution into a staining jar and equilibrate in a 37°C water bath. Prepare this solution during PBS washes (step 15).
17. Transfer slide(s) to pepsin solution and incubate 10 min at 37°C. Wash slides two times, 2 min each, in PBS.
18. Repeat the fix and wash steps (steps 14 and 15 above).
19. Dehydrate slide(s) in an ethanol series: 5 min each in 70%, 90%, and 100% ethanol.

Hybridize PNA probe

20. Leave slides in a vertical position to air dry or use air stream to dry.
21. Deposit two 10- μ l drops hybridization mixture, with a micropipet, onto a clean 22 \times 60-mm coverslip.
22. Gently place the slide(s) with fixed cells (upside down) on top of the coverslip (placed on a bench or glass plate). Turn slide so that coverslip faces up prior to complete spreading of the hybridization mixture between the slide and the coverslip.

Avoid introducing air bubbles by holding the slide under a slight angle prior to contacting the drops of hybridization mixture on the coverslip.

Heat denature DNA

23. Denature chromosomal DNA by placing the slide(s), coverslip up, on a thick metal plate equilibrated at 80°C in a preheated oven for 3 min.
24. Place the slide(s) in a plastic slide box and place this box into a plastic beaker containing a damp paper towel.
25. Cover the beaker with Parafilm and let the sample hybridize for 1 to 2 hr at room temperature.

Wash slides

26. Prepare 500 ml of wash solution I and 500 ml of wash solution II.
27. In the fume hood, remove coverslips by soaking the slides in 100 ml of wash solution I. Use gloves and forceps to place slides back into a glass slide rack that fits into glass staining jars. Wash the slide(s) two times, 15 min each, in the remaining solution I.
Do not force the coverslips off the slides, as this will damage the cells.
28. Wash the slide(s) three times, 5 min each, in wash solution II on a platform shaker.
29. Dehydrate the cells 5 min each in 70%, 90%, and 100% ethanol and allow the sample to air dry.
30. Place two 10- μ l drops of mounting medium containing antifade and DAPI on a fresh coverslip.
31. Place the sample slide(s) on top of the coverslip (as in step 22).
32. Lift the slide (coverslip attached) and incubate in the dark, in a light-protected slide storage box, for at least 15 min to allow DAPI staining of DNA.

The slides are now ready for analysis (see Support Protocols 1, 2, and 3) and can be stored in a light-protected storage box for several weeks at 4°C prior to fluorescence microscopy and Q-FISH analysis.

IMAGE CAPTURE

The following is a method for capturing images of telomeres and chromosomes prepared using the Basic Protocol. In order to capture images in a constant manner such that they can be used for quantitative analysis, special attention must be given to the steps described herein and in the selection of the appropriate microscope/image capture system. As there are a variety of microscope and image acquisition systems that are used in various laboratories, the method described here has been generalized to encompass the diversity of these systems. Thus, specific instructions of how to use a particular program or how to operate the microscope are not given. For specific details, the reader is referred to the manufacturers' manuals. For example, when instructions to adjust the focus are provided, the user could perform this task manually, using computer control, or via a separate focus controller, all of which would accomplish the same task.

In this protocol, reference images of fluorescent beads are first captured. The bead images indicate proper system set-up and operation. In addition, the fluorescence values extracted from the beads are used to calibrate and normalize subsequently captured telomere fluorescence data. A metaphase cell on the sample slide is then manually selected and placed into the field of view of the camera. Next, the best focus image of the telomeres is captured. The corresponding chromosome image is then captured and these images are stored as a pair for analysis (see Support Protocol 2). In a typical experiment, ~15 metaphase cells from a sample are required.

SUPPORT PROTOCOL 1

**Cell Aging and
Death**

18.4.5

Materials

Immersion oil for fluorescence and general microscopy (NA = 1.5150 ±0.0002, Cargille Laboratories)

Sample slide of labeled telomeres and chromosomes (see the Basic Protocol)

Reference slide of fluorescent beads (see Support Protocol 3)

Fluorescence microscope (Zeiss Axioplan or equivalent) with the following components:

Excitation and emission filters to match the probes used (DAPI/CY3 dichroic mirror/emission filter set, Chroma Technology)

One high-quality objective lens (e.g., Plan Apochromat 63×/1.4, Zeiss or equivalent)

Stable fluorescent light source (e.g., 200 W hybrid mercury/xenon lamp, OptiQuip)

Fine focusing adjustment in ≤0.2-μm increments (e.g., piezo electric focus drive attached to the objective lens or fine focus control provided by the microscope)

Camera (e.g., MicroImager MI1400-12, Q-Imaging or equivalent) with the following characteristics:

Variable exposure times with a maximum time of at least 10 sec to capture weak fluorescence signals

At least 8 bits of photometric resolution (higher bit depth cameras generally are better as the upper 8 bits are less noisy and allow the user to select a sub-range of interest from the full range)

Small pixel size (~7 × 7-μm such that better localization of the small telomere spots can be made)

Large number of pixels (~1000 × 1000 such that the entire metaphase spread is captured within the camera's field of view)

No automatic gain control or the ability to disable this function (e.g., manual gain control)

Image acquisition software with the following features:

Visualization of the captured image on a monitor

Capture and storage of images in the proprietary “.IMG” format or the Windows Bitmap 8 bit/pixel “.BMP” format

Histogram option to display the intensity variation in the image (this feature will give an indication of the values of the minimum and maximum pixel intensity values that are present in the image)

Feature to store the raw image data from the camera (such that unmodified images captured under controlled conditions can be consistently and objectively analyzed later)

Feature to capture and display images in continuous mode (optional)

Feature to switch into “intensity enhancement” mode where low light levels are scaled to appear much brighter and is useful for focusing (optional)

Feature to switch into “binning” mode, an acquisition mode where images are captured at a faster rate (e.g., lower exposure times) but at a lower spatial resolution and is useful for focusing (optional)

CAUTION: Ensure that all automatic gain features are turned off when capturing data for image analysis. This includes the gain control setting on the camera. It also includes the “intensity enhancement”, “binning” modes, and other similar modes that are available on various imaging systems.

NOTE: Ensure that the immersion oil on the slide connects evenly with the objective as this will reduce the optical distortion of the captured and viewed images.

Set focus for reference beads

1. Turn on the fluorescence light source and camera.

These components need to warm up and become stabilized for ≥ 30 min prior to data acquisition. Other components in the system do not need a warm-up period and hence, these other components need only be powered on (if not already powered) just prior to performing the next step.

2. Place a reference slide of fluorescent beads (see Support Protocol 3) onto the fluorescence microscope and view this sample under the microscope with the 63 \times objective using the Cy3 excitation/emission filter set, and an ND3.0 neutral density filter (0.1% transmission) in the light path of the microscope.
3. Focus and capture an image of the beads.
4. Generate a histogram plot of the captured image and ensure that very few pixels, and preferably no pixels, are saturated (above the maximum intensity level of the camera).

In cameras that have defective pixels, make allowances for saturated defective pixels that arise with long camera exposure times. The optimum image should have maximum signal intensities at ~ 230 out of 256 gray levels. For some images, the optimum values could not be achieved without going to higher exposure times that are beyond the capability of the camera. As a result, the lack of sensitivity in the system compromises the precision of the system. If saturation occurs, reduce the camera exposure time, re-capture the image, generate the histogram and verify that the image is not saturated.

5. If there is no continuous acquisition or live capture mode available, repeat the process manually, capturing an image after each time the focus has been changed. Select the focus position that produces the best focus.

If the system supports continuous capture mode, adjust the focus of the object in small increments and observe the image on the monitor until the sharpest image is obtained. Repeat step 4 to ensure that the focused image has not crept into saturation as a result of better focus. If the system supports 'intensity enhancement' and 'binning' modes, use these options as this will speed up the acquisition process and thus will help to reduce the time to get the best focus image. However, remember to switch such modes off when the actual image is captured for analysis (to avoid creating erroneous images for analysis).

6. Capture and store the best focus image for later analysis. Record the exposure time used to capture the image and the mean fluorescence intensity of the captured image.

These numbers should be very similar from experiment to experiment. Hence, they may be used as rough indicators of changes and problems with the overall set-up of the system.

7. Remove the bead slide and place the sample slide on the microscope.

Capture telomere images

8. Switch to the DAPI filter set and find a metaphase cell of interest in the sample.
9. Switch to the Cy3 filter set and perform steps 4 and 5 above to obtain the best focus image of the telomeres for telomere analysis.
10. Capture and store the best focus image of the telomeres for later analysis.

If the software does not store the exposure time in the "IMG" file format, record the exposure time used and the file name of the stored image, as these values will be used later in the analysis program for normalization purposes. Alternatively, the exposure time can be fixed throughout the experiment and, thus, the exposure time only needs to be recorded once. The exposure time will differ between human and mouse telomeres and faint telomeres may require long exposure. As a result, the images acquired with a fixed exposure time may not be optimal. More importantly, there is a risk that some images may have saturated telomere values that will greatly compromise subsequent analysis (see Troubleshooting and Fig. 18.4.1).

- Switch to the DAPI filter set and perform steps 4 and 5 above to obtain the best focus image of the metaphase chromosome image for karyotype analysis.

The best focus is not as crucial for the chromosome images since these objects are larger and occupy a larger focal depth. As the microscope is adjusted for parfocality, the best focus level for the telomeres should be the same or at a fixed offset from the best focus level of the chromosomes.

- Capture and store the image of the chromosomes for later analysis. Choose a file name that is similar to that for the telomere image as these files will be entered as pairs into the image analysis software (e.g., image 001c.bmp for Cy3 stained telomeres and image 001d.bmp for DAPI stained chromosomes).

Only grayscale/8 bit images are accepted. TIFF files can be opened in Adobe Photoshop and changed to gray scale. The best way is to copy the image to clipboard and paste into TFL-Telo and save as .IMG file (TIFF file formats are currently not supported by TFL-Telo but will be supported in later versions of this analysis software program)

Some image acquisition software stores images in the TIFF or other proprietary file formats. In this instance, another utility program such as Adobe Photoshop will be needed to convert the combined TIFF file into individual telomere and chromosome BMP file formats.

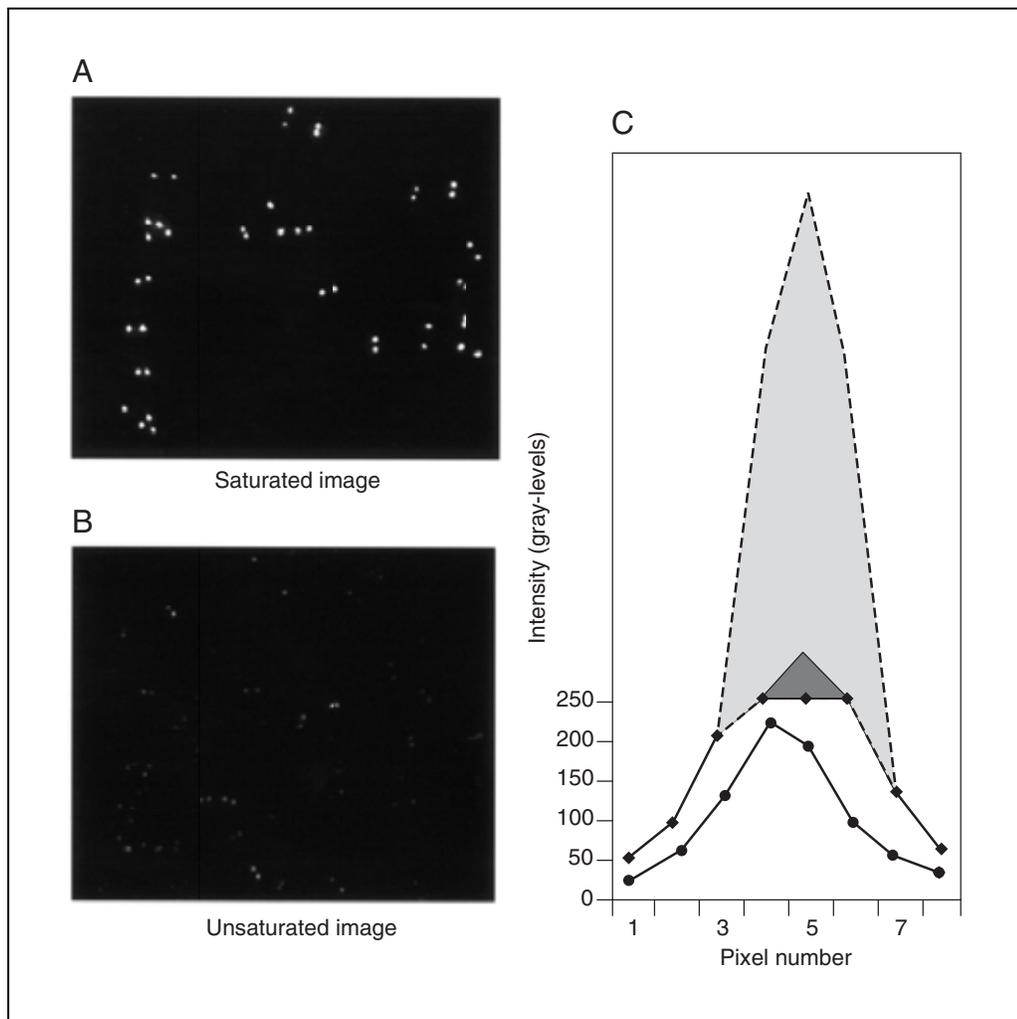


Figure 18.4.1 Intensity profile of saturated (A) and unsaturated (B) telomere signals. (C) There is no ambiguity in determining the total fluorescence of the unsaturated telomere signal (circles). However, there is uncertainty in the true fluorescence of the saturated signal (diamonds). Two possible estimates of the fluorescence signal (shaded regions) are shown.

13. Repeat steps 4 to 12 for ≥ 15 metaphase cells to obtain statistically relevant results for the sample.

The number of metaphases for statistical relevance will vary from sample to sample. Variations in cell preparation (see Basic Protocol) and the quality of the microscope and imaging system components will also reduce the sensitivity of the system. Such variations can only be partially compensated by an increase in the number of metaphases that are analyzed.

14. To verify that the system has not drifted over the course of the experiment, repeat steps 2 to 5 using the reference bead slide.

The fluorescence intensity of the beads should be similar to the values obtained earlier in the experiment and to the values from previous experiments.

IMAGE ANALYSIS

The following method uses the TFL-Telo image analysis software to measure the telomere values of individual chromosomes. A typical telomere/chromosome analysis session is shown in Figure 18.4.1. From the chromosome and telomere images, the program generates a combined image of the detected telomeres and chromosome boundaries superimposed onto the chromosome image. The user then interactively edits the image to correct for mistakes made in the segmentation (definition of the boundaries) of chromosomes, to karyotype the image, to assign and correct for the nomenclature of the telomeres on the p and q arms of the chromosome, and to split nearby telomeres on a specific arm of the chromosome into two. The results are saved into a formatted text file that can be imported into spreadsheets (e.g., Microsoft Excel) or statistical packages (e.g., Microsoft Origin) for analysis. Calibration/normalization of the results can then be performed using the reference bead image and the calibration parameters obtained from Support Protocol 3. To study the variation of the results and compare them to those of other metaphase cells, use a spreadsheet program to cut and paste the results into a combined results file.

Materials

- Images of telomeres (see Support Protocol 1)
- Corresponding images of chromosomes (see Support Protocol 1)
- TFL-Telo (telomere image analysis software, this software is available upon request from the authors: plandor@bccancer.bc.ca)
- Pentium-based personal computer with the following:
 - Minimum of 8 Mb of RAM
 - Graphics card with at least 256 colors and a display resolution of 640×480 pixels
 - High resolution video monitor to match the video card
 - Microsoft Windows 95, 98, or NT operating system

1. From the “Application Menu” in the TFL-Telo program, select the “Chromosome/Telomere” option.
2. First, enter the names of the files associated with the pair of chromosome (DAPI) and telomere images (Cy3) of the metaphase spread that is to be analyzed (i.e., the names assigned to the files stored from Support Protocol 1).

If the files are not stored in the “.IMG” file format, enter the corresponding exposure time used for the telomere image. There is a threshold column value in this window with a default setting of 5. The threshold can be increased or decreased as required (e.g., when telomere signals are too faint or too bright on particular chromosomes).

SUPPORT PROTOCOL 2

3. Finally, enter the name of the file to store the results.

The output file has to be in the ".IMG" format.

4. Double click on the "OK" button when all the fields for the file name have been entered. The program will then automatically determine the borders of the chromosomes and telomeres and calculate the total fluorescence value for each detected telomere in each chromosome.

Once the program finishes its processing, a combined image of the telomere and chromosome results are displayed on the screen (see Fig. 18.4.1). A different color is assigned to the border of each of the telomeres in the chromosome for ease of user recognition (purple, red, blue, cyan, orange, and green are assigned to telomere number 1, 2, 3, 4, 5, and 6, respectively, or more). Similarly, a different color border is used to indicate the number of "telomere" objects in the chromosome (cyan, green, and yellow are assigned to ≤ 3 , 4, and 5, respectively, or more objects). Each chromosome is also labeled with a unique number.

Once the processing has been performed on the telomere/chromosome pair, the processed results can be reloaded into the program. To reload, select the "Open" option from the "File" menu and then enter the file name of the processed results. The combined telomere and chromosome image of the processed results will then be displayed.

5. From the "Options" menu, select the "Chromosome Statistics" option.

A window will then appear showing a table with the following information: the label assigned to the chromosome number, karyotype number of the chromosome, up to five telomere values for the chromosome, and a number of other telomere and chromosome statistics.

6. Select the "Edit" option to open a submenu to edit the parameters for the selected chromosome such as the telomere belonging to the p or q arm of the chromosome and the karyotype number of the selected chromosome.
7. Select the "Save" option to save the changes made (changes are automatically saved in the latest version of the TFL-Telo software).
8. Find the mean fluorescence value of the beads. Perform system calibration by following Support Protocol 3, steps 6 to 8, for the fluorescent bead image and assign the mean value to Bead_{exp} .
9. To normalize the results, import the telomere fluorescence result from the output file with one of the following file extensions "*.LB2" or "*.LB3" (the last four columns are the telomere fluorescence values) into a spreadsheet program such as Microsoft Excel. Multiply the measured telomere fluorescence values using the following formula ($\text{slope}_{\text{cal}}$ and bead_{cal} are obtained from Support Protocol 3):

$$\text{length (base pairs)} = \text{measured fluorescence} \times \text{slope}_{\text{cal}} \times \text{bead}_{\text{exp}} / \text{bead}_{\text{cal}}$$

SUPPORT PROTOCOL 3

Quantitative Fluorescence In Situ Hybridization (Q-FISH)

18.4.10

SYSTEM CALIBRATION

The following method illustrates how data from different experiments can be normalized and how the telomere fluorescence measurements can be converted to telomere length estimates. While slides with fluorescent beads are used on a daily basis to calibrate the microscope, slides with hybridized plasmids are used occasionally to calibrate telomere fluorescence relative to telomere length. For the latter, plasmids (DNA with fixed numbers of telomeric repeat sequences) are used to verify and determine the linearity of the system using these protocols. In order to compensate for differences in illumination and other intensity variations from experiment to experiment, the total fluorescence measurements of fluorescent beads from each experiment is used for normalization purposes.

Materials

0.2- μ m fluorospheres carboxylate-modified microspheres, orange fluorescent (540/560, Molecular Probes)
1:1 (v/v) PBS/FCS
Antifade reagent in glycerol/PBS (Slow Fade Antifade kit, component A, Molecular Probes)
PSXneo plasmids containing fixed numbers of telomere repeats (Hanish et al., 1994; Martens et al., 1998)
22 \times 30-mm coverslips
Slide box
1.5-ml “sure lock” tubes (Rose Scientific)
Thermomixer (e.g., Eppendorf Model 5436) or 80°C water bath
Additional reagents and equipment for PNA probe hybridization (see Basic Protocol), image capture (see Support Protocol 1), and image analysis (see Support Protocol 2)

1. Prepare calibration slides with fluorescence microspheres. Dilute 0.2- μ m fluorospheres carboxylate-modified microspheres 25 \times in a 1:1 mixture of PBS/FCS. Deposit 2 to 3 μ l of microsphere suspension on to periphery of a 22 \times 30-mm coverslip. With a regular slide, produce a thin homogenous smear over the coverslip (like a blood smear). Let the coverslip dry. Keep coverslips in slide box until use.
2. Prepare a calibration slide on the day of acquisition by depositing 5 μ l of slow fade reagent on a slide and adding microsphere-coated coverslips.
Note the different antifade reagent. Vectashield is not optimal for the preparation of calibration slides with fluorescence beads.
3. Prepare calibration slides with fluorescence plasmids. Add PSXneo plasmid DNA to hybridization mixture. For example, add 1 μ l of plasmid DNA with 144 bp of telomere DNA at 0.1 μ g/ml in 10 mM Tris·Cl to 99 μ l of hybridization mixture in a 1.5-ml “sure lock” tube (see Basic Protocol).
4. Denature 10 min at 80°C in a thermomixer or water bath. Incubate 60 min at room temperature and put two 50- μ l drops on a precleaned slide.
5. Incubate 30 min in the dark; some of the plasmids hybridized with fluorescent PNA will noncovalently attach to the glass surface. Proceed with wash steps, alcohol dehydration as described in Basic Protocol, steps 28 to 32.

Calibrate Q-FISH image acquisition and analysis system

6. Set up and prepare the microscope and image acquisition system for image capture (see Support Protocol 1, step 1).
7. Capture and store an image of fluorescence beads (see Support Protocol 1, steps 2 to 6).
8. Capture and store images of plasmids that contain 144 bp of telomere repeats (see Support Protocol 1, steps 9 and 10).

Choose areas on the slide where the plasmids are spread out and not clumped. Adaptation to darkness is essential to see very faint spots of plasmids corresponding to a maximum of eight Cy3 molecules per plasmid. Ensure that a total of ≥ 50 to 100 plasmids are collected in one field. Too low a density of plasmids on the slides complicates acquisition and discrimination between plasmid and background fluorescence. Capture and store more images as required such that the desired minimum number of plasmids are acquired.

9. Repeat step 3 for the slides that contain plasmids with 400, 800, and 1600 bp of telomere repeats and capture the images.

Analyze the data

10. Execute the TFL-Telo program.
11. Select the “Open” option from the “File” menu to open a stored plasmid file.
12. Select the “Spot IOD” option from the “Application” menu.

The program will then automatically find the telomere spots and then calculate the total fluorescence values of each detected spot.

The calculated value needs to be normalized with the exposure time that was used to capture the image. If this value is entered with the image file, the program will make the calculation. Otherwise, divide the value obtained with the manually recorded exposure time for that image.

13. Select “Object Histogram” option from the “Option” menu to obtain the histogram of the fluorescence distribution of all detected objects. Obtain the mean fluorescence number of the telomeres by first rejecting the artifacts from the image as follows. First, point the mouse to the minimum fluorescence value of the objects in the histogram. Hold down the left mouse button and drag the mouse up the intensity scale of the histogram (i.e., towards the right). Release the button to select the maximum value of the intensity range. The selected range in the histogram will change color and the corresponding borders of the objects in the image will also change color. Repeat the range selection until the desired objects are highlighted (i.e., do not include debris or cluster objects). Record the mean fluorescence value for the selected range.
14. Repeat steps 11 to 13 for the other files containing images of plasmids that have different telomere insertion lengths.
15. Repeat steps 11 to 13 for the image of the fluorescent beads. Record the mean bead fluorescence value as bead_{cal} .

This value will be used later for calibrating the results (see Support Protocol 2).
16. Plot the telomere fluorescence values as a function of the number of base pairs of telomere repeats in the plasmid.
17. Find the slope of the plot. Record this value as $\text{slope}_{\text{cal}}$ for use in image analysis (see Support Protocol 2).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

BSA stock solution, 10%

Dissolve 10 g BSA in 100 ml water by gently placing BSA powder on top of the water. Use a magnetic stirrer to dissolve. Do not stir at high speed, slow hydration works best. Filter through 0.4- and 0.2- μm filters to remove bacteria and debris. Store up to several years at -20°C in 1-ml aliquots.

Formamide, deionized

Add 5 g of resin beads to 100 ml of ultrapure formamide, pH 7.0 to 7.5 (Life Technologies). Stir for 2 hr in a fume hood. Filter through Whatmann no. 1 filter paper. Aliquot in 1-ml portions and store at -20°C .

Hybridization mixture

For 100- μ l mix (5 slides):

70 μ l deionized formamide (see recipe)

10 μ l NEN blocking solution (see recipe)

5 μ l 0.1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*)

5 μ l 10 μ g/ml PNA Cy3-telomere probe (see recipe)

5 μ l MgCl₂ buffer (82 mM NaH₂PO₄/9 mM citric acid/20 mM MgCl₂, pH 7.4)

5 μ l distilled H₂O

Store up to a week at 4°C

Mounting medium

To 1 ml of Vectashield, add 1 μ l of DAPI stock solution (0.2 mg/ml in water) to give final concentration of 200 ng DAPI/ml of antifade. Dilute with Vectashield if DAPI staining is too bright and is visible in Cy3 fluorescence filter combination. Store up to a week at 4°C.

NEN blocking solution

Dissolve (layer) 2.5 g of NEN blocking reagent (PE Biosystems or Life Sciences) in 100 ml distilled water in a glass beaker. Stir gently overnight. Filter through 0.4- μ m filter and dispense into 3- to 4-ml aliquots. Store at -20°C.

PBS, 10 \times stock solution (pH 7.4)

Solution A:

16.02 g Na₂HPO₄·2H₂O

73.84 g NaCl

900 ml H₂O

Solution B:

2.76 g NaH₂PO₄·H₂O

16.56 g NaCl

200 ml H₂O

Adjust solution A to pH 7.4 with solution B. Store up to a year at room temperature.

Peptide nucleic acid probes

PNA probes can be synthesized manually or with dedicated equipment by chemists familiar with peptide and/or nucleic acid synthesis. Alternatively Cy3-labeled PNA can be ordered from DAKO as a kit (Telomere PNA FISH/Cy3) or from Boston Probes (www.bostonprobes.com) or Applied Biosystems as a custom order. The latter is the more economical because many hundreds of experiments can be done with just a few OD₂₆₀ units of labeled PNA.

Good results will be obtained with FITC or Cy3-OO-CCCTAACCCCTAACCCCTAA-carboxy-terminus. More information on how to order and use PNA probes can be found at the website of Applied Biosystems (<http://www.appliedbiosystems.com/ds/pna>).

Wash solution I

For 500 ml:

5 ml 1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*, 10 mM final)

350 ml deionized formamide (see recipe, 70% v/v final)

5 ml 10% BSA stock solution (see recipe, 0.1% w/v final)

140 ml H₂O

Check pH and adjust to pH 7.4, if necessary, with 1 M NaOH or 1 M HCl

Store up to a week at 4°C

Wash solution II

For 500 ml:

50 ml 1 M Tris·Cl, pH 7.4 (*APPENDIX 2A*, 0.1 M final)

50 ml 1.5 M NaCl (0.15 M final)

0.5 ml Tween 20 (0.1 % v/v final)

400 ml H₂O

Store up to a week at 4°C.

COMMENTARY

Background Information

PNA probe hybridization

Telomeres are specialized structures at the ends of chromosomes consisting of repetitive DNA and associated proteins (Blackburn, 1994; Zakian, 1995) that are maintained by the reverse transcriptase enzyme telomerase (Greider, 1995). In most somatic cells, telomere repeats are lost with each cell division and with age. Because telomere loss has been shown to limit the replicative life span of cells, the loss of telomere repeats is believed to be important in the aging of tissues with continuous cell turnover. Progressive telomere shortening has, furthermore, been shown to result in chromosome fusions and genetic instability (Blasco et al., 1997). This is increasingly recognized as an important mechanism contributing to malignant progression and the “mutator” phenotype of cancer cells (Wan et al., 1997; Artandi et al., 2000). Questions about the role of telomere shortening in aging and cancer have encouraged the development of novel techniques that can provide information about the length of telomere repeats present in individual chromosomes (Q-FISH; Lansdorp et al., 1996; Zijlmans et al., 1997; Martens et al., 1998) and in individual cells (flow FISH; Rufer et al., 1998, 1999). Such information cannot be obtained by the standard Southern blotting method to measure telomere length because it requires genomic DNA typically isolated from $>10^5$ cells (reviewed in de Lange, 1995).

Fluorescence in situ hybridization (FISH) with PNA probes has some distinct differences with the more established FISH procedures that employ DNA or RNA probes. The major differences are the inclusion of protein in the hybridization mixture to prevent nonspecific binding of the PNA probes and the use of very stringent hybridization conditions that are unsuitable for annealing of DNA or RNA probes to DNA target sequences. The denaturation of chromosomal DNA in the presence of the PNA

probe simplifies the FISH procedure. Because hybridization of PNA probes to specific target sequences is fast and specific, the procedure is furthermore fast relative to conventional FISH procedures. The major advantage of Q-FISH with PNA probes over conventional FISH procedures with conventional telomeric DNA probes is that Q-FISH allows extraction of quantitative information. Such information is much more difficult to obtain using conventional FISH procedures because in conventional FISH procedures the hybridization of probes competes with the renaturation of complementary strands of the denatured genomic DNA. This competition inevitably introduces a stochastic component in conventional FISH experiments with the eventual probe signal depending on the concentration and size of the DNA or RNA probe, the efficiency of the denaturation of the genomic DNA, and the repetitive nature of DNA target sequences. The conditions that favor binding of small oligonucleotide probes to highly repetitive telomeric DNA, unfortunately, also result in efficient renaturation of genomic DNA. Even with multiple rounds of immunological amplification the net result of conventional hybridization for telomere targets is a highly variable signal on some but not all telomeres (Moyzis et al., 1988). Even to the inexperienced, the difference in telomere fluorescence obtained with indirect conventional labeling procedures using small RNA or DNA telomere probes and directly labeled PNA probes will be striking. The use of Q-FISH for quantitative purposes is supported by the overall good correlation between fluorescence from sister chromatids, the good correlation between telomere length data obtained by Southern blotting analysis, and Q-FISH analysis, and the analysis of plasmids with defined T₂AG₃ content (Lansdorp et al., 1996; Martens et al., 1998). While telomere repeats are ideal for use with PNA probes (little or no sequence divergence), other repeat sequences such as chromosome-specific alphoid

repeat sequences, can also be detected efficiently using selected 15- to 18-mer PNA probes (Taneja et al., 2000).

Image capture

Image acquisition and analysis systems have been around for a number of decades (Young and Roberts, 1951; Walton, 1952). However, it was not until the last decade that high-resolution, variable-exposure-time cameras were commercially available; these cameras can detect and measure weak fluorescent telomere signals (Poon and Hunter, 1994). The increased use of FISH analysis has also led to a number of improvements to the fluorescence microscope, such as more efficient and wavelength-specific excitation and emission filters, better quality objective lenses for fluorescence detection, and better and more precise fine focus controls. While, in particular, the light source of standard fluorescence microscopes remains problematic, all these improvements have brought quantitative analysis from capture images of telomeres hybridized to directly labeled PNA probes within reach of dedicated experimentalists. It is expected that further improvements will make quantitative analysis a standard feature of future fluorescence microscope and imaging systems.

Image analysis

Interest in telomere length measurements has led to incorporation of a telomere analysis feature in the current version of a commercial chromosome analysis package (Metasystems V2.0). The automation of karyotyping by this software would greatly reduce the time required to identify chromosomes and assign telomere fluorescence values to specific chromosomes. Although, the accuracy of telomere analysis in this software needs to be validated, there is an option to export the karyotype results and link it with the telomere measurements obtained from the TFL-Telo program. A description of the algorithms used in the TFL-Telo program is presented elsewhere (Poon et al., 1999a,b).

Another new development for estimating the average telomere length in single cells in a sample is flow-FISH. This method uses the speed and power of the flow cytometer to quickly measure the total intensity of the fluorescent telomere probes in the cell. All these methods have specific advantages and disadvantages in studies of telomerase/telomere biology.

System calibration

Data normalization and calibration are the best means of ensuring that data from one experiment can be correlated to that of another. As the objective of the study is to measure telomere lengths, a good choice of test objects to use on the system would be those where the telomere lengths are known. Plasmids containing telomere inserts are good candidates as the telomere length inside the circular DNA sequence structure does not change as a function of cell division. Hence, these test objects can be duplicated for calibration purposes. As there can only be a limited number of telomeres that can be inserted to a plasmid, calibration points for long telomere lengths are extrapolated from the linear results of fluorescence intensities from different smaller-length telomeres. The smallest size insert also gives an indication of the telomere detection limit of the system. While the use of hybridized plasmids will allow conversion of arbitrary fluorescence units to telomere fluorescence units (TFU, with each unit corresponding to 1 kb of telomere repeats), comparisons of telomere length measurements by Southern blot analysis and Q-FISH are required to further validate the use of Q-FISH for telomere length measurements.

Critical Parameters

PNA probe hybridization

A number of issues in the Q-FISH protocol deserve careful consideration and attention. In general, it is recommended to include controls such as a slide with nicely fixed metaphase cells and a slide, done in a previous experiment, with good hybridization signals. These controls will help to identify common errors with metaphase preparation and/or microscope set-up. Sometimes it is difficult to obtain sufficient good quality metaphase spreads from cells. If this is the case, continuing with the hybridization procedure will not produce satisfactory results. Instead, it is probably a better idea to increase the yield of dividing cells and improve the metaphase preparation procedure. There are many tricks of the trade of a cytogeneticist, and there is undoubtedly an aspect of art to the preparation of good metaphase spreads. For example, for a discussion of the synchronization of cell cultures to increase the mitotic index or the climate-dependency of optimal results, the reader is referred to cytogenetic protocols for chromosome preparations. A visit to the local cytogenetic department may greatly help to resolve problems with metaphase prepara-

tions (one may even obtain some slides to do the first Q-FISH experiment).

The following issues are important if slides with satisfactory metaphase spreads have been obtained.

Prior to hybridization, slides with fixed cells should be dried extensively. Drying overnight in a fume hood produces satisfactory results. Shorter periods of drying (i.e., on a 65°C plate) may work, but insufficient dehydration (i.e., for 1 hr at room temperature) increases the nonspecific binding of the PNA probe.

The pepsin treatment can be omitted, but the signals at telomeres will be more variable.

The wash steps with formamide after hybridization can be replaced by washing for 5 min with wash solution II at 65°C. However, for optimal telomere signal and chromosome morphology, the authors recommend washes

with wash solution I as described in the protocol.

Before adding antifade with DAPI, ensure that slides are completely dry again.

Image capture

The microscope set-up and sample preparation play a key role in the success of the experiment. It is important that the illumination over the field of interest (i.e., the metaphase spread) is constant. Otherwise, the fluorescence measurements taken from one area of the image will not be the same as those taken from another area. A slide of fluorescent beads or a plastic fluorescent slide could be used to check on the homogeneity of illumination over the field of view. Use objective features in the acquisition software (such as histograms) to measure the fluorescence intensity distribution over the

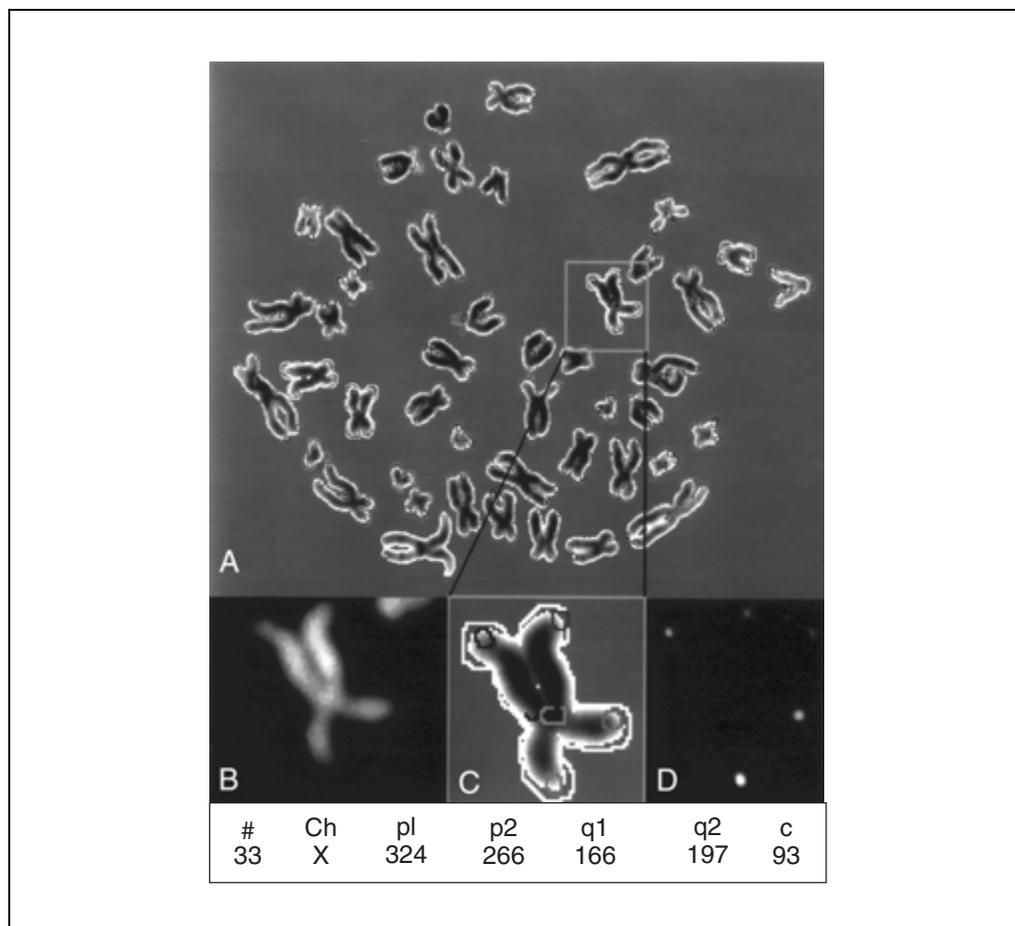


Figure 18.4.2 Output of TFL-Telo telomere length analysis software program. From imported image files the program generates an output image file shown in (A) in which chromosomes are given an arbitrary number. Telomere fluorescence values corresponding to that chromosome are calculated by the program and provided in a table. After editing and karyotyping by the user, the table provides information on the telomere fluorescence values for the two telomeres at the short (p) and long (q) arm of each selected chromosome (Ch X in the figure). The fluorescence intensity at the centromere is also calculated (c in the table). (B) A magnified view of a selected chromosome of the raw DAPI image file, (C) the output of the TFL-Telo program, and (D) the raw telomere fluorescence image are shown.

field of view rather than relying on subjective visual judgment. In instances where the light intensity varies significantly (e.g., >5%) between pixels, an appropriate correction of fluorescence values will increase the accuracy of results. Use flat-field correction in which all fluorescence pixel values are multiplied by a factor depending on the pixel location in the field of view that corrects for uneven illumination of the field.

The sensitivity of the acquisition system to detect the probes that are used has a major impact on the outcome of the experiment. Hence, it is worth the initial effort to experiment and choose the best components to use before starting experiments. For example, some objective lenses allow much more Cy3 fluorescence to pass through than other similar magnification lenses. The spectrum of the excitation and emission filters also plays a key role in the intensity of the observed fluorescence and the amount of interference or overlap of fluorescence from other wavelengths. The sensitivity of the camera and size of the camera pixels also affect the precision of the results obtained. Image fluorescence intensities, which span the 256-level intensity map of the camera, would give higher precision results than if the intensities were hovered around the lower levels. Similarly, smaller size pixels would give higher precision results than larger pixels as the area of the telomere can be defined better.

The focus of the telomeres also has an effect on the precision of the fluorescence measurements. Generally, calculations of total probe fluorescence can be made over a larger area that encompasses the probe, and hence focus variations do not play a major factor. However, telomeres can lie very close to each other, and as a result the signals will interfere with its neighbors' if they are out-of-focus. The TFL-Telo software has been written to identify the area occupied by an individual telomere and distinguish this from other nearby telomeres. A typical output of the TFL-Telo program is shown in Figure 18.4.2. As the algorithm used is focus-sensitive, the objects captured should be within 0.1- μm from best focus. Otherwise, the values obtained would be significantly lower than expected (see Fig. 18.4.3).

Photobleaching is another important aspect to consider in quantitative fluorescence microscopy. Antibleaching agents are often used to minimize the fading effects of probes used. If a sample shows significant photobleaching, a method will need to be incorporated to determine the amount of photobleaching so that the results generated can be correlated or compared with that of other cells. This will add to the complexity of the experiment since an estimate of how long the cells have been exposed to light will need to be determined, and extra care is needed to keep track of the locations on the slide that have been previously exposed to light. To avoid this complexity, it is better to experiment

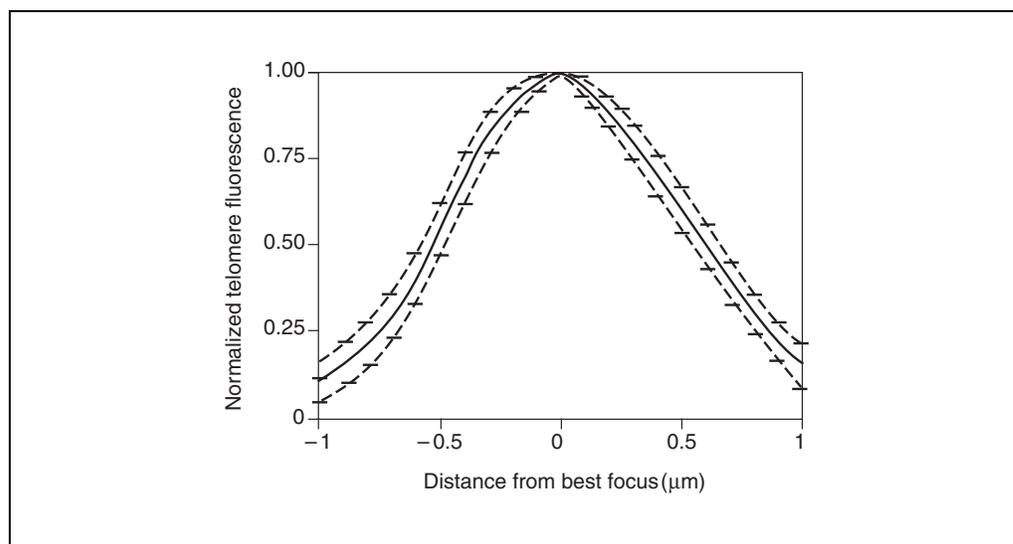


Figure 18.4.3 Variation of measured telomere fluorescence as a function of focus. In this experiment, the focus was varied at precise intervals using a piezzo electric drive connected to the objective lens. The telomere images were acquired and telomere fluorescence values calculated using the TFL-Telo program. Shown is the average (\pm s.d.) telomere fluorescence of all telomeres in the image ($n = 184$). Note that changing the focus by 0.5- μm results in a drop of calculated telomere fluorescence of up to 50%.

with the intensity of the light source and with different antifade agents. For example, the introduction of PNA probes tagged with different fluorescence dyes such as Cy5 that are specific for chromosome-specific alphoid repeats at centromeres may require a different antifade agent. Hence, experimentation with different antifade agents is needed when new sample preparation procedures are introduced. Reducing the intensity of the illumination (i.e., controlling the power applied to the lamp or placing a neutral density filter in the light path) is another method to reduce the effects of photobleaching. Although longer exposure times will be required, the results will typically be less dependent on the timing of the image capture. Closing the shutter between image captures is another good approach to reduce the effects of photobleaching.

The effects of photobleaching, light source stability, and system variability can be checked by repeatedly capturing images of the same metaphase (e.g., every 15 to 30 sec) over a 10-min interval. During this experiment, check and ensure that the focus of the metaphase does not drift over time. The images are then analyzed and the total fluorescence values from a number of selected telomeres are plotted as a function of time (Fig. 18.4.4). The slope of the graph gives an indication of the amount of photobleaching in the sample. The variability between adjacent measurements gives an indication of the amount of variability in the sys-

tem. As a general rule, photobleaching (slope of plot) should be <5% of the total or average intensity over a 5-min working interval. In addition, the amount of variation in fluorescence intensity between consecutive captured images should be <10%. With experience, the acquisition time will typically decrease resulting in reduced photobleaching and improved data.

Troubleshooting

PNA probe hybridization

Poor morphology of chromosomes. The denaturation time and temperature are critical and may vary to some extent between cells from different tissues or between cell lines. Denaturation at too high a temperature or for >3 min will result in blurred chromosomes and poor morphology. Too low a temperature or too short a denaturation step will result in nice DAPI staining of chromosomes but poor telomere fluorescence signals. Consider reducing the amount of pepsin or the duration of the pepsin treatment for improved morphology. Do not skip fixation steps before or after pepsin treatment.

Poor signals. Include pepsin treatment of cells. Increase temperature and/or duration of denaturation step until morphology is compromised. Decrease salt concentration in hybridization mixture. Decrease time or temperature

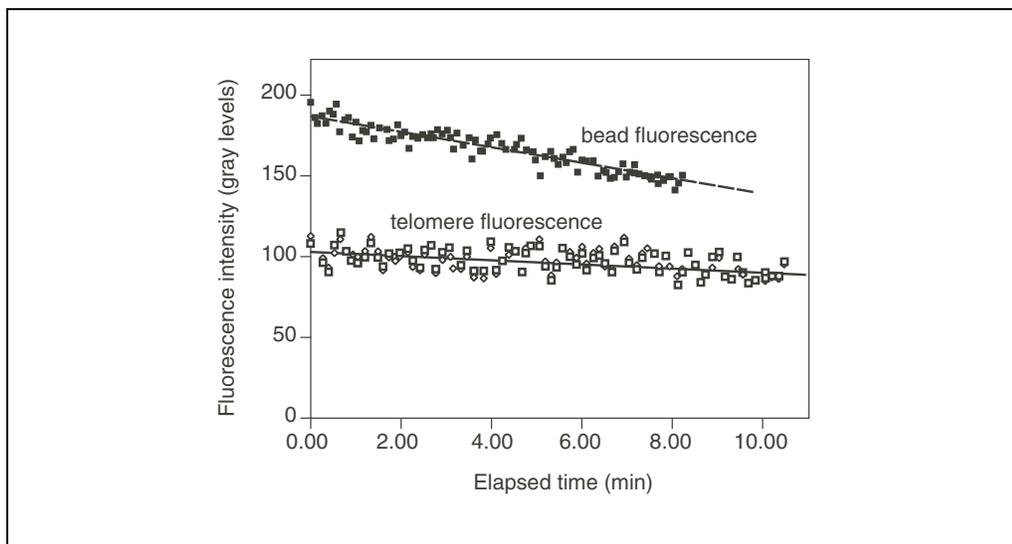


Figure 18.4.4 Photobleaching of fluorescent calibration beads and actual telomere fluorescence spots following PNA FISH and slide preparation as described in the text. Note that photobleaching of calibration beads (acquired at 0.1% of standard illumination) exceeds that of telomeres acquired at full light intensity and that photobleaching of telomere fluorescence over a 5-min period is <10%. The more pronounced variation in fluorescence values of beads and telomeres between sequentially acquired image files almost certainly reflects variation in the output of the light source.

of high-stringency wash with 70% formamide post hybridization.

High background PNA binding. If binding is localized to cytoplasm, increase number of fixation steps with methanol/acetic acid fixative when preparing fixed metaphase cells. If it is over the whole slide area, use different blocking protein or change concentrations of blocking protein in hybridization mixture. Insufficient drying of slides prior to denaturation will also increase background PNA fluorescence.

Image capture

As mentioned earlier, capturing an image of fluorescent beads at the beginning of every acquisition session helps to give an indication of the system setup. If the observed fluorescence intensity of the calibration beads differs significantly from the previous experiment (different intensity or similar intensity but at

different exposure times), the change in the way the system is set up should be identified and corrected. If possible, confirm that the measured values with calibration beads correspond to the values obtained at the previous acquisition session.

If the telomere distribution plot of a sample appears to be skewed to the right of a normal distribution, check the raw image data for saturated pixels. Saturated images are excellent image candidates for the traditional spot counting as all telomeres can readily be seen (Fig. 18.4.5). However, telomere lengths are underestimated in saturated images giving rise to a large number of telomeres with fluorescence values clustered near the upper portion of the telomere length distribution. As illustrated in Figure 18.4.5, the underestimate in telomere length can be a few percent to a few hundred percent of the estimated fluorescence value.

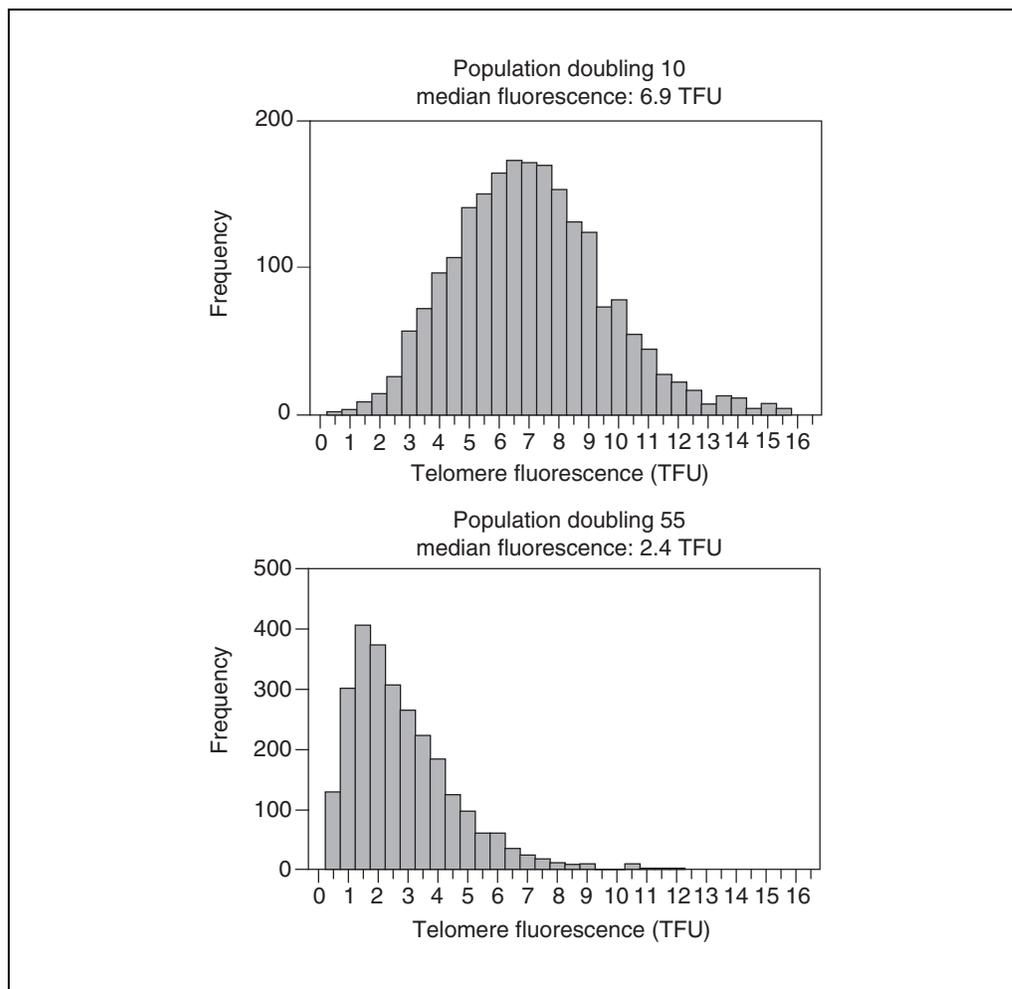


Figure 18.4.5 Accumulation of short telomeres in fibroblasts prior to replicative senescence. Note that the telomere fluorescence in early passage normal human fibroblasts shows a relatively normal distribution whereas at population doubling 55 (close to the Hayflick limit for these cells) the distribution is highly skewed towards the left. These observations indicate that a minimal number of telomere repeats need to be present on all chromosome ends in order for a cell to enter mitosis.

System calibration

The measured fluorescence values should be linearly related to the telomere length of the object. If the plot (see Support Protocol 3, step 12) does not generate a linear relationship, repeat the experiment to ensure that errors were not introduced in the sample preparation and image acquisition steps. For lower-sensitivity systems (microscope, camera), measurements of the smaller-size plasmids (e.g., 144 bp) may not be reliable, and hence this data point should be ignored from the determination of the slope used for calibration. Check also that the exposure time has been incorporated into the fluorescence measurement calculations.

Anticipated Results

PNA probe hybridization

All telomeres in normal human cells should easily be detectable. The signals from sister chromatids should be similar in intensity, and fluorescence of the telomere probe should be restricted to chromosome ends (sometimes a faint spot on human chromosome 2 identifies the area where two short primate chromosomes are fused to give rise to human chromosome 2 during evolution (Ijdo et al., 1991). Chromosomes should be easily identified using DAPI staining. No DAPI staining should be visible when analyzing telomere fluorescence.

Image capture

A typical experiment would give results that show the telomere fluorescence values of individual chromosomes. Figure 18.4.2 shows a raw DAPI image of chromosomes (B), a raw image of corresponding telomere spots (D), the image produced by the TFL-Telo program using the two raw image files (A,C), and the calculated telomere fluorescence of the p and q arms of the selected chromosomes in the metaphase as an example of the tabulated output of the TFL-Telo program.

Image analysis

Typical telomere length distributions of young and old cells are shown in Figure 18.5.5. These distributions resemble those obtained with the Southern blot technique (Oexle, 1998). Studies have been performed to show that telomeres on certain chromosomes are consistently shorter than others (Martens et al., 1998), and that there is a large variation in telomere lengths in mouse cells (Zijlmans et al., 1997). Other examples of telomere length studies using this protocol can be found in the literature (Blasco et al., 1997; Zhu et

al., 1998; d'Adda di Fagagna et al., 1999; Hande et al., 1999a,b; Surralles et al., 1999; Wan et al., 1999; Martens et al., 2000; Niida et al., 2000).

Time Considerations

PNA probe hybridization

The time for the Colcemid treatment for different cell types can vary from 30 min to 24 hr. After the treatment, the preparation of metaphase chromosomes typically takes 2 to 3 hr. The Q-FISH protocol, on the next day, typically takes 5 to 6 hr to perform.

Image capture

The time to capture images also varies from user to user depending on the density of metaphase cells on the slide and one's experience in finding suitable metaphase spreads, focusing objects, as well as other aspects. Typically, after 30 min of initial setup, it takes ~1 hr to capture 15 metaphases on a slide with good quality metaphase spreads.

Image analysis

The time to analyze the data also varies from user to user as well as with the quality of the metaphases. The TFL-Telo program takes <1 min to extract the telomere length measurements (depending on the CPU speed and RAM of the computer). Editing the results can take up to 30 min per metaphase. If karyotyping is to be performed, then up to 1 hr can be added to the time for each metaphase.

System calibration

As mentioned earlier, telomere length measurements of plasmids need not be performed on a daily basis with each experiment. It takes several hours to prepare the plasmid slides, 1 to 2 hr to capture the images, and ~1 hr to extract the results.

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Analysis of Mitochondrial Dysfunction During Cell Death

For many years mitochondria were considered only as a main energy supplier in the cell. In the middle of the 1990s it became clear that mitochondria actively participate in apoptosis, a form of cell death characterized by specific biochemical and morphological changes.

The following protocols represent basic tools widely used in estimating functional activity of mitochondria. The protocols are organized into three groups. The first group describes direct assessment of mitochondria in apoptotic cells—collection of proteins released from isolated mitochondria (see Basic Protocol 1), and from mitochondria within the cell (see Basic Protocol 2), immunoblot analysis of released proteins (see Basic Protocol 3), assessment of the mitochondrial membrane potential in apoptotic cells stained with TMRE (see Basic Protocol 4), and microscopy of apoptotic cells stained with MitoTracker Red (see Basic Protocol 5). The second group of protocols describes measurement of the mitochondrial permeability transition (MPT)—measurement of Ca^{2+} fluxes with an electrode (see Basic Protocol 6) or spectrophotometrically (see Alternate Protocol 1), measurement of the mitochondrial membrane potential with a TPP^+ electrode (see Basic Protocol 7) or spectrophotometrically (see Alternate Protocol 2), estimation of mitochondrial swelling (see Basic Protocol 8), and measuring Ca^{2+} accumulation in permeabilized cells (see Basic Protocol 9). Support Protocols are included for the preparation of mitochondria from a number of sources—liver (see Support Protocol 1), brain (see Support Protocol 2), and cultured cells (see Support Protocol 3)—and for assessing the quality of the prepared mitochondria (see Support Protocol 4).

EVALUATION OF THE RELEASE OF PROTEINS FROM INTERMEMBRANE SPACE OF MITOCHONDRIA

The release of certain proteins from mitochondria in apoptotic cells is believed to be one of the key events in apoptosis. Therefore, estimation of the release of these proteins is important for understanding the role of mitochondria in apoptosis.

Collecting Samples of Proteins From Isolated Mitochondria

This method is based on the estimation of the amount of cytochrome *c* or any other protein released from the intermembrane space of mitochondria that remains in the incubation buffer after the mitochondria are removed by centrifugation. This protocol describes collecting samples of proteins released from isolated mitochondria incubated under different experimental conditions. Such experiments are very helpful in order to estimate a possible effect of certain pro-apoptotic compounds on mitochondria.

Materials

Isolated mitochondria (Support Protocols 1 to 3)
Buffer

1. Incubate mitochondria at a concentration of 1 mg protein/ml (time, conditions of incubation, and buffer to be used depend on the aim of the experiment).

*This buffer can be used for mitochondria isolated from different sources. The composition of the buffer is determined mainly by the aims of the experiment (see also Background Information section, MPT-dependent release of cytochrome *c*). For example, estimation of the release of cytochrome *c* is more suitable to perform in KCl-based ionic buffer, since the*

BASIC PROTOCOL 1

Cellular Aging and Death

18.5.1

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binding of cytochrome c to the inner mitochondrial membrane is significantly weaker as compared to mannitol-sucrose buffer. If the aim of the experiment is to assess MPT-independent release of cytochrome c, a calcium chelator EGTA, or cyclosporin A should be included.

2. Take a 200- μ l aliquot and transfer to a microcentrifuge tube.
3. Microcentrifuge 5 min at $10,000 \times g$, 4°C .
4. Gently remove the supernatant without disturbing the pellet.
5. Resuspend the pellet in the original volume of the same buffer used for the incubation in step 1.
6. Freeze samples of supernatant (step 4) and pellet (step 5) and keep them at -20°C .

The samples are now ready for detection, e.g., of cytochrome c via immunoblotting (see Basic Protocol 3).

BASIC PROTOCOL 2

Evaluation of Cytochrome c Release from the Mitochondria of Apoptotic Cells

In order to analyze the release of certain proteins from mitochondria of apoptotic cells, the cellular plasma membrane should be disrupted and cytosolic fraction should be separated from membrane material. This can be achieved by preincubation of cells in a hypotonic solution that induces cell lysis.

Materials

Cells of interest (e.g., Jurkat cells, U 937, HeLa)
Apoptotic stimuli (e.g., etoposide, staurosporine)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice-cold
S100 buffer (see recipe), ice-cold
Refrigerated low-speed centrifuge and ultracentrifuge
Additional reagents and equipment for immunoblot analysis (*UNIT 6.2* and Basic Protocol 3 in this unit)

1. Incubate cells with apoptotic stimuli (type, concentration, and incubation time determined by cell type).
2. Wash cells twice, each time with 5 ml of ice-cold PBS.
3. Resuspend cells at 75×10^6 cells/ml in ice-cold S100 buffer.
4. Incubate on ice for 10 to 20 min.
5. Centrifuge cells 15 min at $10,000 \times g$, 4°C .
6. Transfer supernatant to ultracentrifuge tube and ultracentrifuge 1 hr at $100,000 \times g$, 4°C .
7. Use supernatant for immunoblot analysis (see Basic Protocol 3 and *UNIT 6.2*) with an antibody specific for cytochrome c.

BASIC PROTOCOL 3

Analysis of Mitochondrial Dysfunction During Cell Death

18.5.2

Immunoblot Analysis of Proteins Released from the Mitochondria During Apoptosis

After separation as in Basic Protocol 1 or 2, the supernatant contains proteins released from mitochondria, while the pellet represents proteins associated with the organelles. The detection of these proteins is performed using electrophoresis with subsequent blotting and probing with specific antibodies.

Materials

Sample collected from apoptotic mitochondria (see Basic Protocol 1 or 2)
4× Laemmli's loading buffer (see recipe)
15% (w/v) SDS-PAGE gel (UNIT 6.1)
5% (w/v) nonfat milk in PBS (see APPENDIX 2A for PBS)
Antibody specific for apoptotic protein of interest (e.g., Becton Dickinson Biosciences)
PBS (APPENDIX 2A) containing 2.5% (w/v)
Nonfat dry milk
Phosphate-buffered saline (PBS; APPENDIX 2A) containing 1% (w/v) bovine serum albumin and 0.01% (w/v) azide (NaN₃)
PBS (APPENDIX 2A) containing 15% (v/v) Tween 20
Horseradish peroxidase–conjugated secondary antibody (e.g., Pierce)
ECL Western Blotting Detection Reagents kit (Amersham Biosciences)
Additional reagents and equipment for SDS-PAGE (UNIT 6.1) and electroblotting (UNIT 6.2)

Perform electrophoresis

1. Mix 40 µl of sample with 12.5 µl of 4× Laemmli's loading buffer. Boil 5 min.
2. Separate the proteins on a 15% SDS-PAGE gel at 130 V (UNIT 6.1).

Perform immunoblotting and detect apoptotic proteins

3. Transfer proteins to nitrocellulose membranes by electroblotting (Basic Protocol 1 in UNIT 6.2) for 2 hr at 100 V.
4. Block membranes for 1 hr with 5% nonfat milk in PBS at room temperature.
5. Probe overnight in a cold room with an antibody (diluted 1:2500 in PBS containing bovine serum albumin and 0.01% NaN₃) specific for the apoptotic protein of interest.
6. Rinse membranes for 10 min in 30 to 40 ml of PBS.
7. Rinse membranes for 15 min in 30 to 40 ml of PBS with 0.15% Tween 20.
8. Rinse membranes for 10 min in 30 to 40 ml of PBS.
9. Incubate with a horseradish peroxidase–conjugated secondary antibody (diluted 1:10,000 in PBS containing 2.5% nonfat dry milk).

Visualize bound antibodies

10. Following incubation with the secondary antibody, rinse the membranes for 10 min in 30 to 40 ml of PBS.
11. Rinse membranes for 15 min in 30 to 40 ml of PBS with 0.15% Tween 20.
12. Rinse membranes for 10 min in 30 to 40 ml of PBS.
13. Detect bound antibodies using enhanced chemiluminescence (ECL kit) according to the manufacturer's instructions.

Assessment of the Mitochondrial Membrane Potential in Apoptotic Cells

The mitochondrial membrane potential ($\Delta\psi$), which drives oxidative phosphorylation (Mitchell and Moyle, 1967) and which also drives mitochondrial calcium uptake, is generated by an electron-transporting chain. When electron transport ceases, for example during ischemia, the inner-membrane potential is developed at the expense of ATP

**BASIC
PROTOCOL 4**

**Cellular Aging
and Death**

18.5.3

hydrolysis by the mitochondrial ATP synthase. An early manifestation of apoptosis is often a decrease in the $\Delta\psi$, which is usually demonstrated using flow cytometry. The relationship between mitochondrial depolarization and apoptosis remains controversial. Some investigators consider a decrease in $\Delta\psi$ an early irreversible signal for apoptosis (Zamzami et al., 1996), while others describe it as a late event (Bossy-Wetzel et al., 1998).

Materials

Cells of interest (e.g., Jurkat cells, U 937, HeLa)
RPMI-1640 medium (Life Technologies) supplemented with 5% (v/v)
heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml),
and streptomycin (100 μ g/ml)
25 mM TMRE stock solution: dissolve 12.8 mg tetramethylrhodamine methyl
ester (TMRE; Molecular Probes) in 1 ml ethanol; store per manufacturer's
instructions
HEPES buffer (see recipe)
Flow cytometer (e.g., FACS; Becton Dickinson)

1. Prepare an aliquot of 10^6 cells in RPMI-1640 medium.
2. Dilute TMRE stock solution 1:1000 with HEPES buffer (for a concentration of 25 μ M).
3. Add an aliquot of the diluted (25 μ M) TMRE to cells for a final concentration of 25 nM.
4. Incubate cells with TMRE 20 min at 37°C.
5. Further dilute the 25 μ M TMRE 1:1000 with HEPES buffer for a final concentration of 25 nM. Centrifuge cells 5 min at $200\times g$, room temperature, and resuspend in fresh HEPES buffer containing 25 nM TMRE.
6. Analyze membrane potential by flow cytometry according to the manufacturer's instructions for the instrument used.

BASIC PROTOCOL 5

Microscopy of Apoptotic Cells Stained with MitoTracker

Fluorescent probes for mitochondria such as rhodamine 123 or tetramethylrhodamine are readily sequestered by mitochondria. However, in experiments that require fixation of the organelles, these stains are easily washed out. In contrast, mitochondria-selective probes—e.g., MitoTracker Red (Molecular Probes)—are concentrated by active, energized mitochondria and retained during fixation. Once mitochondria are labeled, the cells can be fixed for further processing, for example, if cells are going to be subsequently labeled with antibody.

Materials

Cells of interest (e.g., Jurkat cells, U 937, HeLa) and appropriate culture medium
MitoTracker Red (Molecular Probes); store according the manufacturer's
instructions
Dimethylsulfoxide (DMSO)
4% (w/v) paraformaldehyde in serum-free culture medium
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
PBS (*APPENDIX 2A*) containing 0.2% (v/v) Triton X-100
PBS (*APPENDIX 2A*) containing 1% (v/v) fetal bovine serum (FBS)
Mounting medium: 50% (w/v) glycerol/PBS
Fluorescent or scanning confocal microscope (e.g., Bio-Rad), with 581 nm
excitation filter and 644 nm emission filter
Additional reagents and equipment for cell culture (*UNIT 1.1*)

1. Grow cells on coverslips inside a petri dish filled with the appropriate culture medium to the optimal density.

The optimal cell density depends on the cell type.

2. Open fresh vial of MitoTracker Red and add DMSO to create a 1 mM stock. Protect from light.
3. Dilute 1 mM MitoTracker Red to final working concentration in the culture medium used to grow the cells.

Recommended Mito Tracker Red concentration is 100 to 500 nM. Diluted MitoTracker Red can be stored up to several months at -20°C

4. When cells have reached the desired confluence, remove the medium from the dish and add the prewarmed (37°) growth medium containing MitoTracker probe. Incubate for 30 min.
5. Replace the loading solution with fresh prewarmed medium.

Fix and permeabilize cells

6. Remove the growth medium covering the cells and replace it with fresh prewarmed medium containing 4% paraformaldehyde. Incubate 15 min at 37°C .
7. Rinse cells three times, each time for 5 min with room temperature PBS.
8. Incubate fixed cells in PBS containing 0.2% Triton X-100 at room temperature for 5 min.
9. Rinse cells three times, each time for 5 min with PBS containing 1% FBS.
10. Mount coverslips on glass slides in 50% glycerol/PBS and examine under fluorescent or scanning confocal microscope with excitation at 581 nm and emission at 644 nm.

ASSESSMENT OF THE MITOCHONDRIAL PERMEABILITY TRANSITION IN ISOLATED MITOCHONDRIA

The mitochondrial permeability transition (MPT) is one of the mechanisms that can lead to the release of cytochrome *c* from mitochondria during apoptosis. MPT is a consequence of Ca^{2+} overload. Accumulation of Ca^{2+} in mitochondria is driven by the mitochondrial membrane potential supported either by mitochondrial respiration or by ATP hydrolysis. Mitochondria release Ca^{2+} when the membrane potential is dropped either by inhibitors of mitochondrial respiratory chain (if the potential is supported by oxidation of a substrate) or by inhibitors of ATPase (if the source of potential is hydrolysis of ATP).

Retention of Ca^{2+} by mitochondria stimulates processes that result in mitochondrial deterioration. Mitochondria swell and become leaky, whereupon the membrane potential drops and Ca^{2+} is released. Although Ca^{2+} is obligatory for MPT induction, the sensitivity of mitochondria to permeability transition can be enhanced by different factors. Among these factors are elevated levels of phosphate, oxidative stress, and the depletion of adenine nucleotides. When phosphate or organic peroxide is added to Ca^{2+} -loaded mitochondria these organelles swell, membrane potential decays, and accumulated Ca^{2+} is released. All of these manifestations can be prevented by cyclosporin A (CsA), an inhibitor of pore opening. Therefore, MPT in isolated mitochondria can be assessed by monitoring either swelling, membrane potential, or the level of Ca^{2+} in the incubation buffer.

Accumulation of Ca^{2+} by mitochondria, as well as Ca^{2+} retention and release induced by different stimuli, can be monitored using a Ca^{2+} -sensitive electrode or spectrophotometrically with the Ca^{2+} -sensitive dye arsenazo III.

Monitoring of Ca^{2+} Fluxes Across the Inner Mitochondrial Membrane with a Ca^{2+} -Sensitive Electrode

One relatively easy method applicable to mitochondria and permeabilized cells employs a Ca^{2+} -sensitive electrode to follow the Ca^{2+} . Accumulation of Ca^{2+} by mitochondria can be followed by a decrease in the concentration of Ca^{2+} in the incubation buffer.

Materials

Incubation buffer for calcium-sensitive electrode (see recipe)

Isolated mitochondria (Support Protocol 1, 2, or 3)

2.5 mM rotenone: dissolve 1 mg rotenone (Sigma) in 1 ml ethanol; store frozen at -20°C up to 1 month

10 mM CaCl_2 : dissolve 1.47 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 1 ml H_2O ; store frozen at -20°C up to 1 month

0.5 M KH_2PO_4 : dissolve 136.1 mg KH_2PO_4 in 2 ml H_2O and adjust pH to 7.4 with KOH; store frozen at -20°C up to 1 month

Apparatus for membrane potential measurement consisting of:

Glass (or plastic) sample chamber large enough for incubation volume, which can be warmed by being connected to a water bath and can be constantly stirred
 Ca^{2+} -sensitive electrode (e.g., Orion)

pH meter as source of current

Chart recorder with variable input and chart speed

Any commercially available reference electrode for pH measurements

1. Add 2 ml of incubation buffer for calcium-sensitive electrode to the sample chamber of the measurement apparatus and set to conditions of constant stirring. Add a volume of mitochondrial suspension (which will depend on the concentration of the mitochondrial preparation) containing 2 mg of protein to the sample chamber.
2. After 30 sec, add 2 μl of 2.5 mM rotenone, for a final concentration of 2.5 μM .

When mitochondria are energized with the complex II substrate succinate, rotenone prevents formation of oxaloacetate, an inhibitor of succinate dehydrogenase that suppresses mitochondrial respiration.

3. Turn on the chart recorder.
4. After 1 min (or when stabilization has been achieved as observed by recorder trace) add 50 to 60 nmol Ca^{2+} (from 10 mM CaCl_2 stock) per milligram of mitochondrial protein.

The addition of Ca^{2+} to mitochondria leads to a rapid increase in the level of this cation in the reaction buffer, followed by a return to the initial level as mitochondria accumulate the excess Ca^{2+} .

5. After 1 min, add 20 μl of 0.5 M KH_2PO_4 , for a final concentration of 5 mM.

Inorganic phosphate is a trigger for permeability transition in mitochondria loaded with calcium.

6. Follow the retention of Ca^{2+} by its release as seen from the recorder trace.

Monitoring of Ca²⁺ Fluxes Across the Inner Mitochondrial Membrane with a Spectrophotometer

ALTERNATE
PROTOCOL 1

Spectrophotometric estimation of Ca²⁺ fluxes across the mitochondrial membrane should be performed using a dual-wavelength spectrophotometer, in order to avoid interference from the turbidity of mitochondrial suspension.

Additional Materials (also see Basic Protocol 6)

25 mM arsenazo III: dissolve 19.4 mg arsenazo III (Sigma) in 1 ml H₂O; store frozen at -20°C up to 1 month

Dual-wavelength recording spectrophotometer set at 675 versus 685 nm, with appropriate cuvettes

1. Place 2 ml of incubation buffer for calcium-sensitive electrode in a spectrophotometric cuvette and set to conditions of constant stirring. Add 2 µl of 25 mM arsenazo III, for a final concentration of 25 µM.
2. Add an aliquot of mitochondrial suspension containing 2 mg of protein, for a final concentration of 1 mg/ml.

The volume of the mitochondrial aliquot will depend on the concentration of the mitochondrial preparation.

3. After 30 sec add 2 µl of 2.5 mM rotenone, for a final concentration of 2.5 µM.
4. After 1 min add 50 to 60 nmol Ca²⁺ (from 10 mM CaCl₂ stock) per mg of mitochondrial protein.

The addition of Ca²⁺ to mitochondria leads to a rapid increase in the level of this cation in the reaction buffer, followed by a return to the initial level as mitochondria accumulate the excess Ca²⁺.

5. After 1 min, add 20 µl of 0.5 M KH₂PO₄, for a final concentration of 5 mM.

The retention of Ca²⁺ is followed by its release upon induction of MPT as can be seen from the recorder trace.

DETERMINATION OF MITOCHONDRIAL MEMBRANE POTENTIAL

Estimation of the mitochondrial membrane potential ($\Delta\psi$) can be performed using an electrode sensitive to the lipophilic cation tetraphenylphosphonium (TPP⁺) or spectrophotometrically with a $\Delta\psi$ -specific dye, safranin.

Measurements of Mitochondrial Membrane Potential with a TPP⁺-Sensitive Electrode

BASIC
PROTOCOL 7

Energized mitochondria rapidly accumulate TPP⁺ from the incubation buffer and release this cation as $\Delta\psi$ decays. Accumulation of TPP⁺ by mitochondria can be followed by a decrease in the concentration of TPP⁺ in the incubation buffer.

Materials

Incubation buffer for TPP⁺-sensitive electrode (see recipe)

TPP⁺ stock solution: dissolve 3.75 mg tetraphenylphosphonium chloride (Aldrich) in 10 ml H₂O; store up to 1 month at room temperature

Isolated mitochondria (Support Protocol 1, 2, or 3)

2.5 mM rotenone: dissolve 1 mg rotenone (Sigma) in 1 ml ethanol; store frozen at -20°C up to 1 month

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- 10 mM CaCl₂: dissolve 1.47 mg CaCl₂·2H₂O in 1 ml H₂O; store frozen at –20°C up to 1 month
- 0.5 M KH₂PO₄: dissolve 136.1 mg KH₂PO₄ in 2 ml H₂O and adjust pH to 7.4 with KOH; store frozen at –20°C up to 1 month

Apparatus for membrane potential measurement consisting of:

- Glass (or plastic) sample chamber that can accommodate 2 ml of incubation buffer, which can be warmed by being connected to a water bath and can be constantly stirred
- TPP⁺ electrode (purchase from Microelectrodes, Inc. or prepare in the laboratory, Kamo et al., 1979)
- pH meter as source of current
- Chart recorder with variable input and chart speed
- Any commercially available reference electrode

1. Place 2 ml of incubation buffer for TPP⁺-sensitive electrode in the sample chamber of the measurement apparatus and set conditions to constant stirring. Add 4 μl of TPP⁺ stock, for a final concentration of 2 μM TPP⁺, and turn on the chart recorder.

The recorder trace shows the basal level of TPP⁺ in incubation buffer.

2. Add mitochondrial suspension to a final concentration of 1 mg/ml to the incubation buffer under conditions of constant stirring.

The volume of the mitochondrial aliquot will depend on the concentration of mitochondrial preparation.

Mitochondria accumulate TPP⁺ according to the membrane potential so that the level of TPP⁺ in the incubation buffer decreases.

3. After 30 sec add 2 μl of 2.5 mM rotenone, for a final concentration of 2.5 μM.

4. After 1 min, add 50 to 60 nmol Ca²⁺ (from 10 mM CaCl₂ stock) per milligram of mitochondrial protein.

Upon addition of Ca²⁺, the mitochondrial membrane potential decreases, with subsequent restoration when Ca²⁺ is accumulated.

5. After restoration of the membrane potential, add 20 μl of 0.5 M KH₂PO₄ for a final concentration of 5 mM and monitor the release of TPP⁺ from mitochondria, which is indicative of the drop of the membrane potential upon induction of MPT.

If Ca²⁺ loading is not sufficient to induce permeability transition, mitochondria will maintain membrane potential. In this case Ca²⁺ loading should be increased.

ALTERNATE PROTOCOL 2

Measurement of Mitochondrial Membrane Potential Using a Spectrophotometer

Spectrophotometric measurement of the membrane potential can be performed using safranin, a cationic dye, because of its accumulation in mitochondria and a shift in absorption spectrum that occurs when membrane potential is high. In these experiments, a dual-wavelength spectrophotometer should be used in order to avoid a contribution by the turbidity of the mitochondrial suspension to light absorption.

Additional Materials (also see Basic Protocol 7)

- Incubation buffer for calcium-sensitive electrode (see recipe)
- 10 mM safranin: dissolve 3.5 mg safranin in 1 ml H₂O; store frozen at –20°C up to 1 month
- Dual-wavelength recording spectrophotometer set at 511 versus 533 nm, with appropriate cuvettes

1. Place 2 ml of incubation buffer for calcium-sensitive electrode in a spectrophotometric cuvette and set to conditions of constant stirring. Add 2 μ l of 10 mM safranin, for a final concentration of 10 μ M.
2. Add an aliquot of mitochondrial suspension containing 2 mg of protein, for a final concentration of 1 mg/ml.

The volume of the mitochondrial aliquot will depend on the concentration of the mitochondrial preparation.

3. After 30 sec add 2 μ l of 2.5 mM rotenone, for a final concentration of 2.5 μ M.
4. After 1 min add 50 to 60 nmol Ca^{2+} (from 10 mM CaCl_2 stock) per milligram of mitochondrial protein.

Upon addition of Ca^{2+} , the mitochondrial membrane potential decreases, with subsequent restoration when Ca^{2+} is accumulated.

5. After restoration of the membrane potential, add 20 μ l of 0.5 M KH_2PO_4 , for a final concentration of 5 mM, and monitor the decrease in the membrane potential upon induction of MPT.

Estimation of Mitochondrial Swelling

One of the most reliable manifestations of mitochondrial permeability transition is swelling of the organelles. Nephelometric or turbidometric techniques have generally been used to monitor mitochondrial swelling. Mitochondrial swelling can be monitored continuously as changes in OD_{540} .

Materials

Incubation buffer for swelling (see recipe)

Isolated mitochondria (Support Protocol 1, 2, or 3)

10 mM CaCl_2 : dissolve 1.47 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 1 ml H_2O ; store frozen at -20°C up to 1 month

2.5 mM rotenone: dissolve 1 mg rotenone (Sigma) in 1 ml ethanol; store frozen at -20°C up to 1 month

0.5 M KH_2PO_4 : dissolve 136.1 mg KH_2PO_4 in 2 ml H_2O and adjust pH to 7.4 with KOH; store frozen at -20°C up to 1 month

Spectrophotometer with 540-nm filter, or multiwavelength spectrophotometer
Chart recorder with variable input and chart speed

1. Place 2 ml of incubation buffer for swelling in a spectrophotometric cuvette and set to conditions of constant stirring. Add a volume of mitochondrial suspension (which will depend depends on the concentration of mitochondrial preparation) for a final concentration of 0.5 mg/ml.
2. After 30 sec, add 2 μ l of 2.5 mM rotenone, for a final concentration of 2.5 μ M.
3. After 1 min add 50 to 60 nmol Ca^{2+} (from 10 mM CaCl_2 stock) per milligram of mitochondrial protein.
4. After 1 min, add 20 μ l of 0.5 M KH_2PO_4 .
5. Monitor gradual decrease in OD_{540} nm due to mitochondrial swelling.

BASIC PROTOCOL 8

Estimation of Mitochondrial Ca^{2+} Accumulation in Digitonin-Permeabilized Cells

Permeabilization of the plasma membrane allows measurement of mitochondrial activity in situ, without isolation of these organelles and the accompanying potential risk of mitochondrial damage. For the permeabilization, a steroid glycoside, digitonin, is widely used. The affinity of digitonin for cholesterol allows a selective disruption of the plasma membrane, making mitochondria accessible to specific substrates and Ca^{2+} . The concentration of digitonin must be chosen carefully and usually should not exceed 0.005% (w/v), since higher concentrations might affect the outer mitochondrial membrane barrier functions.

Permeabilization of the plasma membrane allows estimation of the main functional parameters of mitochondria, such as respiration, membrane potential, and accumulation and release of Ca^{2+} . A description of the monitoring of Ca^{2+} fluxes is given below.

Induction of MPT in apoptotic cells is thought to be one of the reasons for the decrease in $\Delta\psi$. Pore opening during apoptosis (even in a subpopulation of mitochondria) will impair the ability of the total mitochondrial population to accumulate Ca^{2+} ; therefore estimation of a threshold level of Ca^{2+} loading (the Ca^{2+} capacity of mitochondria) in cells might be a criterion for changes on the mitochondrial level.

Materials

Cells of interest (Jurkat cells, U 937, HeLa)

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Incubation buffer for digitonin-permeabilized cells (see recipe)

1% (w/v) digitonin: dissolve 10 mg digitonin in 1 ml H_2O ; shake vigorously before adding to cells; store up to 2 to 3 months at room temperature

2.5 mM rotenone: dissolve 1 mg rotenone (Sigma) in 1 ml ethanol; store frozen at -20°C up to 1 month

10 mM CaCl_2 : dissolve 1.47 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 1 ml H_2O ; store frozen at -20°C up to 1 month

Apparatus for membrane potential measurement consisting of:

Glass (or plastic) sample chamber large enough for incubation volume, which can be warmed up by being connected to a water bath and can be constantly stirred

Ca^{2+} -sensitive electrode (e.g., Orion)

pH meter as source of current

Chart recorder with variable input and chart speed

Any commercially available reference electrode for pH measurements

1. Centrifuge $3\text{--}5 \times 10^6$ cells 5 min at $200 \times g$, room temperature. Remove supernatant, resuspend cells in PBS, and centrifuge again under the same conditions.
2. Resuspend cells in 50 μl of incubation buffer for digitonin-permeabilized cells, then add the resuspended cells to 450 μl of the same buffer in sample chamber with Ca^{2+} electrode at constant stirring. Turn on the chart recorder.
3. Following a 2-min stabilization period, add 1% digitonin to a final concentration of 0.005% and 2.5 mM rotenone to a final concentration of 5 μM .
4. Load mitochondria with pulses of Ca^{2+} (20 to 25 nmol each, added from 10 mM stock) to induce MPT, which can be observed by the release of accumulated Ca^{2+} as measured by the Ca^{2+} -sensitive electrode.

The calcium capacity is calculated as a sum of the amount of Ca^{2+} in each addition.

ISOLATION OF MITOCHONDRIA

Experiments with isolated mitochondria are a helpful supplement to experiments on a cellular level. The former type of experiments provide better understanding of the mechanisms responsible for the mitochondrial changes in apoptotic cells. All the methods of isolation of mitochondria are based on the disruption of tissue and subsequent differential centrifugation of homogenate.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: Mitochondria should be kept on ice at all times.

Isolation of Rat Liver Mitochondria

Isolation of liver mitochondria represents a relatively “easy” procedure giving a high yield. The liver from a 150 to 200-g rat weighs 5 to 6 g. Liver can be easily disrupted and homogenized. The most important thing is keeping the temperature not higher than 4 to 6°C.

Materials

150- to 200-g rat

Buffer A for liver (see recipe)

Buffer B: Buffer A (for liver) without EDTA

Dissecting equipment

Motor-driven glass Dounce homogenizer and tight Teflon pestle

Refrigerated centrifuge and 50-ml centrifuge tubes

Additional reagents and equipment for protein assay (*APPENDIX 3H*) and determination of the respiratory control ratio for mitochondria (Support Protocol 4)

NOTE: All operations should be done on ice, using ice-cold buffers and instruments.

Harvest the liver

1. Let the rat starve overnight.

This step is necessary to decrease the content of fat and glycogen in the liver.

2. Sacrifice rat in accordance with institutional guidelines and the aims of the experiment.

Donovan and Brown (1995) provide a variety of protocols for rodent euthanasia.

3. Remove liver and immerse immediately in ice-cold buffer A for liver.
4. Cut liver into pieces with scissors and wash several times by replacing the ice-cold buffer A with fresh ice-cold buffer, in order to remove as much blood as possible.

Homogenize the liver

5. Homogenize the liver in 60 ml of buffer A with 5 to 6 up-and-down strokes of the tight Teflon pestle.

Homogenization occurs as the piston of the homogenizer rotates (driven by an electrical motor) and the pestle is slowly moved up and down 5 to 6 times, so that there are no pieces of liver big enough to be seen by eye.

6. Transfer homogenate into two cold centrifuge tubes and balance them.

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7. Centrifuge the homogenate 8 min at $600 \times g$, 4°C , to remove nuclei and unbroken cells.

Isolate mitochondria

8. Discard the pellet and centrifuge supernatant 15 min at $5500 \times g$, 4°C , to form a mitochondrial pellet.
9. Remove floating fat droplets with a paper towel or piece of tissue paper.
10. Detach the pellet from the bottom of the tube with the piston of the homogenizer, add 0.2 to 0.3 ml of buffer A for liver, resuspend the pellet using the pestle, and pass mitochondria gently 3 to 4 times through the tip of a 1-ml pipet tip using automatic pipettor.
11. Dilute the mitochondrial suspension with 30 ml of buffer B, then centrifuge again as in step 9.
12. Resuspend the final mitochondrial pellet to a volume of ~ 1 ml in buffer B by gentle passing through a 1-ml pipet tip, then transfer into a 10-ml tube and keep on ice.

Assess the preparation

13. Determine the protein concentration using Lowry's method (or any other method available; see *APPENDIX 3H*) with bovine serum albumin as a standard.

The protein concentration of a final suspension usually is about 90 to 100 mg/ml.

14. As a control of the mitochondrial preparation, estimate the respiratory control ratio (see Support Protocol 4). Use within 4 hr.

Measurements can be started directly after mitochondrial isolation.

SUPPORT PROTOCOL 2

Isolation of Brain Mitochondria

Another source of mitochondria frequently used in the investigation of mitochondrial functioning (e.g., during apoptosis) is the brain. The method for isolation of brain mitochondria is similar to that for liver mitochondria (see Support Protocol 1); however, in order to get a purer preparation, a purification of homogenate with a Ficoll gradient is involved.

Materials

150- to 200-g rat
SET buffer (see recipe), ice-cold
3% and 6% (w/v) Ficoll solutions (see recipe)
MSH buffer (see recipe)

Motor-driven glass Dounce homogenizer and tight Teflon pestle
Refrigerated centrifuge and 50-ml centrifuge tubes

Additional reagents and equipment for protein assay (*APPENDIX 3H*) and determination of the respiratory control ratio for mitochondria (Support Protocol 4)

Dissect brain

1. Sacrifice rat in accordance with institutional guidelines and the aims of the experiment.
2. Open the skull with scissors, gently remove the brain, place it in ice-cold SET buffer, and chop it into pieces with scissors.

3. Wash pieces of brain several times in ice-cold SET buffer in order to remove as much blood as possible.

Homogenize brain

4. Homogenize manually chopped brain in 30 ml of SET buffer at 4°C using a Dounce homogenizer with six up-and-down strokes of the tight pestle.
5. Dilute homogenate to a final volume of 80 ml with SET buffer.
6. Place the homogenate in two cold centrifuge tubes, balance them, and centrifuge 3 min at $2000 \times g$, 4°C.
7. Decant supernatant and centrifuge again for 3 min at $2000 \times g$, 4°C.

Isolate mitochondria

8. Collect the supernatant and centrifuge 15 min at $12,000 \times g$, 4°C, to obtain a crude mitochondrial fraction.
9. Resuspend the pellet in 6 ml of ice-cold 3% Ficoll solution and layer onto 25 ml of ice-cold 6% Ficoll solution.
10. Centrifuge 10 min at $11,500 \times g$, 4°C.
11. Resuspend the final pellet in MSH buffer to a final volume of ~1 ml and keep on ice.

Assess preparation

12. Determine the protein concentration (*APPENDIX 3H*).
13. As a control of the mitochondrial preparation estimate the respiratory control ratio (see Support Protocol 4). Use within 4 hr.

Measurements can be started directly after mitochondrial isolation.

Isolation of Mitochondria from Cultured Cells

Isolation of mitochondria from cultured cells is more problematic than isolation from tissue, since it is difficult to disrupt the cell membrane without affecting the mitochondrial membrane. In order to make the plasma membrane more vulnerable, different tactics are used. The plasma membrane can be sensitized to disruption by using a buffer that contains digitonin, a steroid glycoside with a high affinity for cholesterol that selectively disrupts the cholesterol-rich plasma membrane. Another approach is based on passing cells through a needle. Both methods are followed by a differential centrifugation commonly used for preparation of mitochondria.

Materials

Tissue culture cells (e.g., Jurkat cells, U 937, HeLa; *UNIT 1.1*)

Complete RPMI medium (*UNIT 1.2*) or appropriate medium for cells

Buffer A for cultured cells (see recipe), ice-cold

Buffer B: Buffer A (for cultured cells) without EGTA

1% (w/v) digitonin: dissolve 10 mg digitonin in 1 ml H₂O; shake vigorously before adding to cells; store up to 2 to 3 months at room temperature

Refrigerated centrifuge and tubes

1. Take an aliquot containing 5×10^7 cells growing in culture in complete RPMI medium and centrifuge 5 min at $200 \times g$, 4°C. Remove supernatant.
2. Resuspend cells in buffer A for cultured cells. Centrifuge 4 min at $200 \times g$, 4°C.

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3. Add 1% digitonin to buffer A for a final concentration of 0.01%. Resuspend cells in 5 ml of the buffer A containing 0.01% digitonin and incubate 10 min on ice.
4. Add 5 ml of ice-cold buffer A and mix well. Sediment lysed cells by centrifuging 7 min at $500 \times g$, 4°C .
5. Discard the pellet and centrifuge supernatant 15 min at $5500 \times g$, 4°C , to form a mitochondrial pellet.
6. Detach the pellet from the bottom of the centrifuge tube with a pipet tip and resuspend the pellet in 0.1 ml of buffer B using an automatic pipettor.
7. Dilute the mitochondrial suspension with 2 ml of buffer B and centrifuge again for 15 min at $5500 \times g$, 4°C .
8. Resuspend the final mitochondrial pellet in buffer B by gently passing through a pipet tip, then transfer into a microcentrifuge tube and keep on ice.

Measurements can be started directly after mitochondrial isolation.

Unfortunately, the yield from this protocol is sufficient material for an experiment but not enough to assess protein concentration and respiratory control ratio as well.

**SUPPORT
PROTOCOL 4**

Estimation of the Quality of Isolated Mitochondria: Measuring the Respiratory Control Ratio (RCR)

One of the keystones of the chemiosmotic theory of energy transduction is impermeability of the inner mitochondrial membrane to protons. Oxidation of substrates results in extrusion of protons from the mitochondrial matrix to generate the mitochondrial membrane potential. High membrane potential suppresses further extrusion of protons and therefore inhibits respiration. Under resting conditions, the rate of respiration of mitochondria is quite low and determined by a passive leakage of protons into the matrix space.

Phosphorylation of ADP requires translocation of protons into the matrix through mitochondrial ATP synthase. This results in a decrease in membrane potential that stimulates respiration. Comparison of the rates of respiration in the resting state and during phosphorylation of ADP is a useful measure of the efficiency of mitochondrial functioning. The most reliable and widely used criterion of the quality of a mitochondrial preparation is the respiratory control ratio (RCR). RCR is defined as the rate of respiration in the presence of ADP (phosphorylating respiration, state 3) divided by the rate obtained following the expenditure of ADP, state 4.

Materials

Mitochondria (Support Protocol 1, 2, or 3)

Incubation buffer for testing mitochondrial quality (see recipe)

2.5 mM rotenone: dissolve 1 mg rotenone (Sigma) in 1 ml ethanol; store frozen at -20°C up to 1 month

0.5 M sodium succinate: dissolve 135 mg of sodium succinate in 1 ml of H_2O , store frozen at -20°C up to 1 month

50 mM ADP: dissolve 23 mg of ADP in 1 ml H_2O and adjust pH to 7.5 with KOH; store frozen at -20°C up to 1 month

1 mM CCCP: dissolve 0.2 mg CCCP in 1 ml ethanol and mix thoroughly; store frozen at -20°C up to 1 month

Biological oxygen monitor equipped with a Clark-type oxygen electrode (Yellow Spring Instrument Co.) or similar equipment (operate per manufacturer's instructions)

Chart recorder with variable input and chart speed

10- and 50- μl Hamilton syringes (e.g., Sigma)

1. Place 2 ml of incubation buffer for testing mitochondrial quality into the sample chamber of the oxygen monitor and set conditions to constant stirring. Add an aliquot of mitochondrial suspension (the volume of which will depend on the concentration of the mitochondrial preparation) for a final concentration of 2 mg/ml mitochondrial protein.
2. After 30 sec add 2 μ l of 2.5 mM rotenone, for a final concentration of 2.5 μ M.
3. Stop stirring and insert the electrode into the sample chamber. Expel all air through the slot in the plunger (slight twisting of the electrode helps to gather the bubbles at the slot) and set the chart speed.

The desirable chart speed should be 10 to 15 mm/min.

4. After 1 min stabilization observed by recorder trace add 20 μ l of 0.5 M sodium succinate, for a final concentration of 5 mM, through the slot on electrode's body with a Hamilton syringe.

Mitochondria will now start to consume oxygen. The slope of the curve shows the rate of respiration.

5. After stabilization of respiration (~2 min) add 10 μ l of 50 mM ADP.

The rate of respiration will increase (state 3) with subsequent restoration of the initial rate of respiration (state 4) when all added ADP is phosphorylated.

6. Add 2 μ l of 1 mM CCCP, for a final concentration of 1 μ M.

CCCP lowers the mitochondrial potential that stimulates oxygen consumption.

7. Calculate the rate of respiration from the recorder trace as the amount of oxygen consumed in 1 min.

Assume that at room temperature and normal atmospheric pressure the concentration of oxygen in the incubation buffer is 250 μ M.

8. Calculate the RCR by dividing the state 3 respiration rate by the state 4 respiration rate.

Mitochondria with a respiratory control ratio above 4 are acceptable for use in the experiments.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Buffer A for cultured cells

38.26 g/liter mannitol (210 mM final)
 23.96 g/liter sucrose (70 mM final)
 0.2g/liter MgCl₂·6 H₂O (1 mM final)
 1.2 g/liter HEPES (5 mM final)
 0.38 g/liter EGTA (1 mM final)
 Adjust pH to 7.5 with KOH
 Store up to 3 to 4 days at 4°C

Buffer A for liver

38.26 g/liter mannitol (210 mM final)
 23.96 g/liter sucrose (70 mM final)
 1.2 g/liter HEPES (5 mM final)
 2 ml/liter 0.5 M EDTA (1 mM final)
 Adjust pH to 7.5 with KOH
 Store up to 3 to 4 days at 4°C

Ficoll dilution buffer

0.25 M mannitol
60 mM sucrose
100 μ M potassium EGTA
10 mM Tris-Cl, pH 7.5 (APPENDIX 2A)
Store up to 3 to 4 days at 4°C

Ficoll solutions, 3% and 6% (w/w)

Prepare 20% (w/v) Ficoll (Sigma) stock solution in Ficoll dilution buffer (see recipe). Store 3 to 4 days at 4°C. Before the experiment prepare 6% (v/v) and 3% (v/v) solutions by diluting 20% solution with Ficoll dilution buffer.

HEPES buffer

2.38 g/liter HEPES (10 mM final)
8.76 g/liter NaCl (150 mM final)
0.37 g/liter KCl (5 mM final)
0.2 g/liter MgCl₂·6H₂O (1 mM final)
Adjust pH to 7.4 with NaOH
Store up to 2 to 3 days at 4°C

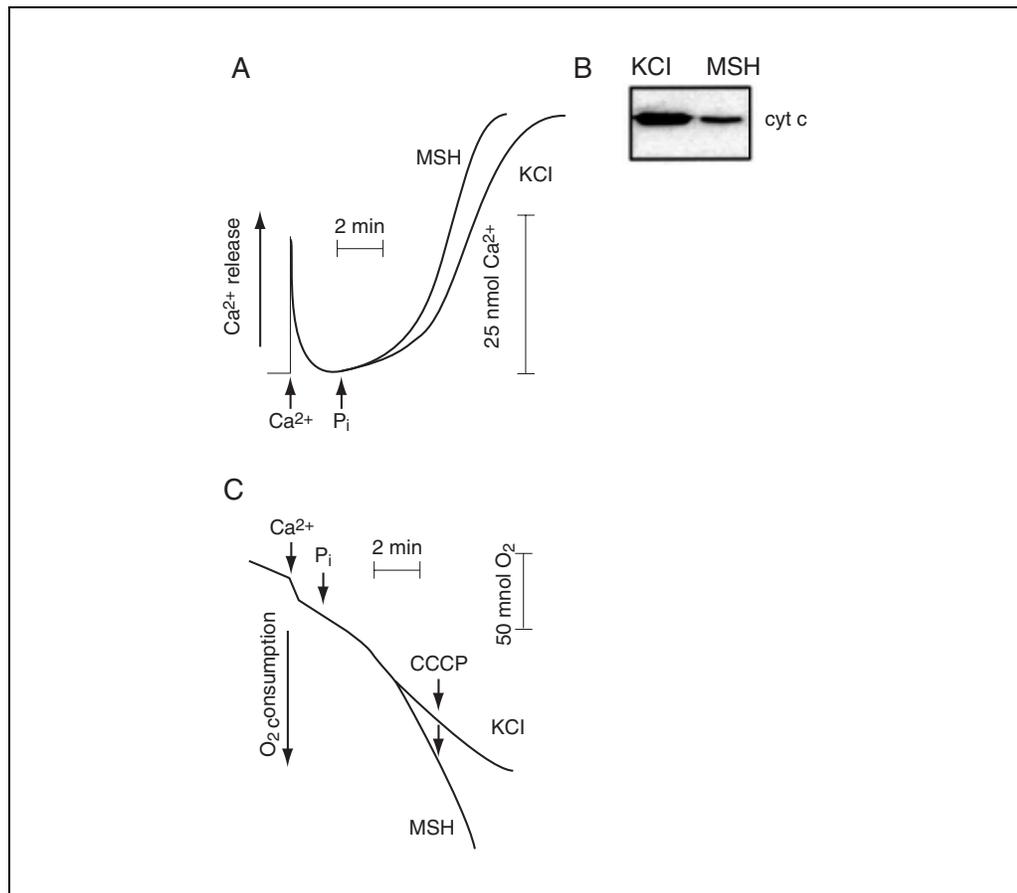


Figure 18.5.1 MPT-induced release of cytochrome c from mitochondria incubated in either MSH or KCl buffer. **(A)** Mitochondria were added to buffer at a concentration of 1 mg/ml. After a 2-min stabilization period, mitochondria were loaded with Ca²⁺ (50 nmol/mg protein), and MPT was induced by adding 5 mM inorganic phosphate (P_i). **(B)** Mitochondrial suspensions from **(A)** were centrifuged and the resulting supernatants were separated by SDS-PAGE and immunoblotted as described in Basic Protocol 3. **(C)** Mitochondrial respiration following MPT induction in either MSH or KCl buffer was analyzed as described in Support Protocol 4. The concentration of CCCP was 1 μ M.

Incubation buffer for calcium-sensitive electrode

1.11 g/100 ml KCl (150 mM final)
13.1 mg/100 ml KH_2PO_4 (1 mM final)
60 mg/100 ml Tris (5 mM final)
135 mg/100 ml sodium succinate (5 mM final)
Adjust pH to 7.4 with HCl
Store up to 2 to 3 days at 4°C

Incubation buffer for digitonin-permeabilized cells

150 mM KCl
5 mM KH_2PO_4
1 mM MgCl_2
5 mM sodium succinate
5 mM Tris·Cl, pH 7.4 (APPENDIX 2A)
Store up to 2 to 3 days at 4°C

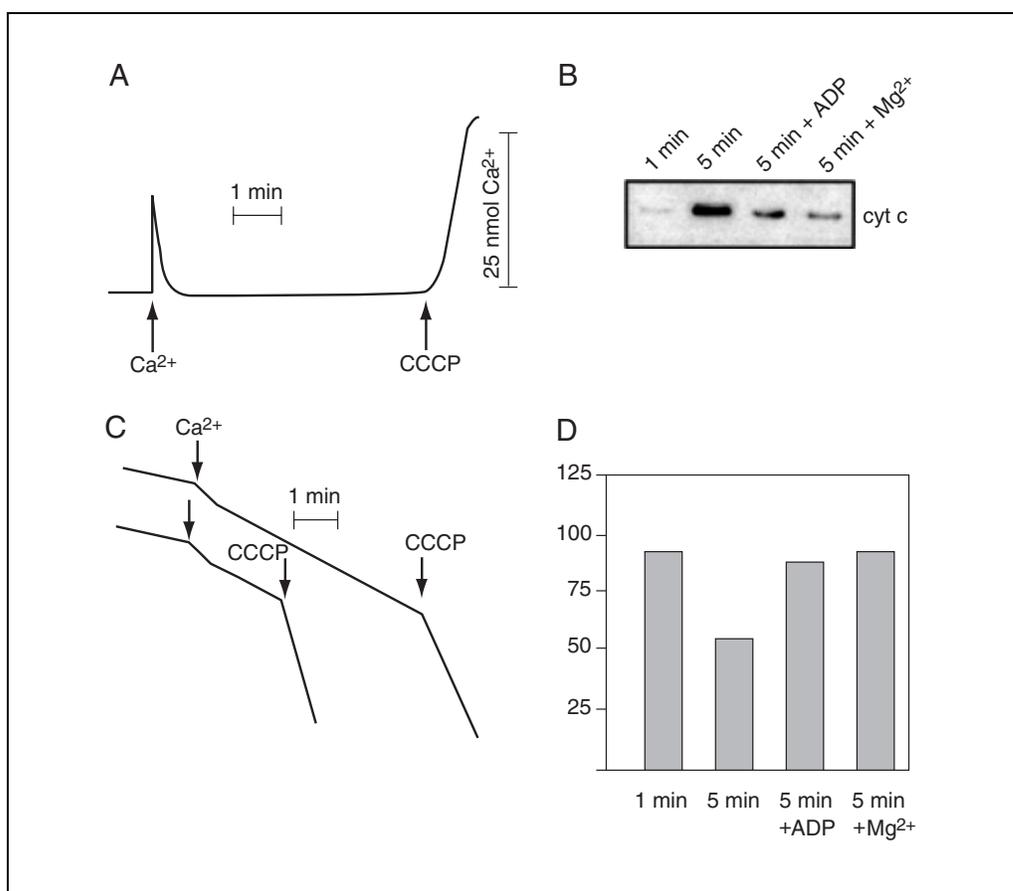


Figure 18.5.2 Ca^{2+} -induced release of cytochrome *c* from mitochondria in the absence of observable MPT. **(A)** Mitochondria (1 mg/ml) were incubated in KCl-based buffer. After a 2-min stabilization period, mitochondria were loaded with Ca^{2+} (25 nmol/mg protein) prior to the addition of 1 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) at 5 min. **(B)** The amount of cytochrome *c* released from mitochondria after 1 and 5 min of Ca^{2+} retention in the presence and absence of 0.5 mM ADP or 1 mM Mg^{2+} . **(C)** Samples incubated under the same conditions as (A) were used to evaluate the rate of uncoupled respiration after 1 and 5 min. **(D)** Samples incubated under the same conditions as conditions as (B) were used to determine the effect of inhibitors of MPT on the rate of uncoupled respiration of Ca^{2+} -loaded mitochondria.

Incubation buffer for swelling

1.11 g/100 ml KCl (150 mM final)
6.8 mg/100 ml KH₂PO₄ (0.5 mM final)
60 mg/100 ml Tris (free base; 5 mM final)
135 mg/100 ml sodium succinate (5 mM final)
Adjust pH to 7.4 with HCl
Store up to 2 to 3 days at 4°C

Incubation buffer for testing mitochondrial quality

1.11 g/100 ml KCl (150 mM final)
13.1 mg/100 ml KH₂PO₄ (1 mM final)
60 mg/100 ml Tris (free base; 5 mM final)
Adjust pH to 7.4 with HCl
Store up to 3 to 4 days at 4°C

Incubation buffer for TPP⁺-sensitive electrode

1.11 g/100 ml KCl (150 mM final)
27 mg/100 ml KH₂PO₄ (2 mM final)
60 mg/100 ml Tris (free base; 5 mM final)
135 mg/100 ml sodium succinate (5 mM final)
Adjust pH to 7.4 with HCl
Store up to 2 to 3 days at 4°C

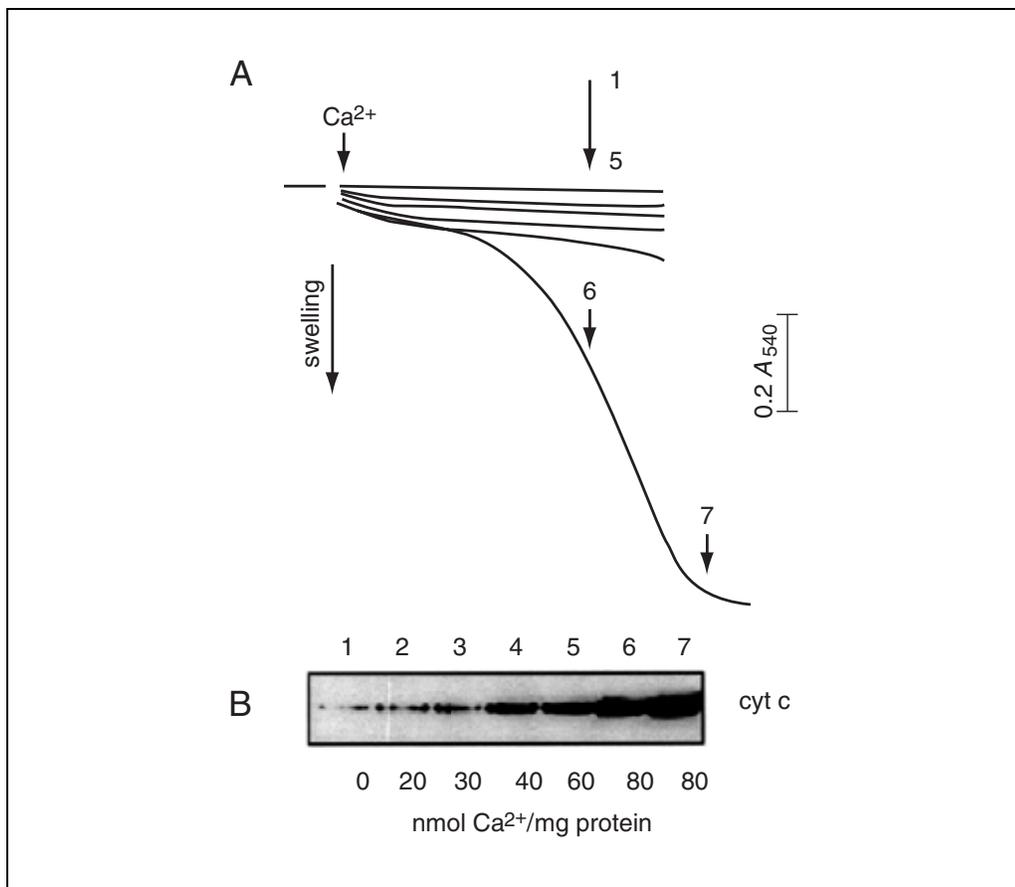


Figure 18.5.3 The effect of Ca²⁺ loading on mitochondrial swelling and the release of cytochrome c. Mitochondria (0.5 mg/ml) were incubated in 2 ml of KCl-based buffer. **(A)** Mitochondria were loaded sequentially with varied amounts of Ca²⁺ until MPT was induced. **(B)** Samples were taken after 5 min (lanes 1 to 6) or 8 min (lane 7) of incubation and the supernatants evaluated for cytochrome c content.

Laemmli's loading buffer, 4×

3.125 ml 1 M Tris·Cl, pH 6.8 (APPENDIX 2A)
4 ml glycerol
0.8 g sodium dodecyl sulfate
8 mg Bromphenol blue
0.8 ml 2-mercaptoethanol
H₂O to 10 ml
Store up to several weeks at 4°C

MSH buffer

210 mM mannitol
70 mM sucrose
5 mM HEPES
Adjust pH to 7.4 with KOH
Store up to 2 to 3 days at 4°C

S100 buffer

20 mM HEPES, pH 7.5
10 mM KCl
1.5 mM MgCl₂
1 mM EGTA
1 mM EDTA
0.1 mM PMSF
10 mg/ml leupeptin
5 mg/ml pepstatin A
2 mg/ml aprotinin
25 mg/ml calpain I inhibitor
1 mM DTT
Store at 4°C; prepare weekly

SET buffer

8.58 g/100 ml sucrose (0.25 M final)
0.5 ml of 100 mM potassium EGTA stock solution/100 ml (0.5 mM final)
120 mg/100 ml Tris (free base; 10 mM final)
Adjust pH to 7.4 with HCl

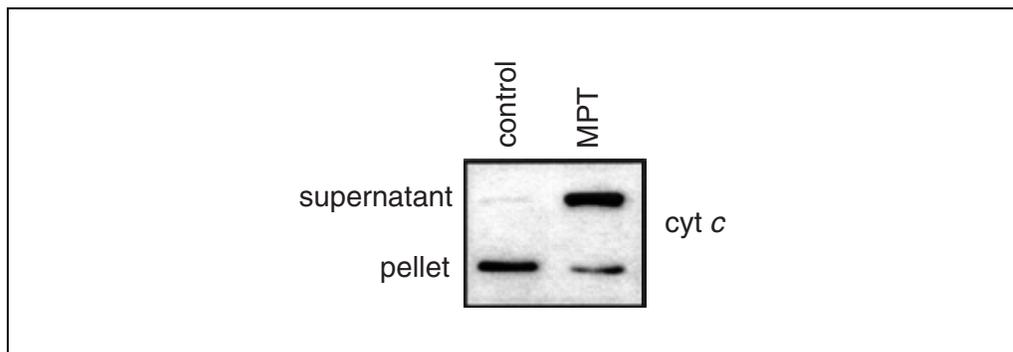


Figure 18.5.4 The release of cytochrome c from isolated rat liver mitochondria as a result of mitochondrial permeability transition (MPT). Mitochondria (1 mg/ml) were incubated in KCl-based buffer. After a 2-min stabilization period, mitochondria were loaded with Ca²⁺, and, when accumulation was complete, MPT was induced by 5 mM KH₂PO₄. A 200- μ l aliquot of mitochondrial suspension was centrifuged and the resulting supernatant and pellet were separated by SDS-PAGE and immunoblotted as described under in Basic Protocol 3. Control mitochondria were incubated for the same time but without Ca²⁺ loading.

COMMENTARY

Background Information

Mitochondrial function in apoptotic cells

Attempts to identify a common, unifying step in the apoptotic program, in response to various cytotoxic stimuli, have focused on the role of mitochondrial participation in this form of cell death (Petit et al., 1997). Specifically, the release of several proteins, normally located in the intermembrane space of mitochondria, has been observed during the early stages of apoptotic cell death (Green and Reed, 1998). Among these proteins are cytochrome *c* and other intermembrane-space proteins, including AIF (apoptosis-inducing factor), HSPs (heat shock proteins), DIABLO/Smac (direct IAP-binding protein with low pI/Second mitochondria-derived activator of caspase), AK-2 (adenylate kinase-2), several pro-caspases (Mancini et al., 1998; Köhler et al., 1999; Samali et al., 1999a; Susin et al., 1999; Zhivotovsky et al., 1999; Du et al., 2000; Verhagen et al., 2000), endonuclease G, and sulfite oxidase. Although the role of many of these proteins in the apoptotic process is well characterized, the involvement of others (AK-2, sulfite oxidase) is still unclear. It is important to note that the function of the released proteins in the cytoplasm is different from their functions within mitochondria. The best-studied of these proteins is cytochrome *c*.

Cytochrome *c*, is a component of the electron transport chain that shuttles electrons between complexes III (bc1) and IV (cytochrome oxidase). Apo-cytochrome *c* is encoded by a nuclear gene, synthesized in the cytosol, and imported into the mitochondrial intermembrane space in an unfolded configuration where it receives its heme group. Covalent attachment of this heme group stimulates a conformational change, and holo-cytochrome *c* subsequently assumes its functional role as a component of the electron transport chain.

During the early phase of apoptosis, cytochrome *c* is released into the cytosol and interacts with Apaf-1 to form the apoptosome complex together with dATP and pro-caspase-9 (Li et al., 1997).

The mechanisms regulating cytochrome *c* release remain obscure. However, two distinct models for cytochrome *c* release have emerged, and these can be distinguished on the basis of whether Ca^{2+} is required for the event. In one instance, mitochondrial Ca^{2+} overload results in opening of a pore in the inner mitochondrial membrane, with subsequent swelling and rupture of the outer membrane followed by the release of cytochrome *c* and other intermembrane space proteins (Crompton, 1999). The Ca^{2+} -independent model asserts that a more selective protein release occurs without changes in mitochondrial volume. This mecha-

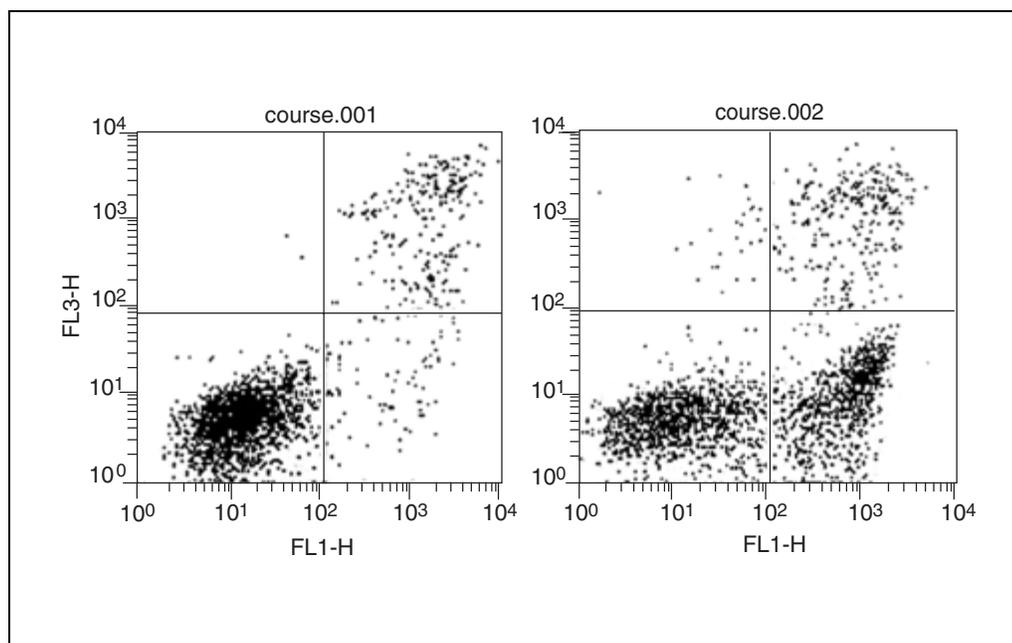


Figure 18.5.5 A typical image of FACS analysis of mitochondrial membrane potential in the cells undergoing apoptosis. Apoptosis was induced in Jurkat cells by 1 μM staurosporine and samples were analyzed 3 hr after initiation of apoptosis.

nism involves specific channels/pores in the outer mitochondrial membrane that may be opened and regulated by certain pro-apoptotic members of the Bcl-2 family of proteins, including Bax (Robertson and Orrenius, 2000). The precise manner in which Bax modulates cytochrome *c* release is controversial. In particular, the authors have reported recently that depending on the experimental conditions this protein can either act directly on mitochondria to stimulate the release of cytochrome *c* by forming a selective pore in the outer membrane, or it may facilitate opening of the permeability transition pore (Gogvadze et al., 2001). Recent evidence indicates that truncated Bid induces a conformational change in Bax that allows this protein to insert in the outer membrane, oligomerize, and stimulate cytochrome *c* release (Eskes et al., 2000).

Mitochondrial permeability transition

Energized mitochondria can take up and retain added Ca^{2+} . However this ability is not limitless. Mitochondria swell and become leaky; the membrane potential, which is the driving force for Ca^{2+} accumulation, drops, and accumulated Ca^{2+} is released. This process is called mitochondrial permeability transition (MPT). These changes are identified as a result of the opening of Ca^{2+} -dependent pore in the mitochondrial inner membrane.

MPT-dependent release of cytochrome *c*

Although the kinetics of MPT are unaffected by the composition of the incubation buffer (KCl versus MSH; Fig. 18.5.1A), the amount of cytochrome *c* released is much higher when mitochondria are incubated in the physiologically more relevant KCl buffer as compared to MSH buffer. Immunoblot analysis revealed that most cytochrome *c* was present in the supernatant fraction following MPT induction in KCl buffer, whereas only partial release of cytochrome *c* was observed in MSH buffer (Fig. 18.5.1B). Further evidence of this comes from the different effects that cytochrome *c* release has on mitochondrial respiration in the two buffers (Fig. 18.5.1C). Specifically, the induction of MPT in MSH buffer resulted in a time-dependent acceleration of oxygen consumption that persisted until all oxygen was consumed. In contrast, incubation of mitochondria in KCl buffer resulted in an initial acceleration of respiration that was followed by its suppression, and no additional stimulation of oxygen consumption was observed upon the addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Taken together, these data indicate that while MPT-induced uncoupling of mitochondria occurs in both buffers, respiration is suppressed only in KCl buffer, an effect most likely due to a loss of cytochrome *c*.

It should be taken into account that mitochondrial preparations are heterogeneous. At a given concentration of Ca^{2+} , a certain subpopu-

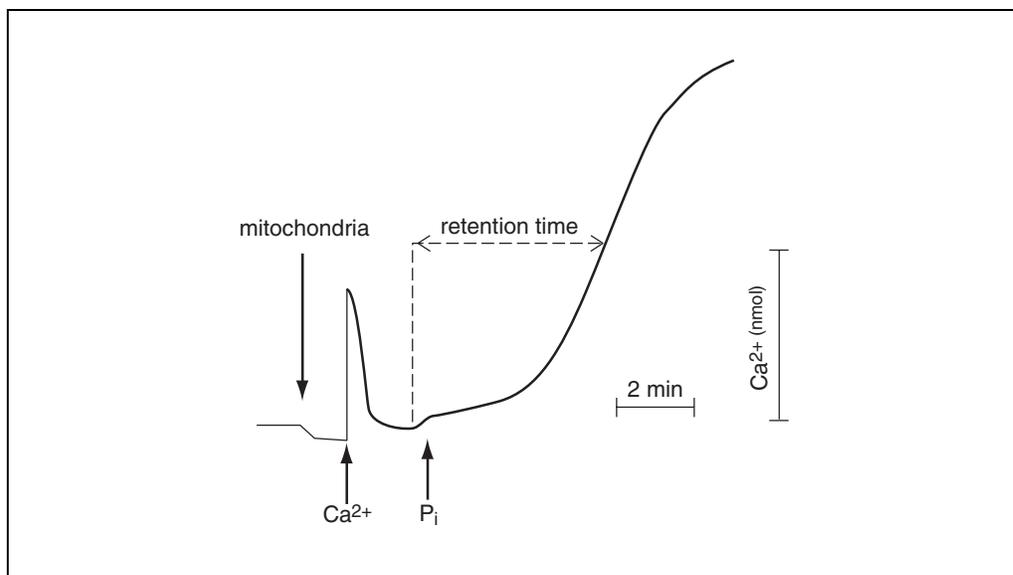


Figure 18.5.6 MPT monitored with a Ca^{2+} -sensitive electrode. Mitochondria (1 mg/ml) were incubated in KCl-based buffer. After a 1-min stabilization period, mitochondria were loaded with Ca^{2+} , and, when accumulation was complete, MPT was induced by 5 mM KH_2PO_4 .

lation (more susceptible) of mitochondria have undergone permeability transition at a given time, while the released Ca^{2+} is accumulated by more resistant mitochondria, which thereby increase their load until they also undergo permeabilization (Zoratti and Szabo, 1995).

In the aforementioned protocols, cytochrome *c* release was a consequence of MPT induction, accompanied by swelling of mitochondria and rupture of the outer membrane. When Ca^{2+} loading is insufficient to induce observable manifestations of MPT, the release of cytochrome *c* can still occur despite the absence of changes characteristic of MPT.

As seen in Figure 18.5.2A, moderate Ca^{2+} loading of mitochondria did not induce MPT, and this cation was retained by mitochondria unless the uncoupler CCCP was added. The accumulation of Ca^{2+} by mitochondria in the absence of observable MPT was nevertheless sufficient to stimulate a release of cytochrome *c*, an effect that was considerably more pronounced the longer mitochondria retained the accumulated Ca^{2+} . Inhibitors of MPT, such as Mg^{2+} and ADP, suppressed this release (Fig. 18.5.2B). In addition, Ca^{2+} -loaded mitochondria exhibited controlled respiration (Fig. 18.5.2C), although increasing the Ca^{2+} retention time from 1 to 5 min prominently diminished the rate of CCCP-directed uncoupled respiration (upper trace versus lower

trace). Inhibitors of MPT, such as Mg^{2+} and ADP, suppressed this release (Fig. 18.5.2D) and restored the rate of uncoupled respiration.

Additional evidence of cytochrome *c* release in the absence of observable MPT is presented in Figure 18.5.3A, where the sequential addition of Ca^{2+} pulses to isolated mitochondria led to a step-wise decrease in the overall optical density without the large-amplitude swelling characteristic of MPT. Meanwhile, the amount of cytochrome *c* released from mitochondria was enhanced as Ca^{2+} loading increased (Fig. 18.5.3B). This release reflected MPT induction in a subpopulation of mitochondria, which was further supported by the fact that cotreatment of isolated mitochondria with 1 μM CsA (cyclosporin A) eliminated cytochrome *c* release induced by Ca^{2+} additions ranging between 20 to 80 nmol/mg protein (data not shown). Cytochrome *c* release ultimately reached a pinnacle at 80 nmol Ca^{2+} /mg protein (Fig. 18.5.3B, lanes 6 and 7) as all mitochondria underwent MPT (Fig. 18.5.3A).

MPT-independent release of cytochrome c

Although it was originally believed that MPT induction was the root mechanism responsible for cytochrome *c* release in response to different cytotoxic stimuli, more recently this notion has been challenged and the precise mechanisms regulating the release of this pro-

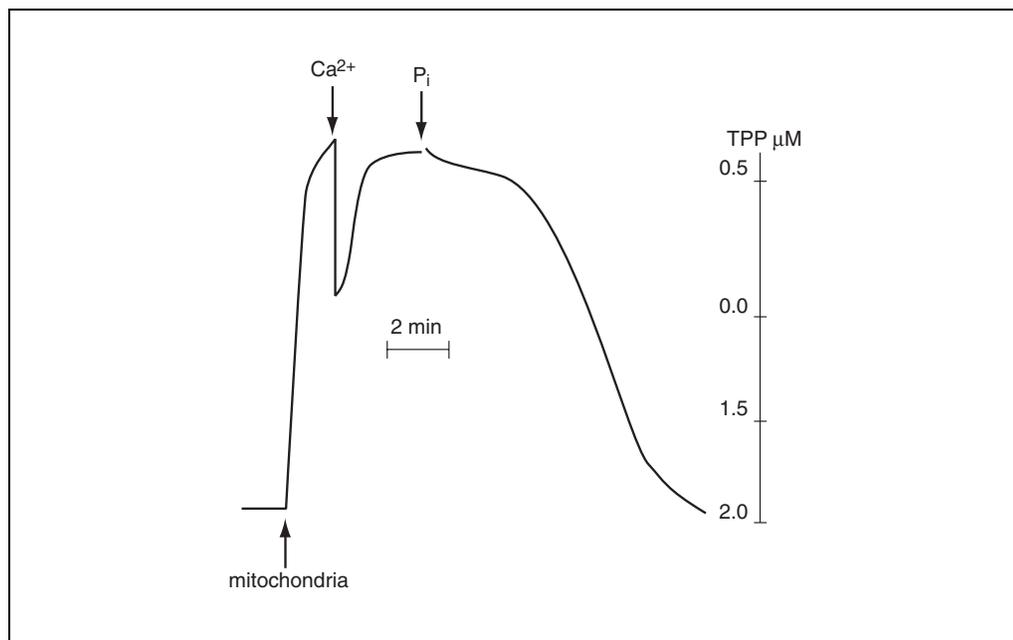


Figure 18.5.7 MPT monitored with a $\Delta\psi$ -sensitive electrode. Mitochondria (1 mg/ml) were incubated in KCl-based buffer. After a 1-min stabilization period mitochondria were loaded with Ca^{2+} , and, when accumulation was complete and potential was restored, MPT was induced by 5 mM KH_2PO_4 . The concentration of TPP^+ was 2 μM .

tein are unclear. In fact, many of the early results on mechanisms of cytochrome *c* release were generated using cell-free systems wherein isolated mitochondria and nuclei were treated with different MPT pore activators, which, in turn, led to mitochondrial swelling, the release of cytochrome *c* (and other proteins), and subsequent changes in nuclear morphology that were characteristic of apoptosis. However, ample evidence from more recent studies suggests that, while MPT is likely to be a mechanism responsible for cytochrome *c* release, it is no longer regarded as *the* mechanism.

In case of MPT induction, mitochondrial swelling leads to a rupture of the outer membrane; without MPT, permeabilization can be achieved by Bcl-2 family proteins Bax or Bid that form pores in the outer membrane, leaving the inner mitochondrial membrane intact.

Discrimination between MPT-dependent and MPT-independent release of cytochrome *c*

While analyzing the release of cytochrome *c* from mitochondria, one should keep in mind that even without added Ca^{2+} , MPT may occur in a subpopulation of mitochondria if the trace amount of Ca^{2+} in buffers is enough to induce MPT in “susceptible” mitochondria. Therefore the release of cytochrome *c* observed in the absence of added Ca^{2+} can be mistaken for MPT-independent release of cytochrome *c*. In order to distinguish between the MPT-depend-

ent and -independent release of cytochrome *c*, experiments should be performed in the presence of the Ca^{2+} chelator EGTA, which sequesters Ca^{2+} and therefore prevents MPT induction. Similar results can be achieved with CsA.

Critical Parameters and Troubleshooting

Collecting released proteins (Basic Protocols 1 and 2)

Separation of the mitochondria from the supernatant should be done thoroughly. Aliquots of supernatant should be taken without disturbing the pellet.

Immunoblot analysis of released proteins (Basic Protocol 3)

For immunoblot analysis, too many bands on the membrane may indicate too high a concentration of primary and/or secondary antibodies. If the background of nonspecific binding of immunological probes is unacceptably high, increase the length of the time of washing with PBS. If there is no band on the film, increase the duration of incubation with primary antibodies. If a white spot on the film makes it difficult to detect the protein band of interest, a bubble may have been introduced during blotting and the whole procedure must be repeated. If a band on the film is too strong, decrease exposure time of X-ray film to the ECL-stained membrane. If the gel runs too fast

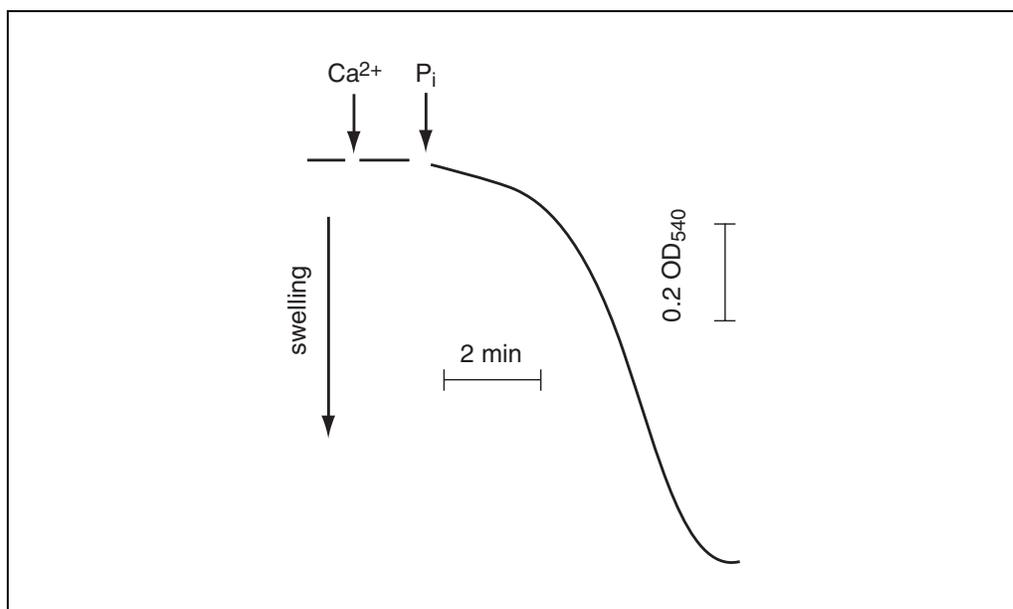


Figure 18.5.8 MPT assessed by mitochondrial swelling. Mitochondria (0.5 mg/ml) were incubated in 2 ml of KCl-based buffer. Mitochondria were loaded with Ca^{2+} and 5 mM phosphate was added. Swelling was monitored by a decrease of optical density at 540 nm.

or too slow and poor resolution of protein occurs, decrease or increase voltage, respectively, or check the buffer recipe. If no pre-stained markers appear on the nitrocellulose filter, check the transfer buffer recipe, or reposition the location of gel towards the cathode and anode.

FACS analysis of TMRE-stained cells (Basic Protocol 4)

FACS analysis needs an appropriate gating of the cells—a selection of the population of interest, especially if the initial population is a mixture of different types of cells (for example when blood samples are analyzed).

Another critical parameter is time. The analysis of the membrane potential should be done shortly after staining of the cells. TMRE is only accumulated by mitochondria with high membrane potential and any delay in analysis may negatively affect the functional state of the mitochondria and therefore cause leakage of the dye.

Microscopy of apoptotic cells stained with MitoTracker Red (Basic Protocol 5)

To reduce potential artifacts from overloading, the concentration of dye should be kept as low as possible. However if cells are not sufficiently stained, the concentration of dye or time of incubation should be increased.

Measurement of calcium release and membrane potential (Basic Protocols 6 and 7)

The release of Ca^{2+} from mitochondria may result not from opening of the pore, but simply because of a drop of the membrane potential. This may happen, for instance, if the mitochondria consume all available oxygen. However at a mitochondrial protein concentration of 1 mg/ml (or less), and with rapid stirring of the suspension, MPT usually occurs before the exhaustion of oxygen. If higher concentrations of mitochondrial suspension are used, possible anaerobiosis can be prevented by fine flow of oxygen blown onto the surface of the suspension.

Mitochondrial swelling (Basic Protocol 8)

Mitochondrial swelling assessed by a decrease in optical density of mitochondrial suspension represents one of the most reliable parameters of MPT induction in isolated mitochondria. However it should be kept in mind that anaerobiosis and subsequent release of accumulated Ca^{2+} due to the drop in membrane potential may prevent pore opening and terminate swelling. Anaerobiosis can be prevented by a fine flow of oxygen blown onto the surface of the suspension. On the other hand, a relatively low concentration of mitochondria (up to 1 mg/ml) should not lead to anaerobiosis.

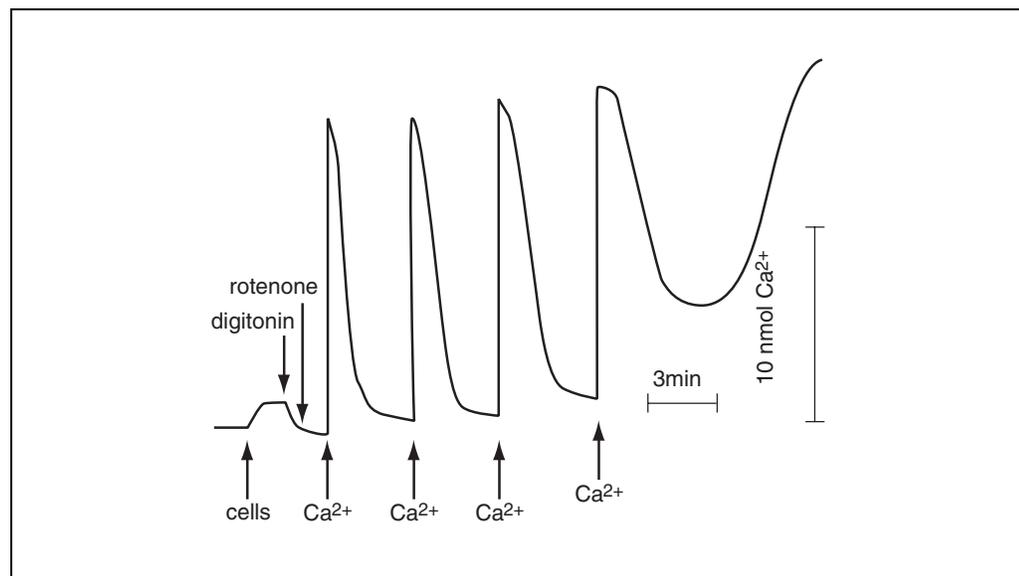


Figure 18.5.9 Estimation of mitochondrial Ca^{2+} accumulation in digitonin-permeabilized cells. Jurkat cells (2.5×10^6) were washed in PBS, resuspended in 500 μl of KCl-based buffer, and added to the incubation chamber. Following a 2-min stabilization period, cells were permeabilized with 0.005% digitonin, and 5 μM rotenone was added in order to maintain pyridine nucleotides in a reduced form. MPT was induced by sequential additions of Ca^{2+} and changes in the level of this cation were monitored using a Ca^{2+} -selective electrode.

Estimation of mitochondrial Ca²⁺ accumulation in digitonin-permeabilized cells (Basic Protocol 9)

Digitonin generally permeabilizes plasma membrane, which contains cholesterol; therefore, this detergent should be used with caution because relatively high concentrations of digitonin can affect also the outer mitochondrial membrane. The minimal concentration of digitonin that is necessary for the outer-membrane permeabilization can be detected with a Ca²⁺-sensitive electrode. Cells do not accumulate added Ca²⁺, since plasma membrane is permeable neither to Ca²⁺ nor to succinate, oxidation of which provide a driving force for Ca²⁺ accumulation. Upon addition of digitonin, the plasma membrane becomes permeable and the mitochondria start to accumulate Ca²⁺ from the incubation buffer.

Preparation of mitochondria (Support Protocols 1, 2, and 3)

The temperature during isolation should be kept around 4°C. Elevation of the temperature may result in activation of catabolic enzymes, in particular, mitochondrial phospholipase A₂, which leads to accumulation of free fatty acids in mitochondria. Free fatty acids are known as natural protonophores (agents that facilitate transport of protons through membrane); therefore, accumulation of fatty acids will decrease the mitochondrial membrane potential and destabilize mitochondria.

Anticipated Results

Analyzing functioning of isolated mitochondria and mitochondria in apoptotic cells: collection of released proteins and immunoblot analysis (Basic Protocols 1, 2, and 3)

Depending on the mechanisms involved in the release of cytochrome *c* or other proteins from mitochondria, the final result will be different. If the release was associated with mitochondrial swelling and the rupture of the outer membrane, most of the cytochrome *c* will be found in the supernatant after sedimentation of mitochondria. In such a situation, it would be informative also to present the data concerning changes in the content of cytochrome *c* in the pellet. Figure 18.5.4 represents a typical blot showing the release of cytochrome *c* from isolated liver mitochondria upon induction of MPT in KCl-based buffer.

FACS analysis of TMRE-stained cells (Basic Protocol 4)

A typical image of FACS analysis of mitochondria in the cells undergoing apoptosis is present in Figure 18.5.5. The lower left quadrant contains living cells, the lower right, apoptotic cells, and upper right, necrotic cells.

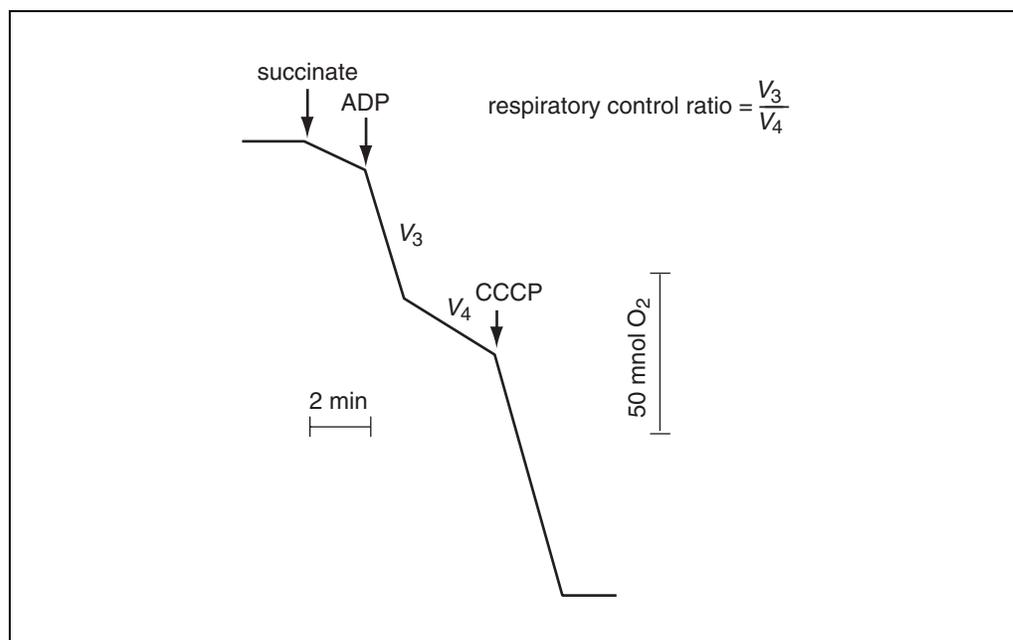


Figure 18.5.10 Estimation of mitochondrial respiration as described in Support Protocol 4.

Measurement of calcium release (Basic Protocol 6)

A typical response of mitochondria to Ca^{2+} is shown in Figure 18.5.6. Addition of Ca^{2+} to isolated mitochondria results in a quick rise in the basal level of Ca^{2+} in the incubation buffer, followed by restoration of the initial level due to accumulation of Ca^{2+} in mitochondria, whereupon a new steady state is reached. After addition of phosphate, mitochondria start releasing accumulated Ca^{2+} . The lag time before the onset of MPT induction, as well as the rate of release, depend on the Ca^{2+} load. If Ca^{2+} and other conditions are held constant, the effect of phosphate on MPT induction is dose-dependent. Therefore, the opening of the pore can be modulated either by varying the Ca^{2+} concentration while keeping the concentration of phosphate constant, or by varying the concentration of phosphate.

Both methods (spectrophotometric and ion-selective electrodes) are equally sensitive, and the choice is dictated by the availability of the equipment.

Measurement of membrane potential (Basic Protocol 7)

Energized mitochondria rapidly accumulate TPP^+ from the incubation buffer and release this cation as $\Delta\psi$ decays. A typical change in mitochondrial membrane potential upon MPT induction is shown on Figure 18.5.7. As can be seen in that figure, upon induction of MPT, two distinct phases of decrease in membrane potential occur. First, a relatively slow decrease starts when an MPT-inducing agent (P_i) is added, which is followed by a second, rapid phase, which reflects the induction of MPT in the whole population of mitochondria.

Measurement of mitochondrial swelling (Basic Protocol 8)

Figure 18.5.8 shows a typical example of results from measurement of mitochondrial swelling. When mitochondria are added to incubation buffer at a concentration of 0.5 mg/ml, the initial light absorption at 540 nm is about 1.3 to 1.4. Addition of Ca^{2+} to the mitochondria, followed by inorganic phosphate, results in a time-dependent decrease in the optical density (OD) of the mitochondrial suspension.

Estimation of mitochondrial Ca^{2+} accumulation in digitonin-permeabilized cells (Basic Protocol 9)

The addition of Ca^{2+} to a permeabilized cell suspension leads to a rapid increase in the level

of this cation in the reaction buffer, followed by a return to the initial level as mitochondria accumulate the excess Ca^{2+} (Fig. 18.5.9); this is an effect that can be completely abrogated by 2 $\mu\text{g}/\text{ml}$ antimycin, an inhibitor of mitochondrial respiratory chain (data not shown). Mitochondria accumulate sequential additions of Ca^{2+} until MPT is induced and the accumulated Ca^{2+} is released.

Preparation of mitochondria and assessing their quality (Support Protocols 1 to 4)

Isolation of mitochondria usually takes about 60 to 75 min. For liver the yield is about 20 to 25 mg protein of mitochondria per 1 g of tissue. High concentration of the final mitochondrial preparation is a prerequisite of mitochondrial stability.

A typical respiratory response of mitochondria and the method of calculating RCR are shown in Figure 18.5.10. Addition of CCCP when added ADP is phosphorylated and state 4 respiration is stabilized allows one to determine the rate of uncoupled respiration that represents the activity of the mitochondrial respiratory chain.

Time Considerations

The time of collecting samples and preparing for immunoblotting depends on the incubation conditions as well as the experience of a researcher. Basic Protocol 1 requires 45 to 60 min, Basic Protocol 2, 2 to 2.5 hr, and Basic Protocol 3, 2 days. TMRE (Basic Protocol 4) and MitoTracker Red (Basic Protocol 5) staining takes 1 hr and 1.5 hr, respectively. Measurement of the mitochondrial permeability transition (Basic Protocols 6, 7, 8, and 9 and Alternate Protocols 1 and 2) can be completed in 5 to 20 min.

Mitochondrial preparation (Support Protocols 1, 2, and 3) takes 60 to 80 min. Assessment of respiratory control ratio takes 20 to 35 min.

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Analysis of Telomeres and Telomerase

UNIT 18.6

This unit describes techniques to analyze telomeric length (Basic Protocol 1) and telomerase activity (Basic Protocol 2) in human cells. These assays can be used to study the in vitro cellular effects of aging and cancer treatments on telomere biology and telomerase activity.

Telomere length can be determined by a modification of Southern blotting in which the analysis of chromosome terminal restriction fragments (TRFs), as visualized with a radiolabeled telomeric repeat probe (Support Protocols 2 and 3), provides the average lengths of all telomeres in a cell population. A labeled molecular weight marker (Support Protocol 1) is used to determine telomere length.

Telomerase activity can be measured in vitro by a sensitive and efficient polymerase chain reaction (PCR)-based detection method, also known as telomeric repeat amplification protocol (TRAP). This method can use either radiolabeled or fluorescently labeled (Alternate Protocol) primers. Support Protocol 4 describes the preparation of tissue samples for TRAP; the preparation of other reagents that are needed for TRAP are described in Support Protocols 5 and 6.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and surroundings. Carry out the experiments and dispose of wastes in appropriately designated area, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

TERMINAL RESTRICTION FRAGMENT SIZE DETERMINATION TO MEASURE AVERAGE TELOMERE LENGTHS

**BASIC
PROTOCOL 1**

The simplified method for isolating DNA described below was developed in the authors' laboratory to efficiently process a large number of samples by eliminating phenol/chloroform extractions and avoiding alcohol precipitation. Because there are no steps where significant losses can occur, DNA concentrations can be calculated based on initial cell numbers, a more reproducible method than optical density measurements, which are frequently distorted by contaminating RNA. The measurement of telomere length relies on the absence of restriction enzyme recognition sites within the TTAGGG tandem repeat sequences. The length of telomeric repeats can be indirectly measured by analyzing the length of the terminal restriction fragment (TRF), the large DNA fragments remaining after the rest of the genomic DNA has been reduced to small sizes by digestion with frequent cutters (restriction enzymes with recognition sequences of four bases, such as *Hin*I). There is also a region of mostly repetitive subtelomeric DNA, probably ranging from 2 to 5 kb in length in humans, that lacks functional restriction sites and contributes to the measured size of telomeres on gels. The DNA is probed with a labeled oligonucleotide containing TTAGGG repeats. The measured size of the TRF corresponds to the sum of the sizes of the subtelomeric DNA that lacks functional restriction sites and the length of the telomere repeats. Because small oligonucleotides easily penetrate dried agarose gels, it is more rapid and convenient to perform in-gel hybridization rather than Southern blot transfers.

Materials

- Cell pellet from $\geq 300,000$ cells and appropriate medium
- Quick-prep lysis buffer (see recipe)
- Triton X-100
- 20 mg/ml proteinase K

Cell Aging and
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Contributed by Brittney-Shea Herbert, Jerry W. Shay, and Woodring E. Wright

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18.6.1

Supplement 20

TE buffer, pH 7.5 to 8.0 (*APPENDIX 2A*)

Restriction enzyme mix: equal volumes of *HinfI*, *RsaI*, *MspI*, *CfoI*, *HaeIII*, and *AluI* restriction enzymes (Boehringer Mannheim; 10 U/μl each before mixing, final 1.67 U/μl each)

10× TAE buffer (*APPENDIX 2A*)

10× TBE buffer (*APPENDIX 2A*)

10,000 cpm radiolabeled molecular weight markers mixed with ≥500 ng unlabeled, *SlyI*-digested λ DNA (or other appropriate unlabeled, digested DNA; see Support Protocol 1)

Denaturing solution: 0.5 M NaOH/1.5 M NaCl

Neutralizing solution: 1.5 M NaCl/0.5 M Tris-Cl, pH 8.0 (see *APPENDIX 2A* for Tris-Cl)

Hybridization solution (see recipe)

Radiolabeled telomeric repeat probe (see Support Protocols 2 and 3)

20× SSC (see recipe)

0.1× SSC/0.1% (w/v) SDS

2-ml polypropylene screw-cap tubes (Sarstedt) with an optional extra set of screw caps to be cut vertically with jigsaw to form open screw-cap ring (Fig. 18.6.1A)

37°, 55°, and 70°C water baths heating blocks, or PCR machine

Dialysis membrane sheets or tubing (e.g., Spectra/Por; Spectrum), molecular weight cutoff (MWCO) ≥6000 to 8000

Wide-bore pipet tips

Whatman 3MM filter paper

Hybridization oven, 42°C, and appropriate hybridization containers, or equivalent

PhosphorImager, including phosphor screens and ImageQuant software (Molecular Dynamics)

Additional reagents and equipment for agarose gel electrophoresis (*APPENDIX 3A*)

Lyse cells

1. Resuspend cell pellet from ≥300,000 trypsinized cells in medium and pellet cells in a 2-ml polypropylene screw-cap tube. Remove supernatant and freeze pellet at –80°C or proceed to next step.

It is not necessary to wash cells with phosphate-buffered saline (PBS), as the small amount of medium in the cell pellet does not interfere with subsequent steps. At least 300,000 cells should be used; however, the collection of more cells is desirable (~3 million).

Cell pellets can simply be placed at –80°C; it is not necessary to snap-freeze them.

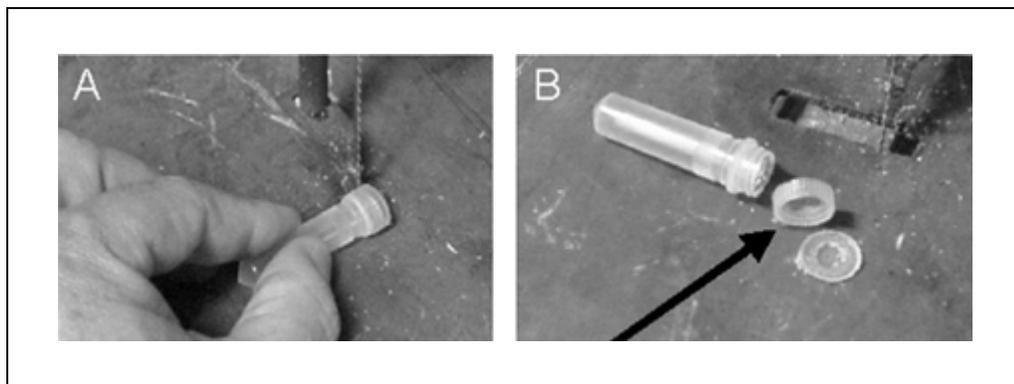


Figure 18.6.1 Construction of the dialysis tube for the terminal restriction fragment (TRF) protocol (see Basic Protocol 1). Large numbers of these open-ended caps can easily be prepared as follows. (A) Screw the caps onto a tube so that the tube functions as a handle and cut off the end of the cap using a jigsaw. (B) The threads of the cap are attached as a ring on the tube (arrow). These rings are reusable as tube closures.

2. Thoroughly resuspend cell pellet (fresh or just after thawing from -80°C) in quick-prep lysis buffer, using 30 μl per million cells.

100 mM EDTA is used in the lysis buffer to very rapidly inhibit nucleases in these concentrated cell suspensions. The lysis buffer should be added immediately to cells as they are thawing on ice to prevent nucleases from degrading the DNA.

3. Add Triton X-100 to 1% (v/v) final concentration and 20 mg/ml proteinase K to 2 mg/ml final concentration.

Triton X-100 rather than SDS is used so that residual detergent present after dialysis does not inhibit digestion with restriction enzymes.

4. Incubate samples ≥ 2 hr at 55°C and inactivate proteinase K by heating 30 min at 70°C .

Heat inactivation of proteinase K avoids the need to phenol/chloroform extract the DNA, and the residual protein present after dialysis does not inhibit enzyme digestion.

After digestion with proteinase K, DNA should only be handled using wide-bore tips to prevent shearing of the DNA.

Dialyze sample

- 5a. *To dialyze sample in screw-cap tube:* Place a 1- to 2- cm^2 piece of dialysis membrane over top of tube and secure with an open screw-cap ring. Invert tube, making sure that sample (and not a bubble) is at the membrane, and place on floating rack in ≥ 200 vol TE buffer (Fig. 18.6.2). Dialyze 1 to 2 hr at 4°C with constant stirring.

For larger volumes (>0.3 ml for >10 million cells), conventional dialysis (step 5b) can be used.

Dialysis reduces salt and EDTA so that digests will not be inhibited later on.

- 5b. *To dialyze sample in dialysis tubing:* Wet dialysis tubing with distilled water and clip one end shut. With a wide-bore pipet tip, carefully collect sample and place in tubing without creating bubbles. Smooth any bubbles out and clip the other end shut, leaving room for buffer exchange and volume expansion. Place tubing in ≥ 200 vol TE buffer and dialyze 1 to 2 hr at 4°C with constant stirring.

Dialysis reduces salt and EDTA so that digests will not be inhibited later on.

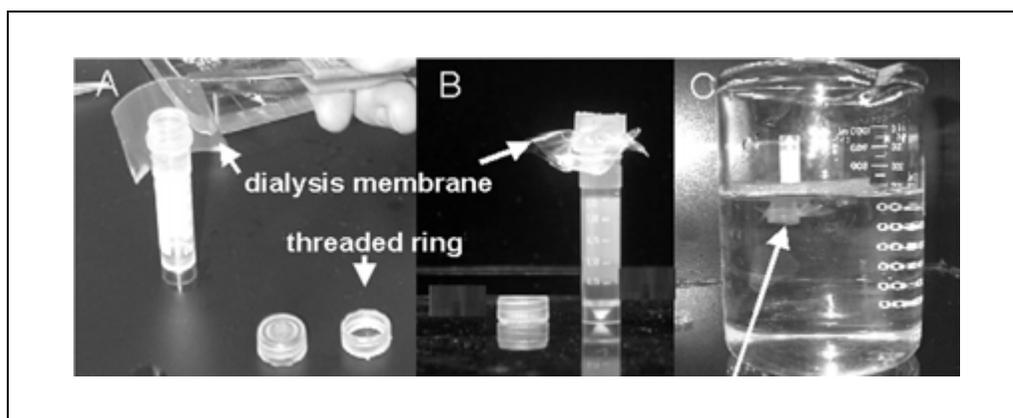


Figure 18.6.2 Assembly of the dialysis tube for the terminal restriction fragment (TRF) protocol (see Basic Protocol 1). **(A)** A wet 1- to 2- cm^2 piece of dialysis membrane is placed on the tube containing the sample (save the original screw cap). **(B)** An open-end screw cap (threaded rings) is carefully threaded on the tube over the membrane without tearing. **(C)** The tube (arrow) is inverted and placed in a floating rack in $1\times$ TE buffer. There should be no droplets remaining on the walls of the tube and no bubbles on the membrane when inverted, as this will prevent dialysis.

6. Change buffer to ≥ 200 vol fresh TE buffer and dialyze samples overnight at 4°C with constant stirring.
7. Discard dialysis membrane, replace threaded ring with a closed cap, and save threaded rings for re-use. Alternatively, remove sample from dialysis tubing and place in a fresh tube. Calculate the DNA concentration.

DNA concentration is calculated at 6 $\mu\text{g}/10^6$ diploid cells or 10 $\mu\text{g}/10^6$ aneuploid cells divided by the final volume after dialysis. The samples should be viscous and they can now be stored for 1 year at 4°C (or for ≥ 1 year at -20°C) until they are digested.

Digest and electrophorese DNA

8. Digest 1 μg DNA to completion for 4 hr to overnight at 37°C with ≥ 1 U/ μg restriction enzyme mix.

The six enzymes ensure that genomic DNA, but not telomeric DNA, is digested. The authors generally use 1 μl enzyme mix/1 μg DNA (1.67 U each enzyme/ μl) with 1 \times buffer A (supplied with the enzyme; Boehringer Mannheim).

9. Separate digested DNA on a 0.7% (w/v) agarose gel stained with ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) for 16 hr at 70 V for a 25-cm-long gel in 1 \times TAE or 0.5 \times TBE buffer. Load 20,000 cpm radiolabeled molecular weight markers mixed with ≥ 250 ng unlabeled, *StyI*-digested λ DNA on either side of the samples.

0.5 \times TBE can be used to analyze telomeres less than ~ 8 kb, whereas 1 \times TAE buffer allows for better separation in the 8- to 20-kb range. The gel should be handled carefully as it may be fragile. Electrophoresis should be stopped temporarily before the < 1 -kb molecular weight markers migrate off the gel to document complete digestion of the DNA by photographing the gel. Actual time and voltage varies with each power supply and gel apparatus. Digested DNA will run as a smear below the 1-kb molecular weight marker. Anything above this size is incompletely digested. If the sample is not digested completely, it is usually sufficient to use an additional 1 μl enzyme mix for a longer amount of time. If the sample is completely undigested, the viscous sample may have floated up out of the well when the gel was loaded. Electrophoresis (at higher voltage) should be continued until the 1- to 1.5-kb marker is near the bottom of the gel.

Radiolabeled molecular weight markers will allow the markers to be seen after hybridization with the telomere sequence-specific probe. The unlabeled, digested λ DNA is included so the markers can be visualized with ethidium bromide after electrophoresis.

Hybridize DNA

10. Denature gel 20 min in enough denaturing solution to cover the gel, rinse 10 min in distilled water, and dry gel upside down (so that the openings of the wells are against the paper) on two sheets of Whatman 3MM filter paper under vacuum for 1 hr at 50°C.

Denaturing the DNA before rather than after drying the gel increases the signal intensity ~ 3 - to 5-fold, presumably by permitting a much greater diffusion of the denatured DNA strands in the 0.7% agarose gel than in the very high percentage dried and rehydrated gel, and thus inhibiting the reannealing of the parental strands, which would compete with the probe.

Rinsing with distilled water gets rid of the NaOH and substantially reduces sticking of the gel to the Whatman paper. Some loss of lower-molecular-weight DNA (particularly < 400 bp) occurs during drying.

Because most of the DNA samples do not fill the wells and are thus in the bottom half of the gel, the gels are flipped and dried with the upper surface against the filter paper support. This increases the distance between the DNA samples and the filter paper, and significantly reduces the loss of lower-molecular-weight DNA.

11. Neutralize gel 10 to 15 min in enough neutralizing solution to cover the gel, rinse 10 min in distilled water.

12. Prehybridize gel ≥ 10 min with 10 ml hybridization solution.
13. Hybridize gel overnight at 42°C in hybridization solution containing all of the radiolabeled telomeric repeat probe made in Support Protocol 2.

The volume of the hybridization solution used depends on the gel size and hybridization setup. For a 20 × 25-cm gel rotating inside a glass cylinder in a hybridization oven, 10 ml is sufficient.

Using DNA polymerase fill-in of uracil-containing templates (see Support Protocol 3) results in a probe with higher specific activity.

14. Wash gel 15 min at room temperature in 2× SSC and then one to three times for 10 min each in 0.1× SSC/0.1% SDS at room temperature. Expose gel ≥ 4 hr to a phosphor screen and then scan with a PhosphorImager and visualize using ImageQuant software.

The number of washes can be adjusted as needed. Two 15-min washes with 0.1× SSC are generally sufficient.

15. Calculate mean TRF lengths from PhosphorImager scans of gels hybridized to the telomeric repeat probe. Position a grid of 30 boxes over each lane and determine the signal intensity and size (kb) corresponding to each box. Calculate the mean TRF length as the weighted mean using the following formula: $\Sigma(OD_i)/\Sigma(OD_i/L_i)$, where OD_i is the PhosphorImager output (signal intensity) and L_i is the length of the DNA at position i . Calculate sums over the range of the molecular weight marker used in the hybridized gel.

TELORUN, a program that performs these calculations, is available (see Internet Resource).

MAKING A LABELED MOLECULAR WEIGHT MARKER FOR RUNNING ON AN AGAROSE GEL

SUPPORT PROTOCOL 1

Running a radiolabeled molecular weight marker on either side of the gel allows for quantitation of the telomere lengths.

Materials

- ~250 ng/ μ l DNA molecular weight marker (e.g., λ DNA digested with *Sty*I)
- 10× React 2 buffer (Roche)
- 100 μ M dAGT mix: 100 μ M each dATP, dGTP, dTTP in H₂O, stored up to 1 year at -20°C
- [α -³²P]dCTP (3000 Ci/mmol)
- 2 U/ μ l Klenow fragment of *E. coli* DNA polymerase I
- QIAquick nucleotide removal kit (Qiagen)

1. In a tube, mix the following:

- 10 μ l of ~250 ng/ μ l DNA molecular weight marker
- 2 μ l of 10× React 2 buffer
- 5 μ l of 100 μ M dAGT mix
- 5 μ l [α -³²P]dCTP
- 1 μ l of 2 U/ μ l Klenow fragment

Incubate 30 min at 37°C.

*The authors use Roche React 2 buffer because it is the same buffer used to digest the λ DNA with *Sty*I. Any other buffer compatible with the Klenow fragment of DNA polymerase can be used.*

Cell Aging and Death

18.6.5

**SUPPORT
PROTOCOL 2**

2. Remove unincorporated label with QIAquick nucleotide removal kit.
3. Determine the cpm of the radiolabeled marker using a scintillation counter.

MAKING KINASED RADIOLABELED TELOMERIC REPEAT PROBE

Empirically, the G-rich probe tends to have higher signals, for unknown reasons, but both probes work reasonably well. G-rich probes generally give about 2-fold better signals than C-rich probes (probably due to possible G-quartet formation by the target G-strands which inhibits hybridization), so making a G-rich probe with 3000 Ci/mmol radioisotope is recommended if maximum sensitivity is needed.

Materials

(T₂AG₃)₄ or (C₃TA₂)₄ oligonucleotide (20 pmol/μl or 0.16 μg/μl)
[γ-³²P]ATP (3000 Ci/mmol)
5× forward or exchange reaction buffer (GIBCO/BRL)
10 U/μl T4 polynucleotide kinase (GIBCO/BRL)
QIAquick nucleotide removal kit (Qiagen)

1. In a tube, mix the following:

2 μl (T₂AG₃)₄ or (C₃TA₂)₄ oligonucleotide
24 μl [γ-³²P]ATP
10 μl of 5× forward reaction buffer
12 μl H₂O
2 μl T4 polynucleotide kinase.

Incubate 30 min at 37°C.

2. Remove unincorporated label with QIAquick nucleotide removal kit (Qiagen).
3. Determine the cpm of the radiolabeled marker using a scintillation counter (≥5000 cpm/μl). Store <2 weeks at -20°C.

**SUPPORT
PROTOCOL 3**

SYNTHESIS OF A HIGH-SPECIFIC ACTIVITY TELOMERIC REPEAT PROBE

In this protocol, the template oligonucleotide contains uracils so that it can be degraded later with uracil deglycosylase, thus leaving a single-stranded probe. The template also contains a small mismatch at one end (GGGAAA rather than GGGTTA), so that the priming oligonucleotide will preferentially anneal in the correct spot rather than anywhere along the template. The template, GTU4, is an oligonucleotide of four G-rich telomeric repeats with uracil. The oligonucleotide T3C3+9 begins with TTTCCC followed by nine nucleotides of correct telomeric sequence. The annealed product of these two oligonucleotides is shown in Figure 18.6.3. Thus, six cytosines will be incorporated per synthesized product. If 50 μCi of [α-³²P]dCTP (3000 Ci/mmol) is used, then one has 16.7 pmol of [α-³²P]dCTP available. Assuming the target is to incorporate 10 pmol so that one gets mostly full-length products, then 1.7 pmol of annealed target is needed. In general, the efficiency of incorporation is so high that it should not be necessary to remove unincorporated dCTP. If desired, one can use plug dialysis on 20% (w/v) acrylamide. The authors currently use [α-³²P]dCTP as the radioisotope because they routinely have it on hand. G-rich probes generally give about 2-fold better signals than C-rich probes (probably because of possible G-quartet formation by the target G-strands, which inhibits hybridization), so making a G-rich probe with 3000 Ci/mmol radioisotope is recommended if maximum sensitivity is needed.

5'-TTTCCCTAACCCCTAA
3'-AAAGGGAUUGGGAUUGGGAUUGGG

Figure 18.6.3 Annealed priming and template oligonucleotides for synthesis of a high-specific activity telomeric repeat probe. The template contains a small mismatch at its 3' end (GGGAAA rather than GGGTTA) so the priming oligonucleotide will anneal in the correct spot.

Materials

10 pmol/μl GTU4 oligonucleotide dissolved in TE buffer:
5'-GGGUUAGGGUUAGGGUUAGGGAAA-3'
100 pmol/μl T3C3+9 oligonucleotide dissolved in TE buffer:
5'-TTTCCCTAACCCCTAA-3'
1 M NaCl
10× buffer M: 10 mM Tris·Cl (pH 7.5)/10 mM MgCl₂/50 mM NaCl/1 mM dithioerythritol (DTE; Roche)
2 M Tris·Cl, pH 7.4 to 7.6 (APPENDIX 2A)
10 mg/ml BSA (e.g., Ambion)
1.25 mM dAdT: 1.25 mM each of dATP and dTTP
[α-³²P]dCTP (3000 Ci/mmol)
5 U/μl Klenow large fragment of *E. coli* DNA polymerase I
1 U/μl uracil deglycosylase (UDG)
37°, 95°, and 99°C water baths heating blocks, or PCR machines
PhosphorImager, including phosphor screens (Molecular Dynamics)
Additional reagents and equipment for DNA acrylamide gel electrophoresis (APPENDIX 3A)

1. To make 1.7 pmol/μl annealed template oligonucleotide stock, mix the following:

3.4 μl 10 pmol/μl GTU4 oligonucleotide
15.6 μl 100 pmol/μl T3C3+9 oligonucleotide
1 μl 1M NaCl (50 mM final salt concentration).

Incubate 1 min at 99°C, 15 min at 37°C, and 15 min at room temperature.

The template oligonucleotide can be stored indefinitely at -20°C.

2. To make 8× buffer M, mix the following (total volume 625 μl):

500 μl 10× buffer M
100 μl 2 M Tris·Cl, pH 7.4 to 7.6
25 μl 10 mg/ml BSA.

Buffer M is the equivalent of Klenow buffer (50 mM Tris·Cl, pH 7.5; 10 mM MgCl₂; 1 mM DTT; 50 μg/ml BSA; 50 μM dNTP) without dNTP and with extra salt so that the oligonucleotides do not melt. It is very stable at -20°C.

3. To prepare the reaction mixture, mix together:

3.125 μl of 8× buffer M (step 2)
1 μl annealed template oligo (step 1)
1 μl of 1.25 mM dAdT (final 50 μM)
13.875 μl dH₂O
5 μl [α-³²P]dCTP
1 μl Klenow large fragment.

Incubate reaction mixture (25 μ l total) 30 min at room temperature and then 5 min at 95°C.

The incubation at 95°C is important because if the Klenow is not inactivated, then the probe is degraded during UDG treatment.

4. Add 0.5 μ l of 1 U/ μ l UDG and incubate 10 min at 37°C and then 10 min at 95°C.

This step is to cleave deglycosylated uracils and release probe as a single-stranded molecule after denaturing the UDG.

5. To check probe, run 30 fmol labeled probe on a 20% (w/v) acrylamide minigel (170 V, 20 min), wrap gel in cellophane (while still attached to one glass plate), and expose 10 min to a phosphor screen. Visualize with a PhosphorImager to confirm that adequate signal is present. Store the probe <2 weeks at -20°C.

The screen is saturated after a 1-hr exposure. A 10-bp ladder can be used for size markers, or a kinased probe or free dCTP can be used for reference, if desired.

Approximately 40% of the [α -³²P]dCTP should be incorporated into the full-length probe.

Either all or half the probe is used for the assay (>20,000 cpm/ μ l).

BASIC PROTOCOL 2

MEASUREMENT OF TELOMERASE ACTIVITY BY THE TELOMERIC REPEAT AMPLIFICATION PROTOCOL

The telomeric repeat amplification protocol (TRAP) is a highly sensitive in vitro assay used for the detection of telomerase activity by the polymerase chain reaction (PCR) amplification of the products produced by the processive elongation of an oligonucleotide primer (Fig. 18.6.4). The current TRAP is a modification of the assay first described by Kim et al. (1994). This improved version allows for the elimination of the need for a wax barrier hot start, reduction of amplification artifacts, and better estimation of telomerase processivity by using a modified reverse primer sequence. Incorporation of an internal standard control allows for quantitation of telomerase activity. The TS primer is a nontelomeric sequence that telomerase can nonetheless recognize as a substrate. The reverse primer ACX contains several modifications (an anchor sequence at its 5' end and two mismatches within the telomerase repeats) that both reduce PCR artifacts and more faithfully amplify the distribution of telomerase-elongated products than occurs with unmodified telomeric sequence primers.

Materials

- 100,000 cells grown in culture or tissue lysate containing 6 μ g protein (see Support Protocol 4)
- Liquid nitrogen
- NP-40 lysis buffer (see recipe), ice cold
- RNase
- 50 \times dNTP mix: 2.5 mM each dATP, dTTP, dGTP, and dCTP in RNase-free water
- 10 \times TRAP buffer (see recipe)
- Radiolabeled TS primer (see Support Protocol 5)
- Primer mix (see Support Protocol 6)
- RNase-free H₂O (DEPC-treated)
- 50 mg/ml BSA, ultrapure (Ambion)
- 5 U/ μ l *Taq* DNA polymerase
- Loading dye (see recipe)
- 0.5 M NaCl/50% (v/v) ethanol/40 mM sodium acetate (pH 4.2), optional

3. Resuspend sample (before it is fully thawed) in 100 μ l ice-cold NP-40 lysis buffer and incubate 30 min on ice.
4. *Optional:* Centrifuge sample 20 min at 14,000 \times g, 4°C, after lysis. Collect 80% of the supernatant in a fresh tube, making sure that no traces of cell debris from pellet are withdrawn.

The whole cell lysate can be used instead of pelleting the insoluble material. The authors have found that recovery of telomerase activity is higher and more reproducible when the samples are not centrifuged and the entire lysate is analyzed. Centrifugation is usually needed only when tissues are used (see Support Protocol 4).

5. Snap-freeze lysate in liquid nitrogen and store at -80°C until analyzed by TRAP assay.

Lead shot beads can be used as an alternative to snap freezing in liquid nitrogen. The beads are poured into a container and placed at -80°C . Samples added to the beads are snap-frozen in a couple of minutes.

The TRAP assay can be performed using a radioactive or nonradioactive (see Alternate Protocol) primer.

To prevent contamination with PCR-amplified products, the TRAP assay should be performed in a separate room from which the lysis took place.

Perform primer extension

6. As a negative control, incubate 5 μ l lysate with 1 μ g RNase for 20 min at 37°C or heat inactivate 10 min at 85°C prior to telomerase assay.

A lysis-buffer-only control should also be included to check for presence of PCR contamination in the lysis buffer.

7. Place 1 to 2 μ l lysate (equal to 100 to 1000 cells) in the bottom of an RNase-free PCR tube on ice and add the following:

- 1 μ l of 50 \times dNTP mix (50 μ M final)
- 5 μ l of 10 \times TRAP Buffer
- 2 μ l radiolabeled TS primer
- 1 μ l primer mix
- 38.6 μ l RNase-free H₂O.

Mix and add 0.4 μ l of 50 mg/ml ultrapure BSA (20 μ g final) and 0.4 μ l of 5 U/ μ l Taq DNA polymerase (2 U final). Incubate 30 min at room temperature.

This recipe is designed for one sample. A stock reaction mixture can be made just before assembling the reaction by combining the appropriate amount of dNTP mix, 10 \times TRAP buffer, BSA, primer mix, and water. The radiolabeled TS primer and Taq DNA polymerase should be added immediately before the reaction mixture is added to each sample.

To prevent contamination by PCR products, the preparation of samples and the assembly of the TRAP assay should take place in a separate room from that in which the PCR and electrophoresis are performed.

During the initial 30-min incubation in the presence of the TS primer, varying numbers of hexameric repeats are added by telomerase. Subsequent amplification will yield a 6-bp incremental ladder.

- Amplify extension products by polymerase chain reaction (PCR) in a thermal cycler as follows:

Initial step:	90 sec	94°C (to inactivate telomerase)
24 to 27 cycles:	30 sec	94°C
	30 sec	60°C.

Analyze extension products

- Add 5 μ l loading dye to 20 μ l TRAP reaction mixture and run on a 10% (w/v) nondenaturing acrylamide gel in 0.5 \times TBE.
- Optional:* Fix gel 30 min in 0.5 M NaCl/50% ethanol/40 mM sodium acetate, pH 4.2.
Fixing the gel to prevent diffusion of the bands may be performed when overnight exposure times are expected.
- Wrap gel in plastic wrap and expose gel, without drying, to phosphor screens 30 min to overnight and visualize on a PhosphorImager using ImageQuant software.
- Sum the intensities of the bands in the TRAP ladder and divide by the intensity of the internal standard control band to determine relative TRAP activity. Compare the activity of each sample with the negative and positive controls.

MEASUREMENT OF TELOMERASE ACTIVITY BY THE TELOMERIC REPEAT AMPLIFICATION PROTOCOL USING FLUORESCENT PRIMERS

**ALTERNATE
PROTOCOL**

Measurement of telomerase activity using fluorescent primers rather than radiolabeled primers can be used as an alternative to Basic Protocol 2 to reduce the exposure to radioactivity. This alternative procedure may be slightly less sensitive than the radioactive method.

Additional Materials (also see Basic Protocol 2)

100 ng/ μ l fluorescently labeled TS primer: 5'-Cy5-AATCCGTCGAGCAGAGTT (Integrated DNA Technologies), HPLC or PAGE purified

- Prepare cells or tissue lysate as described (see Basic Protocol 2, steps 1 to 5).
- Set up a negative control and TRAP reaction mixture as described (see Basic Protocol 2, steps 6 and 7), but use 100 ng/ μ l fluorescently labeled TS primer instead of radiolabeled TS primer in step 7.
- Amplify extension products by polymerase chain reaction (PCR) in a thermal cycler as follows:

Initial step:	90 sec	94°C (to inactivate telomerase)
30 cycles:	30 sec	94°C
	30 sec	52°C
	45 sec	72°C.

- Electrophorese the TRAP reaction mixture and fix the gel, if desired, as described (see Basic Protocol 2, steps 9 and 10).

Fixing of the gel for nonradioactive TRAP allows for easier handling when the gel is placed on a PhosphorImager.

- Wrap gel in plastic wrap and scan under Cy5 (Blue) fluorescence with a PhosphorImager using ImageQuant software (Molecular Dynamics). Analyze the TRAP ladder as described (see Basic Protocol 2, step 12).

**Cell Aging and
Death**

18.6.11

**LYSIS OF TISSUE SAMPLES FOR THE TELOMERIC REPEAT
AMPLIFICATION PROTOCOL**

This procedure can be used to obtain lysates to analyze telomerase activity from tissue samples. Because the sample will be used in an RNase-sensitive procedure, standard precautions to avoid RNase contamination should be observed.

Additional Materials (also see Basic Protocol 2)

50 to 100 mg tissue sample, frozen at -80°C
Washing buffer (see recipe), ice cold
BCA protein assay kit (Pierce)
Kontes tubes and disposable pestles (VWR)
Hand-powered drill

1. *Optional*: Wash 50 to 100 mg frozen tissue sample in enough ice-cold washing buffer to cover the tissue. Centrifuge and remove buffer from tissue. Mince tissue with scalpel.
2. Homogenize tissue with 200 μl ice-cold NP-40 lysis buffer in a Kontes tube on ice using a disposable pestle rotating at 450 rpm with a hand-powered drill. Use short (<10- to 15-sec) pulses.

Care must be taken not to overheat the samples and destroy the telomerase activity in the tissue.

3. Incubate sample 25 min on ice, centrifuge lysate 20 min at $16,000 \times g$, 4°C . Collect 80% of the supernatant in a fresh tube. Save an aliquot for protein assay. Rapidly freeze the supernatant in liquid nitrogen. Store up to ~ 1 year at -80°C .
4. Measure the concentration of protein in the lysate using a BCA protein assay kit (APPENDIX 3H).

An aliquot of the extract containing 6 μg of protein will be used for each telomerase assay (see Basic Protocol 2, step 7).

**MAKING RADIOLABELED TS PRIMER FOR TELOMERIC REPEAT
AMPLIFICATION PROTOCOL**

For the radioactive TRAP assay (see Basic Protocol 2), the TS primer (5'-AATCCGTCGAGCAGAGTT-3') is end-labeled with T4 polynucleotide kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The following protocol makes enough primer for ten samples, and the amounts can be adjusted appropriately for varying number of samples.

Materials

100 ng/ μl TS primer (5'-AATCCGTCGAGCAGAGTT-3')
 $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol), sterile
10 U/ μl T4 polynucleotide kinase and 5 \times forward kinase buffer (GIBCO/BRL)
Sterile, RNase-free H_2O
RNase-free 1.5-ml microcentrifuge tubes
 37° and 85°C water baths or heating block, or PCR machine

1. Mix together the following in a RNase-free 1.5-ml microcentrifuge or PCR tube:

10 μl of 100 ng/ μl TS primer
2.5 μl [γ - ^{32}P]ATP
4 μl of 5 \times forward kinase buffer
3 μl sterile, RNase-free H_2O
0.5 μl of 10 U/ μl T4 polynucleotide kinase.

Incubate 20 min at 37°C.

2. Heat inactivate enough kinase for one assay 5 min at 85°C and use immediately or freeze up to 2 weeks at -20°C. Do not freeze and thaw more than once.

MAKING THE PRIMER MIX FOR TELOMERIC REPEAT AMPLIFICATION PROTOCOL

SUPPORT
PROTOCOL 6

The primer mix includes the reverse primer (ACX), the substrate for the 36-bp internal standard control (TSNT), and the reverse primer for the internal standard (NT). Preparation of the stock TSNT primer should be done in a separate room from the other TRAP steps and prepared with separate micropipettors. The TSNT primer should be purchased from a different company from the other two primers because it is possible to contaminate them at the production facilities with the TSNT oligonucleotide. This protocol makes enough primer mix for 100 sample reactions.

Materials

TSNT oligonucleotide:

5'-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3'

RNase-free H_2O

1 $\mu\text{g}/\mu\text{l}$ ACX primer: 5'-GCGCGGCTTACCCTTACCCTTACCCTAACC-3'

1 $\mu\text{g}/\mu\text{l}$ NT primer: 5'-ATCGCTTCTCGGCCTTTT-3'

10% (v/v) bleach

RNase-free 0.5-ml PCR tubes, including some that are siliconized

1. Dilute dry TSNT oligonucleotide in RNase-free water to 100 μM (1.0×10^{-10} mol/ μl) in a siliconized, RNase-free PCR tube. Perform three dilutions (1:100, 1:1000, and 1:1000) in siliconized tubes so that the final concentration of the TSNT stock is 1.0×10^{-18} mol/ μl .

This step should be done in a separate room with separate micropipettors and tips from the other steps. Because extremely dilute solutions of the TSNT substrate are required, siliconized tubes should be used for all dilutions. The actual amount added to the primer mix below is empirically adjusted to compensate for experimenter error.

2. Mix 10 μl each of the 1 $\mu\text{g}/\mu\text{l}$ ACX and NT primers (100 ng/ μl final) and 79.0 μl RNase-free water together in an RNase-free tube.
3. Move to the area where the TSNT was prepared and add 1 ml dilute TSNT oligonucleotide (0.01×10^{-18} mol/ml final) to the mix. Clean the outside of the tube with 10% bleach.
4. Return to the nonTSNT work area and divide the primer mix into 25- μl aliquots. Use aliquots immediately or store up to 1 year at -20°C. Do not freeze and thaw more than once.

Cell Aging and
Death

18.6.13

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

Denhardt solution, 100×

10 g Ficoll 400
10 g polyvinylpyrrolidone
10 g BSA (Pentax Fraction V; Miles Laboratories)
H₂O to 500 ml
Filter sterilize and store at –20°C in 25-ml aliquots

Hybridization solution

5× SSC buffer (see recipe)
5× Denhardt solution (see recipe)
10 mM Na₂HPO₄
1 mM Na₂H₂P₂O₇
Store up to 6 months at –20°C

Loading dye

0.25% (w/v) each of bromophenol blue and xylene cyanol in 50% (v/v) glycerol/50 mM EDTA. Store up to 1 year at 4°C.

For nonradioactive TRAPs, the xylene cyanol should be removed, as this shows up while imaging the gel.

NP-40 lysis buffer

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1 mM MgCl₂
1 mM EDTA
1% (v/v) NP-40
0.25 mM sodium deoxycholate
10% (v/v) glycerol
150 mM NaCl (*APPENDIX 2A*)
5 mM 2-mercaptoethanol
0.1 mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride)
Store up to 6 months at –20°C

Quick-prep lysis buffer

100 mM NaCl
100 mM EDTA, pH 8.0 (*APPENDIX 2A*)
10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
Store up to 1 month at room temperature

SSC (sodium chloride/sodium citrate), 20×

3 M NaCl (175 g/liter)
0.3 M Na₃citrate·2H₂O (88 g/liter)
Adjust pH to 7.0 with 1 M HCl (*APPENDIX 2A*)
Store up to 6 months at room temperature

TRAP buffer, 10×

200 mM Tris·Cl, pH 8.3 (*APPENDIX 2A*)
15 mM MgCl₂
630 mM KCl
0.5% (v/v) Tween 20
10 mM EGTA
Store up to 6 months at –20°C

Washing buffer

10 mM HEPES (adjusted to pH 7.5 with KOH)

1.5 mM MgCl₂

10 mM KCl

1 mM DTT (*APPENDIX 2A*)

Store up to 6 months at 4°C

COMMENTARY

Background Information

Cells contain repeated TTAGGG DNA sequences, called telomeres, at the ends of chromosomes to provide genomic stability. Telomeres provide a source of expendable DNA, which alleviates the end-replication problem in which one DNA strand cannot complete copying all the way to its end during cell division (reviewed in Collins, 2000). An in-gel Southern hybridization analysis of the chromosome terminal (i.e., telomeres) restriction fragments (TRFs) allows for the determination of all telomere lengths in a cell population (Harley et al., 1990; Ouellette et al., 2000). Telomere length has been shown to decrease with time and with increasing age (Allsopp et al., 1992). Most normal human cells lack telomerase activity, and their telomeres shorten with each cell division until they enter replicative senescence, where cells cease to proliferate (Harley et al., 1990; Wright et al., 1996). Cells are mortal unless a rare survivor activates a mechanism to maintain its telomeres.

The mechanism predominately used to maintain human telomeres is the addition of telomeric sequences by telomerase. Human telomerase is an RNA-dependent DNA polymerase complex consisting of a reverse transcriptase catalytic subunit (hTERT), which uses the integral RNA component (hTR) of the complex as a template for adding TTAGGG repeats to the end of the chromosome (Fig. 18.6.4; reviewed in Greider and Blackburn, 1985; Cong et al., 2002). Telomerase is expressed in only a small number of normal proliferating cell types such as germline and somatic stem cells, but it is found in ~90% of human cancer cells. Once telomerase is activated, it stabilizes telomere length and permits continued cell division (Counter et al., 1992).

The development of a highly sensitive assay to detect telomerase activity in cells and tissues enhanced the evaluation of a wide variety of human tissue and tumor cells (Kim et al., 1994). The telomeric repeat amplification protocol (TRAP) measures in vitro telomerase activity by a primer extension assay in which telom-

erase synthesizes telomeric repeats onto oligonucleotide primers (Fig. 18.6.5; Kim et al., 1994). The products can then be amplified by the polymerase chain reaction (PCR) and run on an acrylamide gel. There are several publications describing modifications and alternative methods of extracting and detecting telomerase activity in human samples using the TRAP assay (Piatyszek et al., 1995; Wright et al., 1995; Holt et al., 1996; Norton et al., 1998; Gollahon and Holt, 2000). The protocol described in this unit incorporates the modifications suggested in these reports. Together with the TRF analysis, telomerase has been shown to be directly involved in telomere maintenance and has been linked with immortality (Kim et al., 1994).

Critical Parameters

Terminal restriction fragments

It is desirable to obtain $\geq 300,000$ cells for the determination of telomere length so that 1 to 2 μg DNA can be assayed for each TRF analysis. The amount of DNA is estimated at 6 $\mu\text{g}/10^6$ diploid cells or 10 $\mu\text{g}/10^6$ aneuploid cells; therefore, 300,000 cells yields 1.8 μg DNA. Triton X-100 rather than SDS is used in the lysis so that the residual detergent present after dialysis does not inhibit subsequent digestion by restriction endonucleases. High concentrations of EDTA (100 mM) are present to inhibit nucleases very rapidly in these concentrated cell suspensions. Frozen cell pellets are first suspended in lysis buffer to quench nuclease activity and obtain a uniform distribution of cells before adding Triton X-100. Including Triton X-100 in the initial suspension buffer leads to a rapid clumping of cells. Lysis buffer should be added before cell pellets are fully thawed to prevent nuclease digestion. Heat inactivation of the proteinase K avoids the need to phenol/chloroform extract the DNA, and the residual salt, EDTA, and peptides present after dialysis do not inhibit enzyme digestion. After digestion with proteinase K, DNA should be handled using only wide-bore pipet tips to

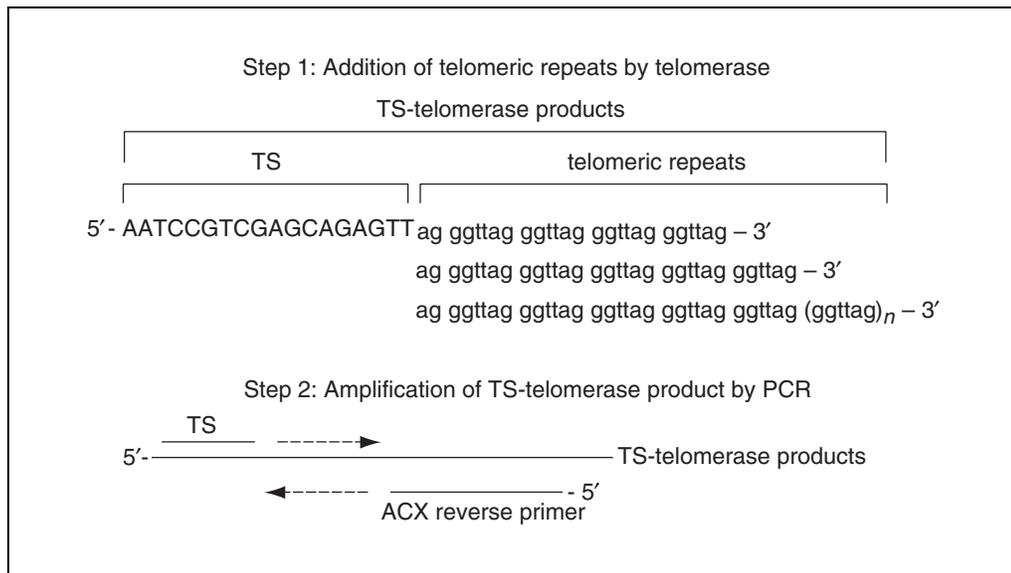


Figure 18.6.5 The telomeric repeat amplification protocol (TRAP; see Basic Protocol 2). In the first step, telomerase-mediated extension occurs on a nontelomeric oligonucleotide that serves as a substrate for telomerase (TS). In the second step, the TS-telomerase products are specifically amplified by the polymerase chain reaction (PCR) using the TS as an upstream primer and ACX as the reverse, downstream primer. The addition of telomeric repeats in the first step results in a TRAP ladder of 6-bp increments as seen on a 10% (w/v) acrylamide gel. Adapted from the manual for the TRAPeze Telomerase Detection Kit (InterGen).

prevent shearing of the DNA. The final DNA solution should be viscous. Lack of viscosity suggests nonspecific nuclease degradation of the DNA, which is easily tested by running 100 ng undigested DNA on a 0.7% (w/v) agarose gel. Degraded DNA is frequently seen the first time an investigator attempts this DNA purification procedure, but generally it is rarely present thereafter. If the DNA concentration is too low after dialysis to load a sufficient quantity on a gel, then concentration on a 30,000-molecular-weight spin-filter or ethanol precipitation of the DNA may be necessary. This is rarely necessary when starting with $\geq 1 \times 10^6$ cells.

Digested DNA should run as a smear below the 1-kb molecular weight marker. Anything above this size is incompletely digested. If the sample is not digested completely, an additional microliter of enzyme mix can be added to the sample and allowed to digest for a longer amount of time. It is essential to have the genomic DNA completely digested to have interpretable results after hybridization with the telomeric probe. If digestion problems persist, completion of the restriction digest can be checked ahead of time by electrophoresing 250 ng DNA on a small agarose gel stained with ethidium bromide.

TRF gels should be handled carefully as they are fragile until they are dried. The dried gel

can be hybridized directly without the need to transfer the DNA to a membrane. Denaturing the DNA in the gel before rather than after drying the gel increases the signal intensity ~3- to 5-fold, presumably by permitting a much greater diffusion of the denatured DNA strands in the agarose gel than in the very-high-percent-age dried and rehydrated gel, and thus inhibiting the reannealing of the parental strands, which would compete with the probe. Rinsing the denatured gel with distilled water gets rid of the NaOH used in the denaturing solution and substantially reduces sticking of the gel to the Whatman 3MM paper. Because most of the DNA samples do not fill the wells and are thus in the bottom half of the gel, the gels are flipped and dried with the upper surface against the filter paper support. This increases the distance between the DNA samples and the filter paper and significantly reduces the loss of lower-molecular-weight DNA. Some loss of lower-molecular-weight DNA (particularly <400 bp) occurs during drying, and this results in the lower limit of detection in the TRF analysis.

Telomeric repeat amplification protocol

Because the TRAP assay is a highly sensitive PCR-based assay, precautions must be taken to eliminate RNases and PCR product contamination. The following guidelines en-

Table 18.6.1 TRAP Troubleshooting Guide^a

Problem	Possible cause	Solution
No visible products in any lane, including the internal standard control band	PCR not initiated	Check that all assay components were added, especially <i>Taq</i> DNA polymerase. Check thermal cycler for proper settings and operation.
Few visible TRAP ladder bands and weak internal control band	Cell or tissue extract contains <i>Taq</i> inhibitors	Dilute sample with lysis buffer.
No visible TRAP products in any lane but internal standard control band is present	Telomerase activity not initiated Possible presence of RNase contamination	Always use RNase-free tips, tubes, and solutions. Use fresh aliquots of solutions. Do not let pipet tips touch anything except the solutions. Keep samples on ice and reactions below 25°C. Add RNase inhibitor to lysis buffer.
Visible TRAP product in all lanes including lysis-buffer-only control	PCR carryover contamination	Use fresh aliquots of each component of the assay. Perform each step in a separate room with separate micropipettors and tips.
Several bands in the lysis-buffer-only control lane	Primer-dimer artifacts	Incorporate hot start into the PCR reaction: Assemble reaction mixture as described, except eliminate <i>Taq</i> polymerase, heat reaction 2 min at 94°C, add <i>Taq</i> , and continue PCR procedure as described.
Products in the heat-treated extracts	Insufficient heat inactivation, primer-dimer artifacts, or contamination of extract with TRAP products	Check temperature of heating block. Make and test new extract and check for PCR carryover contamination. Extreme care must be taken to prevent pipetting into multiple samples or solutions.
Extra bands between internal standard control band and TRAP ladder bands		
Weak intensity of internal standard control band	Telomerase activity too high	Dilute samples.

^aAdapted from the manual for the TRAPeze Telomerase Detection Kit (Intergen).

sure that contamination is kept to a minimum. Gloves must be worn at all times and each step of the protocol should be done in separate rooms or at least in areas separated by barriers. DEPC-treated water must be used for all solutions, solutions should be kept from other solutions in the laboratory, and small aliquots of the solutions should be made and used only once. Separate micropipettors should be designated to be used only for the TRAP assay, and only aerosol-resistant pipet tips should be used with these micropipettors. Samples and solutions should also be kept on ice or snap-frozen, unless otherwise specified, to prevent degradation of telomerase.

Troubleshooting

The most common problem associated with the TRF assay is that the genomic DNA is not completely digested. This results in a smear or tight band >1 kb as visualized by ethidium bromide staining, and this interferes with the interpretation of telomere lengths after hybridizing the gel with a telomeric probe. A test gel using 200 ng of DNA digested with the enzyme mix can show whether the samples have been completely digested. If the DNA is not completely digested, the usual cause is that the sample contains too much EDTA, which interferes with the restriction enzymes. This usually results from dialyzing too many samples against an insufficient volume, or from a failure to ensure that all the liquid rests against the membrane, resulting in a few microliters of 100

mM EDTA failing to get dialyzed. Other occasional failures result from using too much DNA because of an inaccurate measurement of the viscous DNA. Another problem is that the sample may not contain any detectable DNA because of a tear in the dialysis membrane. Weak signals after scanning the hybridized gel may be due to too stringent washing or insufficiently labeled probe. Use a Geiger counter to ensure that the background signal of the gel is low but that the areas expected to have telomeres contain a higher signal before exposing the gel. Check to make sure that the probe is specific for telomeric sequences and that the radioisotope and other components are fresh.

Table 18.6.1 lists some common problems and solutions associated with the TRAP assay. The most common problems associated with the TRAP assay are RNase and PCR contami-

nation or forgetting to add one of the components in the reaction, such as the *Taq* polymerase. Careful handling of the samples and reagents in the TRAP assay ensures successful detection of telomerase activity.

Anticipated Results

The results of an in-gel Southern hybridization of digested genomic DNA with a telomeric probe yields a smear that corresponds to the distribution of all telomere lengths within the population of cells collected (Fig. 18.6.6). Including a labeled molecular weight marker on the hybridized gel allows for calculation of the average telomere length as well as the longest and shortest telomere lengths.

Once the conditions for the TRAP assay have been optimized and RNase contamina-

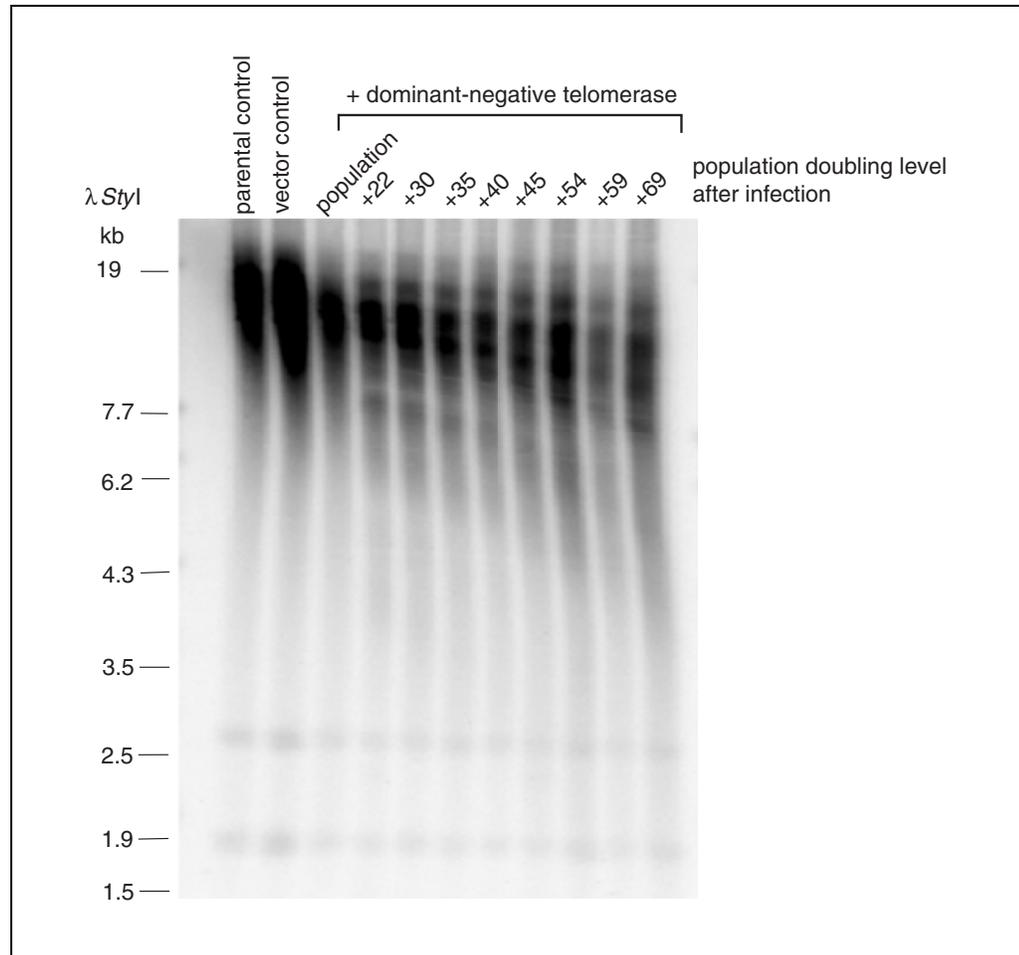


Figure 18.6.6 Determination of telomere length using the terminal restriction fragment (TRF) protocol in sequentially passaged (indicated by the population doubling level, or the number of doublings the population has accrued, after infection) H1299 lung carcinoma cells infected with a dominant-negative mutant of human telomerase to inhibit telomerase and induce telomere shortening. Telomere length was determined by digesting genomic DNA with a mixture of six restriction enzymes having four-base recognition sites (see Basic Protocol 1), and then analyzing the DNA on a 0.7% (w/v) agarose gel. λ DNA cut with *StyI* was used as a molecular weight marker.

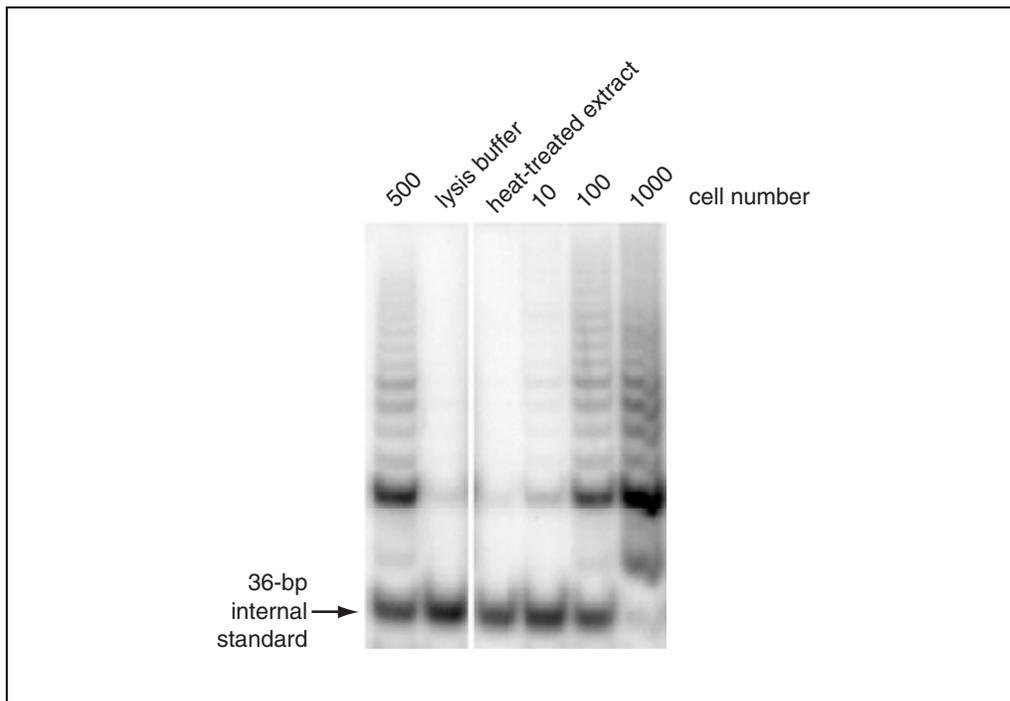


Figure 18.6.7 Analysis of telomerase activity using the telomeric repeat amplification protocol (TRAP; see Basic Protocol 2). Human cancer cell extracts (500 cell equivalents of H1299 lung carcinoma cells) are positive for telomerase activity as evidenced by the 6-bp incremental TRAP ladder. Treating the extract with heat inactivates the telomerase activity as evidenced by no TRAP ladder. Lysis buffer only serves as a negative control. Each TRAP reaction includes a 36-bp internal standard control. Ten-fold increases of H1299 cell equivalents (100 to 1000 cell equivalents) results in increased intensity of the TRAP ladder. The 1000 cell sample in this gel cannot be readily quantitated because of the faint signal in the internal standard (due to competition by excess telomerase products).

tion is eliminated, the amplified telomerase-extended products can be seen as an incremental ladder (Fig. 18.6.7). Because human telomerase is processive, the 30-min incubation yields a TS primer extended by a variable number of hexameric repeats. When the resulting 10% (w/v) acrylamide gel is visualized by a PhosphorImager, a 6-bp DNA incremental ladder is seen as well as the internal standard control band. The lysis buffer only and the heat-treated sample lanes should contain only the internal standard control band. The intensities of each of the bands in the TRAP ladder can be summed together and divided by the internal standard control band to determine relative TRAP activity. Too much telomerase activity will result in intense ladders and a failure to amplify the internal standard. The actual amount of the TSNT primer used is empirically adjusted so that one gets an internal standard signal roughly equivalent to the most intense first band of the telomerase ladder using 100 to 1000 positive tumor cells as a control. The

activity of each sample can then be compared with the negative and positive controls.

Time Considerations

The entire TRF procedure can be performed in 4 days (day 1: harvest and digest cells with proteinase K and dialyze overnight; day 2: restriction digest DNA and run gel overnight; day 3: dry gel and hybridize overnight; day 4: wash and expose gel). Alternatively, isolated genomic DNA can be stored at 4°C (or at -20°C for longer storage) until it is digested. The TRAP assay can be performed in 1 day or separated into a 3-day procedure: one day for lysing the samples, a second day for the PCR assay, and a third day for running and exposing the gel.

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Internet Resource

http://www.swmed.edu/home_pages/cellbio/shay-wright/

Includes TELORUN for calculating mean TRF.

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Nonisotopic Methods for Determination of Poly(ADP-Ribose) Levels and Detection of Poly(ADP-Ribose) Polymerase

The techniques presented in this unit are designed to allow quantification and characterization of pADPr synthesized *in vitro*, in cultured cells, or in tissues from whole animals. Treatment of cells with DNA-damaging agents such as hydrogen peroxide or DNA-alkylating compounds stimulates PARP activity. After treatment, activated PARPs cleave nicotinamide adenine dinucleotide (NAD) into nicotinamide and ADP-ribose and synthesize pADPr polymers covalently attached to nuclear proteins. Following TCA precipitation of poly(ADP-ribosyl)ated proteins, the acid-insoluble fraction is used to purify pADPr in order to determine pADPr levels and its size distribution in intact cells (see Basic Protocol 1). This section presents novel applications for the immunodetection of pADPr in intact cells, such as immunodot blot analysis (see Basic Protocol 2), ELISA detection (see Alternate Protocol 1), size-distribution analysis by immunodetection (see Basic Protocol 3), detection of modified protein by immunoblot (see Basic Protocol 4), and cellular localization by immunocytochemistry (see Basic Protocol 5). In addition to determining the presence of pADPr, methods are also provided for detection of the PARPs themselves by immunoblot (see Basic Protocol 6), proteolytic cleavage during cell death (see Alternate Protocol 2), and nonisotopic-activity immunoblot (see Basic Protocol 7).

NONISOTOPIC METHODS FOR DETERMINATION OF POLY(ADP-RIBOSE) LEVELS IN INTACT CELLS

Poly(ADP-ribose) polymerase-1 (PARP-1) is the main enzyme responsible for poly(ADP-ribose) (pADPr) synthesis in response to DNA strand breaks caused by genotoxic agents. Analysis of pADPr is the easiest method that provides direct proof of PARP activation following genotoxic treatment in cells. Furthermore, monitoring changes in pADPr metabolism could serve as an important diagnostic tool to determine the impact of low-dose exposure to DNA-damaging agents.

The main challenge in pADPr research is its very low concentration in cells: ~2 to 10 pmol/mg DNA in untreated cells. To bypass this problem, some investigators have used isolated nuclei or permeabilized cells in order to study poly(ADP-ribosylation) of proteins following a DNA-damaging treatment; however, while this procedure provides higher levels of pADPr due to the loss of cellular integrity, the results are, by definition, nonphysiological. More recently, the availability of highly specific anti-poly(ADP-ribose) antibodies has become a powerful tool to investigate pADPr metabolism in cultured cells. The establishment of nonisotopic methods to study pADPr synthesis has provided safer and simpler laboratory procedures.

Poly(ADP-Ribose) Preparation from Cell Cultures

pADPr synthesis is a useful biological parameter for the investigation of cellular processes such as DNA repair and cell death. Cell treatments with DNA-damaging agents such as hydrogen peroxide or DNA alkylating compounds cause PARP-1 and -2 activation. It must be kept in mind that these pADPr-inducing treatments must be optimized for each cell line. The following protocol was first described by Aboul-Ela et al. (1988) and was subsequently modified in the authors' laboratory (Shah et al., 1995). These procedures are based on the principle that pADPr is covalently bound to proteins present in the TCA precipitate and released under alkaline conditions. Once released from proteins, the

BASIC PROTOCOL 1

Cellular Aging and Death

18.7.1

Contributed by Jean François Haince, Guy G. Poirier, and Jim B. Kirkland

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pADPr can be further purified by dihydroxyboryl Bio-Rex 70 (DHBB) chromatography (Alvarez-Gonzalez et al., 1983). This purification step is required to eliminate major contaminants such as DNA, RNA, and residual proteins present in the cell extract. In practice, it is useful to eliminate these contaminants to decrease background when using immunodetection techniques for pADPr analysis.

Materials

Cells of interest in appropriate medium
DNA-damaging agent
PBS, pH 7.4 (APPENDIX 2A), ice cold
20% (w/v) trichloroacetic acid (TCA), ice cold
100% ethanol, ice cold
1 M KOH/50 mM EDTA
AAGE9 buffer (see recipe)
HCl, concentrated
50% (w/v) dihydroxyboryl Bio-Rex 70 (DHBB) affinity resin (Alvarez-Gonzalez et al., 1983)
1 M ammonium acetate, pH 9.0
H₂O, 37°C

Rubber policeman
37° and 60°C water baths or heat blocks
10-ml Econocolumn (Bio-Rad)

Prepare acid-insoluble fraction from cultured cells

1. Treat cells of interest in medium with an appropriate DNA-damaging agent and incubate for sufficient time to allow for PARP activation.

For example, treat confluent C3H10T^{1/2} mouse fibroblasts (ATTC# CCL-226) with 200 μM hydrogen peroxide or 100 μM N-methyl-N-nitro-N-nitrosoguanidine (MNNG) for 20 to 30 min at 37°C. This is a typical procedure for PARP-1 activation. An optimal incubation time must be determined by the investigator if using other DNA-damaging stimuli or other cell types. This time should be determined carefully, as pADPr can decrease rapidly at longer time points due to NAD depletion or repair processes.

2. Remove and discard the medium from the cell culture and quickly wash the monolayer with 5 ml ice-cold PBS, pH 7.4.
3. Remove the PBS, add 5 ml ice-cold 20% (w/v) TCA, and incubate cells 15 min on ice.

This step is critical to prevent catabolism of polymer by poly(ADP-ribose) glycohydrolase and must be done as quickly as possible.

4. Scrape the TCA-insoluble material with a rubber policeman and transfer cells to a centrifuge tube. Pellet by centrifuging 10 min at 1800 × g, 4°C.
5. Wash the cell pellet once with ice-cold 20% TCA and twice with 5 vol ice-cold 100% ethanol.
6. Air dry the cell pellet.

The pellet may be stored up to 6 months at -30°C or -80°C or directly processed as follows.

Isolate pADPr from cell pellet

7. Dissolve the TCA precipitate in 1 ml of 1 M potassium hydroxide/50 mM EDTA and mix by vortexing.

In the case where TCA has not been thoroughly removed from the pellet, it may be necessary to utilize a glass homogenizer to disperse the pellet as much as possible.

8. Incubate the alkali-digest reaction mixture 1 hr at 60°C with occasional vortexing to promote separation of the pADPr from the proteins.

Following this incubation, a small aliquot can be removed from each sample for determination of DNA content, if required.

Purify pADPr by DHBB chromatography

9. Add 9 ml AAGE9 buffer to the alkali-digested pADPr and adjust the pH to 9.0 ± 0.2 with concentrated hydrochloric acid.

AAGE9 buffer contains 250 mM ammonium acetate, pH 9.0, 6 M guanidine-hydrochloride, and 10 mM EDTA.

10. Transfer 1 ml of 50% (w/v) DHBB suspension (0.5 ml packed affinity resin) into a 10-ml Econocolumn (Bio-Rad). Equilibrate with 5 ml AAGE9 buffer, 10 ml water, and 10 ml AAGE9 buffer, successively. Verify that the column pH is 9.0.

The description and detailed preparation of DHBB resin is presented in Alvarez-Gonzalez et al. (1983).

11. Pass the alkali digest sample twice through the DHBB column. Wash with 25 ml AAGE9 buffer followed by 10 ml of 1 M ammonium acetate, pH 9.0.

12. Elute the pADPr with 0.5 ml water prewarmed to 37°C. Repeat four more times.

The pADPr will be in fractions 2 and 3.

13. Lyophilize the eluted pADPr and keep frozen until further analysis (up to 1 year).

Quantification of pADPr by Immunodot Blot

The following protocol describes the utilization of an immunodot blot technique for the determination of DHBB-purified pADPr from cell extracts. This nonisotopic immunodetection method, developed in the authors' laboratory (Affar et al., 1999a), is based on the covalent binding of pADPr to a positively charged nylon membrane under alkaline conditions. The detection is carried out using an anti-poly(ADP-ribose) primary antibody, followed by an appropriate peroxidase-conjugated secondary antibody. Quantification is done with chemiluminescence reagents for HRP-conjugated secondary antibodies. This technique can be used routinely for rapid quantification of pADPr at femtomolar concentrations, so it provides great advantages over other procedures. In addition to a great sensitivity, the immunodot blot procedure is applicable to in vitro synthesized polymer as well as to cell culture isolated pADPr. More samples can be processed simultaneously by this method than is possible using HPLC methods.

Materials

DHBB-purified pADPr (see Basic Protocol 1)
0.4 M NaOH/10 mM EDTA
0.4 M NaOH
PBS-MT (see recipe)
Anti-pADPr primary antibody
Peroxidase-conjugated secondary antibody
PBS-T: PBS containing 0.1% (v/v) Tween 20
PBS (APPENDIX 2A)
Chemiluminescence reagents for HRP-conjugated secondary antibodies
7.8 × 11.5-cm Hybond N⁺ nylon membrane (Amersham Bioscience)
Dot blot manifold system (Life Technologies)
Orbital platform shaker

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Cooled CCD camera with ChemiImager 4000 and Digital Imaging and Analysis System (Alpha Innotech) *or* X-ray film

Additional reagents and equipment for autoradiography and densitometry (*UNIT 6.3*; optional)

1. Dilute DHBB-purified pADPr in a vol of 0.4 M sodium hydroxide/10 mM EDTA.
2. Load samples onto a 7.8 × 11.5-cm Hybond N⁺ nylon membrane using a dot blot manifold system.
3. Wash the membrane with 50 ml 0.4 M sodium hydroxide, remove from the manifold, and keep in water for further processing.
4. Incubate the membrane in 50 ml PBS-MT on a orbital platform shaker for at least 1 hr at room temperature.
5. Dilute anti-pADPr primary antibody in PBS-MT and incubate 50 ml with the membrane on a orbital platform shaker overnight at room temperature.

Two antibodies, 10H and LP96-10, are commercially available for the detection of polymer ranged between 1 and 1000 fmol. Generally, experiments are performed with 10H antibody diluted 1:250 or LP96-10 at 1:5000. Under these specific conditions, both antibodies recognize pADPr in a dose-dependent manner, but LP96-10 polyclonal antibody shows a higher sensitivity (also see Critical Parameters).

6. Wash the membrane three times with 50 ml PBS-MT for 15 min each.
7. Dilute an appropriate peroxidase-conjugated secondary antibody in PBS-MT according to supplier's instructions.
8. Incubate the membrane 30 min with 50 ml secondary antibody on a orbital platform shaker at room temperature.
9. Discard the secondary antibody and wash the membrane as follows:
 - Twice with 50 ml PBS-MT for 5 min each
 - Three times with 50 ml PBS-T for 15 min each
 - Twice with 50 ml PBS for 5 min each.
10. Illuminate the blot using chemiluminescence reagents for HRP-conjugated secondary antibodies according to instructions.
- 11a. *Quantify using digital imaging:* Quantify the amount of pADPr using a cooled CCD camera equipped with ChemiImager 4000. Analyze the data with the Digital Imaging and Analysis Systems.

This is the technique used in the authors' laboratory. It removes the nonlinear steps involved in film exposure and developing, but traditional use of X-ray film and densitometry techniques (see below) are also effective, if the film is exposed correctly. With either technique, pADPr standards should be used to ensure that quantification is linear and accurate.

- 11b. *Quantify by densitometry:* Use standard X-ray densitometry techniques (*UNIT 6.3*) to quantify the signal from the secondary antibody.

Quantification of pADPr by Enzyme-Linked Immunosorbant Assay

The enzyme-linked immunosorbent assay (ELISA) is an interesting alternative method to the immunodot blot procedure (see Basic Protocol 2) for quantification of the pADPr synthesized following DNA-damaging or apoptosis-inducing treatments. This technique was developed to provide a simple procedure to investigate pADPr metabolism without radiolabeled compounds. The conditions of fixation and washing to detect low cellular levels of pADPr were optimized in the authors laboratory (Affar et al., 1999a). Briefly, pADPr isolated from cell cultures are alkali digested and fixed in a 96-well microplate. This cellular pADPr is incubated with a specific anti-poly(ADP-ribose) primary antibody. Following incubation with appropriate secondary antibodies, the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) coloration is measured and compared to a standard curve made with known amounts of DHBB-purified pADPr synthesized in vitro.

Additional Materials (also see Basic Protocol 2)

- 1 µg/ml poly-L-lysine in PBS-T₂
- PBS (APPENDIX 2A)
- DHBB-purified pADPr samples (see Basic Protocol 1) in PBS-T₂
- PBS-T₂: PBS (APPENDIX 2A)/0.05% (w/v) Tween 20
- PBS-MT₂ (see recipe)
- Anti-pADPr primary antibody diluted in PBS-MT₂
- Peroxidase-conjugated secondary antibody
- ABTS/H₂O₂ solution (see recipe)
- 96-well microtiter plate (Falcon 3912)
- Microtiter plate reader

Prepare a microtiter plate

1. Coat a 96-well microplate with 200 µl per well of 1 µg/ml poly-L-lysine in PBS-T₂.
2. Incubate the plate overnight at 4°C.
3. Wash the plate twice with 200 µl/well PBS for 5 min each.

Add sample

4. Add each DHBB-purified pADPr sample prepared in PBS-T₂ (final volume 50 µl) to a well.
5. Incubate samples overnight at 37°C.
6. Wash the plate three times with 200 µl/well PBS-T₂ for 2 min each.

Block plate

7. Saturate the plate for 1 hr with PBS-MT₂ at 37°C.

This blocking step should be done with 250 µl per well of PBS-MT₂ to efficiently saturate unoccupied binding sites.

8. Remove PBS-MT₂ from the plate and add 50 µl/well of anti-pADPr primary antibody diluted in PBS-MT₂.

Generally, the authors use LP96-10 polyclonal antibody at a dilution of 1:5000. Other investigators have used monoclonal 10H antibody at a dilution of 1:250. The LP96-10 antibody is preferred because some studies have revealed that the 10H monoclonal antibody fails to recognize poly(ADP-ribose) smaller than 25 residues (also see Critical Parameters).

Incubate plate with antibody

9. Incubate pADPr samples 1 hr with the primary antibody at 37°C.
10. Wash the plate three times with 200 µl/well PBS-T₂ for 2 min each.
11. Dilute an appropriate peroxidase-conjugated secondary antibody 1:1000 in PBS-MT₂.
12. Incubate the plate 30 min with 50 µl/well secondary antibody at 37°C.
13. Discard the secondary antibody and wash the plate as follows:

Three times with 200 µl/well PBS-T for 2 min each
Twice with 200 µl/well PBS for 5 min each.

At this stage, it is important to remove all traces of Tween 20 because it can interfere with the peroxidase assay.

Visualize bound antibody

14. Add 100 µl ABTS/H₂O₂ solution to each well and let the color develop 15 min at room temperature.
15. Read the absorbance at 405 nm using a microtiter plate reader.

High-Resolution Gel Electrophoresis and Immunodetection of pADPr

It has been observed that the size distribution of pADPr varies between unstimulated cells and cells subjected to DNA-damaging agents (Malanga and Althaus, 1994). With this information in mind, it can be useful to measure the levels of pADPr synthesized following DNA-damaging treatments. In order to characterize the size distribution of pADPr synthesized in treated cells, the authors have developed a novel immunodetection method involving the transfer of electrophoresed pADPr onto a positively charged membrane, followed by blotting with specific anti-pADPr antibodies to evaluate the size distribution of pADPr synthesized *in vivo* (Affar et al., 1999a). All pADPr larger than octamers were detected with this technique. Careful analysis of the blot has shown that short polymers (3 to 8 residues) have a weak binding capacity for the positively charged membrane. This class of pADPr is lost during washing in the immunodetection procedure.

Materials

20% (w/v) polyacrylamide gel (see recipe)
Electrode buffer: 54 mM Tris-borate buffer (pH 8.3)/1.2 mM EDTA
Alkali-digested pADPr sample isolated from cell culture (see Basic Protocol 1, step 8) or lyophilized DHBB-purified sample (see Basic Protocol 1)
Modified Laemmli sample buffer (see recipe)
Transfer buffer: 35 mM Tris-borate buffer (pH 8.3)/1 mM EDTA
PBS-MT (see recipe)
Anti-pADPr primary antibody
Peroxidase-conjugated secondary antibody
PBS-T: PBS containing 0.1% (v/v) Tween 20
PBS (APPENDIX 2A)
Chemiluminescence reagents for HRP-conjugated secondary antibodies
Purified pADPr standard (see Basic Protocol 1)
Nitrocellulose Hybond N⁺ membrane (Amersham Bioscience)
Hair dryer
312-nm UV source
X-Omat blue film (Kodak)

Additional reagents and equipment for preparing and running acrylamide gels (*UNIT 6.1*), immunoblot detection (see Basic Protocols 2, steps 5 to 10, and (*UNIT 6.2*), and autoradiography and densitometry (*UNIT 6.3*)

Prepare polyacrylamide gel

1. Prepare a 20 × 20 × 0.15-cm 20% polyacrylamide gel.
2. Prerun the gel 1 to 2 hr in electrode buffer at 400 V.

Prepare pADPr

3. *Optional:* Air dry the alkali-digested pADPr isolated from cell culture.
4. Dilute the dried polymer (i.e., from alkali digested pADPr or lyophilized DHBB-purified sample) in modified Laemmli sample buffer.

Do not heat the sample as pADPr can be degraded at higher temperatures. The use of bromophenol blue and xylene cyanol as standards is useful since they co-migrate with poly(ADP-ribose) molecules of ~8 and 20 residues, respectively.

5. Load a volume of sample containing 25 to 50 pmol pADPr per lane on the prerun polyacrylamide gel (step 2).

pADPr is quantitated by immunodot blot (Basic Protocol 2) or ELISA (Alternate Protocol 1).

Electrophoresis sample

6. Replace the electrophoresis buffer and electrophorese the sample at a constant voltage of 400 V for a period varying with the pADPr size of interest.

The ratio of acrylamide/bisacrylamide (19:1) is not the same as that usually prepared for the SDS-PAGE. Consequently, use the recipes given in Reagents and Solutions.

If the size is unknown, run the gel until the bromophenol blue reaches 5 cm from the end of the gel.

7. Electrotransfer the sample onto a nitrocellulose Hybond N⁺ membrane 1.5 hr in transfer buffer at a constant current of 0.4 A at room temperature (*UNIT 6.2*).
8. Dry the membrane with a hair dryer. Cross-link the pADPr by exposing the membrane to a 312-nm UV source for 5 min.

Immunoblot and detect pADPr

9. Incubate the membrane in 50 ml PBS-MT on a orbital platform shaker for at least 1 hr at room temperature.
10. Perform immunoblot detection with an appropriate anti-pADPr antibody as described (see Basic Protocol 2, steps 5 to 10).

This procedure was optimized to immunodetect quantities of 1 pmol pADPr isolated from cells when using the LP96-10 (1:5000) as anti-pADPr antibody (see Critical Parameters). The authors noticed that, with this technique, the 10H (1:250) anti-pADPr antibody failed to detect pADPr below 25 mers.

11. Expose the membrane to X-Omat blue film (Kodak; *UNIT 6.3*) for an appropriate length of time.

Detection of Poly(ADP-Ribosyl)ated Proteins by Immunoblotting

Poly(ADP-ribosyl)ation is a post-translational modification occurring on glutamate residues of nuclear acceptor proteins, including PARP-1 itself. A large number of acceptor proteins have been identified in a variety of cellular processes. The existence of pADPr in intact cells is a transient event, because poly(ADP-ribose) glycohydrolase (PARG) rapidly catabolizes pADPr by virtue of its exo- and endo-glycohydrolase activities. Under normal growth conditions, cultured cells display low basal levels of polymer, which can dramatically increase when cells are exposed to DNA-damaging agents. On immunoblots, basal protein-bound pADPr generally appears in concentrated smears that originates at ~116 and 89 kDa, likely representing automodification of PARP-1 and its apoptotic fragment (Boyonoski et al., 2002a,b); however, other molecular weights are observed, and the patterns vary between tissues. As a complementary approach to determine the presence of active PARPs, the following protocol provides a simple assay to monitor the accumulation of pADPr in a cellular or tissue extract. This assay uses a specific anti-pADPr primary antibody to quantify the accumulation of pADPr covalently bound to proteins. This technique provides an estimation of the total quantity of pADPr, chain lengths, and molecular weights of major acceptor proteins.

Materials

Cells in appropriate medium *or* pretreated anesthetized animal
DNA-damaging agents, oxidative stress, or apoptosis-inducing stimulus
HeBS (*APPENDIX 2A*), ice cold
HeBS containing 1 mM PMSF and Complete protease inhibitor cocktail (Roche),
ice cold
Extraction buffer (see recipe)
2× SDS sample buffer (*APPENDIX 2A*)
Reducing sample buffer (see recipe)
Ponceau S dye (see recipe)
PBS-MT (see recipe)
Anti-pADPr primary antibody
Peroxidase-conjugated secondary antibody
PBS-T: PBS containing 0.1% (v/v) Tween 20
PBS (*APPENDIX 2A*)
Chemiluminescence reagents for HRP-conjugated secondary antibodies
Prestained molecular weight standards (optional)

Cell scraper (optional)
15-ml conical centrifuge tube
Sonicator
65°C water bath
0.45- μ m nitrocellulose membrane (Amersham Bioscience)
Orbital platform shaker
X-Omat blue film (Kodak)

Additional reagents and equipment for cell trypsinization (*UNIT 1.1*; optional), gel
electrophoresis (*UNIT 6.1*), electrotransfer (*UNIT 6.2*), and immunoblotting (see
Basic Protocol 2, steps 5 to 10)

Prepare samples

For cultured cell samples

- 1a. Subject cells of interest in appropriate medium to DNA-damaging agents, oxidative stress, or apoptosis-inducing treatments to stimulate pADPr synthesis.

- 2a. Remove and discard the medium from the cell culture and quickly wash the monolayer with 5 ml ice-cold HeBS.
- 3a. Harvest adherent cells in ice-cold 5 ml HeBS containing 1 mM PMSF and Complete protease inhibitor cocktail. Use a cell scraper or trypsinization (*UNIT 1.1*) to completely release cells from the dish.

The protease inhibitors may be excluded if direct immunoblotting is to be carried out.

Prepare tissue sample

- 1b. Remove tissues of interest as rapidly as possible from anesthetized pretreated animals. Flash freeze in liquid nitrogen and store up to 1 year at -80°C .
- 2b. Add chunks of frozen tissue to ice-cold extraction buffer. Homogenize on ice using a power unit.

The authors use ~0.25 g of tissue/ml buffer.

- 3b. Prepare ~100 to 200 μl tissue homogenate per milliliter of $2\times$ SDS sample buffer and proceed immediately to step 9.

Lyse cells

4. Transfer harvested cultured cells to a 15-ml conical centrifuge tube and centrifuge 10 min at $600\times g$, 4°C .
5. Wash the cell pellet twice with 5 ml of ice-cold HeBS, centrifuging as described (step 4).
6. Remove supernatant and lyse cells in reducing sample buffer at a concentration of 2×10^6 cells per milliliter.

The presence of urea in the sample buffer favors the dissociation of PARP-1 from DNA.

7. Sonicate resuspended cells on ice for three 20-sec bursts at 40% maximal output to shear the DNA.
8. Immediately freeze sample in liquid nitrogen and store up to 3 months at -80°C . Alternatively, proceed immediately to step 9.

This procedure was designed to obtain a cell extract inactivated as quickly as possible, because the pADPr is rapidly degraded by poly(ADP-ribose) glycohydrolase in whole cells.

Electrophoresis sample

9. Incubate samples 15 min in a 65°C water bath.
10. Load a volume of sample equivalent to $1-2\times 10^5$ cells or 20 μg protein/well onto a $6\times 8\text{-cm}$ 8% resolving SDS-polyacrylamide minigel (*UNIT 6.1*).

Do not forget to add appropriate molecular weight standards at this stage.

11. Electrophorese the gel (*UNIT 6.1*) and electrotransfer (*UNIT 6.2*) samples onto a 0.45- μm nitrocellulose membrane.
12. Stain the membrane with Ponceau S dye for 1 min and then wash extensively in deionized water to remove all residual dye.

This step is useful to identify molecular weight standards, confirm equal transfer of proteins, and visualize the protein profile of the samples. This step can be excluded if prestained molecular weight standards are used, although prestained standards are also less accurate if a close identification of molecular weight is required.

13. Incubate the membrane at least 1 hr in 50 ml PBS-MT on an orbital platform shaker at room temperature to block unoccupied protein-binding sites.

Immunoblot

14. Perform immunoblot detection with an appropriate anti-pADPr antibody as described (see Basic Protocol 2, steps 5 to 10) except incubate the membrane in anti-pADPr primary antibody at 4°C rather than room temperature (see Basic Protocol 2, step 5).
15. Expose the membrane to X-Omat blue film (Kodak; *UNIT 6.3*) for an appropriate length of time.

Immunocytochemical Detection of pADPr

Some investigators have proposed that the synthesis of pADPr could be a useful parameter to identify apoptotic cells (Donzelli et al., 1997; Scovassi and Poirier, 1999; Chang et al., 2002). The combination of pADPr synthesis with other apoptotic markers and the presence of PARP-1 cleavage (see Alternate Protocol 2) facilitates the determination of apoptosis in cell culture. pADPr synthesis is also a sensitive indicator of DNA damage from a variety of sources, such as alkylating agents and oxidative stress. The following procedure provides a sensitive and rapid method for identification of endogenous cellular pADPr synthesis, following DNA damage, by immunocytochemistry (see Fig. 18.7.1B). Alternatively, this procedure can be used to localize PARP family members in cultured cells.

Materials

Cells of interest in appropriate medium
DNA-damaging stimulus (e.g., 100 μ M MNNG, 200 μ M H₂O₂)
PBS (*APPENDIX 2A*), ice cold
70:30 (v/v) methanol/acetone, ice cold
PBS containing 1% (w/v) Triton X-100 and 10% (v/v) FBS
Anti-pADPr primary antibody
Fluorophore-conjugated secondary antibody
Fluoromount-*G* (Southern Biotech)

Coverslips and slides
Humid chamber (i.e., a petri dish lined with a wet paper towel)

Additional reagents and equipment for fluorescence microscopy (*UNIT 4.2*)

1. Grow cells of interest in appropriate medium on coverslips and expose them to DNA-damaging stimulus.

Most DNA-damaging agents, oxidative stress, and apoptosis-inducing treatments can induce pADPr synthesis in different cell types. The authors prefer to treat mouse fibroblast C3H10T^{1/2} cells either with 100 μ M MNNG for 15 min or with 200 μ M hydrogen peroxide for 15 min. These two methods both give good results.

2. Wash the cells with 2 ml ice-cold PBS.
3. Fix cells by incubating them 15 min on ice in 2 ml ice-cold 70:30 methanol/acetone.

A large number of fixation methods can be used at this stage. The authors have also obtained good results when cells were fixed for 10 min on ice with 10% ice-cold TCA (Lankenau et al., 1999); however, methanol/acetone fixation is preferred because traces of residual TCA may alter the integrity of the antibody used to detect pADPr.

4. Remove the methanol/acetone solution and wash four to five times with 2 ml ice-cold PBS.

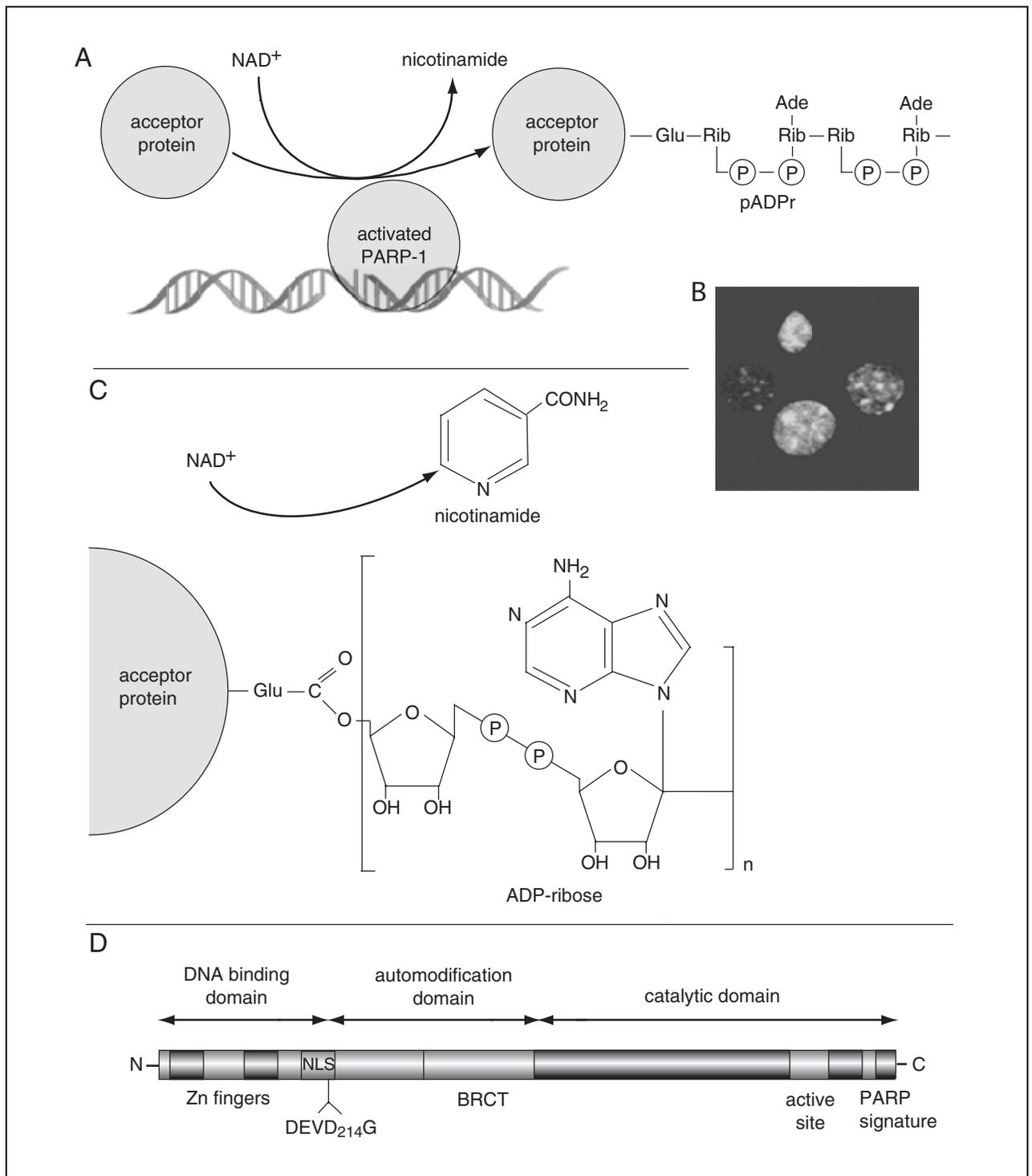


Figure 18.7.1 Poly(ADP-ribosyl)ation reaction and poly(ADP-ribose) polymerase-1 (PARP-1) structure. **(A)** PARP-1 is selectively activated by DNA strand breaks and transfers ADP-ribose from NAD⁺ to a set of acceptor proteins. **(B)** Immunocytochemical detection of pADPr synthesized in mouse fibroblast C3H10T_{1/2} cells exposed 15 min to 200 μM H₂O₂. Immunodetection was carried out with LP96-10 anti-pADPr polyclonal antibody (1:100) and revealed with 1:100 Texas Red-conjugated anti-rabbit second antibody (1:100). **(C)** The sequential addition of ADP-ribose units to a glutamic acid residue of an acceptor protein results in the formation of a long linear and branched chain of pADPr that is structurally similar to nucleic acids. **(D)** Schematic representation of PARP-1 domains. PARP-1 enzyme can be divided into three major domains (also see Fig. 18.7.2): a 46-kDa DNA binding domain, a central 22-kDa automodification region that contains the BRCT domain, and a 54-kDa C-terminal region that comprises the active site and the PARP signature. During caspase-dependent apoptosis, caspase-3 and -7 recognize a DEVD motif in the nuclear localization signal (NLS) and cleave PARP-1 into a p89 and a p24 fragment. (Adapted from the PARP link Web site: <http://parplink.u-strasbg.fr/index2.html>).

5. Permeabilize cells in 2 ml ml PBS containing 1% (w/v) Triton X-100 and 10% (v/v) FBS for 15 min at room temperature.

FBS is used to block unspecific epitopes.

6. Remove permeabilizing solution and wash once with 2 ml ice-cold PBS.
7. Dilute the appropriate anti-pADPr primary antibody in PBS containing Triton X-100 and FBS according to supplier's instructions.
8. Incubate coverslips with 80 μ l diluted primary antibody overnight at 4°C in a humid chamber.

A petri dish lined with wet paper toweling is a suitable humid chamber.

9. Wash coverslips three to five times with 2 ml PBS.
10. Dilute an appropriate fluorophore-conjugated secondary antibody in PBS containing 1% Triton X-100 and 10% FBS according to supplier's instructions.
11. Incubate the coverslips 30 min with diluted secondary antibody at room temperature.

This step should be done in darkness to avoid fluorochrome quenching.

12. Wash the coverslips several times with 2 ml PBS.
13. Mount coverslips on microscope slides with mounting medium (i.e., Fluoromount-G) according to manufacturer's instructions.

Mounting slides with Fluoromount-G reduces fluorochrome quenching during analysis of slides by fluorescence microscopy. This mounting medium also provides a semipermanent seal for long-term storage of slides.

14. Allow mounted slides to air dry 5 min before analyzing the localization of cellular pADPr by fluorescence microscopy using an appropriate filter (*UNIT 4.2*).

DETECTION OF POLY(ADP-RIBOSE) POLYMERASE FAMILY MEMBERS IN CELLS

Poly(ADP-ribosyl)ation is a post-translational modification catalyzed mostly by the 116-kDa enzyme poly(ADP-ribose) polymerase-1 (PARP-1), a nuclear enzyme that transfers an ADP-ribose moiety onto a limited number of nuclear proteins, including itself (D'Amours et al., 1999). When cells are exposed to environmental stresses such as alkylating agents or free radicals, there is up to a 500-fold increase in net pADPr synthesis in response to DNA strand breaks. The enzyme responsible for 80% to 90% of this stimulated pADPr synthesis is PARP-1, while other PARPs are responsible for the remaining 10% to 20%. The physiological meaning of these phenomena is not clear; however, it can be interpreted as a way of translating an event occurring on DNA to the nucleus by protein modification and finally to cytoplasm via NAD⁺ depletion (Chiarugi, 2002). It has also been proposed that the presence of negatively charged pADPr at the site of DNA damage may play several roles in regulation of base excision repair, p53 functions, and apoptosis.

In the past few years, several additional proteins catalyzing poly(ADP-ribosyl)ation have been identified (Fig. 18.7.2). These new members of the PARP family (Shall, 2002) include PARP-2, PARP-3, vPARP (PARP-4), tankyrase-1 (PARP-5) and -2 (PARP-6), and finally Ti-PARP (PARP-7). However, recent characterization of PARP-2 demonstrated that it possesses a nuclear distribution similar to PARP-1. PARP-2 also possesses an N-terminal DNA-binding domain and is activated in response to DNA damage (Ame et al., 1999). The similarities between the activity and functions of PARP-1 and -2, and the fact that PARP-1 knockout cells still have the ability to undergo apoptosis, suggest that PARP-2

PARP Members	Molecular organization	MW (kDa)	Activity dependent on DNA damage	Antibody	Specificity	Used for
PARP-1	N [DBD] [BRCT] [PD] C	113	Yes	PARP-1 Mab (C2-10)	Recognizes both the full length & the 85 kDa cleavage fragment of PARP-1	EL, IF, IP, WB
sPARP-1	[PD]	55	No	sPARP-1 Pab	Recognizes human and mouse sPARP-1 and PARP-1	IF, WB
PARP-2	[DBD] [PD]	62	Yes	mouse recombinant PARP-2	Human, mouse or monkey PARP-2	EL, IF, IP, IB
PARP-3	[PD]	60	Yes			
VPARP (PARP-4)	[BRCT] [PD] [MVPD]	193	No	human VPARP (aa 24-38)	Human Vault PARP	IB
Tankyrase 1 (PARP-5)	[HPS] [ANKYRIN] [PD]	142	No	tankyrase Mab (19A449)	Human tankyrase	IB
Tankyrase 2 (PARP-6)	[ANKYRIN] [PD]	130	?			
Ti PARP (PARP-7)	[TIL] [PD]	76	?			
pADPr		--	--	poly(ADP-ribose) Pab (LP96-10)	poly(ADP-ribose) synthesized in vivo or in vitro	EL, IF, IP, IB
pADPr		--	--	poly(ADP-ribose) Mab (10H)	poly(ADP-ribose), fail to recognize pADPr smaller than 10 mers.	IF, IB

Figure 18.7.2 Poly(ADP-ribose) polymerase family members and their antibodies. Abbreviations: EL, ELISA; IB, immunoblotting; IF, immunofluorescence; IP, immunoprecipitation.

may also play a role in apoptosis. Recent data suggest that PARP-3 is involved in centrosome duplication and cell cycle progression. The nucleoplasmic PARPs tankyrase-1 and -2 possess an N-terminal ankyrin domain and a C-terminal PARP domain and their PARP domains contain all of the amino acids necessary for catalysis (Cook et al., 2002). These PARPs have been described as being strand-break independent and are probably not responsive to DNA damage. A predominantly cytoplasmic PARP, vPARP, is a component of vault particles (Kickhoefer et al, 1999), but it is also observed in nuclear locations at some points of the cell cycle.

Detection of Poly(ADP-Ribose) Polymerase Enzymes by Immunoblot

Recently, identification of novel poly(ADP-ribose) polymerase enzymes with distinct structures has led to a reconsideration of the role of pADPr in a large number of cellular processes, such as DNA damage response, genomic stability, apoptosis, and chromatin modulation. PARP-1 has been shown to be stimulated directly or indirectly by free radicals or agents causing free radical release (Herceg and Wang, 2001). It is likely that PARP-1 is involved in the response to several different types of cell toxicity, because it can bind with very high affinity to double- or single-stranded breaks, and it consumes the NAD⁺ pool in cells following genotoxic insults. Immunoblotting provides a useful method for the detection of various PARPs in cultured cells. Detailed below is a protocol for detection of PARP-1, but this procedure can also be applied to the detection of any PARP family member when an appropriate primary antibody is used (Fig. 18.7.2).

BASIC PROTOCOL 6

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18.7.13

Materials

Tissue, fresh
Extraction buffer (see recipe), ice cold
2× SDS sample buffer (APPENDIX 2A)
HeBS (APPENDIX 2A), ice cold
Reducing sample buffer (see recipe)
Anti-PARP primary antibody
PBS-MT (see recipe) with and without 1 mM NaN₃
Horseradish peroxidase–conjugated secondary antibody
PBS-T: PBS/0.1% (v/v) Tween 20
PBS (APPENDIX 2A)
Chemiluminescence reagents for HRP-conjugated secondary antibodies

Glass homogenizer with Teflon pestle
15-ml conical centrifuge tube
Sonicator
65°C water bath
0.45-µm nitrocellulose membrane
Orbital platform shaker
X-Omat blue film (Kodak)

Additional reagents and equipment for preparation of cell extracts (see Basic Protocol 4, steps 2 to 11), electrophoresis (UNIT 6.1), electrotransfer (UNIT 6.2), and autoradiography (UNIT 6.3)

Prepare tissue extracts

1. Homogenize fresh tissue in ice-cold extraction buffer using 10 to 20 strokes of a Teflon pestle in a glass homogenizer. Dilute the homogenized tissue to a final concentration of 2.5 to 5 mg/ml by adding enough 2× SDS sample buffer.

A small aliquot should be kept after homogenization for subsequent protein determination.

Alternatively, frozen tissue can be pulverized in liquid nitrogen using a mortar and pestle and then suspended in extraction buffer and diluted in 2× SDS sample buffer. Note that frozen tissues may display partial proteolysis of enzymes and give poor results.

Prepare cell extracts, electrophoresis and transfer

2. Perform cell extraction, electrophoresis, and transfer as described above (see Basic Protocol 4, steps 2 to 11).

Immunoblot

3. Dilute an appropriate anti-PARP primary antibody according to the supplier's recommendation in PBS-MT supplemented with 1 mM sodium azide. Incubate along with the membrane on an orbital platform shaker overnight at room temperature.

A large number of antibodies against PARP-1 are commercially available. The monoclonal antibodies F1-23 (BioMol Research) and the C2-10 (BioMol Research) recognize the N-terminal region of PARP-1, which is not present in other PARP. Thus, they are currently used for specific detection of PARP-1. In mouse tissues, where blood contamination is frequent, the authors recommend the use of LP96-72 (BioMol Research), polyclonal antibody raised against affinity-purified PARP-1; however, in all cases, it is often useful to test the conditions of immunoblotting to obtain optimal results.

C-2-10 is a mouse monoclonal antibody generated by immunization of mice with purified calf thymus PARP-1 that recognizes full-length (116 kDa) PARP as well as the 85 to 90 kDa apoptosis-related cleavage fragment of PARP.

4. Wash the membrane three times with 50 ml PBS-MT for 15 min each.

5. Dilute an appropriate horseradish peroxidase-conjugated secondary antibody in PBS-MT according to supplier's recommendations.
6. Incubate the membrane with 50 ml secondary antibody on a orbital platform shaker for 30 min at room temperature.
7. Discard the secondary antibody and wash the membrane as follows:
 - Three times with 50 ml PBS-MT for 10 min each
 - Three times with 50 ml PBS-T for 15 min each
 - Twice with 50 ml PBS for 5 min each.
8. Analyze the blot using chemiluminescence reagents for HRP-conjugated secondary antibodies according to the manufacturer's instructions.

When extracts from limited quantities of cells are used for the immunoblotting procedure, the authors recommend using of the SuperSignal West Dura Extended Duration Substrate system (Pierce) for low signal detection.
9. Expose the membrane to X-Omat blue film (UNIT 6.3) for an appropriate length of time.

Detection of PARP-1 Cleavage During Cell Death

It may be useful to monitor several apoptotic parameters in a unique sample. Recently, several authors have reported that poly(ADP-ribosyl)ation plays a critical role in the apoptotic process (Soldani and Scovassi, 2002). They suggested that proteolysis of PARP-1 is a sensitive marker for identifying apoptotic cells (Scovassi and Poirier, 1999). During apoptosis induced by a variety of chemotherapeutic agents, two caspases, caspase-3 and -7, cleave PARP-1 into its apoptotic 24- and 89-kDa fragments (Germain et al., 1999). PARP-1 was among the first caspase substrates to be identified. In particular, its cleavage by caspase-3 is well documented. The appearance of the 89-kDa proteolytic product is an early event in the apoptotic process (Kaufmann et al., 1993). It displays DNA-independent enzyme activity, and it may be poly(ADP-ribosyl)ated and thus recognized by an anti-polymer antibody in a variety of processes that lead to cell death. This protocol describes a method for the detection of PARP-1 cleavage that can also be used for the detection of cleavage of other caspase substrates.

Prepare apoptotic cell extracts

1. Treat cells of interest with an appropriate apoptosis-inducing agent and incubate for a sufficient length of time.

For example, 1×10^6 HL-60 human promyelocytic leukemia cells may be treated with 68 μM etoposide for 0 to 12 hr. This procedure constitutes a positive control for immunoblotting of caspase cleavage targets including PARP-1 in etoposide-induced apoptosis. A variety of apoptosis-inducing agents can induce the cleavage of PARP-1, although with different kinetics; therefore, the incubation time producing maximal PARP-1 cleavage must be determined for each of these agents.

2. After drug treatment, remove and discard medium from cell culture. Quickly wash the monolayer with 5 ml ice-cold HeBS.
3. Harvest adherent cells and prepare apoptotic cell extracts as described (see Basic Protocol 4, steps 4 to 8).

The apoptotic cell extract may be stored at -30°C or -80°C at this stage or directly processed on an SDS-PAGE gel (UNIT 6.1).

**ALTERNATE
PROTOCOL 2**

**Cellular Aging
and Death**

18.7.15

Electrophoresis and immunoblot

4. Perform electrophoresis and immunoblotting of samples as described (see Basic Protocol 4, steps 9 to 16).

Nonisotopic-Activity Immunoblot Detection of Poly(ADP-Ribose) Polymerases

This technique involves automodification of nitrocellulose-immobilized poly(ADP-ribose) polymerases using nonisotopic NAD⁺ as the substrate. Detection of pADPr synthesis is done using anti-pADPr antibodies to immunoblot. This technique may be useful to study the activity of apoptosis-associated fragments of PARPs that remain active upon cleavage by caspases. The use of anti-pADPr antibodies to detect polymer synthesis on nitrocellulose-immobilized PARPs enables the detection of these enzymes isolated from many species for which anti-polymerase antibodies are not available. In brief, this procedure is divided into four major steps: (1) reduction of oxidized cysteines in the gel, (2) renaturation of proteins resolved on nitrocellulose membrane, (3) synthesis of polymer, and (4) detection of pADPr by 10H or LP96-10 antibodies.

Materials

Tissue, fresh
Extraction buffer (see recipe), ice cold
2× SDS sample buffer (*APPENDIX 2A*)
HeBS (*APPENDIX 2A*), ice cold
Reducing sample buffer (see recipe)
6 × 8-cm 8% or 10% resolving SDS-polyacrylamide minigel (*UNIT 6.1*)
Prestained or biotinylated molecular weight standards
Running buffer (*UNIT 6.1*) containing freshly added 0.7 M 2-mercaptoethanol
Renaturation buffer (see recipe) with and without 100 μM NAD
Nonisotopic-activity immunoblot reducing buffer (see recipe)
PBS-MT (see recipe)
Anti-pADPr primary antibody
Horseradish peroxidase-conjugated secondary antibody
PBS-T: PBS/0.1% (v/v) Tween 20
PBS (*APPENDIX 2A*)
Chemiluminescence reagents for HRP-conjugated secondary antibodies

Glass homogenizer with Teflon pestle
15-ml conical centrifuge tube
Sonicator
65°C water bath or heat block
Orbital platform shaker
X-Omat blue film (Kodak)

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), and autoradiography (*UNIT 6.3*)

Prepare cellular and tissue extracts

1. Homogenize fresh tissue in ice-cold extraction buffer using 10 to 20 strokes of a Teflon pestle in a glass homogenizer. Dilute the sample to a final concentration of 2.5 to 5 mg protein/ml in 2× SDS sample buffer.

A small aliquot should be kept after homogenization for subsequent protein determination.

Alternatively, frozen tissue can be pulverized in liquid nitrogen using a mortar and pestle then suspended in extraction buffer and diluted in 2× SDS sample buffer. Note that frozen tissues may display partial proteolysis of enzymes and give poor results.

Lyse cells

2. Transfer cells to a 15-ml conical centrifuge tube and centrifuge 10 min at $600 \times g$, 4°C .
3. Wash the cell pellet twice with 5 ml of ice-cold HeBS, centrifuging as described (step 2).
4. Remove supernatant and lyse cells in reducing sample buffer at a concentration of 2×10^6 cells per milliliter.

The presence of urea in the sample buffer favors the dissociation of PARP-1 from DNA.

5. Sonicate resuspended cells on ice for three 20-sec bursts at 40% maximal output to shear the DNA.
6. Immediately freeze sample in liquid nitrogen and store up to 6 months at -80°C . Alternatively, proceed immediately to step 7.

Electrophoresis and transfer

7. Incubate sample 15 min in a 65°C water bath or heat block.
8. Load a volume of sample equivalent to 75,000 cells or 20 μg protein in a well of a $6 \times 8\text{-cm}$ 8% or 10% resolving SDS-polyacrylamide minigel. Also load prestained or biotinylated standards.

Prestained or biotinylated molecular weight standards are used to avoid staining under acidic conditions, which may be detrimental to the polymerase activity (step 11).

9. Electrophorese gel as for a classical SDS-PAGE (UNIT 6.1).
10. After electrophoresis, soak gel 1 hr in running buffer containing freshly added 0.7 M 2-mercaptoethanol at 37°C .

This treatment is necessary to avoid cross-linking between proteins and reduction of the cysteines present in poly(ADP-ribose) polymerases.

11. Transfer proteins to a nitrocellulose membrane as for a classical immunoblotting (UNIT 6.2).

Do not stain the membrane with Ponceau S dye.

Renature proteins

12. Incubate the membrane on an orbital platform shaker 1 hr at room temperature in renaturation buffer without NAD.

This step is important because it allows the restoration of the active PARP conformation.

13. Soak the nitrocellulose membrane on an orbital platform shaker for 1 hr at room temperature in 50 ml of renaturation buffer with 100 μM NAD.
14. Wash the membrane in 50 ml renaturation buffer without NAD four times for 15 min each at room temperature.
15. Wash the membrane in 50 ml nonisotopic-activity immunoblot reducing buffer to remove noncovalently bound pADPr.

Immunoblot

16. Wash the membrane three times with 50 ml PBS-MT for 15 min each.
17. Dilute an appropriate horseradish peroxidase-conjugated secondary antibody in PBS-MT.

18. Incubate the membrane with 50 ml of an appropriate horseradish peroxidase–conjugated secondary antibody on a orbital platform shaker for 30 min at room temperature.
19. Discard the secondary antibody and wash the membrane as follows:
 - Three times with 50 ml PBS-MT for 10 min each
 - Three times with 50 ml PBS-T for 15 min each
 - Twice with 50 ml PBS for 5 min each.
20. Analyze the blot using chemiluminescence reagents for HRP-conjugated secondary antibodies according to the manufacturer's instructions.

When extracts from limited quantities of cells are used for the immunoblotting procedure, the authors recommend using the SuperSignal West Dura Extended Duration Substrate system (Pierce) for low signal detection.
21. Expose the membrane to X-Omat blue film (*UNIT 6.3*) for an appropriate length of time.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

AAGE9 buffer

250 mM ammonium acetate, pH 9.0
6 M guanidine hydrochloride
10 mM EDTA
Store 6 months at room temperature

ABTS/H₂O₂ solution

Just before use, prepare 100 ml citrate-phosphate buffer by dissolving 0.103 g anhydrous citric acid (MW 192.1) and 0.108 g anhydrous dibasic sodium phosphate in 100 ml water. Adjust to pH 4.6 with 1 N HCl. To this buffer, add 50 mg of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 50 μ l of 30% (v/v) hydrogen peroxide.

Acrylamide/bisacrylamide (19:1), 30% (w/v)

28.5 g acrylamide
1.5 g bis(acrylamide)
100 ml H₂O
Store for a maximum of 4 weeks at 4°C in a brown bottle.

Extraction buffer

50 mM glucose
25 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
10 mM EDTA
1 mM PMSF
1× Complete protease inhibitor cocktail (Roche)
Prepare fresh

Modified Laemmli sample buffer

6 M urea
25 mM NaCl
4 mM EDTA
0.02% (w/v) xylene cyanol
0.02% (w/v) bromophenol blue
Store up to 6 months at room temperature

Nonisotopic-activity immunoblot reducing buffer

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)

100 mM NaCl

1 mM dithiothreitol (DTT)

2% (w/v) SDS

Prepare fresh

PBS-MT

PBS (*APPENDIX 2A*)

5 % (w/v) nonfat dried milk

0.1% (v/v) Tween 20

Prepare fresh

PBS-MT₂

PBS, pH 7.4 (*APPENDIX 2A*)

5% (w/v) nonfat dried milk

0.05% (v/v) Tween 20

Prepare fresh

Polyacrylamide gel, 20% (w/v)

100 ml 30% 19:1 (w/v) acrylamide/bisacrylamide (see recipe)

30 ml TBE electrophoresis buffer 5× (see recipe)

19 ml H₂O

Store up to 4 weeks at 4°C

Just before use add 0.75 ml 10% (w/v) ammonium persulfate (APS)

0.06 ml *N,N,N',N'*-tetramethylethylenediamine (TEMED)

Ponceau S dye

2 g Ponceau S

30 g trichloroacetic acid (TCA)

30 g sulfosalicylic acid

100 ml H₂O

Store up to 1 year at room temperature

Reducing sample buffer

62.5 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)

4 M urea

10% (v/v) glycerol

2% (w/v) SDS

0.00125% (w/v) bromophenol blue

Store up to 6 months at room temperature

Just before use, add 2-mercaptoethanol to 5% (w/v)

Renaturation buffer

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)

100 mM NaCl

1 mM dithiothreitol (DTT)

0.3% (v/v) Tween 20

2 mM MgCl₂

Prepare fresh

TBE electrophoresis buffer, 5×

54 g Tris base (890 mM)

27.5 g boric acid (890 mM)

980 ml H₂O

20 ml 0.5 M EDTA, pH 8.0 (20 mM final; see APPENDIX 2A)

Store up to 6 months at room temperature

*TBE stands for Tris/borate/EDTA.***COMMENTARY****Background Information*****PARP-1 structure and function***

The enzyme poly(ADP-ribose) polymerase-1 (PARP-1; EC 2.4.2.30) is a multifunctional enzyme that can be divided into three major domains (see Fig. 18.7.1D). A 41.5-kDa N-terminal region comprises the DNA binding domain (DBD), which contains two zinc-finger motifs and a nuclear localization signal (NLS). This region recognizes both double- and single-stranded DNA breaks in a nonsequence-dependent manner through the first and second zinc finger, respectively. A 16-kDa central auto-modification domain contains fifteen highly conserved glutamate residues thought to be the targets of self-poly(ADP-ribosylation), and a 55.4-kDa C-terminal region contains both the NAD binding site and the catalytic domain which synthesizes poly(ADP-ribose) (pADPr). The primary structure of the enzyme is highly conserved in eukaryotes (D'Amours et al., 1999; Pieper et al., 1999; Burkle, 2001; Herceg and Wang, 2001; Virag and Szabo, 2002).

Poly(ADP-ribosylation) is a post-translational modification of nuclear proteins, in which PARP-1 itself is the main poly(ADP-ribosylated) protein *in vivo*. PARP-1 is selectively activated by DNA strand breaks to catalyze the addition of pADPr from its substrate, nicotinamide adenine dinucleotide (NAD), to a set of acceptor proteins (see Fig. 18.7.1A). After initiation, PARP catalyzes elongation and branching reactions to synthesize a highly branched and complex structure of over 200 ADP-ribose residues into a large homopolymer that is structurally similar to nucleic acids (see Fig. 18.7.1C). The massive auto-modification of PARP-1 dramatically changes its physical properties. Thus, PARP-1 molecules carrying long, highly negatively charged, branched ADP-ribose polymers lose their affinity for DNA and are then inactivated. The automodification process represents a major regulatory mechanism leading to the down-regulation of the enzyme activity (reviewed in Lindahl et al., 1995).

Signaling function of pADPr

pADPr that is synthesized in response to massive DNA damage has a short half-life. It is rapidly hydrolyzed at ribose-ribose bonds and converted to free ADP-ribose units by the enzyme poly(ADP-ribose) glycohydrolase (PARG; Davidovic et al., 2001). The rapid response of PARG to pADPr synthesis indicates that pADPr degradation is also an important nuclear response to DNA damage. PARG constitutes an important component in the control of nuclear pADPr levels, which will in turn have direct effects on the activity of poly(ADP-ribosylated) proteins and on the levels of free pADPr. If there is a pADPr concentration threshold required to trigger apoptosis, the level at which PARG controls pADPr levels becomes critical for the decision point of apoptosis versus DNA repair and cell survival. The possible function of PARG in apoptosis and DNA repair is also supported by the fact that free pADPr generated by PARG may target many DNA damage checkpoints and DNA repair proteins. These cytoplasmic and nuclear proteins could interact strongly with free pADPr, by noncovalent interactions possibly mediated by a pADPr binding motif and alter their functions (Pleschke et al., 2000).

Poly(ADP-ribosylation) is involved in a variety of physiological events such as chromatin decondensation, DNA replication, DNA repair, apoptosis, and cellular differentiation (reviewed in Virag and Szabo, 2002). For example, poly(ADP-ribosylation) of histones decondenses chromatin structure, whereas subsequent degradation of the polymer restores chromatin to its condensed form. Relaxation of chromatin by poly(ADP-ribosylation) might facilitate the access of DNA regulatory and repair enzymes at sites of damage as well as at origins of replication and transcription initiation sites. In addition to modifying chromatin structure, PARP-1 has been shown to affect the function of several transcription factors, DNA replication factors and signaling molecules (i.e., NF- κ B, AP-2, Oct-1, DNA-PK, p53, Topoi-

somerase-1, Lamin B) by noncovalent protein-protein interactions and by active poly(ADP-ribosyl)ation.

PARP-1 activation appears to be a sensitive marker of DNA damage at an early stage of the DNA damage response (see Fig. 18.7.3). Poly(ADP-ribosyl)ation is considered to be a major player in the cellular response to DNA damage because a large number of nuclear proteins are covalently modified with pADPr immediately following DNA breakage (Donzelli et al., 1997; Scovassi and Poirier, 1999; Duriez and Shah, 1997; Soldani and Scovassi, 2002). Moreover, the binding of PARP-1 to DNA strand breaks and the resulting synthesis of pADPr might constitute an emergency signal of damaged DNA to other compartments of cells, such as the mitochondrion (Chiarugi and Moskowitz, 2002).

PARP cleavage during cell death

When programmed cell death is activated, caspase-7 and -3 efficiently cleave and inactivate PARP-1 into a 24-kDa fragment containing the N-terminal DNA binding domain and a 89-kDa peptide comprising the central auto-modification domain, the C-terminal domain, and the catalytic domain (see Fig. 18.7.2). Proteolytic cleavage of PARP-1 into these two characteristic fragments was first recognized by Kaufmann and colleagues at an early stage of etoposide-induced apoptosis in treated human HL-60 cells (Kaufmann et al., 1993). The site of PARP-1 cleavage (DEVD₂₁₃ in human PARP-1) is located within the nuclear localization signal and is highly conserved. Separating the two zinc fingers from the catalytic domain renders the 89-kDa fragment insensitive to activation by additional DNA strand breaks, resulting in the inactivation of PARP-1 activity. However, several studies have shown that this 89-kDa apoptotic fragment can also serve as an early marker of apoptosis induced by a variety of chemotherapeutic agents.

The concept of PARP-mediated cell death following excessive DNA damage is supported by a number of studies that show prevention of cell death by specific PARP inhibitors and in mice deficient in PARP-1 (Shall and de Murcia, 2000; Burkle, 2001; Chiarugi, 2002; Virag and Szabo, 2002). For example, the PARP inhibitor, 3-aminobenzamide, has been shown to slow down the rejoining of DNA strand breaks and to increase the frequency of sister chromatid exchanges in MNNG-induced DNA damage (Oikawa et al., 1980). PARP inhibition also

rendered cells more sensitive to cytotoxicity induced by DNA-damaging agents.

PARP-2

Studies from many laboratories have demonstrated the presence of additional proteins which are able to synthesize pADPr. This observation explains why PARP-1-deficient cells have some residual PARP activity. However, research on the biological role of these novel PARP enzymes is at an early stage. Moreover, some interesting differences in their structural domain, subcellular localization, and ability to be activated by DNA strand breaks have already been established (Fig. 18.7.2). PARP-1 is the best characterized of a growing family of enzymes; however, recent data have provided a model for the biological role of PARP-2 (Ame et al., 1999; Schreiber et al., 2002). This 62-kDa protein is capable of auto-poly(ADP-ribosyl)ation but could not poly(ADP-ribosyl)ate histones. Despite the fact that the BRCT domain is missing in PARP-2 and that the DNA binding domain (DBD) is very short, this enzyme also localizes to nuclei and is activated by DNA strand breaks. This led to the hypothesis that PARP-2 might be implicated in PARP-mediated cell death. Recently, PARP-2 has been demonstrated to be cleaved by caspase-8 in ischemia-induced neuronal apoptosis (Benchoua et al., 2002). Interestingly, cleavage of PARP-2 by caspase-8 dissociates the DNA binding domain (DBD) of PARP-2 from its catalytic domain, resulting in the inactivation of its DNA-dependent pADPr synthesis activity.

PARP-1 activation time course

Several investigators have examined the time course of PARP-1 activation and cleavage during apoptosis. They used immunofluorescence microscopy (see Basic Protocol 5 and Alternate Protocol 2) as well as immunoblot analysis with antibodies to PARP-1 and pADPr in intact cells or tissues (see Basic Protocols 4 and 6). The authors' laboratory has been actively involved in the establishment and improvement of several methods to study pADPr synthesis and catabolism (Shah et al., 1995; Affar et al., 1999a,b; Boucher et al., 2001). The development of standardized procedures to analyze pADPr synthesis and PARP-1 cleavage during cell death using in vitro models and in cultured cells yield specific tools to study many cell functions influenced by pADPr metabolism. One or more of the techniques presented

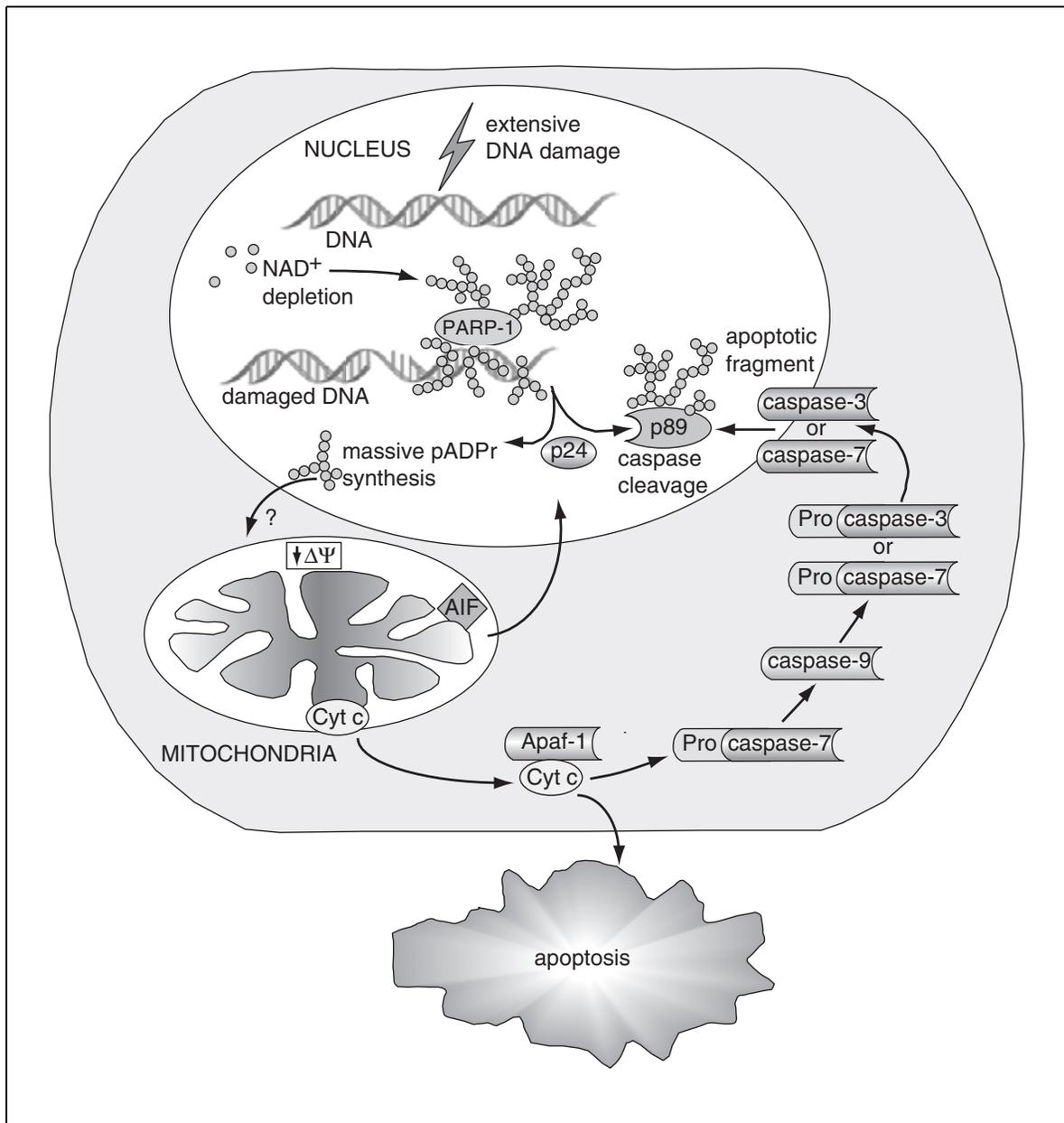


Figure 18.7.3 The concept of PARP-1-mediated cell death. Excessive DNA damage activates PARP-1 in the nucleus, leading to massive pADPr formation and resulting in a NAD⁺ depletion. This high concentration of pADPr may trigger mitochondrial depolarization ($\downarrow\Delta\Psi$) and drive apoptosis through an unknown mechanism. Activated PARP-1 can mediate cell death by caspase-dependent and -delayed pathways. In the caspase-delayed pathway, the insults to DNA, produced by oxidative stress or DNA alkylating agents, induces the release of mitochondrial factors, such as AIF, that translocates to the nucleus and induces caspase-independent chromatin fragmentation into large 50-kDa fragments. However, in caspase-dependent apoptosis, caspase-3 and -7 cleave PARP-1 into two fragments: p89 and p24. Generation of 24-kDa apoptotic fragment contribute to PARP-1 inactivation. The early cleavage PARP-1 serves as preserving cellular ATP for the proper function of further energy-dependent stage of apoptosis.

**Determination of
pADPr Levels
and Detection of
PARP**

18.7.22

in this unit has been used routinely for the detection of apoptotic events in numerous models of cell death. There are many reasons for using PARP-1 cleavage and pADPr synthesis as a sensitive assay for apoptosis. Although PARP-1 is not the only protein cleaved during apoptosis, it is a convenient tool to study caspase activity, and a wide range of detection reagents are currently available from various commercial suppliers. Nevertheless, most of the protocols presented in this unit can be used for studies of other PARPs in order to determine their possible role in cell death.

Critical Parameters

Immunodetection of pADPr

Several points should be considered when using the immunodot method to quantify the levels of pADPr in intact cells. To successfully quantify polymer levels in intact cells, a known concentration of pADPr sample should be used to make a standard curve. This will ensure a more accurate quantification. Quantities loaded should stay within the linear, or mildly curvilinear portion of the response curve. Measurement of polymer concentrations following DNA damage has been described in cultured cells (Affar et al., 1998), but quantitation in animal tissues may be more difficult (Boyonoski et al., 2002a,b). To efficiently quantify the concentration of pADPr in animal tissues, a known amount of protein-bound pADPr from rat liver samples, analyzed by HPLC methods, and/or automodified PARP-1 can be used as standards. These standards reflect chain length and branching similar to that found in other tissues from the whole animal. pADPr made *in vitro* and in many cultured cells appears to be larger and more complex than observed *in vivo*.

Although only a limited number of anti-pADPr antibodies are commercially available, monoclonal (10H) and polyclonal (LP96-10) anti-pADPr antibodies can serve for immunodetection of pADPr. The authors prefer LP96-10 antibody because some studies have revealed that the 10H monoclonal antibody fails to recognize pADPr smaller than 25 residues. The specificity of the anti-pADPr antibody LP96-10 or LP98-10 was confirmed in an *in vitro* competition study (Affar et al., 1999a), and it was shown that NAD, AMP, and ADP-ribose (10 nM) did not significantly interfere with antibody-pADPr binding. Cross-reactivity with nucleic acids is minimal with genomic and nicked DNA, transfer RNA, and total RNA,

in contrast to the strong signal obtained with pADPr.

Detection of PARP-1 cleavage

The relationship between PARP-1 cleavage and apoptosis has been observed by a number of investigators. When immunoblotting detection methods are used for PARP-1 cleavage (see Alternate Protocol 2), one should always keep in mind the possibility of cross-reactivity between the anti-mouse IgG secondary antibodies and polypeptides present in cell lysates. Thus, the detection of PARP-1 cleavage should always be assessed along with morphological changes and DNA fragmentation observation, two other well defined markers of apoptosis. In cases where immunoblotting remains equivocal, it might even be necessary to use a PARP activity immunoblot (see Basic Protocol 7). When poly(ADP-ribose) polymerases are subjected to SDS-PAGE, transferred to nitrocellulose, renatured, and incubated with radioinert NAD, the synthesis of pADPr polymer can be detected using anti-pADPr antibodies. One advantage of this simple nonradioactive activity immunoblot technique is that it can detect any PARP activity in any sample.

Preparation of cell samples

Other critical parameters should be considered when preparing the cell extract. The pADPr synthesized following DNA damage has a very rapid turnover, due to its hydrolysis by PARG (see Basic Protocol 4). Consequently, work as quickly as possible during preparation of cell extracts to avoid the action of activated PARG. This can be achieved by retaining cell extracts on ice or using a specific inhibitor of the PARG enzyme. Note that the procedures described in this unit were optimized to have minimal pADPr degradation during processing of cell samples.

Anticipated Results

Quantitation of pADPr

Most cell lines have a basal level of pADPr in the range of 2 to 30 pmols/mg DNA or 10^8 cells (Affar et al., 1999b); however, in some cell lines, such as human keratinocytes, basal pADPr levels are ~50 to 65 pmols/mg DNA or 10^8 cells (Malanga and Althaus, 1994). Treatments with alkylating agents, such as MNNG, generally induce a 10- to 100-fold stimulation in pADPr synthesis following the formation of DNA strand breaks. The two quantitation methods described in this unit (see Basic Protocol 2

and Alternate Protocol 1), allow the detection of femtomolar concentrations of pADPr.

Immunodetection of pADPr

High-resolution gel electrophoresis, combined with immunodetection of pADPr (see Basic Protocol 3), provides a valuable method to quantify different sizes of pADPr in cultured cells. With this protocol, as little as 1 pmol pADPr may be detected. This high sensitivity can be used for monitoring changes in pADPr size distribution following DNA damage. Although Basic Protocol 2 and Alternate Protocol 1 could also serve to quantify the concentration of pADPr, these techniques do not provide much information about size distribution.

Immunocytochemical detection

The typical results obtained with immunocytochemical detection of pADPr are presented in Figure 18.7.1B. Generally, when mouse fibroblast C3H10T^{1/2} cells are grown on coverslips and stained for pADPr with pAb LP96-10, basal levels of polymer are not detected. However, the burst of pADPr synthesis, which occurs rapidly following DNA damage, peaks and can be visualized within 15 min (see Basic Protocol 5). The use of TCA as the method of fixation gives reliable and reproducible results, since pADPr is insensitive to TCA treatment. However, this method may be incompatible with colocalization experiments due to the possible loss of epitopic sites in some proteins. For pADPr colocalization experiments, methanol/acetone may be a better method of fixation.

Detection of PARP-1 Cleavage

It is generally accepted that PARP-1 proteolysis is a hallmark of caspase-dependent apoptosis. PARP-1 is cleaved by caspases 3 and 7 in the DEVD⁺G₂₁₄ during DNA damage-induced cell death. Both fragments can be easily recognized by immunoblot (see Alternate Protocol 2) with several antibodies. The visualization of PARP-1 and its apoptotic fragment is facilitated by the presence of urea in the sample buffer. However, due to the strong association of PARP-1 and DNA stands, it has been found that DNA-bound PARP-1 may be resistant to caspase-3 cleavage (D'Amours et al., 1998). The cleavage of PARP-1 remains a useful parameter to identify apoptotic cells when it is used in combination with other parameters like DNA fragmentation and morphological change.

Time Considerations

Preparation and quantification of pADPr

Several days (3 to 5) are necessary to perform accurate quantification of pADPr by ELISA or by immunodot blot. The entire quantification involves pADPr induction in cells, preparation of cell lysates, isolation and purification of pADPr from cell pellets, and quantification by immunological methods. Therefore, the process can be stopped at many specific steps and some steps can be done simultaneously. For example, during the induction of pADPr synthesis (~15 to 30 min) the DHBB resin could easily be equilibrated. The TCA precipitate cell pellets and lyophilized DHBB-purified samples can be stored for several months at -80°C. Also note that when the incubation time for the primary antibody is reduced to 2 hr in the immunodot blot procedure (see Basic Protocol 2, step 5) the signal is not sharp.

High-resolution gel electrophoresis

The entire electrophoresis and immunodetection procedure can be completed in 2 days.

After pre-electrophoresis of the 20% polyacrylamide gel, the time required to obtain a fine separation for each size class of pADPr is ~3 to 4 hr. The electrotransfer onto a nitrocellulose membrane takes 1.5 hr, blocking takes 1 hr, incubation with primary antibody is done overnight and finally, washes and incubation with secondary antibody takes ~2 hr. However, pADPr separated by high-resolution electrophoresis can be stained directly on the gel using GELCODE color silver stain kit (Pierce), a procedure less sensitive than the immunodetection method presented in Basic Protocol 3 but allowing the detection of polymer sizes up to 3 mers within the same day. This method has been improved by the group of Althaus for pADPr size quantification (Malanga et al., 1995).

Immunoblotting

This procedure usually takes more than a single working day to treat cells, prepare cell lysates, perform SDS-PAGE, transfer to nitrocellulose, and probe immunoblots. Therefore, samples could be prepared on the first day and the gels run on the second day. Here again, the incubation time for the primary antibody could be reduced to 2 hr. The process could also take advantage of minigels and a minitransfer apparatus, but note that the resolution of the smaller blots will be limited especially if the sample is highly complex.

Immunocytochemical detection of pADPr

The entire immunocytochemical detection of pADPr extends over 2 days. After cells are growing on coverslips, a 15-min DNA-damaging treatment will induce pADPr synthesis. The limiting step after treatment is the overnight incubation in primary antibody. Subsequently, second antibody incubation, washes, and microscopy analysis take no more than 1 hr.

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Internet Resources

<http://parplink.u-strasbg.fr/index2.html>

The PARP Link Web Resource contains useful biological resources and pertinent information to the poly(ADP-ribose) polymerases (PARP) family of proteins and to their role in maintaining the integrity of the eukaryotic genome. This site offers instant access to the latest information and data on the PARP proteins and poly(ADP-ribosylation) and provides a valuable resource to the scientific community.

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Figure 18.7.1 Poly(ADP-ribose)ation reaction and poly(ADP-ribose) polymerase-1 (PARP-1) structure. **(A)** PARP-1 is selectively activated by DNA strand breaks and transfers ADP-ribose from NAD^+ to a set of acceptor proteins. **(B)** Immunocytochemical detection of pADPr synthesized in mouse fibroblast C3H10T $^{1/2}$ cells exposed 15 min to 200 μM H_2O_2 . Immunodetection was carried out with LP96-10 anti-pADPr polyclonal antibody (1:100) and revealed with 1:100 Texas Red-conjugated anti-rabbit second antibody (1:100). **(C)** The sequential addition of ADP-ribose units to a glutamic acid residue of an acceptor protein results in the formation of a long linear and branched chain of pADPr that is structurally similar to nucleic acids. **(D)** Schematic representation of PARP-1 domains. PARP-1 enzyme can be divided into three major domains (also see Fig. 18.7.2): a 46-kDa DNA binding domain, a central 22-kDa automodification region that contains the BRCT domain, and a 54-kDa C-terminal region that comprises the active site and the PARP signature. During caspase-dependent apoptosis, caspase-3 and -7 recognize a DEVD motif in the nuclear localization signal (NLS) and cleave PARP-1 into a p89 and a p24 fragment. (Adapted from the PARP link web site: <http://parplink.u-strasbg.fr/index2.html>).

Figure 18.7.2 Poly(ADP-ribose) polymerase family members and their antibodies. Abbreviations: EL, ELISA; IB, immunoblotting; IF, immunofluorescence; IP, immunoprecipitation.

Figure 18.7.3 The concept of PARP-1-mediated cell death. Excessive DNA damage activates PARP-1 in the nucleus, leading to massive pADPr formation and resulting in a NAD^+ depletion. This high concentration of pADPr may trigger mitochondrial depolarization ($\downarrow\Delta\Psi$) and drive apoptosis through an unknown mechanism. Activated PARP-1 can mediate cell death by caspase-dependent and -delayed pathways. In the caspase-delayed pathway, the insults to DNA, produced by oxidative stress or DNA alkylating agents, induces the release of mitochondrial factors, such as AIF, that translocates to the nucleus and induces caspase-independent chromatin fragmentation into large 50-kDa fragments. However, in caspase-dependent apoptosis, caspase-3 and -7 cleave PARP-1 into two fragments: p89 and p24. Generation of 24-kDa apoptotic fragment contribute to PARP-1 inactivation. The early cleavage PARP-1 serves as preserving cellular ATP for the proper function of further energy-dependent stage of apoptosis.

This unit describes the most common methods applicable to flow cytometry that make it possible to: (1) identify and quantify dead or dying cells, (2) reveal a mode of cell death (apoptosis or necrosis), and (3) study mechanisms involved in cell death. Gross changes in cell morphology and chromatin condensation, which occur during apoptosis, can be detected by analysis with laser light beam scattering. An early event of apoptosis, dissipation of the mitochondrial transmembrane potential, can be measured using a number of fluorochromes that are sensitive to the electrochemical potential within this organelle (see Basic Protocol 1). Another early event of apoptosis, caspase activation, can be measured either directly, by immunocytochemical detection of the epitope that characterizes activated caspase (see Basic Protocol 2), or indirectly by immunocytochemical detection of the caspase-3 cleavage product, the p85 fragment of poly(ADP-ribose) polymerase (see Basic Protocol 4). Exposure of phosphatidylserine on the exterior surface of the plasma membrane can be detected by the binding of fluoresceinated annexin V (annexin V-FITC); this assay is combined with analysis of the exclusion of the plasma membrane integrity probe propidium iodide (PI; see Basic Protocol 5). Also described are methods of analysis of DNA fragmentation based either on DNA content of cells with fractional (sub-G₁) DNA content (see Basic Protocol 6 and Alternate Protocol 1) or by DNA strand-break labeling (Terminal deoxynucleotidyltransferase-mediated dUTP Nick End Labeling, TUNEL; or In Situ End Labeling, ISEL; see Basic Protocol 7). Still another hallmark of apoptosis is the activation of tissue transglutaminase (TGase), the enzyme that cross-links protein and thereby makes them less immunogenic. Methods for analyzing TGase activation are presented in Basic Protocol 8 and Alternate Protocol 2.

STRATEGIC PLANNING

The choice of a particular method often depends on the cell type, the nature of the inducer of apoptosis, the desired information (e.g., specificity of apoptosis with respect to the cell cycle phase or DNA ploidy), and technical restrictions. For example, sample transportation or prolonged storage before the measurement requires prior cell fixation, thereby eliminating the use of “supravital” methods that rely on analysis of freshly collected live cells.

Positive identification of apoptotic cells is not always simple. Apoptosis was recently defined as a caspase-mediated cell death (Blagosklonny, 2000). Activation of caspases, therefore, appears to be the most specific marker of apoptosis (Shi, 2002). The detection of caspase activation, either directly (e.g., by antibody that is reactive with the activated enzyme; see Basic Protocol 2) or indirectly by the presence of poly(ADP-ribose) polymerase (PARP) cleavage product (PARP p85; see Basic Protocol 4), provides the most definitive evidence of apoptosis. Extensive DNA fragmentation is also considered as a specific marker of apoptosis. The number of DNA strand breaks in apoptotic cells is so large that intensity of their labeling in the TUNEL reaction (see Basic Protocol 7) ensures their positive identification and discriminates them from cells that have undergone primary necrosis (Gorczyca et al., 1992). However, in the instances of apoptosis when internucleosomal DNA degradation does not occur (Collins et al., 1992; Catchpole and Stewart, 1993; Ormerod et al., 1994; Knapp et al., 1999), the number of DNA strand breaks may be inadequate to distinguish apoptotic cells by the TUNEL method. Likewise, in some instances of apoptosis, DNA fragmentation stops after the initial DNA cleavage to fragments of 50 to 300 kb (Collins et al., 1992, Oberhammer et al., 1993). The frequency of DNA strand breaks in nuclei of these cells is low, and therefore, they may not be easily detected by the TUNEL method.

The ability of cells to bind annexin V is still another marker considered to be specific to apoptosis. One should keep in mind, however, that use of the annexin V binding assay is hindered in some instances, e.g., when the plasma membrane is damaged during cell preparation or storage, leading to the loss of asymmetry in distribution of phosphatidylserine across the membrane. Furthermore, macrophages and other cells engulfing apoptotic bodies may also be positive in the annexin V assay (Marguet et al., 1999).

Apoptosis can be recognized with greater certainty when the cells are subjected to several assays probing different apoptotic attributes (Hotz et al., 1994). For example, the assay of plasma membrane integrity (exclusion of PI) and annexin V binding combined with analysis of PARP cleavage or DNA fragmentation may provide a more definitive assessment of the mode of cell death than can be determined by each of these methods used alone.

A plethora of kits designed to label DNA strand breaks and applicable to flow cytometry are available from different vendors. Most of these kits were designed by the authors (Gorczyca et al., 1992; Li and Darzynkiewicz, 1995). For example, Phoenix Flow Systems, BD PharMingen, and Alexis Biochemicals provide kits to identify apoptotic cells based on a single-step procedure utilizing either TdT and FITC-conjugated dUTP (APO-DIRECT; Li et al., 1995) or TdT and BrdUTP, as described in Basic Protocol 7 (APO-BRDU; Li and Darzynkiewicz, 1995). A description of the method, which is nearly identical to the one presented in this unit, is included with the kit. Another kit (ApopTag), based on a two-step DNA strand-break labeling with digoxigenin-16-dUTP by TdT, also designed by the authors (Gorczyca et al., 1992), was initially offered by ONCOR, later by Intergen, and most recently by Serologicals.

**BASIC
PROTOCOL 1**

**MITOCHONDRIAL TRANSMEMBRANE POTENTIAL ($\Delta\psi_m$) MEASURED
BY RHODAMINE 123 OR DiOC₆(3) FLUORESCENCE**

The critical role of mitochondria during apoptosis is associated with the release of two intermembrane proteins, cytochrome *c* and apoptosis-inducing factor (AIF), that are essential for sequential activation of pro-caspase 9 and pro-caspase 3 (Liu et al., 1996; Yang et al., 1997). AIF is also involved in proteolytic activation of apoptosis-associated endonuclease (Susin et al., 1997). Still another protein, Smac/Diablo, that interacts with the inhibitors of caspases, thereby promoting apoptosis, is released from mitochondria (Deng et al., 2002). Dissipation (collapse) of mitochondrial transmembrane potential ($\Delta\psi_m$), also called the permeability transition (PT), likewise occurs early during apoptosis (Cossarizza et al., 1994; Kroemer, 1998; Zamzani et al., 1998). However, a growing body of evidence suggests that this event may be transient when associated with the release of cytochrome *c* or AIF, and mitochondrial potential may be restored for some time in the cells with activated caspases (Finucane et al., 1999; Scorrano et al., 1999; Li et al., 2000).

The membrane-permeable lipophilic cationic fluorochromes such as rhodamine 123 (R123) or 3,3'-dihexyloxacarbocyanine iodide [DiOC₆(3)] can serve as probes of $\Delta\psi_m$ (Johnson et al., 1980; Darzynkiewicz et al., 1981, 1982). When live cells are incubated in their presence, the probes accumulate in mitochondria, and the extent of their uptake, as measured by intensity of cellular fluorescence, reflects $\Delta\psi_m$. A combination of R123 and PI discriminates among live cells that stain only with R123, early apoptotic cells that have lost the ability to accumulate R123, and late apoptotic/necrotic cells whose plasma membrane integrity is compromised and that stain only with PI (Darzynkiewicz et al., 1982; Darzynkiewicz and Gong, 1994). The specificity of R123 and DiOC₆(3) as $\Delta\psi_m$ probes is increased when they are used at low concentrations (<0.5 $\mu\text{g/ml}$). Still another probe of $\Delta\psi_m$ is the J-aggregate-forming lipophilic cationic fluorochrome 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). Its uptake by

charged mitochondria driven by the transmembrane potential is detected by the shift in color of fluorescence from green, which is characteristic of its monomeric form, to orange, which reflects its aggregation in mitochondria (Cossarizza and Salvioli, 2001). In light of the recent evidence that the collapse of $\Delta\psi_m$ may not be a prerequisite for release of cytochrome c, AIF, and other apoptotic events (Finucane et al., 1999; Scorrano et al., 1999; Li et al., 2000), one should be cautious in interpreting the lack of collapse of $\Delta\psi_m$ as a marker of non-apoptotic cells.

Materials

Cells of interest in appropriate complete culture medium
10 μM rhodamine 123 (R123; see recipe) *or* 10 μM DiOC₆(3) (see recipe for 0.1 mM stock solution) *or* 0.2 mM JC-1 stock solution (see recipe)

Phosphate-buffered saline (PBS; APPENDIX 2A)

1 mg/ml propidium iodide (PI; Molecular Probes) in distilled water; store at 4°C in the dark

12 × 75-mm tubes suitable for flow cytometer

Flow cytometer with 488-nm excitation and filters for collection of green, orange, and red fluorescence

Stain with R123 or DiOC₆(3) and PI

- 1a. Add either 20 μl of 10 μM R123 (200 nM final) or 5 μl of 10 μM DiOC₆(3) (50 nM final) to $\sim 10^6$ cells suspended in 1 ml complete tissue culture medium (with 10% serum), and incubate 20 min at 37°C in the dark.
- 2a. Centrifuge cells 5 min at $300 \times g$, room temperature. Resuspend cell pellet in 1 ml PBS.
- 3a. Add 10 μl PI solution and incubate 5 min at room temperature in the dark.
- 4a. Analyze cell fluorescence on the flow cytometer. Excite fluorescence with blue (488-nm) laser. Set the signal-triggering threshold on forward- and side-scatter signals. Collect green fluorescence [R123 or DiOC₆(3)] at 530 ± 20 nm and red fluorescence (PI) above 600 nm.

Stain with JC-1

- 1b. Suspend cell pellet ($\sim 10^6$ cells) in 1 ml complete tissue culture medium with 10% serum.
- 2b. Add 10 μl of 0.2 mM JC-1 stock solution. Vortex cells intensely during addition and for the next 20 sec. Wash cells two times with PBS; centrifuge each time 5 min at $200 \times g$, room temperature.

Addition of JC-1 to the cell suspension without vortexing may lead to formation of precipitate. Vortexing too vigorously, on the other hand, may cause cell damage.

- 3b. Incubate cells 15 min at room temperature in the dark.
- 4b. Analyze cell fluorescence on the flow cytometer, using 488-nm excitation. Collect green fluorescence at 530 ± 20 nm and orange fluorescence at 570 ± 20 nm with a band-pass filter or above 570 nm with a long-pass filter.

**IMMUNOCYTOCHEMICAL DETECTION OF ACTIVATED CASPASES BY
ZENON TECHNOLOGY**

Caspases are activated by transcatalytic cleavage of their zymogen procaspase molecules into large and small subunits. The subunits then assemble to form the heterotetramer consisting of two small and two large subunits, which is the active caspase (Budihardjo et al., 1999; Earnshaw et al., 1999). Antibodies that are specific to activated caspase-3, caspase-8, and caspase-9 are now commercially available and one expects that antibodies reactive with other active caspases will soon be developed as well. It is possible, therefore, to detect caspase activation by immunocytochemical means. This protocol combines the use of activated caspase-specific antibody with staining of cellular DNA by propidium iodide (PI) to concurrently detect cells with activated caspases and relate caspase activation to the cell-cycle position.

The immunocytochemical detection of caspase-3 in this protocol makes use of Zenon technology (Haugland, 2002). Zenon technology consists of a labeling complex that is formed by a fluorochrome-labeled Fab fragment (Zenon Alexa Fluor 488) of an anti-IgG antibody that is directed against the Fc portion of a mouse (or rabbit) IgG₁ antibody. Mixing of the labeled Fab fragment with the primary antibody forms the labeling complex. Excess unbound labeled Fab fragments is removed by admixture of nonspecific mouse (or rabbit) IgG. The labeling complex is then used to stain cells in the same manner as a covalently labeled primary antibody (Haugland, 2002).

Materials

Cells of interest, both untreated (control) and induced to apoptosis (e.g., exponentially growing HL-60 cells incubated 2 to 4 hr with 0.15 μ M camptothecin)

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Fixatives:

1% (v/v) methanol-free formaldehyde (Polysciences) in PBS, 0° to 5°C

4% (v/v) methanol-free formaldehyde (Polysciences) in PBS, room temperature

70% (v/v) ethanol, -20°C

Rinse solution (see recipe)

Primary antibody: cleaved (activated) caspase-3 antibody (Cell Signaling Technology, cat. no. 9661)

Zenon Alexa Fluor 488 rabbit IgG labeling kit (Molecular Probes, cat. no. Z-25302)

10% (v/v) Triton X-100 in PBS

DNA staining solution with PI (see recipe)

12 \times 75-ml tubes suitable for use on flow cytometer

Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Suspend $\sim 10^6$ cells in 0.5 ml PBS.
2. Fix cells by transferring the above cell suspension into tubes containing 4.5 ml of 1% methanol-free formaldehyde in PBS at 0° to 5°C. Let stand 15 min at 0° to 5°C.
3. Centrifuge 5 min at $300 \times g$, room temperature. Decant supernatant.
4. Resuspend cell pellet in 3 ml of 70% ethanol at -20°C. Allow to sit at least 2 hr (cells can be stored several days in 70% ethanol at -20°C).
5. Bring the cell suspension in 70% ethanol to room temperature, add 2 ml PBS to this suspension, and let sit 5 min at room temperature.
6. Centrifuge 5 min at $300 \times g$, room temperature. Decant supernatant.

7. Resuspend cell pellet in 5 ml PBS and let sit 5 min at room temperature.
8. Centrifuge 5 min at $300 \times g$, room temperature. Decant supernatant.
9. Resuspend cell pellet in rinse solution. Let stand 30 min at room temperature.
10. Prepare the staining solution as follows.
 - a. Mix 4 μ l primary antibody (anti-caspase-3) with 16 μ l rinse solution and with 5 μ l solution A Zenon (from kit) in a 1.5-ml microcentrifuge tube.
 - b. Keep 5 min in the dark at room temperature.
 - c. Add 5 μ l solution B Zenon (from kit).
 - d. Keep 5 min in the dark at room temperature.
 - e. Add 0.3 μ l of 10% Triton X-100 in PBS.
11. Centrifuge the cell suspension (from step 9) 5 min at $300 \times g$, room temperature. Thoroughly drain the rinse solution by blotting on filter paper. Add 15 μ l of the staining solution prepared in step 10, and 85 μ l rinse solution, for a final volume of 100 μ l. Resuspend the cell pellet.
12. Incubate cells with the staining solution 1 hr in the dark at room temperature.
13. Add 5 ml PBS, centrifuge 5 min at $300 \times g$, room temperature, and decant supernatant.
14. Resuspend cell pellet in 1 ml of 4% methanol-free formaldehyde in PBS and let stand 5 min at room temperature.
15. Centrifuge cells 5 min at $300 \times g$, room temperature. Decant supernatant.
16. Resuspend cell pellet in 1 ml DNA staining solution with PI.
17. Analyze cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green Alexa 488 fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

DETECTION OF APOPTOTIC CELLS USING FLUOROCHROME-LABELED INHIBITORS OF CASPASES (FLICAs)

Exposure of live cells to fluorochrome-labeled inhibitors of caspases (FLICAs) results in uptake of these reagents by apoptotic cells (Smolewski et al., 2001). Unbound FLICAs are removed from the nonapoptotic cells by rinsing the cells with wash buffer. The cells may also be fixed with formaldehyde; after fixation only apoptotic cells retain the label. Cells labeled with FLICAs can be examined by fluorescence microscopy, or their fluorescence can be measured by flow cytometry. FLICAs are convenient markers of apoptotic cells, and when used in combination with PI as described in the protocol below, they reveal three sequential stages of apoptosis. FAM-VAD-FMK, the inhibitor designed to react with all caspases, except perhaps caspase-2, is used in this protocol. It should be stressed, however, that FLICAs appear to react in apoptotic cells also with targets other than activated caspases. Cell labeling with FLICAs, therefore, although perhaps reflecting caspase activity and although reflecting caspase activation, cannot be interpreted as indicating reactivity with active enzyme centers of caspases only.

**BASIC
PROTOCOL 3**

**Cellular Aging
and Death**

18.8.5

Materials

Cells of interest

Medium supplemented with 10% (v/v) serum or 1% (w/v) serum albumin

FLICA kit (Immunochemistry Technologies) containing:

FAM-VAD-FMK reagent (see recipe)

Fixative

Hoechst stain

Rinse solution: 1% (w/v) BSA in PBS (APPENDIX 2A)

1 mg/ml propidium iodide (PI; Molecular Probes) in distilled water; store at 4°C in the dark

12 × 75-ml tubes suitable for use on flow cytometer

Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Suspend $\sim 10^6$ cells in 0.3 ml medium containing 10% serum or 1% serum albumin.
2. Add 10 μ l FAM-VAD-FMK working solution to this cell suspension (final concentration 10 μ M). Mix gently and incubate 1 hr at 37°C.

Sulforhodamine-labeled FLICA (SR-VAD-FMK) may be used instead of FAM-VAD-FMK to make apoptotic cells fluorescence in the red.

3. Add 2 ml rinse solution, mix gently, and centrifuge 5 min at $200 \times g$, room temperature.
4. Resuspend cell pellet in 2 ml rinse solution and centrifuge as in step 3.

Cells may be fixed 15 min in 1% formaldehyde in PBS, then suspended in 70% ethanol and stored for several days. A fluorochrome of a different color than FLICA may be used to counterstain other cellular components (e.g., DNA) or other markers of apoptosis (e.g., DNA strand breaks).

5. Resuspend cell pellet in 1 ml rinse solution. Add 1.0 μ l of 1 mg/ml PI stock solution. Keep 5 min at room temperature.

Protect samples from light at all times.

Staining with PI is optional. It allows one to distinguish the cells that have compromised plasma-membrane integrity (e.g., necrotic and late apoptotic cells, cells with mechanically damaged membranes, or isolated cell nuclei) to the extent that they cannot exclude PI.

6. Analyze cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green FAM-VAD-FMK fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

BASIC PROTOCOL 4

DETERMINATION OF POLY(ADP-RIBOSE) POLYMERASE (PARP) CLEAVAGE

PARP is a nuclear enzyme that is involved in DNA repair and activated in response to DNA damage (de Murcia and Menissier-de Murcia, 1994). Early in apoptosis, PARP is cleaved by caspases, primarily by caspase-3 (Kaufmann et al., 1993; Lazebnik et al., 1994; Alnemri et al., 1996). The specific cleavage of this protein results in distinct 85-kDa and 24-kDa fragments, usually detected electrophoretically, and this is considered to be a hallmark of the apoptotic mode of cell death.

The development of antibodies that recognize the cleaved PARP products prompted their use as immunocytochemical markers of apoptotic cells. The antibody that recognizes the 85-kDa fragment (PARP p85) was initially used to score the frequency of apoptosis in tissue sections (Sallman et al., 1997; Kockx et al., 1998). Recently, this antibody has been adapted to label apoptotic cells for detection by flow cytometry and laser scanning

cytometry (LSC; Li and Darzynkiewicz, 2000; Li et al., 2000). A good correlation was observed between the frequency of apoptosis detected cytometrically with PARP p85 Ab and that detected by the DNA strand-break (TUNEL) assay. However, at least in some cell systems, the cleavage of PARP occurs prior to the onset of DNA fragmentation (Li and Darzynkiewicz, 2000). In these instances, the correlation may not be apparent at early stages of apoptosis because the apoptotic index estimate based on PARP cleavage may exceed the estimate based on the TUNEL reaction. Cytometric analysis of cells differentially stained for PARP p85 and DNA, similar to the TUNEL assay, makes it possible not only to identify and score apoptotic cell populations but also to correlate apoptosis with the cell cycle position or DNA ploidy.

The classic immunocytochemical indirect (two-step) method to detect the 85-kDa PARP fragment is presented below. Alternatively, however, one can use the Zenon technology as described above (see Basic Protocol 2) for detection of activated caspases.

Materials

Cells of interest

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

1% methanol-free formaldehyde (Polysciences) in PBS (*APPENDIX 2A*)

70% ethanol

0.25% (v/v) Triton X-100 (Sigma) in PBS (*APPENDIX 2A*); store at 4°C

PBS/BSA solution: 1% (w/v) bovine serum albumin (Sigma) in PBS; store at 4°C

Anti-PARP p85 antibody (Promega anti-PARP-85 fragment, rabbit polyclonal)

Fluorescein-conjugated anti-rabbit immunoglobulin antibody (Dako)

1 mg/ml propidium iodide (PI; Molecular Probes) in distilled water; store at 4°C in the dark

RNase stock solution (see recipe)

12 × 75-mm centrifuge tubes suitable for use on the flow cytometer

Pasteur pipets

Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Suspend ~10⁶ cells in 0.5 ml PBS. Transfer this suspension to a 12 × 75-mm (5-ml) tube containing 4.5 ml of 1% methanol-free formaldehyde and fix cells 15 min on ice. Centrifuge the cells 5 min at 300 × g, 4°C, wash once with 5 ml PBS, centrifuge 5 min at 300 × g, and resuspend the cell pellet in 0.5 ml PBS. With a Pasteur pipet, transfer this cell suspension into a new 12 × 75-mm centrifuge tube containing 4.5 ml of ice-cold 70% ethanol.

The cells may be stored several days in ethanol at -20°C.

2. Centrifuge cells 5 min at 200 × g, room temperature, and resuspend the cell pellet in 5 ml PBS; repeat centrifugation.
3. Resuspend cells in 5 ml 0.25% Triton X-100/PBS for 10 min.
4. Centrifuge cells 5 min at 300 × g, room temperature, and resuspend in 2 ml BSA/PBS solution for 10 min.
5. Centrifuge cells 5 min at 300 × g, room temperature, and resuspend in 100 μl BSA/PBS containing anti-PARP p85 Ab diluted 1:200. Incubate 2 hr at room temperature, or overnight at 4°C.
6. Add 5 ml BSA/PBS solution, let sit 5 min, and centrifuge 5 min at 300 × g, room temperature.

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7. Resuspend cell pellet in 100 μ l PBS/BSA containing fluorescein-conjugated secondary Ab [F(ab')₂ fragment, swine anti-rabbit immunoglobulin] diluted 1:30. Incubate 1 hr in the dark at room temperature.
8. Add 5 ml BSA/PBS, centrifuge 5 min at 200 \times g, room temperature, and resuspend cell pellet in 1 ml PBS. Add 20 μ l of 1 mg/ml PI and 50 μ l RNase solution. Incubate 20 min in the dark at room temperature.
9. Analyze cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green FITC-anti-PARP p85 fluorescence at 530 \pm 20 nm and red PI fluorescence above 600 nm.

ANNEXIN V BINDING

Phospholipids of the plasma membrane are asymmetrically distributed between the inner and outer leaflets of the membrane. Phosphatidylcholine and sphingomyelin are exposed on the external leaflet of the lipid bilayer, while phosphatidylserine is located on the inner surface. During apoptosis, this asymmetry is disrupted and phosphatidylserine becomes exposed on the outside surface of the plasma membrane (Fadok et al., 1992; Koopman et al., 1994; van Engeland et al., 1998). Because the anticoagulant protein annexin V binds with high affinity to phosphatidylserine, fluorochrome-conjugated annexin V has found an application as a marker of apoptotic cells, in particular for their detection by flow cytometry (van Engeland et al., 1998). The cells become reactive with annexin V prior to their loss of plasma membrane ability to exclude cationic dyes such as PI. Therefore, by staining cells with a combination of annexin V-FITC and PI, it is possible to detect unaffected, non-apoptotic cells (annexin V-FITC negative/PI negative), early apoptotic cells (annexin V-FITC positive/PI negative), and late apoptotic (“necrotic stage” of apoptosis) as well as necrotic cells (PI positive).

Materials

Cells of interest

Fluorescein-conjugated annexin V (see recipe) in binding buffer (see recipe)

1 mg/ml propidium iodide (PI; Molecular Probes) in distilled water; store at 4°C in the dark

Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Suspend 10⁵ to 10⁶ cells in 1 ml fluorescein-conjugated annexin V in binding buffer for 5 min at room temperature in the dark.
2. Prior to analysis, add an appropriate volume of 1 mg/ml PI solution to the cell suspension to have a final PI concentration of 1.0 μ g/ml. Incubate 5 min at room temperature in the dark.
3. Analyze cells on the flow cytometer, using 488-nm excitation. Set gates based on light scatter. Collect green annexin V fluorescence at 530 \pm 20 nm and red PI fluorescence above 600 nm.

DNA FRAGMENTATION: DETECTION OF CELLS WITH FRACTIONAL (SUB-G₁) DNA CONTENT USING PI

Endonucleases activated during apoptosis target internucleosomal DNA sections and cause extensive DNA fragmentation (Kerr et al., 1972; Arends et al., 1990; Nagata, 2000). The fragmented, low-molecular-weight DNA can be extracted from the cells following their fixation in precipitating fixatives such as ethanol. Conversely, fixation with cross-linking fixatives such as formaldehyde results in the retention of low-molecular-weight DNA in the cell and therefore should be avoided. Generally, the extraction occurs during the process of cell staining in aqueous solutions after transfer from the fixative. As a result, apoptotic cells often end up with deficient DNA content, and when stained with a DNA-specific fluorochrome, they can be recognized by cytometry as cells having less DNA than G₁ cells. On the DNA content frequency histograms, they form a characteristic sub-G₁ peak (Umansky et al., 1981; Nicoletti et al., 1991; Gong et al., 1994). It should be noted that loss of DNA may also occur as a result of the shedding of apoptotic bodies containing fragments of nuclear chromatin.

The degree of DNA degradation varies depending on the stage of apoptosis, cell type, and often the nature of the apoptosis-inducing agent. Hence, the extractability of DNA during the staining procedure also varies. A high-molarity phosphate-citrate buffer enhances extraction of the fragmented DNA (Gong et al., 1994). With some limitations, this approach can be used to extract DNA from apoptotic cells to the desired level in order to achieve their optimal separation by flow cytometry.

Materials

Cells of interest
PBS (APPENDIX 2A)
70% ethanol
DNA extraction buffer (see recipe)
DNA staining solution with PI (see recipe)

12 × 75-mm tubes suitable for use on the flow cytometer
Flow cytometer with 488-nm excitation and filter for collection of red fluorescence

1. Suspend 1–2 × 10⁶ cells in 0.5 ml PBS and fix cells by adding suspension to 4.5 ml of 70% ethanol in a 12 × 75-mm tube on ice.

Cells may be stored several weeks in fixative at –20°C.

2. Centrifuge cells 5 min at 200 × g, decant ethanol, suspend the cell pellet in 5 ml PBS, and centrifuge 5 min at 300 × g, room temperature.
3. Suspend cell pellet in 0.25 ml PBS. To facilitate extraction of low-molecular-weight DNA, add 0.2 to 1.0 ml DNA extraction buffer.
4. Incubate 5 min at room temperature, then centrifuge 5 min at 300 × g, room temperature.
5. Suspend cell pellet in 1 ml DNA staining solution with PI. Incubate cells 30 min at room temperature in the dark.
6. Analyze cells on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect forward light scatter and red fluorescence above 600 nm.

**DNA FRAGMENTATION: DETECTION OF CELLS WITH FRACTIONAL
(SUB-G₁) DNA CONTENT USING DAPI**

Cellular DNA may be stained with other fluorochromes instead of PI, and other cell constituents may be counterstained in addition to DNA. The following is the procedure used to stain DNA with DAPI. This protocol requires a UV laser.

Additional Materials (also see *Basic Protocol 6*)

DNA staining solution with DAPI (see recipe)

Flow cytometer equipped with UV excitation and filter for collection of blue fluorescence

1. Follow *Basic Protocol 6*, steps 1 to 4. Then, suspend the cell pellet in 1 ml DNA staining solution containing DAPI. Keep on ice 20 min.
2. Analyze cells on the flow cytometer, using UV excitation (e.g., 351-nm line from an argon-ion laser, or mercury lamp with a UG1 filter). Collect blue DAPI fluorescence in a band from 460 to 500 nm.

**DNA FRAGMENTATION: DETECTION OF DNA STRAND BREAKS (TUNEL
ASSAY)**

DNA fragmentation during apoptosis, particularly when it progresses to internucleosomal regions (Arends et al., 1990; Oberhammer et al., 1993), generates a multitude of DNA strand breaks in the nucleus. The 3'-OH ends of the breaks can be detected by attaching a fluorochrome. This is generally done directly or indirectly (e.g., via biotin or digoxigenin) using fluorochrome-labeled deoxynucleotides in a reaction catalyzed preferably by exogenous terminal deoxynucleotidyltransferase (TdT; Gorczyca et al., 1992, 1993; Li and Darzynkiewicz, 1995; Li et al., 1995). The reaction is commonly known as TUNEL, from TdT-mediated dUTP-biotin nick-end labeling (Gavrieli et al., 1992). This acronym is actually a misnomer, since double-stranded DNA breaks are labeled, rather than single-stranded nicks. Of all the markers used to label DNA breaks, BrdUTP appears to be the most advantageous with respect to sensitivity, low cost, and simplicity of reaction (Li and Darzynkiewicz, 1995). When attached to DNA strand breaks in the form of poly-BrdU, this deoxynucleotide can be detected with a FITC-conjugated anti-BrdU Ab, the same Ab commonly used to detect BrdU incorporated during DNA replication. Poly-BrdU attached to DNA strand breaks by TdT, however, is accessible to the Ab without the need for DNA denaturation, which otherwise is required to detect the precursor incorporated during DNA replication.

It should be stressed that the detection of DNA strand breaks by this method requires pre-fixation of cells with a cross-linking agent such as formaldehyde. Unlike ethanol, formaldehyde prevents the extraction of small pieces of fragmented DNA. Thus, despite cell permeabilization and the subsequent cell washings required, the DNA content of early apoptotic cells (and the number of DNA strand breaks) is not markedly diminished through extraction. Labeling of DNA strand breaks in this procedure, which utilizes fluorescein-conjugated anti-BrdU Ab, can be combined with staining of DNA by a fluorochrome of another color (PI, red fluorescence). Cytometry of cells that are differentially stained for DNA strand breaks and for DNA allows one to distinguish apoptotic from non-apoptotic cell subpopulations and reveals the cell cycle distribution in these subpopulations (Gorczyca et al., 1992, 1993).

Materials

Cells of interest
1% (v/v) methanol-free formaldehyde (Polysciences) in PBS (*APPENDIX 2A*), pH 7.4 (primary fixative)
PBS (*APPENDIX 2A*)
70% ethanol (secondary fixative)
5× TdT reaction buffer (see recipe)
2 mM BrdUTP (Sigma) in 50 mM Tris·Cl, pH 7.5
TdT in storage buffer (both from Roche Diagnostics), 25 U in 1 μl
10 mM cobalt chloride (CoCl₂; Roche Diagnostics)
Rinsing buffer: PBS with 0.1% (v/v) Triton X-100 and 0.5% (w/v) BSA
FITC-conjugated anti-BrdU MAb (see recipe)
PI staining buffer: PBS with 5 μg/ml PI and 200 μg/ml DNase-free RNase
Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

Fix cells

1. Fix 1–5 × 10⁶ cells in suspension 15 min in 1% methanol-free formaldehyde in PBS, pH 7.4, on ice.
2. Centrifuge 5 min at 200 × *g*, 4°C, resuspend cell pellet (~2 × 10⁶ cells) in 5 ml PBS, centrifuge 5 min at 200 × *g*, 4°C, and resuspend cells in 0.5 ml PBS.
3. Add the 0.5-ml cell suspension from step 2 to 5 ml ice-cold 70% ethanol.
The cells can be stored several weeks in ethanol at –20°C.
4. Centrifuge 5 min at 200 × *g*, 4°C, remove ethanol, and resuspend cells in 5 ml PBS. Repeat centrifugation.

Stain cells

5. Resuspend the pellet (not more than 10⁶ cells) in 50 μl of a solution that contains:
 - 10 μl 5× TdT reaction buffer
 - 2.0 μl 2 mM BrdUTP stock solution
 - 0.5 μl TdT in storage buffer (12.5 U final)
 - 5 μl 10 mM CoCl₂ solution
 - 32.5 μl dH₂O.
6. Incubate cells in this solution 40 min at 37°C.
Alternatively, incubation can be carried out overnight at 22° to 24°C.
7. Add 1.5 ml rinsing buffer and centrifuge 5 min at 200 × *g*, room temperature.
8. Resuspend cells in 100 μl FITC-conjugated anti-BrdU MAb solution.
9. Incubate 1 hr at room temperature or overnight at 4°C. Add 2 ml rinsing buffer and centrifuge 5 min at 200 × *g*, room temperature.
10. Resuspend cell pellet in 1 ml PI staining solution containing RNase. Incubate 30 min at room temperature in the dark.
11. Measure cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green FITC-anti BrdU MAb fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

**DETECTION OF TISSUE TRANSGLUTAMINASE ACTIVATION BY CELL
RESISTANCE TO DETERGENTS**

Extensive protein cross-linking takes place during apoptosis. The ubiquitous transglutaminase TGase 2 (also called “tissue transglutaminase”; tTGase) was identified as the enzyme responsible for this reaction (Fesus et al., 1987; Melino and Piacentini, 1998). It is presumed that activation of TGase 2 during apoptosis prevents release of soluble and immunogenic proteins from dying cells because protein cross-linking makes these proteins less soluble and thereby decreases a possibility of induction of autoimmune reaction. Furthermore, protein packaging into apoptotic bodies may be facilitated when proteins remain in solid state rather than in solution. The additional role of TGase 2 as one of the “executor enzymes” during apoptosis is still being debated.

This protocol is a simple and rapid approach to identify apoptotic cells with activated TGase 2. The method is based on the propensity of cross-linked protein to withstand treatment with detergents. The authors have noticed that when live, nonapoptotic cells are subjected to treatment with solutions of nonionic detergents, lysis of their plasma membrane and release of the content of cytoplasm is complete, resulting in preparation of isolated nuclei. In contrast, apoptotic cells resist the detergent treatment; their cytoplasmic protein remains insoluble and attached to the nucleus in the form of a shell-like cover (Grabarek et al., 2002). It is possible, therefore, by flow or laser scanning cytometry to distinguish apoptotic cells from the nuclei isolated from nonapoptotic cells, by means of the abundance of protein in the former. In addition, bivariate gating analysis of cellular DNA and protein content makes it possible to reveal the cell cycle distribution separately for the population of cells with cross-linked protein (activated TGase 2) and for the population of cells that did not show protein cross-linking (Grabarek et al., 2002).

Alternate Protocol 2 combines the detection of TGase 2 activity by binding of fluoresceinated cadaverine (F-CDV) with analysis of the cell cycle.

Materials

- Cells of interest
- DAPI/sulforhodamine 101/detergent solution (see recipe)
- Flow cytometer equipped with UV excitation and filters for collection of blue and red fluorescence

1. Collect $\sim 10^6$ cells from the culture and centrifuge 5 min at $300 \times g$, room temperature.
2. Suspend the cell pellet in 1 ml DAPI/sulforhodamine 101/detergent solution and vortex 20 sec.
3. Analyze cells on the flow cytometer, using UV excitation (e.g., 351-nm line from an argon-ion laser, or mercury lamp with a UG1 filter). Collect blue DAPI fluorescence in a band from 460 to 500 nm and red fluorescence of sulforhodamine 101 above 600 nm.

**DETECTION OF TGase 2 ACTIVATION BY FLUORESCAINED
CADAVERINE (F-CDV) BINDING**

This alternate protocol is based on the covalent attachment, by the activated TGase 2, of the fluorescein-tagged cadaverine to the respective protein substrates within the cell (Lajemi et al., 1998). This assay was adapted to flow cytometry and combined with concurrent analysis of cellular DNA content (Grabarek et al., 2002). Like the detergent-based assay, this method is simple and also offers good distinction between apoptotic cells with activated versus nonactivated TGase 2.

It should be noted that when the cost of the reagents for the procedure is taken into account, the detergent-based assay (see Basic Protocol 8) is less expensive by several orders of magnitude than this F-CDV assay.

Materials

Fluoresceinated cadaverine solution (F-CDV; see recipe)
Cells of interest
100% methanol
DNA staining solution with PI (see recipe)
Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Add aliquots of F-CDV stock solution (1 part per 50) directly to cell cultures (10^6 to 10^7 cells) to obtain 50 μM final F-CDV concentration in the culture.
2. Incubate cells in the presence of F-CDV for the desired time interval during which activity of TGase 2 has to be detected (e.g., one to several hours).
Because cross-linking is a cumulative process, intensity of cell labeling increases with time of incubation.
3. Harvest the culture by centrifuging 5 min at $200 \times g$, room temperature.
4. Suspend the cells in 0.5 ml PBS and fix in 5 ml of 100% methanol on ice. Keep in methanol ≥ 2 hr at 0° to 4°C ; cells may be stored in the fixative for several days.
5. Centrifuge cells 5 min at $200 \times g$, room temperature, decant the fixative thoroughly, and suspend cell pellet in 2 ml DNA staining solution with PI.
6. Keep ≥ 30 min at room temperature.
7. Measure cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green FITC-CDV fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Binding buffer

10 mM HEPES-NaOH, pH 7.4
140 mM NaCl
5 mM CaCl_2
Store several weeks at 4°C

DAPI/sulforhodamine 101/detergent solution

Dissolve 100 μg DAPI, 2 mg sulforhodamine 101 (Molecular Probes), and 0.1 ml Triton X-100 in 100 ml PBS (or 0.1 ml Nonidet NP-40). Store several weeks at 0° to 4°C .

DiOC₆ (3) stock solution

Prepare a 0.1 mM solution of DiOC₆ (3) (Molecular Probes) by dissolving 5.7 mg dye in 10 ml dimethyl sulfoxide (DMSO). Store for weeks in small (e.g., 0.5- or 1-ml) aliquots protected from light at -20°C . Prior to use, dilute ten-fold with PBS to obtain 10 μM concentration.

DNA extraction buffer, pH 7.8

192 ml 0.2 M Na₂HPO₄
8 ml 0.1 M citric acid
Store for months at 4°C

DNA staining solution with DAPI

Dissolve 100 µg DAPI in 100 ml PBS. Store at 0° to 4°C for several weeks.

DNA staining solution with PI

200 µg propidium iodide (PI)
2 mg DNase-free RNase
10 ml PBS
Prepare fresh before use

FAM-VAD-FMK

Stock solution: following kit directions, dissolve FAM-VAD-FMK in dimethyl sulfoxide (DMSO) to obtain 150× solution. Store aliquots protected from light ≤3 months at -20°C.

Working solution: Just prior to use, prepare a 30× solution by diluting FAM-VAD-FMK stock solution 1:5 in PBS. Mix the vial until the solution becomes transparent and homogenous. Protect all FAM-VAD-FMK solutions from light. Discard unused portions. Do not store.

FITC-conjugated anti-BrdU MAb solution

100 µl PBS (APPENDIX 2A)
0.3 µg FITC-conjugated anti-BrdU MAb (Becton Dickinson)
0.3% (v/v) Triton X-100
1% (w/v) BSA
Prepare fresh before use

Fluoresceinated cadaverine solution

Dissolve 5-[(5-aminopentyl)thioureidyl]fluorescein dihydrobromide (F-CDV; Molecular Probes) in distilled water to obtain a 2.5 mM stock solution. Store aliquots (0.2 to 0.5 ml) of this solution several weeks at -20°C.

Fluorescein-conjugated annexin V

Dissolve FITC-conjugated annexin V (1:1 stoichiometric complex; e.g., from BRAND Applications) in binding buffer (see recipe) at a concentration of 1.0 µg/ml. This solution must be prepared fresh just prior to use.

JC-1 stock solution

Prepare a 0.2 mM solution of JC-1 (Molecular Probes) by dissolving 1.3 mg dye in 10 ml *N,N*-dimethylformamide (Sigma). Store for weeks in small (e.g., 0.5- or 1.0-ml) aliquots protected from light at -20°C. Use glass containers; *N,N*-dimethylformamide will dissolve plastics.

Rhodamine 123 (R123) stock solution

Prepare a 0.1 mM solution of R123 (Molecular Probes) by dissolving 0.38 mg dye in 10 ml methanol. Store for months in small aliquots protected from light at -20°C. Prior to use, dilute ten-fold with PBS to obtain 10 µM concentration.

Rinse solution

1 g BSA
0.1 ml Triton X-100
100 ml PBS (APPENDIX 2A)
Store up to 1 week at 4°C

RNase stock solution

Dissolve 2 mg RNase A (e.g., Sigma) in 1 ml distilled water. If the RNase is not DNase free, heat the solution 5 min at 100°C to inactivate any traces of DNase. Store ≤1 year at 4°C.

TdT reaction buffer, 5×

1 M potassium (or sodium) cacodylate
125 mM Tris-Cl, pH 6.6 (*APPENDIX 2A*)
0.125% bovine serum albumin (BSA)
Store for weeks at 4°C

COMMENTARY

Background Information

Applications of flow cytometry in cell neurobiology have two distinct goals (for reviews, see Darzynkiewicz et al., 1992, 1997; Ormerod, 1998; van Engeland et al., 1998; Vermes et al., 2000). One goal is to elucidate molecular and functional mechanisms associated with cell death. For this purpose, flow cytometry is used to measure cellular levels of components involved in the regulation and/or execution of apoptosis or cell necrosis. The most frequently studied are pro- and anti-apoptotic members of the Bcl-2 protein family, caspases, proto-oncogenes (e.g., *c-myc* or *ras*), or tumor suppressor genes (e.g., p53 or pRB). Flow cytometry is also widely used to study functional attributes of the cell such as mitochondrial metabolism, oxidative stress, intracellular pH, or ionized calcium, all closely associated with mechanisms of apoptosis. The major advantage of flow cytometry in these applications is that it provides the possibility of multiparametric measurements of cell attributes. Multivariate analysis of such data reveals the correlation between the measured cell constituents. For example, when one of the measured attributes is cellular DNA content (the parameter that reports the cell cycle position or DNA ploidy), an expression of the other measured attribute can then be directly related to the cell cycle position (or cell ploidy) without a need for cell synchronization. Since individual cells are measured, intercellular variability can be assessed, cell subpopulations identified, and rare cells detected.

The second goal of cytometry applications is to identify and quantify dead cells and discriminate between apoptotic and necrotic modes of death. Dead-cell recognition is generally based on the presence of a particular biochemical or molecular marker that is characteristic for apoptosis, necrosis, or both. Numerous methods have been developed, espe-

cially for the identification of apoptotic cells. Apoptosis-associated changes in the gross physical attributes of cells, such as cell size and granularity, can be detected by analysis of laser light scattered by the cell in forward and side directions (Swat et al., 1981; Ormerod et al., 1995). Some methods rely on apoptosis-associated changes in the distribution of plasma membrane phospholipids (Fadok et al., 1992; Koopman et al., 1994). Others detect the loss of active transport function of the plasma membrane. Still other methods probe the mitochondrial transmembrane potential (Cossarizza et al., 1994; Kroemer, 1998; Zamzani et al., 1998). The detection of DNA fragmentation provides another convenient marker of apoptosis; apoptotic cells are then recognized either by their fractional (subdiploid, sub-G₁) DNA content due to extraction of low-molecular-weight DNA from the cell (Umansky et al., 1981; Nicoletti et al., 1991), or by the presence of DNA strand breaks, which can be detected by labeling their 3'-OH termini with fluorochrome-conjugated nucleotides in a reaction utilizing exogenous terminal deoxynucleotidyl transferase (TdT; Gorczyca et al., 1992, 1993; Li and Darzynkiewicz, 1995; Li et al., 1996). More recently, flow cytometric methods have been developed to detect activation of caspases and tissue transglutaminase (TGase 2; Grabarek et al., 2002). It should be noted, however, that the fluorochrome-labeled inhibitors of caspases (FLICAs), initially described as markers of caspase activation (Smolewski et al., 2001), or serine proteases, although convenient markers of apoptotic cells and most likely detecting activation of these proteases, do not have sufficient specificity to be used as specific probes of their active enzymatic centers (Pozarowski et al., 2003).

A variety of kits are commercially available to identify apoptotic cells using the methods presented in this unit. Since the reagents are

already prepackaged and the procedures are described in cookbook format, the kits offer the advantage of simplicity. Their cost, however, is many-fold higher than that of the individual reagents. Furthermore, the kits do not allow one the flexibility that is often required to optimize procedures for a particular cell system. In many situations, therefore, the preparation of samples for analysis by flow cytometry as described herein may be preferred.

Light-scattering changes during apoptosis

Intersection of cells with the laser light beam in a flow cytometer results in light scattering. Analysis of light scattered in different directions reveals information about cell size and structure. The intensity of the forward light-scatter signal correlates with cell size. Side scatter, on the other hand, yields information on light-refractive and light-reflective properties of the cell and reveals optical inhomogeneity of the cell structure resulting from condensation of cytoplasm or nucleus, granularity, and so on.

As a consequence of cell shrinkage, a decrease in forward light scatter is observed at a relatively early stage of apoptosis (Swat et al., 1981; Ormerod et al., 1995). Initially, there is little change in side scatter during apoptosis. In fact, in some cell systems, an increase in intensity of side-scatter signal may be seen, reflect-

ing perhaps chromatin and cytoplasm condensation and nuclear fragmentation, the events that may lead to an increase in the light-refractive and light-reflective properties of the cell. When apoptosis is more advanced and the cells shrink in size, the intensity of side scatter also decreases (Fig. 18.8.1). Late apoptotic cells, therefore, are characterized by markedly diminished intensity of both forward- and side-scatter signals. In contrast to apoptosis, the early stages of cell necrosis are marked by cell swelling, which is detected by a transient increase in forward light scatter. Rupture of the plasma membrane and leakage of the cytosol during subsequent steps of necrosis correlate with a marked decrease in intensity of both forward- and side-scatter signals.

Analysis of light scatter is often combined with other assays, most frequently surface immunofluorescence (to identify the phenotype of the dying cell), or another marker of apoptosis. It should be stressed, however, that the change in light scatter alone is not a specific marker of apoptosis or necrosis. Mechanically broken cells, isolated nuclei, cell debris, and individual apoptotic bodies may also display diminished light-scatter properties. Therefore, the analysis of light scatter must be combined with measurements that can provide a more definite identification of apoptotic or necrotic cells.

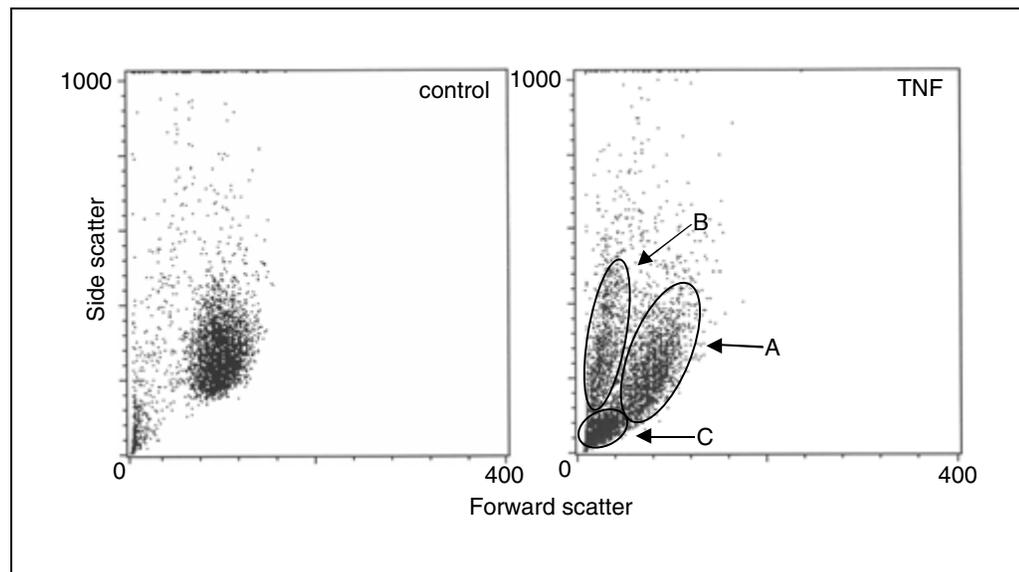


Figure 18.8.1 Changes in light scattering properties of cells undergoing apoptosis. HL-60 cells were untreated (left panel) or treated 3 hr with TNF- α and cycloheximide (CHX) to induce apoptosis (right panel). Cell population **A** in the treated culture (right panel) represents cells that have light scattering properties similar to those of untreated cells. Early apoptotic cells (**B**) have diminished forward scatter and are very heterogenous with respect to side scatter. Late apoptotic cells (**C**) have both forward and side scatter diminished.

Mitochondrial potential

A point to consider in measuring $\Delta\Psi_m$ is that the mitochondrial potential probes lack absolute specificity and also accumulate in the cytosol. Probe distribution in mitochondria versus cytosol follows the Nernst equation, according to which the ratio of mitochondrial to cytosolic free cation concentration should be 100:1 at 120 mV mitochondrial transmembrane potential (Waggoner, 1979). However, the specificity of particular mitochondrial probes towards mitochondria is higher at low probe concentrations. It is advisable, therefore, to use these probes at the lowest possible concentration. The limit for the minimal dye concentration that still provides an adequate signal-to-noise ratio during the measurement is dictated by sensitivity of the instrument (laser power, optics, photomultiplier sensitivity) and by the mitochondrial mass per cell; the latter varies depending on the cell type or upon mitogenic stimulation (Darzynkiewicz et al., 1981).

A series of MitoTracker dyes (chloromethyltetramethylrosamine analogs) of different colors was introduced by Molecular Probes as new mitochondrial $\Delta\Psi_m$ -sensitive probes. Some of these dyes remain attached to mitochondria following cell fixation using cross-linking agents (Poot et al., 1997; Haugland, 2002). It should be noted, however, that because these dyes bind to thiol moieties within mitochondria, their retention after fixation may not be a reliable marker of the transmembrane potential (Ferlini et al., 1998; Gilmore and Wilson, 1999). Furthermore, they are potent inhibitors of respiratory chain I and may themselves induce dissipation of $\Delta\Psi_m$ (Scorrano et al., 1999). Because it is likely that other $\Delta\Psi_m$ probes may predispose the cells to the permeability transition, one has to be cautious in interpreting the data on their use in analysis of apoptosis.

It has been reported that other mitochondrial probes, 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green, are markers of mitochondrial mass and are not sensitive to $\Delta\Psi_m$ (Ratinaud et al., 1988; Poot et al., 1997). It was proposed, therefore, to measure both $\Delta\Psi_m$ and mitochondrial mass by using a combination of $\Delta\Psi_m$ -sensitive and $\Delta\Psi_m$ -insensitive probes (Petit et al., 1995). Recent observations, however, indicate that 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green are quite sensitive to changes in $\Delta\Psi_m$ and therefore, either alone or in combination with $\Delta\Psi_m$ -sensitive probes, cannot be used as markers of mitochondrial mass (Keiji et al., 2000).

Caspases

Caspases (cysteine-aspartic acid specific proteases) are activated in response to different inducers of apoptosis (Kaufmann et al., 1993; Lazebnik et al., 1994; Alnemri et al., 1996). The process of their activation is considered to be the key event of apoptosis, a marker of cell commitment to disassemble the machinery that supports cell life (for reviews, see Budihardjo et al., 1999; Earnshaw et al., 1999; Shi, 2002). Caspases recognize a four-amino-acid sequence on their substrate proteins; within this sequence, the carboxyl end of aspartic acid is the target for cleavage. Detection of caspase activation is of primary interest in oncology as well as in other disciplines of medicine and biology, and several methods have been developed to accomplish this.

One approach that is potentially useful for cytometry utilizes fluorogenic caspase substrates. The peptide substrates are colorless or nonfluorescent, but upon caspase-induced cleavage, they generate colored or fluorescing products (Gorman et al., 1999; Hug et al., 1999; Liu et al., 1999; Komoriya et al., 2000; Telford et al., 2002). Many kits designed to measure activity of caspases using fluorometric or colorimetric assays are commercially available (e.g., from Biomol Research Laboratories or Calbiochem). Some kits can be used to detect activation of multiple caspases, while others are based on the substrates that are specific for caspase-1, caspase-3, or caspase-8.

The second approach in studies of caspase activation applicable to cytometry is based on immunocytochemical detection of the epitope of these enzymes that is characteristic of their active form. This epitope appears as a result of conformational changes that occur during activation of caspases, such as those associated with the transcatalytic cleavage of the zymogen pro-caspases (for reviews, see Budihardjo et al., 1999; Earnshaw et al., 1999). Antibodies developed to react only with the activated caspases have recently become commercially available (e.g., from Promega). These antibodies can be used in standard immunocytochemical assays. Basic Protocol 3 describes the methodology of detection of caspase-3 activation based on this principle, combined with the convenient immunocytochemical Zenon technology (Haugland, 2002). Another approach that was proposed to probe caspase activation utilizes fluorochrome-labeled inhibitors of caspases (FLICAs; e.g., FAM-VAD-FMK), which are reagents designed as affinity labels to the active enzyme center of these enzymes (Smolewski et al.,

2001). It was recently found, however, that these reagents lack the desired specificity with respect to the active enzyme center of caspases (Pozarowski et al., 2003). However, they are convenient and specific markers of apoptotic cells and can be used as such, particularly when there is a need to distinguish between apoptotic and necrotic modes of cell death. Basic Protocol 3 describes application of FAM-VAD-FMK to identify three sequential stages of apoptosis. Finally, caspase activation can be detected indirectly, by identifying specific protein fragments which are the products of cleavage by particular caspases. For example, immunocytochemical detection of the poly(ADP-ribose) polymerase 85-kDa fragment (PARP p85 fragment), which results from cleavage of the PARP substrate by caspase-3, is described in Basic Protocol 4. Concurrent analysis of cellular DNA content allows one to correlate activation of caspases with the cell cycle position or DNA ploidy. Using immunocytochemical methods, however, one has to remember that, often, antibodies that are applicable to immunoblotting or immunoprecipitation may not always be useful in immunocytochemical assays, and vice versa.

Critical Parameters and Troubleshooting

Positive and negative controls

There are instances when cells die by a process of atypical apoptosis that lacks one or more characteristic apoptotic features. Obviously, apoptosis cannot be detected if the feature serves as a marker. It is also possible that the assay (kit) used to identify apoptotic cells malfunctions for technical reasons. For example, the enzyme TdT used in the TUNEL assay may be inactive due to improper storage. A mistake might be made during the staining procedure. It is essential, therefore, to distinguish between the genuine lack of apoptosis and the inability to detect it due to technical causes. The distinction can be made using a positive control consisting of cells known to be apoptotic (confirmed by a standard method and inspection of cell morphology). Such control cells have to be processed in parallel with the investigated sample through all the prescribed protocol steps. Some vendors provide positive and negative control cells with their kits (e.g., APO-BRDU from Phoenix Flow Systems).

The positive control cells can be prepared in large quantity and stored in aliquots to be used during each experiment. Such a convenient

control may be, to give an example, exponentially growing HL-60 or U-937 leukemic cells treated 3 to 6 hr in culture with 0.2 μ M camptothecin (CPT) to induce apoptosis. The cells so treated consist of subpopulations of apoptotic (S-phase) and non-apoptotic (G_1 -phase) cells, present in the same sample. However, to induce apoptosis of S-phase cells with CPT, it is critical to have the cultures in the exponential phase of growth, at relatively low cell density (<800,000 cells/ml); subconfluent cultures are quite resistant to CPT. A large batch of cells treated in such a manner can be appropriately fixed in 70% ethanol and then stored in aliquots at -20°C to be used as a positive and negative control for each assay that utilizes fixed cells. For the assays that require live cells, controls should be freshly made and must not be fixed. Cells from healthy, untreated cultures may also serve as negative controls.

False-positive apoptosis

The exposure of phosphatidylserine on cell surfaces that occurs during apoptosis (Fadok et al., 1992; Koopman et al., 1994) makes apoptotic cells and apoptotic bodies attractive to neighboring cells, which phagocytize them. The ability to engulf apoptotic bodies is not solely the property of professional phagocytes, but is shared by cells from fibroblast or epithelial lineages. It is frequently observed, especially in solid tumors, that the cytoplasm of both nontumor and tumor cells located in the neighborhood of apoptotic cells contains inclusions typical of apoptotic bodies. The remains of apoptotic cells engulfed by neighboring cells contain altered plasma membrane, fragmented DNA, and other constituents with attributes characteristic of apoptosis. Thus, if the distinction is based on any of these attributes, the live, nonapoptotic cells that phagocytized apoptotic bodies cannot be distinguished from genuine apoptotic cells by flow cytometry. For example, nonapoptotic cells that engulf apoptotic bodies become reactive with annexin V (Marguet et al., 1999). Most likely this is due to the fact that during engulfment, the plasma membrane of apoptotic bodies fuses with the plasma membrane of the phagocytizing cell. It has also been shown that nonapoptotic cells (primarily monocytes and macrophages) in bone marrow of patients undergoing chemotherapy have large quantities of apoptotic bodies in their cytoplasm and are strongly positive in the TUNEL reaction (Bedner et al., 1999).

Even after relative mild treatment such as trypsinization, mechanical agitation, detach-

ment with rubber policeman, or electroporation, the plasma membrane of live nonapoptotic cells may have phosphatidylserine, reactive with annexin V, exposed on the surface. Such cells may also be erroneously identified as apoptotic cells.

Distinction between apoptosis, necrosis, and the “necrotic stage” of apoptosis

There are several differences between typical apoptotic and necrotic cells (Kerr et al. 1972; Arends et al., 1990; Majno and Joris, 1995) that provided a basis for development of numerous markers and methods that can discriminate between these two modes of cell death (Darzynkiewicz et al., 1992, 1997). The major difference stems from the early loss of integrity of the plasma membrane during necrosis. This event results in a loss of the cell's ability to exclude charged fluorochromes such as trypan blue or PI. In contrast, the plasma membrane and membrane transport functions remain, to a large extent, preserved during the *early stages* of apoptosis. A cell's permeability to PI or its ability to retain some fluorescent probes, such as products of enzyme activity (e.g., fluorescein diacetate hydrolyzed by esterases), is the most common marker distinguishing apoptosis from necrosis (Darzynkiewicz et al., 1994). A combination of fluorochrome-conjugated annexin V with PI distinguishes live cells (unstainable with both dyes) from apoptotic cells (stainable with annexin V but unstainable with PI) from necrotic cells (stainable with both dyes; Koopman et al., 1994). The same holds true for a combination of PI with FLICA (Smolewski et al., 2001). However, while this approach works well in many instances, it has limitations and possible pitfalls.

(1) Late-stage apoptotic cells resemble necrotic cells to such an extent that the term “apoptotic necrosis” was proposed to define them (Majno and Joris, 1995). This is a consequence of the fact that the integrity of the plasma membrane of late apoptotic cells is compromised, which makes the membrane leaky and permeable to charged cationic dyes such as PI. Since the ability of such cells to exclude these dyes is lost, the discrimination between late apoptosis and necrosis cannot be accomplished by methods based on the use of dye exclusion (PI, trypan blue) or annexin V binding.

(2) The permeability and asymmetry of plasma membrane phospholipids (accessibility of phosphatidylserine) may change, as a result

of prolonged treatment with proteolytic enzymes (trypsinization), mechanical damage (e.g., cell removal from flasks by rubber policeman, cell isolation from solid tumors, or even repeated centrifugations), electroporation, or treatment with some drugs.

(3) Many flow cytometric methods designed to quantify the frequency of apoptotic or necrotic cells are based on the differences between live, apoptotic, and necrotic cells in the permeability of plasma membrane to different fluorochromes such as PI, 7-AAD, or Hoechst dyes. It should be stressed, however, that plasma membrane permeability probed by dye accumulation in the cell may vary depending on the cell type and other factors unrelated to apoptosis or necrosis (e.g., very active efflux mechanism that rapidly pumps dye out of the cell). The assumptions, therefore, that live cells maximally exclude a particular dye, while early apoptotic cells are somewhat leaky and late apoptotic or necrotic cells are fully permeable to the dye, and that these differences are large enough to identify these cells, are not universally applicable.

It is particularly difficult to discriminate between apoptotic and necrotic cells in suspensions from solid tumors. Necrotic areas form in tumors as a result of massive local cell death due to poor accessibility to oxygen and growth factors when tumors grow in size and their local vascularization becomes inadequate. Needle-aspirated samples or cell suspensions from the resected tumors may contain many cells from the necrotic areas. Such cells are indistinguishable from late apoptotic cells by many markers. Because the AI in solid tumors, representing spontaneous or treatment-induced apoptosis, should not include cells from the necrotic areas, one has to eliminate such cells from analysis. Because incubation of cells with trypsin and DNase selectively digests all cells whose plasma membrane integrity is compromised, i.e., primarily necrotic cells (Darzynkiewicz et al., 1994), such a procedure may be used to remove necrotic cells from suspensions. It should be noted, however, that late apoptotic cells have partially leaky plasma membrane and are also expected to be sensitive to this treatment.

In conclusion, examination of cells by microscopy may be the only way to distinguish between apoptosis and necrosis, based on their characteristic differences in morphology.

Preferential loss of apoptotic cells during sample preparation

Cell detachment in culture. Early during apoptosis, cells detach from the surface of culture flasks and float in the medium. The standard procedure of discarding the medium, trypsinizing the attached cells or treating them with EDTA, and collecting the detached cells results in selective loss of those apoptotic cells that are discarded with the medium. Such loss may vary from flask to flask depending on how the culture is handled, e.g., the degree of mixing or shaking, efficiency in discarding the old medium, and so on. Surprisingly, some authors still occasionally report discarding the medium and trypsinizing the cells. Needless to say, such an approach cannot be used for quantitative analysis of apoptosis. To estimate the frequency of apoptotic cells in adherent cultures, it is essential to collect floating cells, pool them with the trypsinized ones, and measure them as a single sample. It should be stressed that trypsinization, especially if prolonged, results in digestion of cells with a compromised plasma membrane. Thus, collection of cells from cultures by trypsinization is expected to cause selective loss of late apoptotic and necrotic cells.

Density-gradient centrifugation. Similarly, density-gradient separation of cells (e.g., using Ficoll-hypaque or percoll solutions) may result in selective loss of dying and dead cells, because early during apoptosis the cells become dehydrated and have condensed nuclei and cytoplasm, and therefore have a higher density than nonapoptotic cells. Knowledge of any selective loss of dead cells in cell populations purified by such an approach is essential when one is studying apoptosis.

Centrifugations, mechanical agitations. Repeated centrifugations lead to cell loss by at least two mechanisms. One involves electrostatic cell attachment to the tubes and may be selective for a particular cell type. For example, preferential loss of monocytes and granulocytes is observed during repeated centrifugation of white blood cells, while lymphocytes remain in suspension (Bedner et al., 1997). Cell loss is of particular concern when hypocellular samples ($<5 \times 10^4$) are processed. In such a situation, carrier cells in excess (e.g., chick erythrocytes) may be added to preclude disappearance of the cells of interest through centrifugation. The second mechanism of cell loss involves preferential disintegration of fragile cells. Because apoptotic cells are very fragile, especially at late stages of apoptosis, they may

selectively be lost from samples that require centrifugation or are repeatedly vortexed or pipetted. Addition of serum or bovine serum albumin to cell suspensions, shortened centrifugation time, and decreased gravity force all may have a protective effect against cell breakage by mechanical factors. Apoptotic cells may also preferentially disintegrate in biomass cultures that require constant cell mixing.

It should be noted that sensitivity of apoptotic cells to mechanical factors depends on activation of TGase 2. Cells with activated TGase 2 have their cytoplasmic protein cross-linked and are resistant to mechanical stress. They also resist treatment with detergents. In contrast, apoptotic cells that do not activate TGase 2 are overly sensitive and easily undergo disintegration. It was observed that cells may often undergo apoptosis without evidence of TGase 2 activation (Grabarek et al., 2002).

Abundance of extractable (fragmented) DNA is not a quantitative measure of apoptosis

A common misconception in analysis of apoptosis is that the amount of fragmented (low-molecular-weight, “extractable”) DNA detected in cultures, tissue or cell extracts, or other samples reflects incidence of apoptosis. Many methods have been developed to estimate the abundance of fragmented DNA and numerous reagent kits are being sold for that purpose. They include direct quantitative colorimetric analysis of soluble DNA, densitometry of DNA ladders on gels, and immunochemical assessment of nucleosomes. These approaches and the related kits are advertised as quantitative, in that they provide information regarding the incidence of apoptosis in cell populations. Such claims are grossly incorrect for the reason that the amount of fragmented (low-molecular-weight) DNA that can be extracted from a *single* apoptotic cell varies over a wide range depending on the *stage* of apoptosis. Although at an early stage of apoptosis only a small fraction of DNA is fragmented and extractable, nearly all DNA can be extracted from the cell that is more advanced in apoptosis. Thus, the total content of low-molecular-weight DNA extracted from the cell population, or the ratio of low- to high-molecular-weight fraction, does not provide information about the *frequency* of apoptotic cells (AI), even in relative terms, e.g., for comparison of cell populations. For this simple reason, biochemical methods based on analysis of fragmented DNA can be used to *quantitatively* estimate the frequency of apop-

tosis only when comparing cell populations that have identical distribution of cells across the stages of apoptosis.

DNA laddering observed during electrophoresis provides evidence of internucleosomal DNA cleavage, which is considered one of the hallmarks of apoptosis (Arends et al., 1990). Analysis of DNA fragmentation by gel electrophoresis to detect such laddering is thus a valuable method to demonstrate the *apoptotic mode* of cell death; however, it should not be used as a means to quantify the *frequency* of apoptosis.

It should be noted that in some cell types, particularly of fibroblast and epithelial lineages, apoptosis may occur without internucleosomal DNA cleavage. The products of DNA fragmentation in these cells are large (50- to 300-kb) DNA sections that cannot be extracted from the cell (Oberhammer et al., 1993). Obviously, in these cases, apoptosis cannot be revealed by the presence of DNA laddering on gels or by analysis of low-molecular-weight products. These large DNA fragments, however, can be identified by pulse-field gel electrophoresis.

Changes in cell morphology, the “gold standard” for identification of apoptosis

Apoptosis was originally defined as a specific mode of cell death based on very characteristic changes in cell morphology (Kerr et al., 1972; Fig. 18.8.2). These changes are still considered the “gold standard” for identification of apoptotic cells. Although particular markers may be used in conjunction with flow cytometry for detection and quantitative assessment of apoptosis in cell populations, the mode of cell death should always be confirmed by inspection of cells by light or electron microscopy. If there is any ambiguity regarding the mechanism of cell death, the morphological changes should be the deciding factor in resolving the uncertainty.

The characteristic morphological features of apoptosis and necrosis are listed in Table 18.8.1. The most specific (and generic to apoptosis) of these changes is chromatin condensation; the chromatin of apoptotic cells is very “smooth” (structureless) in appearance, and the structural framework that otherwise characterizes the cell nucleus is entirely lost. Because of the condensation, chromatin shows strong hyperchromicity with any of the DNA-specific

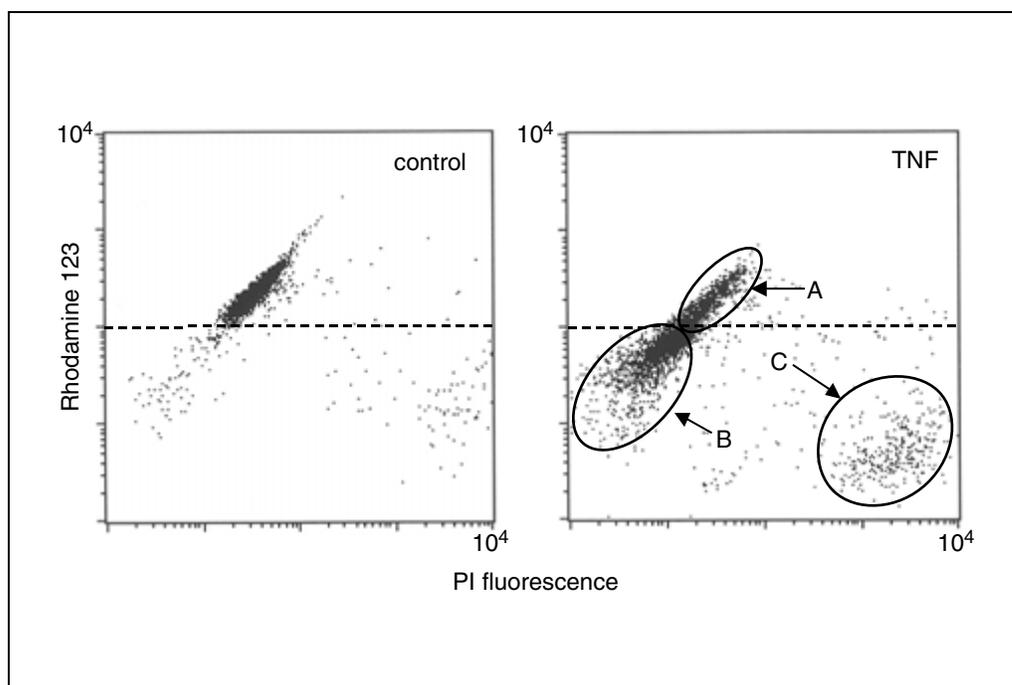


Figure 18.8.2 Detection of the collapse of mitochondrial electrochemical potential (ψ_m) by rhodamine 123 (R123). HL-60 cells were untreated (control; left panel) or treated 3 hr with TNF- α and CHX (right panel) to induce apoptosis. The cells were then incubated with R123 and PI according to Basic Protocol 1. The early apoptotic cells have diminished green fluorescence of R123 but exclude PI (cell population B). The late apoptotic (also necrotic) cells are stained strongly by PI (population C).

Table 18.8.1 Morphological Criteria for Identification of Apoptosis or Necrosis

Apoptosis	Necrosis
Reduced cell size, convoluted shape	Cell and nuclear swelling
Plasma membrane undulations (“blebbing,” “budding”)	Patchy chromatin condensation
Chromatin condensation (DNA hyperchromicity)	Swelling of mitochondria
Loss of the structural features of the nucleus (smooth, planate appearance)	Vacuolization in cytoplasm
Nuclear fragmentation (karyorrhexis)	Plasma membrane rupture (“ghost-like” appearance of lysed cells)
Presence of apoptotic bodies	Dissolution of nuclear chromatin (karyolysis)
Dilatation of the endoplasmic reticulum	Attraction of inflammatory cells
Relatively unchanged cell organelles	
Shedding of apoptotic bodies	
Phagocytosis of the cell remnants	
Cell detachment from tissue culture flasks	

dyes (Hotz et al., 1992). Apart from chromatin condensation, however, other changes are less generic to apoptosis and may not always be apparent. For example, nuclear fragmentation, although commonly observed during apoptosis of hematopoietic-lineage cells, may not occur during apoptosis of some epithelial- or fibroblast-lineage cells. Likewise, cell shrinkage, at least early during apoptosis, is not a universal marker of the apoptotic mode of cell death.

It should be stressed that optimal preparations for light microscopy require cytospinning of live cells followed by fixation and staining on slides. The cells become flat, facilitating assessment of their morphology. On the other hand, when cells are initially fixed and stained in suspension, transferred to slides, and analyzed under the microscope, their morphology is obscured by unfavorable geometry; the cells are spherical and thick, and require confocal microscopy to reveal details such as early signs of apoptotic chromatin condensation.

Differential staining of cellular DNA and protein of cells on slides with DAPI and sulforhodamine 101, respectively, is rapid and simple and provides very good morphological resolution of apoptosis and necrosis (Darzynkiewicz et al., 1997). A combined cell illumination with UV light (to excite the DAPI or other DNA fluorochrome) and light-transmission microscopy utilizing interference contrast (Nomarski illumination) is the authors' favorite method of cell visualization to identify apop-

totic cells. Other DNA fluorochromes, such as PI, 7-aminoactinomycin D, or acridine orange, can be used as well.

In conclusion, regardless of which cytometric assay has been used to identify apoptosis, the mode of cell death should be confirmed by inspection of cells by light or electron microscopy. Morphological changes during apoptosis have a very characteristic pattern (Kerr et al., 1972; Majno and Joris, 1995) and should be the deciding factor in situations where any ambiguity arises regarding the mode of cell death. It should be noted, however, that the laser scanning cytometer (LSC), offering many attributes of flow cytometry and simultaneously providing the means to examine morphology of the measured cells (Darzynkiewicz et al., 1999; Kamensky, 2001), appears to be the instrument of choice in analysis of apoptosis (Bedner et al., 1999).

Membrane potential

It should be stressed that $\Delta\psi_m$, like other functional markers, is sensitive to minor changes in cell environment. The samples to be compared, therefore, should be incubated and measured under identical conditions, taking into an account temperature, pH, time elapsed between the onset of incubation and actual fluorescence measurement, and other potential variables.

Annexin V

Interpretation of the results may be complicated by the presence of non-apoptotic cells with damaged membranes. Such cells may have phosphatidylserine exposed on the plasma membrane and therefore, like apoptotic cells, bind annexin V. Mechanical disaggregation of tissues to isolate individual cells, extensive use of proteolytic enzymes to disrupt cell aggregates, to remove adherent cells from cultures, or to isolate cells from tissue, mechanical removal of the cells from tissue-culture flasks (e.g., with a rubber policeman), or cell electroporation all affect the binding of annexin V. Such treatments, therefore, may introduce experimental bias in the subsequent analysis of apoptosis by this method.

Even intact and live cells take up PI upon prolonged incubation. Therefore, fluorescence measurement should be performed rather shortly after addition of the dye.

DNA fragmentation

It should be stressed that the degree of extraction of low-molecular-weight DNA and consequently the content of DNA remaining in apoptotic cells for flow cytometric analysis varies markedly depending on the extent of DNA degradation (duration of apoptosis), the number of cell washings, and the pH and molarity of the washing and staining buffers. DNA fragmentation is often so extensive that most DNA is removed during the post-fixation rinse with PBS and in the staining solution, and a DNA extraction step is therefore unnecessary. Conversely, when DNA degradation does not proceed to internucleosomal regions but stops after generating 50- to 300-kb fragments (Oberhammer et al., 1993), little DNA can be extracted, and this method may fail to detect such apoptotic cells. It also should be noted that if G₂, M, or even late S-phase cells undergo apoptosis, the loss of DNA from these cells may not be adequate to place them at the sub-G₁ peak, as they may end up with DNA content equivalent of that of G₁ or early S-phase cells and therefore be indistinguishable from the latter.

It is a common practice to use detergents or hypotonic solutions instead of fixation in the process of DNA staining for flow cytometry (Nicoletti et al., 1991). Such treatments cause lysis of plasma membrane and release of the nucleus. Although this approach is simple and yields excellent resolution for DNA-content analysis, when used to quantify apoptotic cells it introduces bias owing to the fact that nuclei

of apoptotic cells are often fragmented. Lysis of cells with fragmented nuclei releases nuclear fragments rather than individual nuclei, and consequently several fragments can be released from a single cell. Likewise, lysis of mitotic cells that happen to be in the specimen releases individual chromosomes or chromosome aggregates. In the case of micronucleation (e.g., after cell irradiation), the micronuclei are released upon cell lysis. Each nuclear fragment, chromosome, or micronucleus is then recorded by the flow cytometer as an individual object with a sub-G₁ DNA content. Such objects are then erroneously classified as individual apoptotic cells. This bias is increased if DNA content is displayed on a logarithmic scale. Such a scale allows one to record objects with DNA content as little as 1% or even 0.1% of that of G₁ cells, which certainly cannot be individual apoptotic cells.

Activation of TGase 2

Although apoptotic cells with activated TGase 2 can be detected using either Basic Protocol 8 or Alternate Protocol 2, the differences between these assays should be underscored. The most distinct is the difference in the length of the time window that may be measured by the respective assay. Namely, the detergent-based assay (see Basic Protocol 8) detects cumulative protein cross-linking, reflecting the integrated cross-linking, from its onset to the time of cell harvesting. In contrast, the assay based on incorporation of F-CDV (see Alternate Protocol 2) detects cross-linking that occurs only during the time interval when this reagent is present in the culture. Thus, if F-CDV is included at time zero, i.e., when the inducer of apoptosis is added, its incorporation is a reflection of the cumulative protein cross-linking and thus is comparable with the detergent-based assay. However, if it is added during the final hour or two, it will reflect the cross-linking that took place only during this 1- or 2-hr time window. This difference between the assays should be kept in mind when comparing frequency of TGase 2-positive cells, which may vary between the assays, depending on the length of the respective time window.

Situations (e.g., following treatment with particular drugs) may occur in which cell proteins may become less soluble and more detergent resistant, not necessarily because of TGase 2 activation, but because of alteration by the drug. Some treatments unrelated to TGase 2 activity may also result in attachment of F-CDV to cellular proteins. Some cell types may be

more resistant to detergents. In all these cases, the assay may detect the “false-positive” TGase 2–positive cells. As with other markers of apoptosis, one has to be careful and additionally identify these cells by microscopy based on the characteristic changes in their morphology.

Apoptosis can be induced and may progress in some cells with no apparent TGase 2 activation (Grabarek et al., 2002). In general, the activation of TGase 2 is seen to occur in cells that show a high degree of chromatin and cytoplasm condensation leading to pronounced nuclear and cell shrinkage, and that either lack or have very limited nuclear fragmentation. In contrast, the apoptotic cells that appear larger and whose nuclei are excessively fragmented do not show activation of TGase 2. Apoptosis without activation of TGase 2 appears to occur more frequently when induced with higher drug doses, i.e., when cells enter the apoptotic process more rapidly following treatment. Thus, a note of caution should be added, that since TGase 2 activation may not be detected in some instances of apoptosis, the absence of its activation should not be considered a marker of nonapoptotic cells.

Anticipated Results

Light scatter. A decrease in forward light scatter characterizes early apoptotic cells (Fig. 18.8.1, cluster B). Late apoptotic cells and perhaps also larger apoptotic bodies show marked

decrease in both forward and side light scatter (cluster C).

Mitochondrial potential. A combination of PI and R123 identifies nonapoptotic cells that stain only green, early apoptotic cells whose green fluorescence is diminished, and late apoptotic or necrotic cells that stain with PI and have red fluorescence only (Fig. 18.8.2). Likewise, a combination of DiOC₆(3) and PI labels live nonapoptotic cells green, early apoptotic cells dim-green, and late apoptotic and necrotic cells red (not shown).

The change in binding of JC-1 is manifested by a loss of the orange fluorescence that represents the aggregate binding of this dye and that characterizes charged mitochondria (Fig. 18.8.3). JC-1 green fluorescence is expected to increase as a result of disaggregation of the complexed JC-1. However, either no change or a decrease in green fluorescence may be seen if JC-1 concentration within the cell is too high, which causes quenching of its fluorescence.

Caspases. Caspase-3 activation during apoptosis induced by topotecan (TPT), a camptothecin (CPT) analog, is reflected by the cells' ability to bind antibody that is reactive with the activated (cleaved) form of this enzyme (Fig. 18.8.4). Concurrent staining of cellular DNA with PI makes it possible to correlate caspase-3 activation with the cell cycle position. Note that activation of caspase-3 occurs preferentially in S-phase cells.

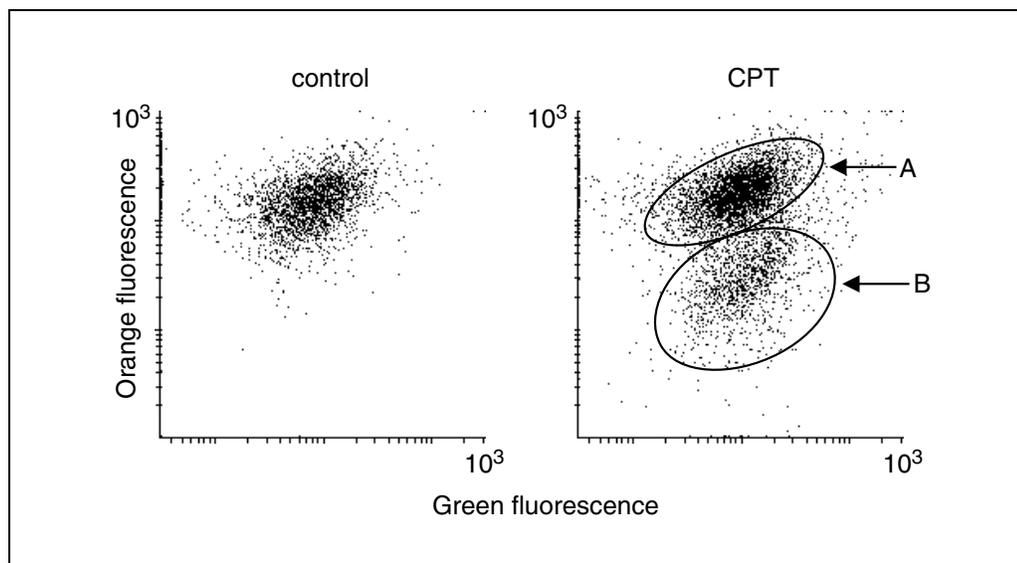


Figure 18.8.3 Detection of the collapse of mitochondrial electrochemical potential using the aggregate dye JC-1. HL-60 cells were untreated (control, left panel) or treated 3 hr with camptothecin (CPT, right panel) to induce apoptosis. Cells were then stained with JC-1 and their orange and green fluorescence was measured by cytometry, as described in Basic Protocol 2. Decreased intensity of orange fluorescence (subpopulation B) characterizes the cell with collapsed potential.

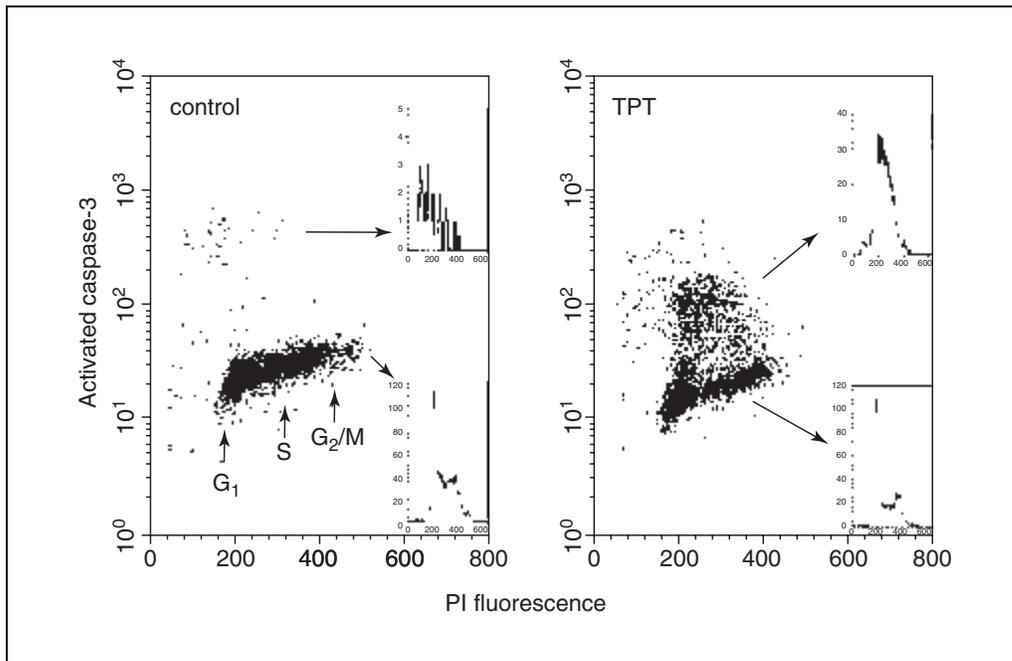


Figure 18.8.4 Immunocytochemical detection of caspase-3 activation using antibody reactive with the activated (cleaved) caspase-3. Apoptosis of HL-60 cells was induced by topotecan (TPT), an analog of CPT. Zenon technology (Haugland, 2002) was used to detect caspase-3 as described in Basic Protocol 2. Top and bottom insets in each panel show cellular DNA content frequency histograms of cells with activated and nonactivated caspase-3, respectively. Note that S-phase cells preferentially contain activated caspase-3 after induction of apoptosis by TPT.

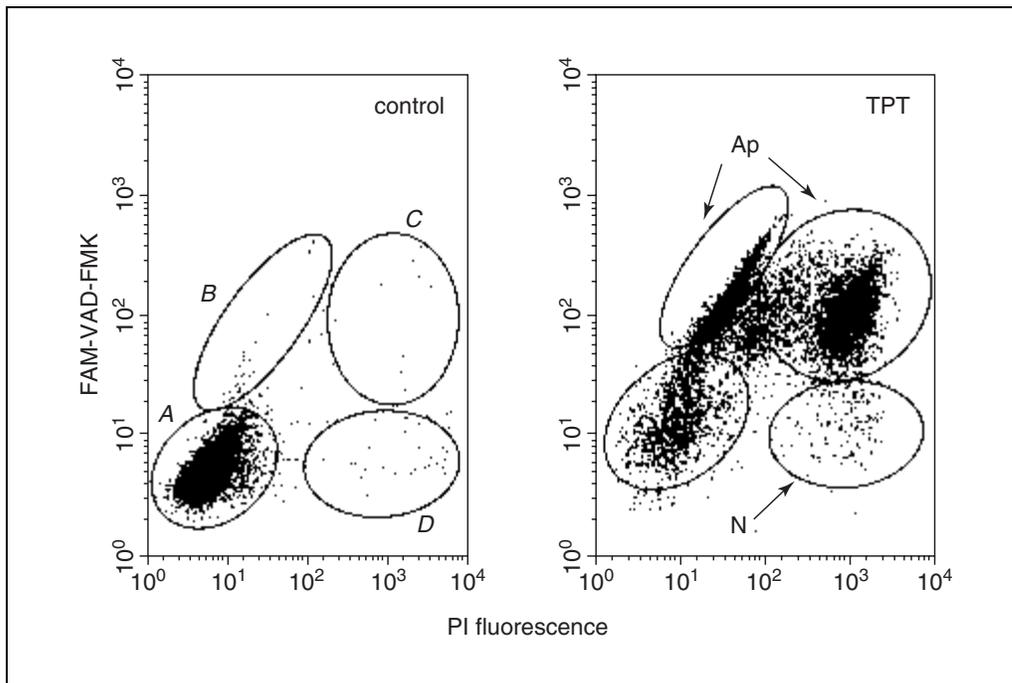


Figure 18.8.5 Binding of fluorochrome-labeled inhibitor of caspases (FLICA; FAM-VAD-FMK) and PI during apoptosis. Apoptosis of HL-60 cells was induced by TPT. The cells were stained according to Basic Protocol 3. Green (FAM-VAD-FMK) and red (PI) cellular fluorescence was measured by flow cytometry. Four cell subpopulations (A to D) can be identified, differing in their capability to bind FAM-VAD-FMK and PI. They represent sequential stages of apoptosis, starting with binding of FAM-VAD-FMK (B), loss of plasma membrane integrity to exclude PI (C), and loss of reactivity with FAM-VAD-FMK (D).

The bivariate distributions (scatterplots) of green and red fluorescence intensity representing cells supravivally stained with FAM-VAD-FMK (FLICA) and PI reveal the presence of four distinct subpopulations (Fig. 18.8.5). Nonapoptotic cells show neither FLICA nor PI fluorescence (FLICA⁻/PI⁻; subpopulation A). Early apoptotic cells bind FAM-VAD-FMK and still exclude PI (FLICA⁺/PI⁻; subpopulation B). More advanced in apoptosis are the cells that bind FAM-VAD-FMK but lose the ability to exclude PI (FLICA⁺/PI⁺; subpopulation C). The cells most advanced in apoptosis are FAM-VAD-FMK negative and are stained with PI (FLICA⁻/PI⁺; subpopulation D). Because the late phase of apoptosis during which the plasma membrane becomes permeable to cationic dyes such as PI or trypan blue has been defined as the “necrotic stage” of apoptosis (Majno and Joris, 1995; Darzynkiewicz et al., 1997), the FLICA⁺/PI⁺ and FLICA⁻/PI⁺ cells thus represent two consecutive phases of the “necrotic stage.” It should be noted that genuine necrotic cells, i.e., cells that die by the mode of necrosis (“accidental” cell death), not having activated caspases and unable to exclude PI (Darzynkiewicz et al., 1997), have the same properties (FLICA⁻/PI⁺) as very late apoptotic cells.

As mentioned earlier, because of lack of specificity, the labeling of apoptotic cells with FAM-VAD-FMK, while likely the marker of caspase activation, is not in and of itself evi-

dence of its binding to the active enzymatic center of caspases (Pozarowski et al., 2003).

PARP cleavage. Differences in intensity of PARP p85 immunofluorescence versus PI fluorescence (cellular DNA content) allow one to identify apoptotic cells and reveal the cell cycle distribution of both apoptotic (PARP p85-positive) and nonapoptotic (PARP p85-negative) cells (Fig. 18.8.6). It is quite evident that predominantly S-phase cells were undergoing apoptosis upon CPT treatment (Li and Darzynkiewicz, 2000).

Annexin V. Live nonapoptotic cells stained according to Basic Protocol 5 have minimal green (annexin V-FITC) fluorescence and also minimal red (PI) fluorescence (Fig. 18.8.7; subpopulation A). At early stages of apoptosis, cells stain green but still exclude PI and therefore continue to have no significant red fluorescence (subpopulation B). At late stages of apoptosis, cells show intense green and red fluorescence (subpopulation C). It should be noted that isolated nuclei, cells with severely damaged membranes, and very late apoptotic cells stain rapidly and strongly with PI and may not bind annexin V (subpopulation D).

DNA fragmentation. Apoptotic cells have decreased PI (or DAPI) fluorescence and diminished forward light scatter relative to cells in the main peak (G₁; Fig. 18.8.8). Optimally, the sub-G₁ peak representing apoptotic cells should be separated from the G₁ peak of the

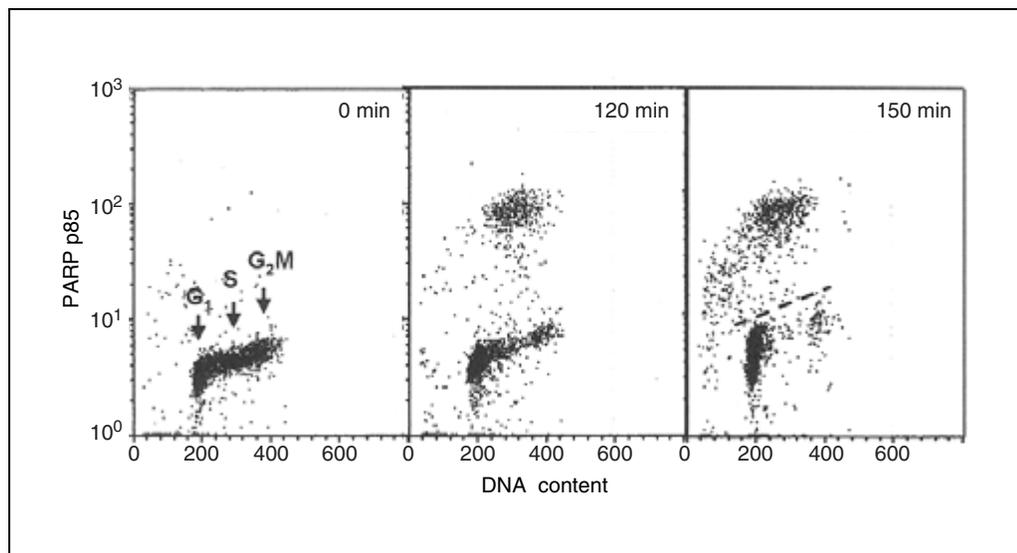


Figure 18.8.6 Identification of apoptotic cells by flow cytometry based on the immunocytochemical detection of the 85-kDa PARP cleavage fragment. To induce apoptosis, HL-60 cells were treated 60 min with TNF- α in the presence of CHX (Li and Darzynkiewicz, 2000). PARPp85 was detected immunocytochemically and DNA was counterstained with PI, as described in Basic Protocol 3.

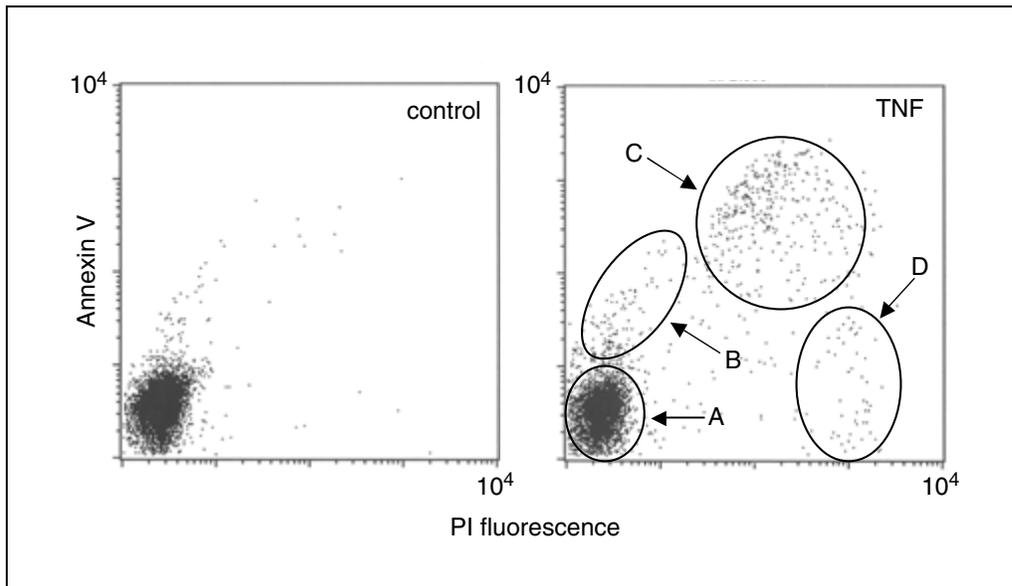


Figure 18.8.7 Detection of early and late apoptotic cells after staining with annexin V-FITC and PI. To induce apoptosis, HL-60 cells were treated 2 hr with TNF- α and CHX. Untreated (control; left panel) and TNF- α -treated (right panel) cells were then stained with annexin V-FITC and PI.

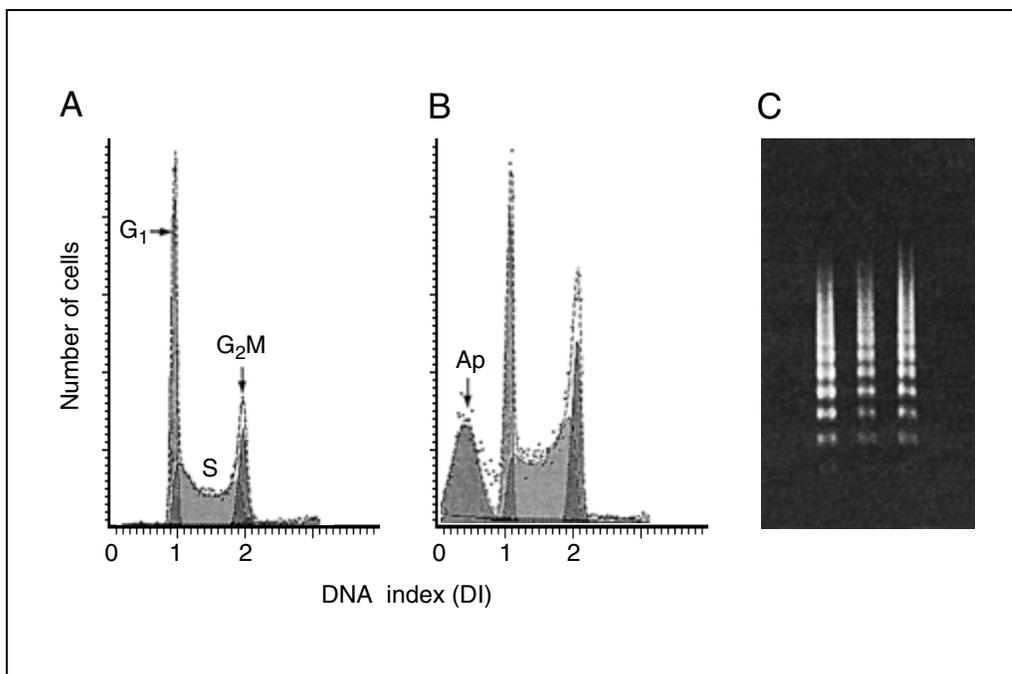


Figure 18.8.8 Detection of apoptotic cells by flow cytometry based on cellular DNA content analysis. (A) Normal cell plot. (B) To induce apoptosis, HL-60 cells were treated with the DNA topoisomerase II inhibitor fostriecin (Hotz et al., 1994). Cells were fixed in 70% ethanol, suspended in high-molarity phosphate buffer to extract fragmented DNA, and then stained with PI. A subpopulation of apoptotic cells (Ap) with fractional (sub-diploid) DNA content, i.e., with DNA index (DI) < 1.0 (sub-G₁ cells), is apparent. Note also the increase in the proportion of S-phase cells in the nonapoptotic population. (C) The fragmented DNA extracted from the apoptotic cells by the buffer was subjected to gel electrophoresis (Gong et al., 1994). Note "laddering" that reflects preferential DNA cleavage at internucleosomal sections, the characteristic feature of apoptosis (Arends et al., 1990).

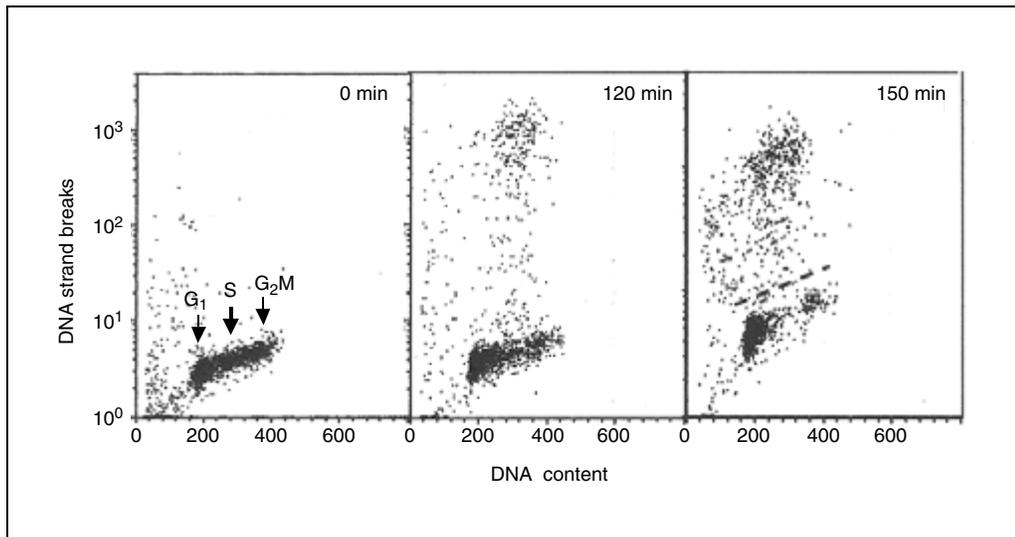


Figure 18.8.9 Detection of apoptotic cells by flow cytometry based on the presence of DNA strand breaks. To induce apoptosis, HL-60 cells were treated 120 or 150 min with CPT (Li and Darzynkiewicz, 2000). DNA strand breaks were labeled with BrdUTP using exogenous terminal deoxynucleotidyl transferase. The cell cycle distribution of both apoptotic and nonapoptotic cell subpopulations can be estimated based on the DNA content of individual cells. Note that in analogy to PARP cleavage (Fig. 18.8.6), preferentially S-phase cells undergo apoptosis following CPT treatment.

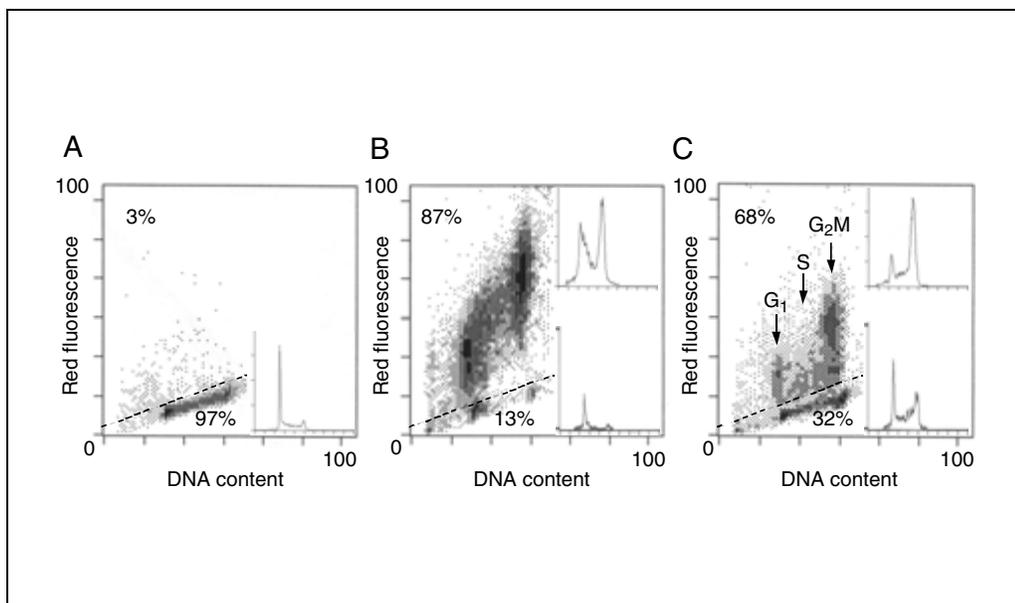


Figure 18.8.10 Detection of tissue transglutaminase (TGase 2) activation during apoptosis by the acquired resistance of the cytoplasmic proteins to detergent. Bivariate distributions illustrating red fluorescence of sulforhodamine 101 (protein content) versus blue fluorescence of DAPI (DNA content) of HL-60 cells, untreated (**A**) or exposed to hyperthermia (72 hr at 41.5°C) in the absence (**B**) and presence (**C**) of the cytotoxic RNase onconase (1.67 μ M; Grabarek et al., 2002). Following cell lysis by Triton X-100 and staining with DAPI and sulforhodamine 101, the isolated nuclei of nonapoptotic cells from control culture (**A**) show low and uniform intensity of the red fluorescence, reflecting low protein content. Subpopulations of apoptotic cells in **B** and **C** have their cytoplasmic protein crosslinked and therefore are resistant to detergent. They stain intensely with sulforhodamine 101. Note differences in DNA content (cell cycle) distribution of the cells with activated (top insets; cells gated above the dashed line) versus nonactivated TGase 2 (bottom insets; cells gated below the dashed line) in **B** and **C**. Percentage of cells with activated and non-activated TGase 2 in the respective cultures is indicated in each panel.

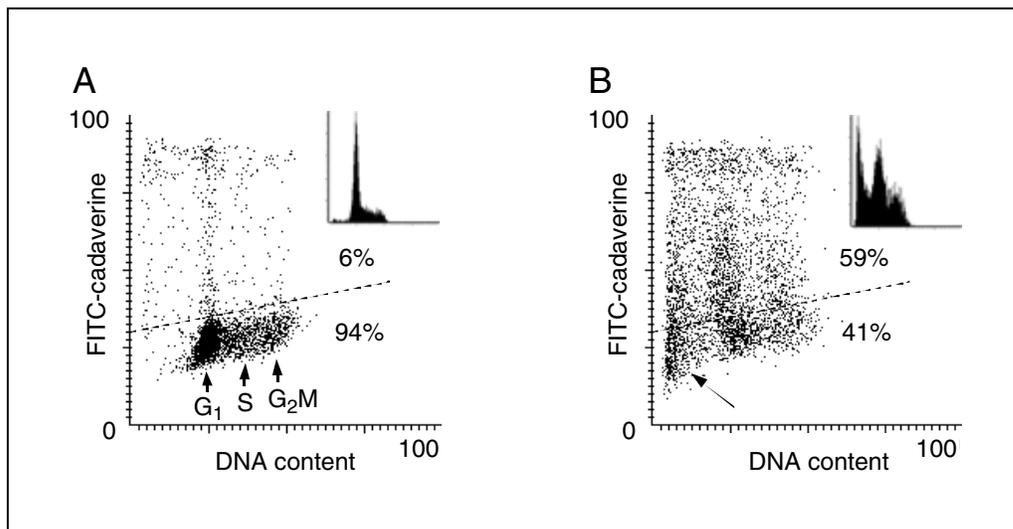


Figure 18.8.11 Detection of TGase 2 activity in HL-60 cells using FITC-conjugated cadaverine (F-CDV) as the enzyme substrate. Cultures of untreated (**A**) and hyperthermia (5 hr at 41.5°C)-treated (**B**) HL-60 cells were incubated 5 hr with 100 μ M F-CDV. Cells were then fixed and their DNA was counterstained with PI in the presence of RNase. Note the presence in the untreated culture (**A**) of few cells that incorporated F-CDV (spontaneous apoptosis), and large numbers of F-CDV-labeled cells in the treated culture (**B**). Note also that some apoptotic cells with fractional DNA content (sub-G₁ subpopulation) in the treated culture do not show incorporation of F-CDV (arrow). Percentage of cells with activated and nonactivated TGase 2 in the respective cultures is indicated. The inset shows the cellular DNA content distribution histogram of all cells (Grabarek et al., 2002).

nonapoptotic cell population with little or no overlap between these two.

TUNEL. DNA strand breaks in apoptotic cells are strongly labeled with fluoresceinated anti-BrdU Ab that distinguishes them from the nonapoptotic cells (Fig. 18.8.9). Because of the high intensity of their green fluorescence, an exponential scale (logarithmic PMTs) often must be used for data acquisition and display. Simultaneous measurement of DNA content makes it possible to identify the cell cycle position of cells in apoptotic and nonapoptotic populations. It should be noted, however, that late apoptotic cells may have diminished DNA content because of prior shedding of apoptotic bodies (which may contain nuclear fragments), or due to such massive DNA fragmentation that small DNA fragments cannot be retained in the cell even after fixation with formaldehyde. Such late apoptotic cells may have sub-G₁ DNA content as shown in Figure 18.8.8.

Tissue transglutaminase (TGase 2). Detergent (Triton X-100) treatment of nonapoptotic cells as well as apoptotic cells without activated TGase 2 results in cell lysis and release of isolated nuclei or nuclear fragments that have minimal protein content. Such isolated nuclei or nuclear fragments have very low red fluorescence after staining with sulforhodamine 101

(Fig. 18.8.10, panel A). Apoptotic cells with activated TGase 2, on the other hand, have cross-linked proteins and resist lysis under these conditions. Intensity of their red fluorescence is several times higher than that of the isolated nuclei (subpopulations represented by the scatter plots above the dashed lines in panels B and C). Note the high heterogeneity among individual TGase 2-positive cells in intensity of their red fluorescence.

Because cellular DNA content (PI fluorescence) is measured concurrently with protein content, induction of protein cross-linking can be correlated with the cell cycle position. It is evident that the effects are cell cycle phase specific. Both hyperthermia and onconase lead to preferential protein cross-linking in G₂/M-phase cells.

Activation of TGase 2 in HL-60 cells, detected by cell labeling with F-CDV combined with cellular DNA content analysis, is shown in Fig. 18.8.11. As in the case of protein content (Fig. 18.8.10), populations of cells with activated TGase 2 either undergoing spontaneous apoptosis in control culture (Fig. 18.8.11A) or subjected to hyperthermia (Fig. 18.8.11B) are heterogeneous in terms of their TGase 2-related fluorescence. In the hyperthermia-treated culture, a large number of cells have fractional

DNA content, forming a characteristic sub-G₁ population typical of apoptotic cells. This population is heterogeneous in terms of intensity of F-CDV fluorescence, with many F-CDV-negative sub-G₁ cells (arrow). Thus, the degree of activation of TGase 2 is uneven, and many cells with apoptotic features (sub-G₁ DNA content) have undetectable level of TGase activity.

Time Considerations

Basic Protocol 1 takes ~15 min to prepare cells for incubation with mitochondrial probes followed by an additional 15- to 30-min incubation.

Basic Protocol 2 (activated caspase detection by Zenon technology) requires ~2 hr to complete (cell fixation not included).

In Basic Protocol 3, FLICA (FAM-VAD-FMK) is added directly to cultures at least 30 min before cell centrifugation. Optimal labeling is achieved after a 1- to 2-hr incubation with FLICA. Cell rinses and staining with PI require an additional ~15 min before cells are measured by flow cytometry.

Basic Protocol 4 requires ~4 hr to process cells from fixation through primary and secondary Ab incubations followed by staining with PI before they are analyzed by flow cytometry.

Basic Protocol 5 is a rapid procedure that can be completed in 15 min.

Basic Protocol 6 requires ~40 min to carry out cell rinsing and staining with PI following removal from fixative.

In Alternate Protocol 1, cell rinsing and staining can be completed in 25 to 30 min.

Basic Protocol 7 takes ~3 hr to carry out all steps of cell rinsing and incubations with the respective reagents after removal of cells from fixative.

Basic Protocol 8 is a rapid procedure. Cells can be analyzed by flow cytometry ~5 min after their removal from cultures.

In Alternate Protocol 2, the reagent F-CDV is added directly to cultures at different time intervals. It takes ~40 min following cell fixation to carry out cell rinsing and labeling with PI.

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Figure 18.8.1 Changes in light scattering properties of cells undergoing apoptosis. HL-60 cells were untreated (left panel) or treated 3 hr with TNF- α and cycloheximide (CHX) to induce apoptosis (right panel). Cell population **A** in the treated culture (right panel) represents cells that have light scattering properties similar to those of untreated cells. Early apoptotic cells (**B**) have diminished forward scatter and are very heterogenous with respect to side scatter. Late apoptotic cells (**C**) have both forward and side scatter diminished.

Figure 18.8.2 Detection of the collapse of mitochondrial electrochemical potential (ψ_m) by rhodamine 123 (R123). HL-60 cells were untreated (control; left panel) or treated 3 hr with TNF- α and CHX (right panel) to induce apoptosis. The cells were then incubated with R123 and PI according to Basic Protocol 1. The early apoptotic cells have diminished green fluorescence of R123 but exclude PI (cell population B). The late apoptotic (also necrotic) cells are stained strongly by PI (population C).

Figure 18.8.3 Detection of the collapse of mitochondrial electrochemical potential using the aggregate dye JC-1. HL-60 cells were untreated (control, left panel) or treated 3 hr with camptothecin (CPT, right panel) to induce apoptosis. Cells were then stained with JC-1 and their orange and green fluorescence was measured by cytometry, as described in Basic Protocol 2. Decreased intensity of orange fluorescence (subpopulation B) characterizes the cell with collapsed potential.

Figure 18.8.4 Immunocytochemical detection of caspase-3 activation using antibody reactive with the activated (cleaved) caspase-3. Apoptosis of HL-60 cells was induced by topotecan (TPT), an analog of CPT. Zenon technology (Haugland, 2002) was used to detect caspase-3 as described in Basic Protocol 2. Top and bottom insets in each panel show cellular DNA content frequency histograms of cells with activated and nonactivated caspase-3, respectively. Note that S-phase cells preferentially contain activated caspase-3 after induction of apoptosis by TPT.

Figure 18.8.5 Binding of fluorochrome-labeled inhibitor of caspases (FLICA; FAM-VAD-FMK) and PI during apoptosis. Apoptosis of HL-60 cells was induced by TPT. The cells were stained according to Basic Protocol 3. Green (FAM-VAD-FMK) and red (PI) cellular fluorescence was measured by flow cytometry. Four cell subpopulations (A to D) can be identified, differing in their capability to bind FAM-VAD-FMK and PI. They represent sequential stages of apoptosis, starting with binding of FAM-VAD-FMK (B), loss of plasma membrane integrity to exclude PI (C), and loss of reactivity with FAM-VAD-FMK (D).

Figure 18.8.6 Identification of apoptotic cells by flow cytometry based on the immunocytochemical detection of the 85-kDa PARP cleavage fragment. To induce apoptosis, HL-60 cells were treated 60 min with TNF- α in the presence of CHX (Li and Darzynkiewicz, 2000). PARPp85 was detected immunocytochemically and DNA was counterstained with PI.

Figure 18.8.7 Detection of early and late apoptotic cells after staining with annexin V-FITC and PI. To induce apoptosis, HL-60 cells were treated 2 hr with TNF- α and CHX. Untreated (control; left panel) and TNF- α -treated (right panel) cells were then stained with annexin V-FITC and PI.

Figure 18.8.8 Detection of apoptotic cells by flow cytometry based on cellular DNA content analysis. **(A)** Normal cell plot. **(B)** To induce apoptosis, HL-60 cells were treated with the DNA topoisomerase II inhibitor fostriecin (Hotz et al., 1994). Cells were fixed in 70% ethanol, suspended in high-molarity phosphate buffer to extract fragmented DNA, and then stained with PI. A subpopulation of apoptotic cells (Ap) with fractional (sub-diploid) DNA content, i.e., with DNA index (DI) <1.0 (sub-G₁ cells), is apparent. Note also the increase in the proportion of S-phase cells in the nonapoptotic population. **(C)** The fragmented DNA extracted from the apoptotic cells by the buffer was subjected to gel electrophoresis (Gong et al., 1994). Note laddering that reflects preferential DNA cleavage at internucleosomal sections, the characteristic feature of apoptosis (Arends et al., 1990).

Figure 18.8.9 Detection of apoptotic cells by flow cytometry based on the presence of DNA strand breaks. To induce apoptosis, HL-60 cells were treated 120 or 150 min with CPT (Li and Darzynkiewicz, 2000). DNA strand breaks were labeled with BrdUTP using exogenous terminal deoxynucleotidyl transferase. The cell cycle distribution of both apoptotic and nonapoptotic cell subpopulations can be estimated based on the DNA content of individual cells. Note that in analogy to PARP cleavage (Fig. 18.8.6), preferentially S-phase cells undergo apoptosis following CPT treatment.

Figure 18.8.10 Detection of tissue transglutaminase (TGase 2) activation during apoptosis by the acquired resistance of the cytoplasmic proteins to detergent. Bivariate distributions illustrating red fluorescence of sulforhodamine 101 (protein content) versus blue fluorescence of DAPI (DNA content) of HL-60 cells, untreated (**A**) or exposed to hyperthermia (72 hr at 41.5°C) in the absence (**B**) and presence (**C**) of the cytotoxic RNase onconase (1.67 μ M; Grabarek et al., 2002). Following cell lysis by Triton X-100 and staining with DAPI and sulforhodamine 101, the isolated nuclei of nonapoptotic cells from control culture (**A**) show low and uniform intensity of red fluorescence, reflecting low protein content. Subpopulations of apoptotic cells in **B** and **C** have their cytoplasmic protein cross-linked and therefore are resistant to detergent. They stain intensely with sulforhodamine 101. Note differences in DNA content (cell cycle) distribution of the cells with activated (top insets; cells gated above the dashed line) versus nonactivated TGase 2 (bottom insets; cells gated below the dashed line) in **B** and **C**. Percentage of cells with activated and non-activated TGase 2 in the respective cultures is indicated in each panel.

Figure 18.8.11 Detection of TGase 2 activity in HL-60 cells using FITC-conjugated cadaverine (F-CDV) as the enzyme substrate. Cultures of untreated (**A**) and hyperthermia (5 hr at 41.5°C)-treated (**B**) HL-60 cells were incubated 5 hr with 100 μ M F-CDV. Cells were then fixed and their DNA was counterstained with PI in the presence of RNase. Note the presence in the untreated culture (**A**) of few cells that incorporated F-CDV (spontaneous apoptosis), and large numbers of F-CDV-labeled cells in the treated culture (**B**). Note also that some apoptotic cells with fractional DNA content (sub-G₁ subpopulation) in the treated culture do not show incorporation of F-CDV (arrow). Percentage of cells with activated and nonactivated TGase 2 in the respective cultures is indicated. The inset shows the cellular DNA content distribution histogram of all cells (Grabarek et al., 2002).

CHAPTER 19

Whole Organism and Tissue Analysis

INTRODUCTION

The ultimate test of cell biological principles is in the intact organism. Ideally, concepts and conclusions based on *in vitro* biochemistry, molecular biology, or tissue culture studies should be validated *in vivo* in intact tissues and organisms. In addition, some studies can be performed only in living animals or plants. There are innumerable examples of conclusions from *in vitro* studies that have needed major qualification after analysis *in vivo*. One common class involves substantially altered conclusions about the necessity of a specific protein after testing whether it is essential by gene knockout analysis. Others include changing concepts about gene regulatory pathways in development and about the mechanisms and inhibitors of malignancy. An obvious reason is that organisms have complex microenvironmental, regulatory, and system-redundancy properties that are not mimicked adequately *in vitro*.

This chapter will provide detailed protocols for studies in whole organisms and tissues. Although experimental analyses in intact systems could theoretically span all of biology, the focus will be on current techniques particularly relevant to cell biology. The study of cancer metastasis shows the particular value, but also the many pitfalls, of analyzing the cell biology of this complex type of process in animals. In *UNIT 19.1*, members of a leading laboratory in the field (Paweletz, Charboneau, and Liotta) provide an insightful and thought-provoking overview of metastasis assays. The unit starts with a review of key cell biological principles and concludes with discussions of what comprises a “good” animal model and some state-of-the-art approaches. As discussed in detail by this overview, metastasis involves a plethora of cell biological steps. Relevant processes include angiogenesis, cell adhesion, cell motility and invasion, and local proteolysis, each of which will need understanding both *in vitro* and in the context of the living organism.

UNIT 19.2 by Elkin and Vlodaysky then provides practical details, focusing on the most frequently used approach, the analysis of metastasis by tail vein assay. This approach, often termed “experimental metastasis,” involves injecting single-cell suspensions of cells into the tail vein of mice or rats. The cells lodge particularly effectively in lungs, where they form tumor colonies that can be quantitated. This procedure permits analyses of the steps of metastasis that occur during and after the extravasation of tumor cells from blood vessels into target organs. It has been used widely, and should continue to provide valuable insight into the mechanisms of metastasis and a system for testing candidate anti-metastatic drugs. An alternative to using adult rodents is the use of chick embryos for analyzing metastasis. Procedures for handling chick embryos are described in *UNIT 19.5*.

UNITS 19.3 & 19.4 provide complementary approaches to solving the difficult problem of how to obtain reliable quantitative determinations of gene expression with very small quantities of material. For example, tiny pieces of embryonic tissue or even laser-microdissected sites from histological sections are often the optimal sources of material for analyzing gene expression patterns in a specific part of a tissue from an intact organism. However, standard RT-PCR approaches for amplifying low quantities of nucleic acids from these sources can introduce distortions in gene expression data resulting from preferential amplification of some transcripts and sequence errors introduced by *Taq* DNA polymerase. *UNIT 19.3* by Sakai, Larsen, and

Contributed by Kenneth M. Yamada

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Yamada provides a procedure for analyzing ultra-micro amounts of sample, such as from laser-microdissected bits of tissue cut out of frozen sections, using high-fidelity T7-based RNA amplification. It also provides a detailed protocol for the Serial Analysis of Gene Expression (SAGE) procedure based on published protocols, which can provide digital quantification of gene expression. If larger amounts of sample (though still relatively tiny) are available, e.g., 1-2 μg of RNA, risks of amplification can be avoided by using the procedures described in *UNIT 19.4* by Cai, Ash, and Jabs, followed by SAGE analysis. In both cases, SAGE provides an approach that can identify novel genes that are not yet present in DNA microarrays and quantify expression levels for the full spectrum of genes expressed in each tissue.

Assays for angiogenesis *in vivo* are important for verifying predictions from *in vitro* angiogenesis models and for studying this complex process in the intact organism. The simplest *in vivo* angiogenesis model is the chick chorioallantoic membrane (CAM) assay. In *UNIT 19.5*, Ponce and Kleinman describe the advantages, caveats, and detailed protocols for this assay, which can be performed in virtually any laboratory without the need for an animal facility.

Although tumor metastasis is most frequently studied in mouse model systems, an attractive alternative is described in *UNIT 19.6* by Wilson and Chambers. The chick embryo provides an inexpensive and convenient system that has the added advantage of being immune-deficient and thus useful for analyzing tumor cells from any species including human without concerns about immunological compatibility. Besides methods for experimental metastasis assays using chick embryos, a protocol is provided for live-cell *in vivo* video microscopy. This procedure for real-time microscopy analysis of the thin, readily visualized chick embryo chorioallantoic membrane permits direct visualization of specific steps of metastasis as the injected tumor cells interact with host vasculature and invade tissues. Future protocols in this Chapter will describe methods for gene targeting *in vivo*.

Kenneth M. Yamada

Overview of Metastasis Assays

Cancer treatment and diagnosis usually begins at a late stage when most patients already have acquired occult or overt metastasis adding significantly to the diminishing success rates for localized surgery and cytotoxic approaches (Astrow, 1994). Consequently, metastatic dissemination is responsible for the majority of treatment failures and deaths.

During tumor progression, cells acquire genetic and proteomic changes as they transform from normal to hyperplastic, through dysplasia, to carcinoma in situ, and finally to invasive and metastatic. The time course of progression may extend as far back as 10 years prior to diagnosis (Fig. 19.1.1). Discerning the mechanism whereby tumor cells execute metastatic dissemination may provide the foundation necessary for successful treatment of the disease. For example, direct genetic evidence has linked in situ breast cancer to invasive carcinoma of the breast (Zhuang et al., 1995) supporting the generally accepted assumption that carcinoma in situ of the breast is a clonal expansion of hyperproliferating cells (Gallager and Martin, 1969; Spratt et al., 1986). This in turn may

provide a more comprehensive and/or functionally directed target strategy for intervention and prevention of breast cancer.

Cancer metastasis is a highly complex process that involves the deregulation of interacting proteins and genes that are responsible for invasion, angiogenesis, circulation of tumor cells in blood vessels, colonization at secondary organ sites, and finally evasion of host defense systems (Fig. 19.1.2). Invasion can, furthermore, be subdivided into cellular adhesion, local proteolysis, and motility. While all cohorts mentioned above are necessary for successful metastasis, none alone is sufficient to cause metastasis.

Angiogenesis

The critical turning point in tumor progression is the initiation of local invasion and neovascularization leading to metastasis. Neovascularization, the formation of new blood vessels, is not only necessary for continuous tumor maintenance by providing adequate nutritional support but is also required for metastasis by providing a mechanism to allow tumor cells to

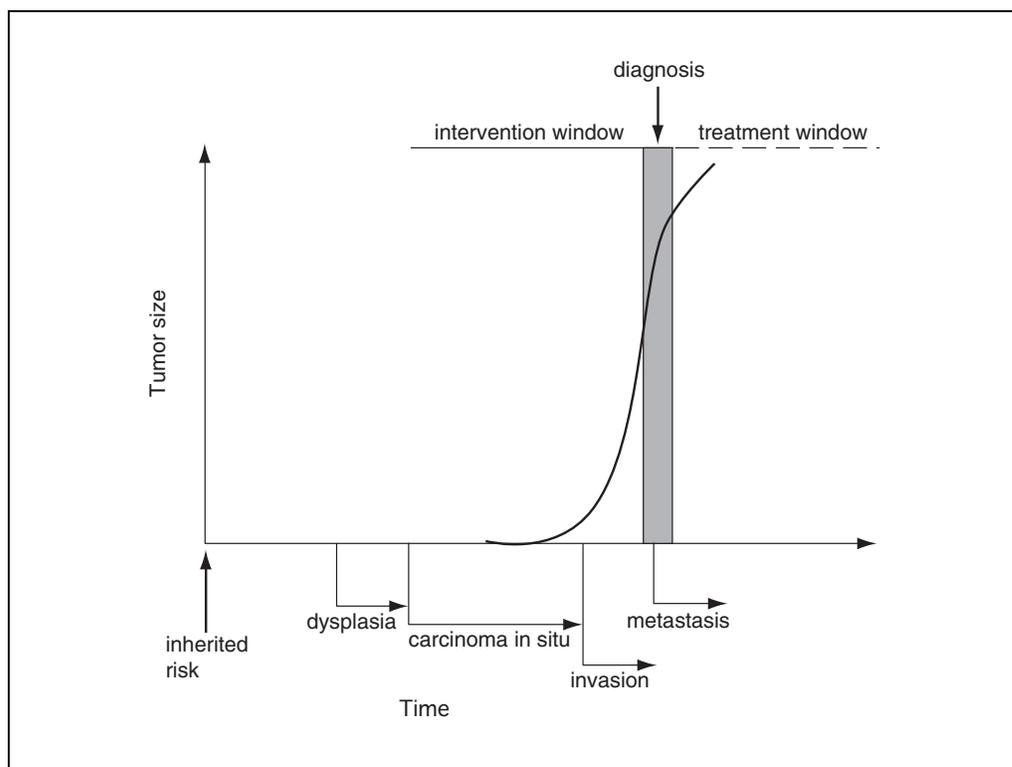


Figure 19.1.1 Theoretical improvement for intervention strategies as a function of time. Diagnosis of cancer usually is performed late into the disease, when most patients already present occult metastasis. However, progression of cancer from dysplasia to metastatic dissemination may extend as far back as 10 years providing a much larger window for intervention strategies before metastasis occurs.

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enter the blood circulation. A vascular entry point to dissemination is achieved by induced proliferation of highly permeable capillaries by tumor cells (Dvorak et al., 1995). These local capillaries mainly arise by angiogenic stimuli released by tumor or host cells. However, angiogenesis will cease when the local stimulus is removed. A variety of molecules have been found to mediate angiogenesis *in vitro* and *in vivo*. Among these are fibroblast growth factors (bFGF), angiogenin, vascular permeability factor (VPF), and transforming growth factor α and β (TGF- α , TGF- β ; Folkman and Klagsbrun, 1987).

Neovascularization is not regulated by a single event but rather consists of tightly regulated molecular events that cause endothelial cells to degrade the basement membranes, proliferate, migrate, and initiate growth of capillaries (Fidler and Ellis, 1994; Folkman, 1995). Angiogenesis occurs in parallel with initiation of invasion. Rapidly growing tumors are capable of shedding up to millions of tumor cells into vascular circulation by angiogenesis and invasion alone (Liotta et al., 1974). Furthermore, experimental studies show that <0.05% of circulating tumor cells are successful in in-

itiation of metastatic colonies making metastatic dissemination a highly inefficient process (Liotta et al., 1974; Nicolson, 1991). These studies are also further validated by the observation that circulating tumor cells are detected in patients who never form a metastasis.

Invasion

Invasion is the active translocation of neoplastic cells across tissue boundaries and through host cellular and extracellular matrix barriers. Cellular adhesion, local proteolysis, and motility characterize successful invasion. While all three cohorts are necessary for successful metastasis, none alone is sufficient enough to cause metastasis. Invasion is not due to growth pressure but involves additional genetic and proteomic deregulation over and above those molecular events that cause uncontrolled proliferation. Instead, cellular invasion is dependent on the coordinated activity of a series of interacting proteins extending from the inside of the cell to the cell surface and the imminent microenvironment.

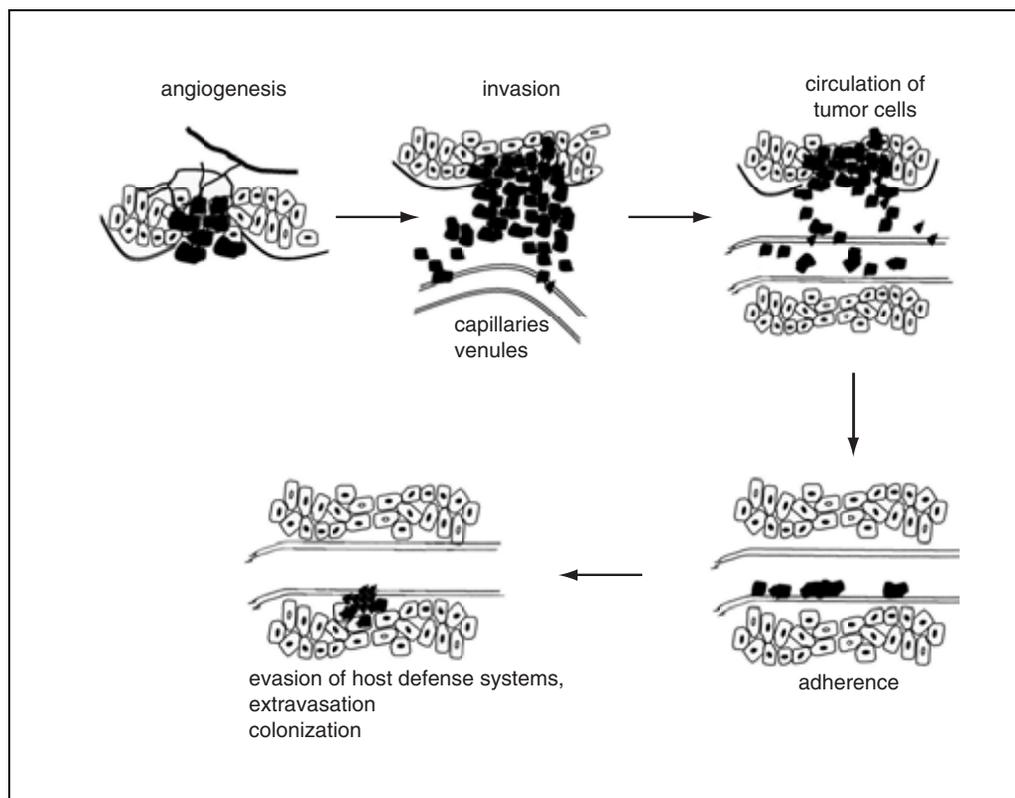


Figure 19.1.2 Pathogenesis of cancer. Cancer metastasis is a highly complex process that involves the deregulation of interacting proteins and genes that are responsible for angiogenesis, invasion, circulation of tumor cells in blood vessels, colonization at secondary organ sites, and finally evasion of host defense systems.

Cellular Adhesion

Maintaining cellular integrity with the extracellular matrix and/or basement membrane involves the rapid ability to respond to extracellular signals at receptors, which in turn affects corresponding intracellular signal transduction pathways that regulate proliferation, differentiation, and migration. These receptors can be membrane surface receptors that become activated by ligands (such as growth factors), or cytosolic/nuclear receptors that transmit hormone signals to regulate gene expression. Among the many types of cell surface receptors several classes have been shown to be predominant during tumorigenesis. These include integrins, cell adhesion molecules (CAMs), and cadherins.

The integrin family of cell surface extracellular matrix proteins consists of heterodimeric units, designated α (140 kDa) and β (95 kDa). An important aspect of this family is that integrins can either exist in an "on" or "off" state, thereby selectively changing its affinity for corresponding ligands, and changing molecular events in doing so (Hynes, 1992; Juliano and Haskill, 1993). Activation of integrins has been shown to be involved in cell migration, cell proliferation, and dissemination. However, loss of stimulus of integrins has been associated with apoptosis. This suggests a diverse switch-on/switch-off function for integrins. Increased expression of integrin receptors on cell surfaces has been associated with an invasive phenotype of melanoma and squamous carcinoma of the head and neck, whereas loss of expression status has been shown in cancers of breast, prostate, and colon (Chammas and Brentani, 1991; Natali et al., 1991).

The cadherins, on the other hand, comprise a family of transmembrane glycoproteins that mediate Ca^{2+} -dependent cell adhesion (Takeichi, 1991). Special intracellular proteins, the catenins, form zipper-like structures to form extracellular cell-cell bonds with the cell cytoplasm. These interactions are regulated by tyrosine phosphorylation as well as additional cell-to-cell communications. Down regulation of epithelial cadherin transcription, E-cadherin, has been shown to cause a more aggressive cancer cell phenotype (Frixen et al., 1991; Vleminckx et al., 1991). For example, transforming Madin-Darby canine kidney cells (MDCK) by *H-ras* not only diminished E-cadherin expression but also increased the invasive behavior of these cells. This effect could be reversed by transfecting E-cadherins back into the transformed cells (Behrens et al., 1989;

Mareel et al., 1991; Vleminckx et al., 1991). Furthermore, overexpression of E-cadherin in highly invasive tumor types (i.e., bladder, breast, lung, and pancreas) showed loss of invasiveness in their respective transformed cell line.

Cellular adhesion receptors (CAMs) have also been studied in the context of cancer invasion. These include but are not limited to: intercellular adhesion molecules (ICAMs), L-, E-, and P-selectins, vascular cell adhesion molecules (VCAM), neural cell adhesion molecules (NCAM), and neuroglial cell adhesion molecules (NG-CAM). These adhesion receptors provide the necessary framework for the interaction of tumor cells with the microenvironment forming a vital part of cancer invasion and metastasis. Unlike other receptors that bind proteins, selectins bind carbohydrate ligands on endothelial cells. While adhesion alone may not be sufficient for metastasis, dysregulation of any of the surface receptors above may alter signaling pathways responsible for positive and negative feedback controls and may therefore result in a more aggressive invasive phenotype.

Local Proteolysis

A positive correlation between tumor aggressiveness and protease levels has been documented (Liotta et al., 1980). Not only are proteases upregulated in tumor cells, and protease inhibitors down regulated, but zymogens are also activated into active protease enzymes. Local proteolysis by itself is not a sole metastatic phenomenon, and it is routinely observed in trophoblast implantation, embryo morphogenesis, wound healing, tissue remodeling, and angiogenesis. Local proteolysis is a tightly regulated process in normal tissue by appropriate protease inhibitors. Furthermore it has been indicated that deregulation between the equilibrium of protease to protease inhibitor regulates vascular morphogenesis and invasion (Ura et al., 1989). Metastasis cannot simply be a product of unbridled production of degradation enzymes as cell dissemination and invasion concomitantly are dependent on adhesion and motility. Deregulation of this tightly regulated process causes secretion of proteases by tumor cells that creates local access points across the basement membrane and/or extracellular matrix. This process takes place by coordinations of proteases including serine, aspartyl, cysteinyl, and metalloproteases (Nakajima et al., 1987; Ostrowski et al., 1988; Reich et al., 1988). Sufficient evidence exists that proteases

inside the cell may also be involved during invasion (Koblinski et al., 2000). Invasion is mostly hindered by collagens, which form with laminin, fibronectin, triple helical proteins, and vitronectin, important structural components of the basement membranes and extracellular matrices. Collagen I and III are located in stromal collagens, and collagen IV and V are predominant in the basement membrane. Among the many types of proteases involved in metastasis such as tissue-type plasminogen activator (tPA), plasmin, cathepsin -D, -B, -L, -G, the urokinase plasminogen activator (uPA), metalloproteinases, and the heparanases deserve special mentioning.

Urokinase plasminogen activator, a serine protease, has been shown to correlate with a metastatic phenotype of cells. Antibodies against uPA block human HEP-3 cell invasion and murine B16-F10 melanoma cell metastasis after tail vein injection (Ossowski and Reich, 1983; Esheicher et al., 1989). Alternatively, overexpression of uPA in *H-ras* transformed cell lines enhances lung metastases (Axelrod et al., 1989).

Enzymatic cleavage of collagens, necessary for invasion of tumor cells into the vascular vessel, is mostly carried out by metalloproteinases (MMP). Inhibition of metalloproteinases has been demonstrated to inhibit cell invasion (DeClerck et al., 1991). These inhibitors have also been shown to block metastasis. MMPs can be divided into three general classes: (1) interstitial collagenases; (2) stromelysins; and (3) gelatinases. Interstitial collagenase, degrade types I, II, III, and VII collagens. Stromelysins degrade types I, III, IV, V, IX collagens, laminin, fibronectin, and gelatin. The third group of the MMP family, the gelatinases (MMP-2 and MMP-9), are capable of degrading collagen types I, II, III, IV, V, VII, IX, and X, as well as fibronectin (Emonard and Grimaud, 1990). An important difference between the gelatinases and the other MMPs is that they are capable of interacting with endogenous tissue inhibitor of metalloproteinases (TIMPs) of which there are two major subgroups, TIMP-1 and TIMP-2. Inhibition of MMP-2 by TIMP-1 has shown to reduce cellular invasion. Involvement of MMP-2 and MMP-9 in invasive phenotypes is abundant. Induction of *H-ras* oncogene enhances expression of MMP-2 and MMP-9. Invasive colonic, gastric, ovarian, and thyroid adenocarcinomas showed positive immunoreactivity for MMP-2, whereas normal colorectal, gastric mucosa, and benign ovarian cysts showed reduced or negative staining

(Monteagudo et al., 1990; Levy et al., 1991). A fragile balance between TIMPs and MMPs may act as a positive and negative feedback control regulating vascular morphogenesis and invasion (Mignatti et al., 1989). Additional functions of MMPs besides degradation of tissue boundaries are also ascribed to its involvement in angiogenesis (Chambers and Matrisian, 1997).

Additional major components of tissue barriers to be degraded by proteases besides collagen, fibronectin, and laminin are heparan sulfate proteoglycans (HSPG). Involvement of heparanases, whose function involves the degradation of ubiquitous expressed heparan sulfate glycosaminoglycan side chains, with respect to metastasis has previously been demonstrated (Nakajima et al., 1988; Vlodaysky et al., 1995). Not only did heparin and similar polysaccharides inhibit metastasis (Parish et al., 1987), increased expression of heparanase genes and proteins were also found in tumor cells compared to normal cells (Vlodaysky et al., 1999). Discerning the fundamental mechanism whereby heparanases cause metastatic cell dissemination has become more achievable with the completion of the cloning of the heparanase gene (Hullet et al., 1999; Vlodaysky et al., 1999). In these studies, transfection of nonmetastatic murine T-lymphoma Eb cell lines with full-length human heparanase cDNA enhanced its metastatic phenotype, as shown by increased invasiveness of cells and a higher mortality rate of tumor-injected mice (Vlodaysky et al., 1999).

In the end, the coordination of families of proteases with adhesion molecules and locomotion is required for successful invasion. None alone can cause invasion, and it is likely that many other proteases will be found in the near future.

Motility

Translocation of individual cells across tissue membranes inevitably is a major part of invasion. However, as with local proteolysis and angiogenesis, cell migration is also part of normal cell development such as gastrulation and organogenesis. Diminishing cell adhesion single cell locomotion, and cohort cell translocations, have been reported to be involved in malignancy and metastasis (Liotta and Stetler-Stevenson, 1993; Nabeshima et al., 1999).

Early events in cell motility consist of extensions by pseudopods prior to translocation of the whole cell body (Stossel, 1993). These nuclear-free vessels would have all the neces-

sary requirements to sense and protrude within the three-dimensional structure of the tumor mass leading cell migration away from the primary organ (Condeelis, 1993; You et al., 1996). However, unregulated protease interactions alone cannot ad infinitum translocate the pseudopod, as the vessel would dig itself into a hole and thereby immobilize itself. Adhesion, interaction, and detachment with the ECM are essential parts of locomotion (Liotta et al., 1991; Damsky and Werb, 1992). Proteinases at the tip are vital for the disruption of the ECM, but as the matrix becomes more and more disrupted, the pseudopod must adhere to the matrix and pull the cell forward. At the rear of the pod, detachment from adjacent cells is necessary.

Movement across tissue boundaries is initiated by a chemotactic (directional) mechanism. A variety of chemoattractive compounds, among them certain cytokines (e.g., hepatocyte growth factor), collagen peptides, formyl peptides, or autocrine growth factors (e.g., autotaxin), have been identified to be important in metastasis locomotion (Anzano et al., 1983; Guirguis et al., 1987; Di Renzo et al., 1995; Nam et al., 2000). It needs to be realized, even if there exists a direct tumor host interaction, chemoattractant release to the migrating cell may only be sporadic at best and could not sustain metastatic dissemination. These factors are secreted by surrounding stroma, the tumor cells themselves, or by peptides affiliated with the extracellular matrix. For example, it has been demonstrated that the human melanoma cell line A2058 as well as human breast cell lines stimulate their own motility factors when treated with serum-free medium. Motility, like adhesion and local proteolysis, is necessary for metastasis but cannot alone cause metastasis.

In Vivo Models and Metastasis

Successful metastasis only occurs if positive and negative regulatory mechanisms that govern angiogenesis, proteolysis, motility, evading host defense systems, and cellular adhesion events are concomitantly deregulated. Metastasis is a multifactorial event that includes deregulations above and beyond those found during disturbances of cell proliferation and homeostasis. Ultimately, there exists a need to investigate proteins and genes involved in metastasis and invasion *in vivo*. While there exists a variety of assays to study any one of those specific characteristics *in vitro*, such as the modified Boyden chamber assay for the study of motility, transgenic, knock-out, and

xenograft animal models are the only accurate tools available to investigate molecular mechanisms responsible for metastasis in an organism (Fidler and Hart, 1982; Guan et al., 2000; Zuber et al., 2000).

Organ tropism varies widely depending on the morphology and anatomic location of primary tumors. Clinical studies indicate that most metastases form micro-colonies in the first capillary bed found by circulating, invasive tumor cells. For example, lung cancer metastasizes predominately to the brain, or colorectal cancers to the liver. However, not all metastatic sites can be predicted by anatomical considerations alone. For example, clear-cell carcinoma of the kidney metastasizes to the bone and thyroid. Animal models that either show preferential organ metastases by purposeful selection or have not undergone purposeful selection have been designed to accompany and simulate these observations (see Table 19.1.1; Nicolson, 1988).

A “good” animal model is based on the following three basic axioms: (1) metastasis is not a random phenomenon; (2) neoplasms are heterogeneous in their cell make up and therefore observe different metastatic outcomes; and (3) the outcome of the metastasis is dependent on both the tumor cell and the host cell interactions (Fidler, 1991).

The human genome project in conjunction with other projects, such as the CGAP initiative of the NCI, is expected to provide a wealth of information of possible tumor (or metastasis) suppressor genes and oncogenes that are recessives and mutated dominants, respectively (Strausberg et al., 1997). However, it is necessary to establish whether these genes are actually involved in metastasis or tumorigenesis *in vivo*. A logical way is to transfect mutant forms of these candidate genes into a noninvasive cell line of the tumor to be studied, inject these modified cell lines intravenously, and determine the number of lung colonizations. Alternatively, the effects of possible oncogenes toward metastasis can be investigated by knock-out mice lacking certain candidate genes (Granovsky et al., 2000). The oncogene-deficient mice can then be compared to nondeficient mice by counting lung colonies of micro metastases and comparing tumor weight. Substantial decrease in colonies and tumor weight is expected if the gene that is “knocked out” is involved in metastasis.

An excellent example of how transfection of a gene provided valuable insights into metastasis was recently demonstrated by Vlo-

davsky et al. (1999). Initially, there existed sufficient evidence that heparanases may be involved in metastasis, as an increased level of heparanase was found in patient's urine and serum samples (Vlodavsky et al., 1999). However, the gene itself was (1) not cloned; and (2) not expressed in an animal model making accurate presumptions about its function difficult. After successful cloning of the gene, full-length human heparanase cDNA was transfected into nonmetastatic murine T-lymphoma Eb cell lines. Injection of transferred cell lines into DBA/2 mice not only increased the metastatic phenotype as shown by liver colonization but also decreased survival rates of injected mice. However, a word of caution is advised because transfection of genes and subsequent ectopic injections may cause "false-negatives," as it is possible to inject cells in such a way that they do not form metastases even if the cell possesses a real metastatic phenotype (Kerbel et al., 1991).

At present, there does not exist a completely accurate animal model that simulates a specific human histologic tumor type and its complete metastatic behavior. While the positive and negative attributes of animal models can be debated, their positive productive role in dissecting molecular mechanisms for metastasis and treatment cannot be disregarded.

Conclusion

Even though studies on metastasis have traditionally lagged behind other fields in cancer research, substantial progress has been made over the last 10 years. This is partly due to the separation of cancer metastasis into distinct fields such as angiogenesis, invasion, metastatic suppressor genes, and others. For each distinct field, novel technologies and methodologies have provided important insights. However, exciting and emerging technologies such as in vivo microscopy of cell motility (Farina et al., 1998) or proteomics combined with laser capture microdissection (Lawson et

Table 19.1.1 Organ Preferences of Metastasis in Some Human and Animal Selected Models^{a,b}

Tumor system subline ^{c,d}	Lung	Liver	Brain	Ovary	Spleen	Lymph node
<i>Murine B16 melanoma (i.v. or i.c.)</i>						
B16-F1	+	±	±	±	–	±
B16-F10	++++	±	–	+	±	±
B16-B15b	+++	–	+++	+	±	±
B16-O13	++	–	–	+++	–	±
<i>Murine RAW117 large cell lymphoma (i.v. or s.c.)</i>						
Raw117-P	±	±	–	–	±	–
Raw117-H10	±	++++	–	–	++	–
<i>Murine MT1 mammary carcinoma (i.v. or s.c.)</i>						
TC3	+++	+	–	±	–	–
<i>Chicken MD lymphoma (i.v.)</i>						
AL-2	–	++++	–	±	–	–
AL-3	–	+	–	++++	–	–
<i>Human A375 melanoma (i.v. in nude mice)</i>						
A375-P	±	±	–	–	–	±
A375-SM	++	±	–	±	–	+
<i>Human PC-3 prostatic carcinoma (i.v. in athymic mice)</i>						
PC-3-125-IN	++++	–	–	–	–	–
PC-3-1-LN	++++	+	–	+	±	+++
<i>Human MeWo melanoma (i.v. in nude mice)</i>						
MeWo	+	–	–	–	–	–
MeWo-70-W	++	–	++	+	–	–

^aModified from Nicolson (1988).

^bMetastasis: –, none; ±, sometimes; +, few; ++, moderate; +++, many; +++++, large numbers and heavy tumor burden.

^cAbbreviations: i.v., intravenous; i.c., intracecum; s.c., subcutaneous.

^dCommon animal models used in the study of metastasis.

al., 1991; Emmert-Buck et al., 2000; see *UNIT 2.5*) will provide valuable insights into the field. For example, it is feasible to microdissect patient-matched normal, tumor, and metastatic tissue cells and analyze their corresponding DNA, mRNA, and protein levels, giving a true cellular snapshot. Information acquired through these studies will almost certainly target signal transduction pathways that may provide alternative intervention and prevention strategies much earlier than is possible today.

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Tail Vein Assay of Cancer Metastasis

UNIT 19.2

**BASIC
PROTOCOL**

The most damaging change during cancer progression is the switch from a locally growing tumor to a metastatic killer. This switch involves numerous alterations that allow tumor cells to complete the complex series of events needed for metastasis (UNIT 19.1). In considering steps required for successful metastasis, extravasation from blood vessels in target organs is regarded as a critical process. Circulating tumor cells arrested in the capillary beds of different organs must invade the endothelial cell lining of blood vessels and degrade its underlying basement membrane in order to escape into the extravascular tissue where they establish metastasis. This unit describes the most common assay applied to evaluate the metastatic potential of blood-borne tumor cells. The protocol is often called “experimental metastasis”, distinct from “spontaneous metastasis”, where the tumor cells are first allowed to form a primary tumor in the site of injection and then escape into lymphatic or blood circulation. The Basic Protocol is to apply intravenous injection as a method for introducing tumor cells into circulation. For this purpose, cells maintained in tissue culture are dissociated into a single cell suspension, counted, and injected into the tail vein of mice or rats. After 12 to 20 days, the animals are euthanized and their lungs are removed, rinsed, and fixed. The number of pulmonary tumor colonies is then counted with the aid of a dissecting microscope. When an organ containing extensive secondary foci is seen, it is also removed and fixed for subsequent enumeration of tumor foci.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 7% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO₂ to maintain a pH 7.4.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Materials

Cells of interest growing in tissue culture (e.g., B16 mouse melanoma, ATCC#CRL-1619; 3LL Lewis lung carcinoma; A375 human melanoma, ATCC#CRL-6322; 13762 MAT rat mammary adenocarcinoma)

Supplemented high-glucose DMEM-10 (UNIT 10.4)

Trypsin/EDTA solution (UNIT 10.4)

CMF-DPBS: divalent cation-free Dulbecco's PBS (CMF-DPBS; Life Technologies; APPENDIX 2A)

Mice for injection (or rats)

70% ethanol

3.8% (v/v) formaldehyde

Bouin's solution (see recipe; optional)

6-cm tissue culture dishes

15-ml polypropylene tubes

Sorvall RT6000D centrifuge and H-1000B rotor or equivalent

Beaker containing warm water (~50° to 60°C)

Lamp with 150-W light bulb (optional)

Restraining device (restrainer)

1-ml syringe, tuberculin type

27-G, 3/4-in. needles

Dissecting microscope

**Whole Organism
and Tissue
Analyses**

19.2.1

Contributed by Michael Elkin and Israel Vlodavsky

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Supplement 12

Additional reagents and equipment for counting cells with hemacytometer (*UNIT 1.1*)

Prepare cells

1. Culture a sufficient number of cells in supplemented high-glucose DMEM-10 in 6-cm tissue culture dishes in a humidified 37°C, 7% CO₂ incubator.

Other size dishes (i.e., 35-mm, 10-cm) can be used.

2. Aspirate medium from subconfluent cultures ($0.5\text{--}1 \times 10^5$ cells/cm²), add 2 ml trypsin/EDTA solution per 6-cm tissue culture dish, and incubate 2 to 3 min at 37°C, or until the cells have rounded up, but are not detached.
3. Aspirate trypsin/EDTA solution, resuspend cells in 5 ml warm supplemented high-glucose DMEM-10, transfer into a 15-ml polypropylene tube, centrifuge 7 min at $\sim 350 \times g$ (1000 rpm Sorvall RT6000D centrifuge, H-1000B rotor, or equivalent), room temperature, and resuspend in 5 ml warm CMF-DPBS.

Trypsin/EDTA solution is commonly used to detach adherent cells. Its action is terminated by resuspending the cells in supplemented high-glucose DMEM-10. Prolonged (and unnecessary) trypsinization of tumor cells can alter their survival and metastatic behavior in vivo. Trypsinization >3 min may affect lung colony formation and should be avoided.

Serum-free DMEM can be used instead of CMF-DPBS.

4. Count cells on a hemacytometer (*UNIT 1.1*), and prepare working cell suspension at 2.5×10^5 to 1×10^6 cells/ml, depending on the metastatic potential of the cells (e.g., 2.5×10^5 B16F10 or A375SM cells/ml; 1×10^6 A375 cells/ml).

It is important to guard against clumping or aggregation of cells. The number of lung colonies is affected not only by the number of tumor cells injected, but also by the aggregation of cells and the tumor embolus size. To avoid clumping of cells, the cell preparations must be free of serum, and cells should be injected in a divalent cation-free balanced salt solution, which also serves to decrease clumping.

Obviously, the trypsinization step is not applied when nonadherent cells (e.g., mouse Eb T-lymphoma) are tested. Cells growing in suspension are washed in CMF-DPBS, or serum-free medium, counted, and suspended at the desired cell density.

Intravenously inject tumor cells

5. Dilate the lateral tail veins by immersing the tail for 1 to 2 min in warm (50° to 60°C) water.

The tail vein is dilated to facilitate the insertion of a 26- or 27-G needle. Alternatively, animals housed in a well-ventilated cage are placed for ~ 5 min under a lamp with a 150-W light bulb. The distance of the lamp from the cage can vary, but 4 to 8 in. is sufficient.

6. Place the mouse in a restrainer by grasping the mouse by the tail out of the cage and placing with tail protruding through the opening in the wall of a restraining device.
7. Fill 1-ml syringe (tuberculin type) with the cell suspension and remove air bubbles.
8. Hold the distal third part of the tail between thumb and middle finger, wipe clean with 70% ethanol, and rotate to position the lateral vein. Stabilize the vein with the index finger. Apply slight pressure to straighten the tail and further dilate the lateral vein.
9. Hold the 1-ml syringe with a 27-G, $\frac{3}{4}$ -in. needle in the other hand. Direct the bevel of the needle upward. Force the needle gently through the skin (at a slight angle) and then immediately thread it almost parallel into the vein. When the vein is cannulated, advance the needle into the lumen an additional 5 mm and slowly inject 0.2 to 0.3 ml of the cell suspension.

No significant pressure should be applied at this stage. If the needle is not in the vein, bleb formation and tissue resistance to the inoculum can easily be felt, indicating failure to cannulate the vein. Perform the first injection at the distal third of the tail. In case of failure, additional attempts may be made proximally in the same or opposite lateral vein. Intravenous injection of anaesthetized mice, or of mice that struggle violently should be avoided.

Tail vein injection is delicate and requires pretraining, usually, 20 to 30 mice may suffice. Locating the lateral vein is relatively difficult with C57BL mice compared to Balb/c mice.

Enumerate pulmonary tumor colonies

- 10a. After 2 to 3 weeks post-injection, euthanize mice (or rats) by CO₂ asphyxiation or cervical dislocation (or other approved method), remove the lungs, rinse in tap water, fix in 3.8% formaldehyde, and count colonies with the aid of a dissecting microscope.

An illuminated colony-counting magnifying lens can be used instead of a dissecting microscope.

When an organ containing extensive secondary foci is observed, it is also removed, rinsed, and fixed for subsequent enumeration of tumor foci. Most pulmonary tumor colonies in mice are found near or on the surface of the lungs. Therefore, pigmented colonies that differ in color from the lung parenchyma (such as melanoma) can be counted when adequately magnified.

Tumor colonies that are not pigmented and do not differ in color from the pulmonary parenchyma are more difficult to enumerate. To induce contrast between tumor colonies and the lung parenchyma perform step 10b.

- 10b. *Optional:* Remove lungs (or other organs) and rinse in tap water. A few minutes later, place the lungs in a beaker containing Bouin's solution. After 24 hr, rinse the lungs in water to remove excess Bouin's solution and count the tumor colonies with the aid of a dissecting microscope.

The lung parenchyma will immediately turn yellow. After 24 hr, the white tumor colonies can readily be distinguished from the yellow lung parenchyma.

11. Specify the actual number of pulmonary colonies counted for each mouse, or show the range of counts by indicating the lowest and highest numbers of colonies observed within a given experimental group. Present the average number of nodules per mouse \pm standard error or standard deviation, using the two-tailed student's *t*-test. Murine lungs with >300 colonies are reported as such.

Instead of actual counting of pulmonary colonies, one can remove and weigh the lungs of each mouse and express the results as average lung weight \pm standard deviation. Normal lung weight is ~200 mg.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Bouin's solution

0.9% (v/v) picric acid

9% (v/v) formaldehyde

5% (v/v) acetic acid

Store up to 1 year at room temperature

COMMENTARY

Background Information

Metastasis is the foremost cause of death from cancer. Fortunately, metastasis is an extremely inefficient process, with few of the many cells shed from a primary tumor successfully forming secondary tumors (Hart and Fidler, 1980; Liotta, 1986; Fidler and Ellis, 1994; Ruoslahti, 1996). Sequential steps in the metastatic process are as follows: escape of cells from the primary tumor, entry of cells into lymphatic or blood circulation (intravasation), survival and transport in circulation, escape of cells from circulation (extravasation), and growth of cells to form secondary tumors in a new organ environment (Hart and Fidler, 1980; Liotta, 1986; Fidler and Ellis, 1994; Ruoslahti, 1996). The majority of cells that successfully escape from a primary tumor will not complete all of the steps necessary to give rise to metastatic tumors. Steps that have been considered to be major contributors to this inefficiency, and thus, rate limiting for metastasis, include cell survival in, and escape from, circulation (Hart and Fidler, 1980; Liotta, 1986; Fidler and Ellis, 1994; Ruoslahti, 1996). Once in circulation, the tumor cells often attach at or near junctions between adjacent endothelial cells, followed by retraction of the endothelial cells borders, and migration toward the exposed underlying basement membrane (BM). The invading tumor cells then degrade the subendothelial BM in order to migrate out of the vascular compartment. These steps are collectively addressed by the tail vein assay, commonly applied in vivo as a route for introducing cells into blood circulation (Fidler, 1973a, b).

Experimental metastasis, measured by the tail vein assay, is inhibited by molecules (i.e., RGD-containing peptides, laminin-derived peptides, sulfated polysaccharides) and antibodies which inhibit cell-cell and cell-matrix interactions (Humphries et al., 1986; Iwamoto et al., 1987). Because basement membranes underlying epithelial and endothelial cells appear to be clear barriers to metastasis, enzymes degrading BM proteins and glycosaminoglycans are regarded as promising targets for cancer therapy (Stetler-Stevenson et al., 1993; Finkel, 1999; Vlodaysky et al., 1999). Invasion through a reconstituted basement membrane (Matrigel; *UNIT 10.2*) is most often used to model extravasation (*UNIT 12.2*). Cell invasiveness in vitro correlates with the metastatic potential (i.e., lung colonization) of intravenously injected cells. Moreover, inhibitors of ECM de-

grading enzymes inhibit both BM invasion in vitro and experimental metastasis in vivo, further emphasizing the significance of the BM as a prominent physical barrier to metastasis (Parish et al., 1987; Stetler-Stevenson et al., 1993; Vlodaysky et al., 1994). Some of these molecules are being examined in cancer patients (Kohn and Liotta, 1995). Taking into account the complexity of the metastatic process, it is now understood that the mechanistic conclusions on the significance of a certain step and/or action of a given molecule cannot be drawn based on the endpoint outcome of the tail vein assay (i.e., number of metastases counted at the end of the experiment; Chambers and Matrisian, 1997). A procedure, intravital videomicroscopy (IVVM), for direct in vivo observation of early steps in metastasis, has recently provided evidence that some of the assumptions about mechanisms of metastasis need to be revised (Chambers and Matrisian, 1997; Al-Mehdi et al., 2000; Cameron et al., 2000). Unlike early studies, direct IVVM observation of intravenously injected cancer cells revealed that a large proportion of the injected cells not only survive injection and arrest in a target organ, but also succeed in extravasation (Chambers and Matrisian, 1997; Al-Mehdi et al., 2000; Cameron et al., 2000). It was suggested that, unlike previous assumptions, extravasation may be a relatively easy process and that metastatic inefficiency depends, to a large extent, on the inhibition of growth in a subset of extravasated cells (Cameron et al., 2000). Availability of a favorable microenvironment in the target organ is critical for seeding and proliferation of the disseminated cells ("seed and soil" theory; Killion et al., 1998).

Previous research has concentrated on the contribution of individual genes to metastasis. Initially, there was hope that a single metastasis-specific gene could be identified to be responsible for conversion to a metastatic phenotype. Although some cells could be converted to a metastatic phenotype by DNA transfection of certain genes, including those encoding ECM-degrading enzymes (Stetler-Stevenson et al., 1993; Vlodaysky et al., 1999), there is no single master "metastasis" gene that regulates cellular abilities necessary for cancer metastasis (Chambers and Matrisian, 1997). Gene-expression profiling, using high-density DNA microassays combined with the tail vein assay, led to the identification of several genes involved in ECM assembly and in regulation of the

actin-based cytoskeleton that are selectively upregulated in metastatic mouse and human melanoma cells (Clark et al., 2000). A particular significance was ascribed to RhoC, which may regulate metastasis by controlling cytoskeletal events essential for cell motility in response to extracellular factors (Clark et al., 2000).

Critical Parameters and Troubleshooting

General considerations for metastasis. The pathogenesis of metastasis begins with the invasion of tissues, blood vessels, and/or lymphatics by cells originating from a primary cancer. Following their release into circulation, most tumor emboli are initially arrested in the lung tissue, simply because the lung capillary bed is the first to be encountered (Fidler, 1973a; Hart and Fidler, 1980), although some cells recirculate and are trapped in other organs. It is because of this that the majority of intravenously injected cells extravasate and form metastatic colonies in the lungs, as in fact evaluated by the tail vein assay described in this unit. It should be noted that this assay, also known as “experimental metastasis”, is restricted to evaluate the metastatic potential of circulating blood-borne cells. In contrast, the more complete cascade can be studied using the “spontaneous metastasis” assay in which the tumor cells are injected subcutaneously to first form a primary tumor. The tumor is either allowed to grow (Vlodavsky et al., 1999), or is resected when it reaches a certain size (Fitzer-Attas et al., 1997). The latter protocol better mimics the situation in patients who undergo resection of their primary tumor, a procedure which is often associated with stimulated vascularization and growth of already spread metastatic nodules (O’Reilly et al., 1994). Briefly, for measurements of spontaneous metastasis, mice are most often inoculated in the footpad with 2×10^5 cells per mouse. Local tumors are measured with calipers. When individual tumors reach a diameter of 8 mm, tumor-bearing legs are amputated (Fitzer-Attas et al., 1997). Two to 3 weeks post-amputation, the mice are euthanized and the number of pulmonary colonies or lung weights are determined as described for intravenously injected tumor cells (Fitzer-Attas et al., 1997).

Single cell suspension. A major problem likely to be encountered with the tail vein assay is the broad variation in the number of pulmonary colonies obtained among mice of the same group. In some experiments, these may vary

between 10 and 100 nodules per mouse. This variation may primarily be due to inappropriate standardization of the cell suspension. Every effort should be made to obtain a single-cell suspension since cell clumping and aggregation often result in an increased number of lung colonies (Fidler, 1973b). Clearly, cell aggregation and clump (embolus) size influence the outcome of experimental metastasis assays. For example, mice injected with 10,000 clumps of 4 to 5 cells will develop more pulmonary foci than mice injected with 50,000 single tumor cells (Fidler, 1973b). The significance of cell aggregation is also demonstrated by the greatly increased number of tumor foci obtained when the tumor cells are injected simultaneously with lethally irradiated tumor cells, or with live syngeneic embryonic cells (Fidler, 1973b; Hart and Fidler, 1980). To avoid clumping, trypsinization and centrifugation of the cultured cells should be gentle and the dissociated cells should be suspended in serum-free, $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free balanced salt solution. Routine viability tests (e.g., trypan blue exclusion) and even plating efficiency in vitro do not necessarily predict or correlate with the in vivo metastatic potential of over-trypsinized cells (Fidler, 1973b). Also, the 15-ml tube, as well as the syringe containing the cell suspension should be gently rotated from time to time and not be left standing for long periods of time (i.e., >30 min) to prevent the cells from settling and aggregating at the bottom of the tube or in the syringe. The cells should be pipetted gently prior to being taken up into the syringe for injection. Failure to obtain a single-cell suspension will result in a diverse and broad range of colony number among mice of the same experimental group, and in differences in colony size in the same mouse.

Cultures. An important parameter is the health of the cells. Cultures should be actively growing, but should be passaged at least 48 hr prior to the day of the tail vein assay. Also, the use of early passaged cultures (<20) is recommended. Relatively poor metastatic potential is often observed with late-passage cells. If the cells tend to lose their metastatic ability, it is advisable to go back and select for highly metastatic cells by means of a repeated isolation of pulmonary metastases and subsequent growth in culture, according to the scheme first described by Fidler (1973a) and applied by many other groups (Clark et al., 2000). Briefly, to select for highly metastatic tumor cells, pulmonary metastatic nodules are removed aseptically, minced, grown in vitro, and re-injected

intravenously into the tail vein of host mice. This procedure is repeated 2 to 5 times.

Anticipated Results

Using highly metastatic cell lines (e.g., B16-F10 or B16-BL6 mouse melanoma, A375SM human melanoma, 13762 MAT rat mammary carcinoma, D122 3LL Lewis lung carcinoma; Fidler, 1973a; Parish et al., 1987; Zhang et al., 1991; Vlodaysky et al., 1994; Fitzer-Attas et al., 1997; Clark et al., 2000), it is anticipated to obtain 200 to 300 pulmonary metastases per mouse (or rat) injected with 5×10^4 cells. The actual number of pulmonary colonies may range from ~80 to 350 in different mice of the same group. The lung weight of a C57BL mouse injected with 5×10^4 D122 3LL cells is expected to be $\sim 800 \pm 140$ mg versus a normal lung weight of ~ 200 mg (Fitzer-Attas et al., 1997). The effect of compounds that inhibit cell adhesion (mediated by selectins and integrin receptors, for example), or certain degradative enzymes (i.e., matrix metalloproteinases, plasminogen activator, heparanase) can be easily evaluated using the tail vein assay. The inhibitory molecule can be added to the cell suspension prior to the intravenous injection, or administered separately, subcutaneously or intraperitoneally. Generally, the test compound can be injected up to 12 hr prior to the cells, depending on its half-life time, but no later than 30 min following the tail vein injection of the cells. Effective compounds may decrease the number of visible pulmonary metastases by ~95% (Parish et al., 1987; Vlodaysky et al., 1994).

An example is presented in figure 19.2.1, showing a profound inhibition of experimental metastasis in mice treated with a chemically modified, non-anti-coagulant fragment of heparin (Vlodaysky et al., 1994). This effect is

attributed primarily to efficient inhibition of heparanase activity (Vlodaysky et al., 1994). The heparanase enzyme has been shown to promote tumor angiogenesis and metastasis (Vlodaysky and Friedmann, 2001).

Time Considerations

Cell harvesting and tail vein injection require 2 to 4 hr, depending on the number of mice and treatment groups. The mice are generally euthanized 14 to 18 days after injection, unless injected with very low metastatic cells (i.e., A375 melanoma) where up to 8 weeks after injection may be required (Clark et al., 2000). The actual removal of lungs and enumeration of pulmonary colonies can be carried out in 1 day for a typical experiment of 4 to 5 groups, 6 to 8 mice each. In the case of tumor colonies that are not pigmented, it is recommended that lungs be first incubated in Bouin's solution for 24 hr.

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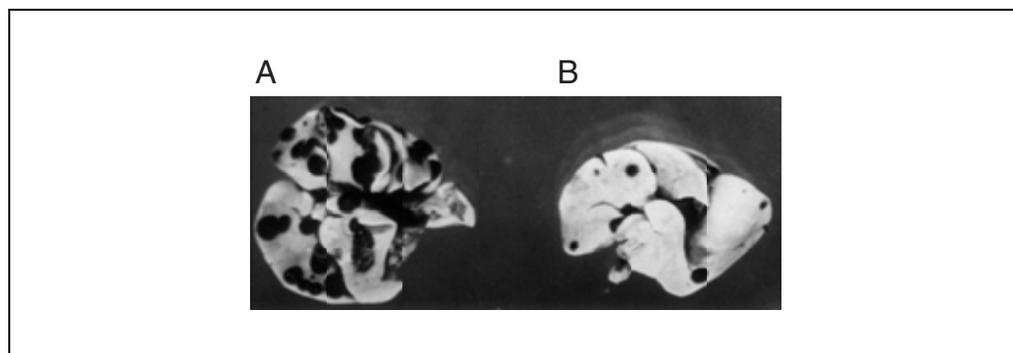


Figure 19.2.1 Lung colonization of B16-BL6 melanoma cells. B16-BL6 melanoma cells were injected into the tail vein of C57BL mice. The mice received a single subcutaneous injection of saline (**A**) or *N*-acetylated heparin fragment, 200 μ g per mouse (**B**), 30 min prior to the cells. After 14 days, the mice were euthanized and evaluated for lung colonization (dark spots).

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Microanalysis of Gene Expression in Tissues Using T7-SAGE: Serial Analysis of Gene Expression After High-Fidelity T7-Based RNA Amplification

Comprehensive gene-expression data obtained from individual cell types in tissues and organs will provide novel insights into the fundamental molecular biology of embryonic development, disease, and other cell biological processes. There are presently two major types of gene expression profiling techniques, microarray analyses and serial analysis of gene expression (SAGE). SAGE has an advantage over microarray analysis in that it provides absolute transcript numbers in a digital format, and can readily identify previously unknown genes (Velculescu et al., 1995, 1997; Ishii et al., 2000). Microarrays have the advantages of being relatively easy to use and more suitable for high-throughput applications; however, mRNA quantitation is more accurate with SAGE than with microarrays (Polyak and Riggins, 2001).

A major disadvantage of SAGE is that it requires microgram quantities of starting poly(A)⁺ mRNA, which prevents its use when mRNA is limited. Several techniques have been developed to overcome this limitation, but they mostly depend on amplification using the polymerase chain reaction (PCR). SAGE-Lite (Peters et al., 1999) and PCR-SAGE (Neilson et al., 2000) convert poly(A)⁺ mRNA to cDNA, which is then amplified by PCR. Alternatively, MicroSAGE (Datson et al., 1999) and SAGE adaptation for downsized extracts (SADE; Virlon et al., 1999) depend on PCR amplification of SAGE ditags. Although the recently reported MicroSAGE protocol does not require a reamplification step (see <http://www.sagenet.org>), it is optimized for between 5×10^4 and 2×10^6 cells (St. Croix et al., 2000), and extra PCR amplifications would be necessary for fewer cells, such as when using laser-capture-microdissected tissue sections (Emmert-Buck et al., 1996).

Although PCR is a powerful method for amplifying rare DNA species from tissue samples, the widely used *Taq* DNA polymerase has relatively low fidelity, and it propagates errors through subsequent cycles of amplification (Van Gelder et al., 1990). The error rate of 1×10^{-3} for *Taq* polymerase (1 mismatch per 1000 bases synthesized) will result, on average, in several erroneous bases in most PCR-amplified cDNAs (Eberwine et al., 1992). Even though SAGE ditags are of approximately equal length for ditag amplification by PCR during SAGE, preferential amplification of some ditags cannot be completely prevented (Datson et al., 1999).

This unit begins with a procedure for T7-based RNA amplification (see Basic Protocol 1). In order to avoid extra PCR or other forms of amplification, the authors incorporate only two cycles of T7-based RNA amplification as the initial step in Basic Protocol 1. This T7-based amplification step has high accuracy, since a combined error rate of 2×10^{-4} has been calculated for avian myeloblastosis virus (AMV) reverse transcriptase and T7 RNA polymerase (Sooknanan et al., 1994). In addition, T7 RNA polymerase has high processivity (Muller et al., 1988) and functions effectively even when broad stretches of nucleotides are being amplified—unlike *Taq* polymerase, which works most efficiently for amplification of small regions of a few hundred nucleotides. Although no protocol that includes an amplification step can claim to permit determination of absolute transcript number, since slight changes in estimated transcript frequency are always possible, T7 procedures appear to be the safest to date (Wang et al., 2000; Bashiardes and Lovett,

2001). A procedure for T7-like amplification of tiny amounts of mRNA using the RiboAmp kit from Arcturus Engineering is also included (see Alternate Protocol).

In this unit, the authors describe a new technique, T7-SAGE (see Basic Protocol 2), in which a high-fidelity T7 amplification step is combined with SAGE analysis. This new T7-SAGE procedure should facilitate application of SAGE for gene-expression profiling using minimal quantities of starting material, such as from embryonic tissues and microdissected cells from histological sections of tissues.

Support Protocols are also provided for: preparation of total RNA (see Support Protocol 1); a phenol/chloroform procedure using Phase Lock Gel Light which is suitable for the numerous DNA extractions in T7-SAGE (see Support Protocol 2); kinasing to generate the linkers used in T7-SAGE (see Support Protocol 3); a gel-shift test (see Support Protocol 4) for completeness of biotinylation, used to evaluate the biotinylated oligo(dT) primer with which the biotinylated cDNA is synthesized in Basic Protocol 1; preparation of gels for the numerous PAGE procedures involved in T7-SAGE (see Support Protocol 5); and direct sequencing of PCR products from a SAGE library (see Support Protocol 6).

NOTE: Investigators should first become familiar with the underlying strategies of SAGE (*UNIT 19.4*) and then practice ahead of time (ideally several times) using larger amounts of starting material before performing the final T7-SAGE procedure with precious samples. Researchers are advised to access the SAGE home page on the internet at <http://www.sagenet.org/> organized by Kenneth Kinzler and co-workers at the Molecular Genetics Laboratory of the Johns Hopkins Oncology Center at Johns Hopkins University, which describes SAGE concepts, new SAGE applications, and recent publications.

NOTE: The quality of the data obtained from T7-SAGE libraries depends on the quality of the RNA and DNA used in its many steps. Consequently, DEPC-treated water should be used for all steps involving RNA. DNase-free water (e.g., obtained from a commercial source) is also recommended.

BASIC PROTOCOL 1

T7-BASED RNA AMPLIFICATION

This protocol describes how to perform high-fidelity T7-based RNA amplification, starting with only small amounts of total RNA, for use in SAGE procedure (Velculescu et al., 1995, 1997). This method can be used with tissue culture cell lines or with homogenates from animal tissues or organs. The protocol is a general method applicable to total RNA isolated in various ways in quantities as small as 4 ng. The Alternate Protocol is specifically designed for laser-microdissected histological samples, where quantities are even lower.

CAUTION: Investigators should wear gloves for all procedures involving RNA and take precautions in order to avoid cross-contamination of samples. See *APPENDIX 2A* for general guidelines when working with RNA. DEPC-treated water (*APPENDIX 2A*) must be used for steps 1 to 41.

Materials

4 to 5 ng purified total RNA from tissues or cells in a volume of 10.5 μ l (see Support Protocol 1)

0.5 mg/ml T7-oligo(dT) primer:

5'-TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGT₂₁-3'
(custom-synthesized, e.g., by Integrated DNA Technologies or core facility)

Superscript Choice System for cDNA Synthesis kit (Invitrogen), including:

- 5× first-strand reaction buffer
- 0.1 M dithiothreitol (DTT)
- 10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
- SuperScript II reverse transcriptase
- 40 U/μl RNaseOUT recombinant ribonuclease inhibitor (Invitrogen)
- 5× second-strand reaction buffer
- 10 U/μl DNA polymerase I
- 60 U/μl RNase H (PanVera)
- 60 U/μl DNA ligase (PanVera)
- DEPC-treated H₂O (Quality Biological or other supplier; also see *APPENDIX 2A*)
- 8 U/μl T4 DNA polymerase (Promega)
- QIAquick PCR Purification Kit (Qiagen)
- Ampliscribe T7 High Yield Transcription Kit (Epicentre Technologies), including:
- Ampliscribe T7 reaction buffer
- 100 mM ATP
- 100 mM CTP
- 100 mM GTP
- 100 mM UTP
- 0.1 M dithiothreitol (DTT)
- T7 RNA polymerase solution
- 10 U/μl DNase I (Boehringer Mannheim)
- RNeasy Mini Kit (Qiagen)
- 1 mg/ml random primers (Invitrogen)
- 0.5 mg/ml biotinylated oligo(dT) primer: 5'- [biotin]T₁₈ -3' (custom-synthesized, e.g., by Integrated DNA Technologies or core facility), gel-purified; test for complete biotinylation before use by gel-shift test (see Support Protocol 4)
- 16°, 42°, 70°, and 95°C water baths
- GeneQuant pro RNA/DNA Calculator (Amersham Pharmacia Biotech) or any other spectrophotometer for determining UV absorbance of small samples (e.g., 20 to 50 μl)

Perform reverse transcription (RT) of RNA

1. Mix 4 to 5 ng purified RNA (in 10.5 μl; see Support Protocol 1) in a 1.5- to 1.7-ml microcentrifuge tube with 1 μl of 0.5 mg/ml T7-oligo(dT) primer to initiate first-strand synthesis.

In general, small samples should be mixed by vortexing for 5 sec, then momentarily microcentrifuged at maximum speed (accelerate to maximal speed of 13,000 to 15,000 rpm, then immediately brake) to collect the sample at the bottom of the tube.

2. Incubate the primer and RNA at 70°C for 10 min, then incubate at 42°C for 5 min.
3. Add the following reagents to the microcentrifuge tube and mix:
 - 4 μl 5× first-strand reaction buffer
 - 2 μl 0.1 M DTT
 - 1 μl 10 mM dNTP mix
 - 0.5 μl 40 U/μl RNaseOUT RNase inhibitor
 - 1 μl SuperScript II reverse transcriptase.

4. Incubate at 42°C for 1 hr.

5. Add the following reagents to the tube and mix:
 - 30 μl 5 \times second-strand reaction buffer
 - 3 μl 10 mM dNTP mix
 - 1.5 μl 10 U/ μl DNA polymerase I
 - 0.25 μl 60 U/ μl RNase H
 - 0.25 μl 60 U/ μl DNA ligase
 - 95 μl DEPC-treated H₂O.
6. Incubate at 16°C for 2 hr.
7. Add 2.5 μl of 8 U/ μl T4 DNA polymerase and incubate for an additional 10 min at 16°C.
8. Purify the double-stranded (ds)-cDNA with a QIAquick PCR purification kit according to the manufacturer's instructions.
9. After collection from the column, vacuum dry the ds-cDNA to \sim 5 μl , then adjust the volume to 8 μl with DEPC-treated H₂O using a micropipettor, in preparation for in vitro transcription.

Samples should not be evaporated to complete dryness, which reduces the efficiency of recovery. Instead, try to reduce the volume to \sim 5 μl , periodically checking the progress of volume reduction. Volumes can be determined using an adjustable 10- μl micropipettor: draw the sample up into a micropipet tip, then adjust the dial that sets the volume of the micropipettor until there is a precise match to the volume of the sample (i.e., after drawing up, no extra volume is left in the tube and no air is left at the bottom of the tip). Once the volume is reduced to $<$ 8 μl , the difference in volume to achieve the final 8 μl can be calculated and added using DEPC-treated water.

Amplify antisense RNA (aRNA) using T7 RNA polymerase

10. Mix the 8 μl ds-cDNA (from step 9) with the following reagents in the tube:
 - 2 μl 10 \times Ampliscribe T7 reaction buffer
 - 1.5 μl each of 100 mM ATP, CTP, GTP, and UTP
 - 2 μl 0.1 M DTT
 - 2 μl T7 RNA polymerase solution.
- All of the above reagents are included with the Ampliscribe T7 High-Yield Transcription kit from Epicentre Technologies.*
11. Incubate at 42°C for 3 hr.
 12. Add 1 μl of 10 U/ μl DNase I, mix, and incubate at 37°C for 15 min.
 13. Purify the resulting aRNA using an RNeasy Mini Kit according to the manufacturer's instructions.
 14. Dry the aRNA down to \sim 5 to 10 μl using a vacuum concentrator, then adjust the volume to 10.5 μl using a micropipettor and DEPC-treated water.

Perform a second round of aRNA amplification

15. To the aRNA from the first-round amplification (step 14), add a 1- μl aliquot of 1 mg/ml random primers in a 1.5- to 1.7-ml microcentrifuge tube, and mix.
16. Incubate the mixture at 70°C for 10 min.
17. Chill the mixture on ice, and then allow it to equilibrate at room temperature for 10 min.

18. Add the following reagents (from the SuperScript kit) to the tube and mix:
 - 4 μ l 5 \times first-strand reaction buffer
 - 2 μ l 0.1 M DTT
 - 1 μ l 10 mM dNTP mix
 - 0.5 μ l 40 U/ μ l RNaseOUT RNase inhibitor
 - 1 μ l SuperScript II reverse transcriptase.
 19. Incubate at room temperature for 5 min and then at 37°C for 1 hr.
 20. Add 0.5 μ l of 60 U/ μ l RNase H, mix, and incubate at 37°C for 20 min.
 21. Heat the reaction mixture to 95°C for 2 min and then chill it on ice.
 22. For second-strand cDNA synthesis, add 1 μ l of 0.5 mg/ml T7-oligo(dT) primer, mix, and incubate the mixture at 70°C for 5 min and then at 42°C for 10 min.
 23. Add the following reagents to the tube and mix:
 - 30 μ l 5 \times second-strand reaction buffer
 - 3 μ l 10 mM dNTP mix
 - 1.5 μ l 10 U/ μ l DNA polymerase I
 - 0.25 μ l 60 U/ μ l RNase H
 - 0.25 μ l 60 U/ μ l DNA ligase
 - 95 μ l DEPC-treated H₂O.
 24. Incubate the mixture at 16°C for 2 hr.
 25. Add 2.5 μ l of 8 U/ μ l T4 DNA polymerase, mix, and incubate at 16°C for 10 min.
 26. Purify the ds cDNA with a QIAquick PCR purification kit according to the manufacturer's instructions.
 27. Vacuum dry the cDNA to ~5 μ l and adjust volume to 8 μ l with DEPC-treated water.

See annotation to step 9, above, for details of this procedure.
 28. Using the same tube, add the following reagents to the 8 μ l of ds cDNA (from step 27) and mix:
 - 2 μ l 10 \times Ampliscribe T7 reaction buffer
 - 1.5 μ l each of 100 mM ATP, CTP, GTP, and UTP
 - 2 μ l 0.1 M DTT
 - 2 μ l T7 RNA polymerase solution.
 29. Incubate at 42°C for 3 hr.
 30. Add 1 μ l DNase I, mix, and incubate at 37°C for 15 min.
 31. Purify the aRNA using an RNeasy Mini Kit according to the manufacturer's instructions.
 32. Dry the aRNA down to 5 to 10 μ l using vacuum concentrator, then adjust volume to 10.5 μ l using a micropipettor and DEPC-treated water.
- Synthesize biotinylated double-stranded cDNA (bds cDNA)***
33. Mix the 10.5 μ l aRNA from the second-round RNA amplification (step 32) with 1 μ l of 1 mg/ml random primers.
 34. Incubate the mixture at 70°C for 10 min, chill it on ice, and then allow it to equilibrate at room temperature for 10 min.

35. Add the following reagents to the tube and mix:
 - 4 μ l 5 \times first-strand reaction buffer
 - 2 μ l 0.1 M DTT
 - 1 μ l 10 mM dNTP mix
 - 0.5 μ l 40 U/ μ l RNaseOUT RNase inhibitor
 - 1 μ l SuperScript II reverse transcriptase
36. Incubate at room temperature for 5 min and then at 37°C for 1 hr.
37. Add 0.5 μ l of 60 U/ μ l RNase H, mix, and incubate the mixture at 37°C for 20 min.
38. Heat this reaction mixture to 95°C for 2 min, and then chill it on ice.
39. Add 1 μ l of 0.5 mg/ml biotinylated oligo(dT) primer (5'- [biotin]T₁₈ -3') and mix.

Before use, the biotinylated oligo(dT) primer should be tested for complete biotinylation by the gel-shift test (see Support Protocol 4).
40. Incubate the mixture at 70°C for 5 min and then at 42°C for 10 min.
41. Add the following reagents to the tube and mix:
 - 30 μ l 5 \times second-strand reaction buffer
 - 3 μ l 10 mM dNTP mix
 - 1.5 μ l 10 U/ μ l polymerase I
 - 0.25 μ l 60 U/ μ l DNA ligase
 - 0.25 μ l 60 U/ μ l RNase H
 - 93.5 μ l DEPC-treated H₂O.
42. Incubate the mixture at 16°C for 2 hr.
43. Purify the bds cDNA using a QIAquick PCR purification kit according to the manufacturer's instructions.
44. Elute with 60 μ l of water.
45. Check the concentration of bds cDNA synthesized using a spectrophotometer appropriate for determining UV absorbance of small samples.

A total of ~1 μ g is needed to proceed to the SAGE protocol.

SUPPORT PROTOCOL 1

PREPARATION OF TOTAL RNA

Total RNA from tissues or cells can be prepared by any of the methods listed in Table 19.3.1, following the company's protocol if a commercial kit is used. A final yield of at least 4 ng total RNA in 10.5 μ l water is needed. The column methods are generally recommended for isolating RNA from small samples, such as those obtained using laser microdissection of histological sections. DNase treatment, as described in the steps below, is needed to avoid any genomic DNA contamination. The integrity of RNA should be checked by gel electrophoresis, RT-PCR, or Northern blot hybridization.

Materials

- Tissues or cells of interest
- Kit or reagents for total RNA isolation (Table 19.3.1)
- DEPC-treated H₂O (*APPENDIX 2A*; or supplied with isolation kit)
- 10 \times DNase I buffer (Ambion)
- 2 U/ μ l DNase I (RNase-free; Ambion)
- 40 U/ μ l RNaseOUT recombinant ribonuclease inhibitor (Invitrogen)
- RNeasy Mini Kit (Qiagen)

Table 19.3.1 Methods for Preparing Total RNA from Tissues or Cells

Method	Supplier or Reference
<i>Standard solution-based methods</i>	
Guanidium isothiocyanate method	Chomczynski and Sacchi (1987)
TRIzol reagenty	Invitrogen
RNAgents Total RNA Isolation System	Promega
Micro RNA Isolation Kit	Stratagene
<i>Column methods</i>	
RNeasy Mini Kit	Qiagen
Pinpoint Slide RNA Isolation System	Zymo Research
PicoPure RNA Isolation Kit	Arcturus
Micro-FastTrack 2.0 mRNA Isolation Kit	Invitrogen

CAUTION: Investigators should wear gloves for all procedures involving RNA and take precautions in order to avoid cross-contamination of samples. See *APPENDIX 2A* for general guidelines when working with RNA.

1. Isolate RNA from tissues or cells of interest using an appropriate method (Table 19.3.1).
2. Adjust the volume of the isolated RNA to 16 μ l using DEPC-treated water.
3. Add the following reagents to the tube and mix:
 - 2 μ l 10 \times DNase I buffer
 - 1 μ l 2 U/ μ l DNase I
 - 1 μ l 40 U/ μ l RNaseOUT RNase inhibitor.
4. Incubate at 37°C for 30 min.
5. Purify the treated RNA using an RNeasy Mini Kit according to manufacturer's instructions.

Follow the RNeasy Mini Protocol for RNA Cleanup.

6. Vacuum dry the eluted RNA solution down to 10.5 μ l.

The product will be ready at this point for T7-based RNA amplification (see Basic Protocol 1).

SUBSTITUTION OF A COMMERCIAL KIT FOR AMPLIFYING TINY QUANTITIES OF RNA

When samples are obtained by laser microdissection for isolation of cells from histological sections, or if investigators prefer to use a commercial kit for the RNA amplification, the RiboAmp kit from Arcturus is recommended for reliable T7-like amplification of tiny amounts of mRNA. This kit, designed to isolate and amplify extremely small amounts of material, can be substituted directly for Basic Protocol 1 through step 32. This kit generates antisense RNA that can be used for the synthesis of biotinylated double-stranded cDNA (bds cDNA).

ALTERNATE PROTOCOL

Whole Organism and Tissue Analysis

19.3.7

Additional Materials (also see *Basic Protocol 1*)

RiboAmp RNA Amplification Kit (Arcturus Engineering)

1. Isolate RNA from samples and synthesize aRNA following the manufacturer's instructions for the RiboAmp RNA Amplification Kit.
2. Dry the aRNA down to 5 to 10 μ l using a vacuum concentrator and then adjust the volume to 10.5 μ l with DEPC-treated water.
3. Use the product in Basic Protocol 1 starting at step 33.
4. Check the concentration of bds cDNA using a spectrophotometer appropriate for determining UV absorbance of small samples.

T7-SAGE PROCEDURE

The following protocol is a modification of the SAGE technique developed by Kinzler and co-workers at Johns Hopkins University (Velculescu et al., 1995, 1997; see also SAGE Detailed Protocol version 1.0e at <http://www.sagenet.org>). Key alterations include the following changes:

- a. The procedure is started with biotin-labeled double-stranded cDNA generated from antisense mRNA produced by T7 amplification.
- b. Use of the commercial mRNA Capture Kit (Roche Molecular Biochemicals) is substituted for the first three steps involving binding of biotinylated double-stranded cDNA (bds cDNA) to streptavidin-magnetic beads, *Nla*III enzyme cleavage of cDNA and ligating of linkers, and release of cDNA tags using *Bsm*FI enzyme. This substitution permits completion of all three steps within two streptavidin-coated microcentrifuge tubes, which significantly reduces losses of material between successive steps.
- c. Phase Lock Gel (PLG, Light; Eppendorf) for optimizing phase separation at several steps in the SAGE procedure for recovering DNA after phenol extraction; this technique significantly increases the recovery and purity of the DNA.

Materials

- 1 μ g of bds cDNA generated by T7-based amplification (see Basic Protocol 1) in ~50 μ l
- mRNA Capture Kit (Roche Molecular Biochemicals) including:
 - Streptavidin-coated PCR tubes
 - Washing solution
- 10 \times NEBuffer 4 (New England Biolabs; supplied with corresponding restriction enzymes)
- 10 U/ μ l restriction endonuclease *Nla*III (New England Biolabs)
- 100 \times (10 μ g/ μ l) BSA (New England Biolabs; supplied with corresponding restriction enzymes)
- DNase-free H₂O
- 1 U/ μ l and 5 U/ μ l T4 DNA ligase and 10 \times ligase buffer (Invitrogen)
- LoTE buffer (see recipe)
- 15 ng/ μ l annealed linker 1 (formed by annealing linkers 1A and 1B; see Support Protocol 3)
- 15 ng/ μ l annealed linker 2 (formed by annealing linkers 2A and 2B; see Support Protocol 3)
- 2 U/ μ l restriction endonuclease *Bsm*FI (New England Biolabs)
- 20 mg/ml glycogen (Boehringer Mannheim)
- 7.5 M ammonium acetate

70% and 100% ethanol
 5× second-strand reaction buffer (Invitrogen; supplied with SuperScript kit, also see Basic Protocol 1)
 10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP; Invitrogen; supplied with SuperScript kit; also see Basic Protocol 1)
 5 U/ml Klenow fragment of DNA polymerase (Amersham Pharmacia Biotech or USB)
 10× PCR buffer (see recipe)
 Dimethyl sulfoxide (DMSO)
 350 ng/μl primer 1: 5'-GGATTTGCTGGTGCAGTACA-3' (custom-synthesized, e.g., by Integrated DNA Technologies or core facility)
 350 ng/μl primer 2: 5'-CTGCTCGAATTCAAGCTTCT-3' (custom-synthesized, e.g., by Integrated DNA Technologies or core facility)
 5 U/μl Platinum *Taq* polymerase (Invitrogen)
 12% and 8% polyacrylamide gels (see Support Protocol 5)
 6× Loading Dye Solution (LDS; Fermentas)
 10- and 25-bp DNA ladders (Invitrogen)
 SYBR Green I (Molecular Probes)
 1× TAE buffer (e.g., Advanced Technologies, or prepare as in APPENDIX 2A)
 TE buffer, pH 7.4 (APPENDIX 2A), cold
 Ready-to-Go 100-bp DNA ladder (Fermentas)
 pZERO-1 plasmid (Invitrogen)
 5 U/μl restriction endonuclease *Sph*I (New England Biolabs)
 10× NEBuffer 2 (New England Biolabs; supplied with corresponding restriction enzymes)
 1% and 3% agarose gels (see APPENDIX 3A)
 One Shot TOP10 Electrocompetent *E. coli* cells (Invitrogen)
 SOC medium (see recipe)
 10-cm Zeocin-containing low-salt LB plates (see recipe)
 350 ng/μl M13 forward primer: 5'-GTAAAACGACGGCCAGT-3' (custom-synthesized, e.g., by Integrated DNA Technologies or core facility)
 350 ng/μl M13 reverse primer: 5'-GGAAACAGCTATGACCATG-3' (custom-synthesized, e.g., by Integrated DNA Technologies or core facility)
 QIAquick 96 Multiwell PCR Purification Kit (Qiagen)
 10 mg/ml ethidium bromide
 16°, 50°, and 65°C water baths
 2-ml PCR tubes
 System for PCR using 96-well plates (e.g., GeneAmp PCR system 9600 or 9700, Applied Biosystems)
 96-well PCR plates (Applied Biosystems)
 Power Pac 200 power source (Bio-Rad)
 18-G needle (1- to 1.5-in. length)
 SpinX columns with collection tubes (Costar)
 Gene Pulser II and Pulse Controller Plus (Bio-Rad) and 0.1-cm electroporation cuvettes
 15-ml polypropylene snap-top tubes
 Bacterial shaker
 Micropipettors with sterile aerosol-barrier tips
 SAGE 2000 software (freely available for noncommercial use; see <http://www.sagenet.org>) and Microsoft Access
 Additional reagents and equipment for phenol/chloroform extraction using Phase Lock Gel Light (see Support Protocol 2), polymerase chain reaction (APPENDIX 3F), polyacrylamide gel electrophoresis (see APPENDIX 3A and Support Protocol 5), and agarose gel electrophoresis (see APPENDIX 3A)

Generation of Linked SAGE Tags

Capture the biotinylated cDNA

1. Add 25 μl of bds cDNA to each of two streptavidin-coated PCR tubes provided in the mRNA Capture Kit ($\sim 0.5 \mu\text{g}$ per tube).

This step can be scaled up by using more bds cDNA and more pairs of tubes, keeping a ratio of approximately 0.5 μg bds cDNA per tube.

2. Incubate at 37°C for 5 min to allow the bds cDNA to attach to the walls of the tubes.
3. Aspirate the solution to remove non-bound bds cDNA and wash the tubes three times, each time with 50 μl of the washing solution provided in the mRNA Capture Kit.

Cleave the biotinylated cDNA with the anchoring enzyme NlaIII

4. After aspirating the washing solution, wash the streptavidin-coated PCR tubes (which contain the bds cDNA bound to the walls) three times, each time with 50 μl 1 \times NEBuffer 4.
5. Add the following reagents to each tube:

2 μl 10 U/ μl NlaIII
0.25 μl 100 \times (10 $\mu\text{g}/\mu\text{l}$) BSA
2.5 μl 10 \times NEBuffer 4
20 μl DNase-free H₂O.

6. Vortex the mixtures very gently (to avoid bubbles) for 10 sec, then momentarily centrifuge at maximum speed in a microcentrifuge.
7. Incubate for 3 hr at 37°C. Mix occasionally by flicking the tube with a finger or by slow vortexing.
8. Heat the tubes at 65°C for 20 min to inactivate the enzyme.

NlaIII is easily inactivated at ambient or high temperature. When ordering NlaIII, request that it be shipped in dry ice. If dry ice shipping is not requested, this enzyme may be sent with an ice pack and arrive at room temperature, with a resulting loss of activity. Aliquots of NlaIII should be stored at -80°C , which helps to prolong its activity. Ensure that the enzyme is fresh, since it appears to have a half-life of only a few months even when stored at -80°C .

Ligate linkers to the bound cDNA

9. Aspirate the solutions, which will contain digested, unbound cDNA, from each of the streptavidin-coated PCR tubes. Keep the tubes and discard the solutions.
10. Wash the PCR tubes three times, each time with 50 μl washing solution (from the kit), then once with 50 μl of 1 \times ligase buffer.
11. Add 5 μl 5 \times ligase buffer and 18 μl LoTE buffer to each tube.
12. Add 1 μl of 15 ng/ μl annealed linker 1 to tube 1.
13. Add 1 μl of 15 ng/ μl annealed linker 2 to tube 2.
14. Mix and heat the tubes for 2 min at 50°C and then let stand for 15 min at room temperature.
15. Add 1 μl of 5 U/ μl T4 DNA ligase to each of the tubes and mix.
16. Allow the mixtures to ligate by incubating at 16°C for 2 hr.

After ligation, the mixture can be stored at 4°C overnight.

Release cDNA tags using the tagging enzyme BsmFI

17. Remove the ligation solutions, and wash each of the PCR tubes three times, each time with 50 μl washing solution (from the mRNA Capture Kit).
18. Wash the PCR tubes once with 50 μl 1 \times NEBuffer 4.
19. Add the following reagents to each tube:
 - 2.5 μl 10 \times NEBuffer 4
 - 0.25 μl 100 \times (1 $\mu\text{g}/\mu\text{l}$) BSA
 - 21.25 μl LoTE buffer.
20. Add 1 μl of 2 U/ μl *BsmFI* to each tube and mix. Digest 1 hr at 65°C. Mix occasionally by flicking the tube with a finger or slow vortexing.

BsmFI is the “tagging enzyme” that cuts cDNA 14 bp away from the enzyme site GGGAC to generate SAGE tags.

Do not discard the supernatant, which contains the SAGE tags after BsmFI digestion.

Generation of 26-mer SAGE Ditags

Prepare the released, linked SAGE tags for blunt-ending

21. After the *BsmFI* digestion, increase the volume of each reaction solution containing the released linked SAGE tags to 200 μl by adding LoTE buffer, then mix.
22. Transfer the reactions to 2-ml tubes numbered 1 and 2, each containing precentrifuged Phase Lock Gel Light (see Support Protocol 2). Perform phenol/chloroform (PC8) extraction as in Support Protocol 2, and finally pipet 200 μl of the SAGE tags contained in the respective aqueous upper phases into fresh 1.5- to 1.7-ml microcentrifuge tubes numbered 1 and 2.
23. Add the following reagents to each of the tubes containing the 200 μl SAGE tag preparation.
 - 3 μl 20 mg/ml glycogen
 - 133 μl 7.5 M ammonium acetate
 - 700 μl 100% ethanol.
24. Mix well by vortexing and place the tube on dry ice for 10 to 20 min.
25. Microcentrifuge 30 min at maximum speed, 4°C.
26. Carefully remove the supernatants and discard them. Be careful not to disturb the pellets.
27. Wash the pellets twice, each time by gently adding 500 to 1000 μl of 70% ethanol solution down the wall of the tube using a micropipet, taking care to avoid disrupting the pellet, and decanting the solution gently.
28. After the final wash, microcentrifuge again briefly at maximum speed to collect any residual ethanol. Carefully remove all of the ethanol with a micropipet and air dry the pellet for 5 to 10 min.
29. Resuspend each pellet in 20 μl LoTE buffer.

Blunt-end the linked SAGE tags

30. Add the following reagents to the tubes 1 and 2 containing 20 μ l of linked SAGE tags.
- 6 μ l 5 \times second-strand reaction buffer
 - 0.3 μ l 100 \times (1 μ g/ μ l) BSA
 - 1.25 μ l 10 mM dNTP mix
 - 1.0 μ l 5 U/ μ l Klenow fragment of DNA polymerase.
31. Mix well, microcentrifuge briefly to collect the solution at the bottom of the tube, and incubate at 37°C for 30 min.
32. Adjust the volume of each blunt-ending reaction to 200 μ l by adding LoTE buffer. Mix, then transfer each mixture (no. 1 and no. 2) to a labeled 2.0-ml tube containing precentrifuged Phase Lock Gel Light. Perform phenol/chloroform (PC8) extraction as in Support Protocol 2, and finally pipet the SAGE tags contained in each aqueous upper phase into 1.5- to 1.7-ml microcentrifuge tubes numbered 1 and 2.
33. Precipitate the SAGE tags and wash and dry the pellets as in steps 23 to 28.
34. Resuspend each pellet in 4 μ l LoTE buffer.

Ligate the linked SAGE tags to form linked ditags

35. Prepare the following reaction mix on ice in a sterile microcentrifuge tube.
- 0.8 μ l 5 U/ μ l T4 DNA ligase
 - 1.2 μ l 5 \times ligase buffer
 - 2 μ l blunt-ended sample 1 from tube 1 (see step 34)
 - 2 μ l blunt-ended sample 2 from tube 2 (see step 34).
36. Prepare a parallel negative control reaction mix without ligase on ice in a sterile microcentrifuge tube.
- 0.8 μ l LOTE buffer
 - 1.2 μ l 5 \times ligase buffer
 - 2 μ l blunt-ended sample 1 from tube 1 (see step 34)
 - 2 μ l blunt-ended sample 2 from tube 2 (see step 34).
37. Mix each of the reactions and incubate overnight at 16°C.
38. After the incubation, make up the volume in each (reaction and control) tube to 20 μ l by addition of 14 μ l LoTE buffer and mix.

Choose the optimal dilution for PCR amplification of the linked ditags

39. Dilute the following samples with sterile water as described below.
- Negative control (prepared in step 36; no ligase) to 1/5 (i.e., 1 vol of sample plus 4 vol of DNase-free H₂O).
 - Template (prepared in step 35; ligated product) to 1/5, 1/10, 1/20, 1/40, and 1/80.

Set up seven PCR reactions on ice using sterile PCR tubes labeled as:

- Tubes 1 to 5: Different dilutions of the template (1/5, 1/10, 1/20, 1/40, and 1/80)
- Tube 6: Negative control (no template, just H₂O)
- Tube 7: Negative control (no ligase, just H₂O).

To each tube, add the following:

- 5 μ l 10 \times PCR buffer
- 3 μ l DMSO
- 7.5 μ l 10 mM dNTP mix
- 1 μ l 350 ng/ μ l primer 1
- 1 μ l 350 ng/ μ l primer 2
- 30.5 μ l DEPC-treated H₂O
- 1 μ l 5 U/ μ l Platinum *Taq* polymerase
- 1 μ l ligation product or negative control at appropriate dilution.

Mix each of the reactions.

Ex Taq (PanVera) appears to produce the same results for SAGE ditag amplification as Platinum Taq, and the former source of Taq polymerase is at present slightly less expensive than the latter.

40. Transfer 50 μ l of each reaction mixture into wells of a 96-well PCR plate. Amplify using the following cycling parameters:

1 cycle:	1 min	94°C	(denaturation)
26-30 cycles:	30 sec	94°C	(denaturation)
	1 min	55°C	(annealing)
	1 min	70°C	(extension)
1 cycle:	5 min	70°C	(final extension).

41. Select the optimal dilution of linked ditag as follows:

- a. Mix 5 μ l of each PCR product (the various amplified dilutions as well as the no-ligase control and no-template control) with 1 μ l of 6 \times Loading Dye Solution (LDS, from Fermentas).
- b. Load the 6 μ l of each sample mixture in a lane of a 12% PAGE gel. Load DNA ladder in additional lanes. Perform electrophoresis (see Support Protocol 5) at 150 V for 1.5 to 2 hr. Stop electrophoresis when the bromphenol blue marker is 1 cm from the bottom of the gel.
- c. Stain gels with a 1:10,000 dilution of SYBR Green I in 1 \times TAE buffer for 20 min.
- d. Wash gels with 1 \times TAE buffer for 5 min.
- e. Examine the gel with a UV transilluminator. Compare the lanes with the various dilutions and pick the lowest dilution (highest concentration of linked ditags) that gives a clear 102-bp band (an 80-bp band represents self-ligated linkers, and cannot be used).

The dilution series and PAGE analysis are necessary to check the successful formation of SAGE ditags (102-bp band) and to identify the optimal concentration for the subsequent large-scale PCR amplification.

42. Perform multiwell large-scale amplification of linked ditag at optimal dilution (selected in step 41) as follows:

- a. Dilute the template (i.e., the remaining template solution from step 38) with water to the optimal dilution (determined in step 41).
For example, 1/10 dilution of 19 μ l will make 190 μ l of diluted PCR product.
- b. Set up 198 to 288 individual PCR reactions in two to three 96-well PCR plates as in step 40, using 50 μ l of the optimally diluted template per well.
- c. Amplify PCR reactions as in step 40.

Experience has shown that, at least with presently available equipment, it is best to divide the reaction mixtures up into tiny (50- μ l) aliquots and to perform PCR in a 96-well

thermocycler configuration to ensure high-efficiency amplification. Usually, 192 PCR reactions (using two 96-well PCR plates) are performed in parallel, although three to four 96-well plates may be needed if the SAGE ditags are diluted to a lower concentration.

Isolate linked ditags

43. Pool the PCR products from all of the wells of the 96-well plates from the large-scale amplification into one 50-ml tube containing precentrifuged Phase Lock Gel Light.

There should be ~4.5 ml from each 96-well-plate.

44. Perform phenol/chloroform (PC8) extraction as in Support Protocol 2, and finally transfer half of the aqueous phase (containing linked ditags) to each of two fresh 30-ml Oak Ridge centrifuge tubes.
45. To ~5 ml of the sample in each tube, add the following reagents:
 - 80 μ l 20 mg/ml glycogen
 - 2 ml 7.5 M ammonium acetate
 - 15 ml 100% ethanol.
46. Mix well by vortexing and place the tubes on dry ice for 20 min. Microcentrifuge 30 min at maximum speed, 4°C.
47. Carefully remove and discard the supernatant. Be careful not to disturb the pellets.
48. Wash the pellets twice, each time with 25 ml of 70% ethanol.
49. Carefully remove the ethanol and air dry the pellets for 5 to 10 min.
50. Resuspend each pellet in 300 μ l LoTE buffer.
51. Add 60 μ l of 6 \times Loading Dye Solution (LDS) to the sample (total volume, 360 μ l) and mix.
52. Load 10 μ l of the sample mixture in each lane of a 10-well, 12% polyacrylamide gel, for a total of 36 lanes on four gels. Use a 10-bp DNA ladder as a marker on each gel. Perform gel electrophoresis at 150 V for 1.5 to 2 hr using a Power Pac 200 power source (or equivalent). Stop electrophoresis when bromophenol blue marker is 1 cm from the bottom of the gels.
53. Stain the gels with SYBR Green I at 1:10,000 dilution in 1 \times TAE for 20 min.
54. Wash gels with 1 \times TAE for 5 min.
55. Make a hole through the bottom of a 0.5-ml sterile microcentrifuge tube using an 18-G needle, and insert the tube into a 1.7-ml microcentrifuge tube.

For safety, the hole should be punched from the inside toward the outside of the tube: insert the sharp end of a 1- to 1.5-in. 18-G needle into the microcentrifuge tube and slide it down to the center of the V-shaped bottom of the tube; push (away from one's hand) through the bottom of the tube to make the hole, then discard the needle in appropriate manner.
56. Visualize the bands in the gel using a UV transilluminator, taking care to keep all exposed areas of skin covered, e.g., with UV eye and face shielding, gloves, and laboratory coat. Using a single-edge razor blade, excise the 102-bp product from all 36 lanes of the gel by cutting out each band as a small rectangle.
57. Scoop up three excised gel pieces with a corner of the razor blade and place into a 0.5-ml sterile tube. Snap the lid of the 0.5-ml tube closed. Repeat this process for the remaining gel pieces. If there is a lid attached to the 1.7-ml microcentrifuge tube, cut through the plastic hinge with scissors or razor blade and discard the lid. Microcentrifuge 3 min at maximum speed, at room temperature. Discard the 0.5 ml tube, which should be empty, leaving behind fragmented gel in the 1.7-ml tube.

The purpose of this step is to fragment the gel by forcing it to pass through the needle hole by centrifugal force. The outer tube needs to be large enough to permit passage of all of the gel through the hole, so a 1.7-ml tube or even a 2.0-ml tube may be preferable to a 1.5-ml tube to permit collection of all gel fragments, depending on the shape of each of the microcentrifuge tubes.

58. Mix 5 vol of LoTE buffer with 1 vol of 7.5 M ammonium acetate. Add 300 μ l of this mixture to the 1.7-ml microcentrifuge tube containing the fragmented gel. Make sure that all gel pieces are covered with buffer. Mix well by vortexing.
59. Incubate at 4°C overnight and then at 65°C for 30 min to elute the DNA from gel.
60. Transfer the contents of the tube to SpinX columns fitted to collection tubes.
61. Microcentrifuge 5 min at maximum speed, at room temperature.
62. Transfer the eluates (each ~300 μ l) to new 1.5 to 1.7 ml microcentrifuge tubes.
63. Precipitate, wash, and air dry the DNA pellet as in steps 23 to 28, above.
64. Resuspend each pellet in 7 μ l LoTE and then combine all samples (~126 μ l).
65. Store the 102-bp ditags at –20°C for up to 1 month.

Purify the SAGE ditags

66. Aliquot the 102-bp product (126 μ l) equally into two separate sterile microcentrifuge tubes (63 μ l per tube). Add the following reagents to each of the two tubes:

15 μ l 10 \times NEBuffer 4
2 μ l 100 \times (1 μ g/ μ l) BSA
12 μ l 10 U/ μ l *Nla*III (10 U/ μ l)
58 μ l DNase-free H₂O.

67. Mix the contents well and incubate for 2 hr at 37°C.
68. Adjust the volume in each tube to 200 μ l by adding 50 μ l LoTE buffer and mix.
69. Analyze 5 μ l of the reaction by electrophoresis on a 12% gel to check the efficiency of *Nla*III digestion (see Support Protocol 5).

Problems with incomplete NlaIII digestion may be due to contaminants, which may be resolved by further purifying the gel-purified 102-bp ditag preparation with a gel-filtration purification step (Angelastro et al., 2000).

70. Transfer the reaction to 200 μ l with LoTE buffer and transfer to a 2-ml tube containing precentrifuged Phase Lock Gel Light. Perform phenol/chloroform (PC8) extraction as in Support Protocol 2, and finally pipet the SAGE tags contained in the aqueous upper phase into a fresh tube.
71. Precipitate the SAGE tags and wash and dry the pellets as in steps 23 to 28.
72. Combine the pellets and resuspend, on ice, in a total volume of 30 μ l using cold TE buffer, pH 7.4.

Note that standard TE buffer, pH 7.4, prepared as in APPENDIX 2A is used through step 76 instead of LoTE buffer. LoTE buffer is again used in step 81. The SAGE ditags are more stable in the presence of the higher-salt TE buffer (see Anticipated Results).

73. Mix with 6 μ l of 6 \times LDS. Load 9 μ l into each of 4 lanes of 10-well 1.5-mm-thick 12% polyacrylamide gel. Use a 10-bp DNA ladder to provide markers. Perform gel electrophoresis at 150 V for 1.5 to 2 hr. Stop electrophoresis when the bromophenol blue marker is 1 cm from the bottom of the gels.

74. Stain gels with SYBR Green I at 1:10,000 dilution in 1× TAE for 20 min. Wash the gels with water for 5 min.
75. Locate and excise each band containing the 26-bp product using the technique described in step 56, above. Referring to steps 55 to 63, above, combine two excised gel bands and fragment through each of two punctured microcentrifuge tubes, elute DNA (except use standard TE buffer in place of LoTE at step 58 and incubate at 37° instead of 65°C at step 59), then purify on SpinX columns and precipitate and wash DNA pellet according to steps 60 to 63.

When eluting, be careful to incubate at 37°C instead of 65°C (see step 59); otherwise, the 26-bp cDNA SAGE tags may be degraded.
76. Resuspend the pellet in a total volume of 6.75 µl cold TE buffer on ice. Proceed immediately to the next step.

Ligate the SAGE ditags to form concatemers

77. Set up the following ligation reaction on ice and mix:

6.75 µl SAGE ditag sample (step 76)
2 µl 5× ligase buffer
1.25 µl 5 U/µl T4 DNA ligase.

Incubate 3 hr at 16°C.

78. After the ligation is complete, add 2 µl of 6× LDS, mix, and heat for 20 min at 65°C.
79. Load the 10-µl sample from step 78 in a single lane of a 10-well 1.5 mm-thick 8% polyacrylamide gel. Also load a 100-bp DNA ladder as a size marker. Perform electrophoresis at 100 V for 3 to 3.5 hr. Stop electrophoresis when bromophenol blue marker reaches a point 1 cm from the bottom of the gel.
80. Stain the gel with SYBR Green I at 1:10,000 dilution in 1× TAE for 20 min. Wash the gel with water for 5 min.
81. Locate and excise the product between 600 and 1200 bp from the sample lane of the gel using the technique described in step 56, above. Referring to steps 55 to 63, above, fragment the excised gel band through a punctured microcentrifuge tube, elute DNA, then purify on SpinX column and precipitate and wash DNA pellet according to steps 60 to 63.

These procedures are performed exactly as in steps 55 to 63; use LoTE buffer and elute with incubation at 65°C.

To be safe, save the gel region between 400 and 600 bp in case it is needed later; discard it if step 105 is successful.
82. Resuspend the pellet in a total volume of 6 µl LoTE buffer on ice. Proceed immediately to the next step.

Cloning and Sequencing of Concatemers (SAGE Tags)

Linearize pZero vector

83. Digest pZero-1 plasmid with *SphI* by combining:

1 µl 1 µg/µl pZero-1
1 µl 5 U/µl *SphI*
0.2 µl 100× (10 µg/µl) BSA
22 µl 10× NEBuffer 2
15.8 µl sterile H₂O.

Incubate at 37°C for 15 min.

84. Add 180 μl LoTE buffer to digestion mix, then transfer to a 2.0 ml tube containing precentrifuged Phase Lock Gel Light. Perform phenol/chloroform (PC8) extraction as in Support Protocol 2, and finally pipet the digest contained in the aqueous upper phase to a fresh tube.

85. Precipitate, wash, and air dry the plasmid DNA as in steps 23 to 28.

86. Resuspend the DNA in a total volume of 50 μl using LoTE buffer.

The final concentration of the digested vector should be about 20 ng/ μl .

87. Check the efficiency of *SphI* digestion by agarose gel electrophoresis (APPENDIX 3A) using a 2- μl aliquot of the digestion mix and a 2- μl aliquot of 100 ng/ μl undigested pZero-1 diluted with water on a 1% agarose gel. Use linearized pZero-1 vector immediately (step 88).

The DNA can be stored for 1 to 2 weeks at -20°C but the cloning efficiency may decrease.

Ligate concatemers into *SphI*-digested vector

88. Set up the following 10- μl ligation reaction on ice and mix:

- 6 μl concatemer sample (step 82)
- 1 μl of ~ 20 ng/ml digested pZero-1 vector (step 87)
- 2 μl 5 \times ligase buffer
- 1 μl 5 U/ μl T4 DNA ligase.

Incubate for 3 hr at 16°C .

Do not ligate at room temperature.

89. Adjust the volume of the above reaction mixture to 200 μl with LoTE buffer, and transfer to a 2-ml tube containing precentrifuged Phase Lock Gel Light. Perform phenol/chloroform (PC8) extraction as in Support Protocol 2, and finally pipet the concatemer ligated to digested contained in the aqueous upper phase, into a fresh 1.5- to 1.7-ml tube.

90. Precipitate, wash, and air dry the DNA pellet as in steps 23 to 28, above.

91. Resuspend the pellet in 12 μl water.

Transform DNA into *E. coli*

92. Add 1 μl of the resuspended DNA to 50 μl of One Shot TOP10 Electrocompetent *E. coli* suspension and mix gently.

93. Carefully transfer the cells and DNA to a chilled 0.1-cm electroporation cuvette on ice.

94. Electroporate the samples using Gene Pulser II and Pulse Controller with the following settings: voltage, 1.8 kV; capacitance, 25 μF ; resistance, 200 Ω .

95. Add 950 μl of room temperature SOC medium to the cuvette, mix, and transfer the contents to a 15-ml polypropylene snap-top tube. Shake the tube in a bacterial shaker at 37°C for 1 hr at 200 to 225 rpm with the lid vented.

96. Plate 50 μl from each transformation on a test 10-cm low-salt Zeocin-containing LB plate.

97. Incubate each of the test plates at 37°C to grow the transformed bacteria for 12 to 16 hr and store the remaining transformation reactions at 4°C .

98. The next day, depending on the number of colonies observed from the previous test plating, plate out all of the transformation reaction or dilute the transformation reaction appropriately to ensure sufficient space between colonies, and plate all bacteria on 10-cm low-salt Zeocin-containing plates.

The ideal number of colonies is roughly 200 to 300 per 10-cm plate.

Identify insert sizes

99. Set up 96 PCR reactions on ice using sterile 0.2 ml PCR tubes containing the following components:

2.5 μ l 10 \times PCR buffer
1.25 μ l DMSO
1.25 μ l 10 mM dNTP mix
0.5 μ l 350 ng/ μ l M13 forward primer
0.5 μ l 350 ng/ μ l M13 reverse primer (350 ng/ μ l)
19 μ l H₂O
0.2 μ l 5 U/ μ l Platinum *Taq* polymerase.

100. Add PCR components to wells of a 96-well PCR plate.
101. Use a micropipettor with a sterile 10- μ l tip containing an aerosol barrier to collect each colony (about 2 μ l of cell suspension). Transfer each colony to a separate well of PCR mix by pipetting the solution in and out several times.
102. Perform PCR using a 96-well thermal cycler using the following cycling parameters:

1 cycle:	2 min	95°C	(denaturation)
25-30 cycles:	30 min	95°C	(denaturation)
	1 min	56°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	5 min	70°C	(final extension).

103. Purify the resulting PCR products using the QIAquick 96 PCR purification kit system according to the manufacturer's instructions.
104. Perform gel electrophoresis using 2 μ l of each sample plus 0.4 μ l of 6 \times LDS on a 3% agarose gel (see APPENDIX 3A). Stain with ethidium bromide (use 1 μ g/ml by mixing 5 μ l 10 mg/ml ethidium bromide with 50 ml water; also see APPENDIX 3A).
105. Examine gel with a UV transilluminator. Select only wells showing PCR products over 600 bp, which should contain at least 25 tags, for sequence analysis.

To avoid low sequencing efficiency, an additional centrifugation step is recommended if the vacuum available for use with the QIAquick 96 PCR purification kit system is not sufficiently strong to remove all residual solution. Before eluting the PCR products with water, centrifuge the 96-column system at 3000 \times g for 5 min using a 96-well plate holder in a low-speed centrifuge.

Sequence the SAGE library and analyze the SAGE tags

106. Sequence the PCR products in 96-well plates by sending them to a DNA sequencing facility or company that can directly sequence PCR products, or sequence in-house (see Support Protocol 6).
107. Extract SAGE tags from sequence files using the newest version of SAGE 2000 Software.

SAGE software is required in order to extract SAGE tag data and to generate quantitative information from the raw sequence data. A widely used software system for this purpose is

SAGE 2000, which is freely available for noncommercial use through a Material Transfer Agreement with Johns Hopkins University. Following the instructions for obtaining the software on the Web site <http://www.sagenet.org>. This software uses the Windows 2000 or Windows 98 operating system and requires the prior installation of Microsoft Access. An alternative SAGE software system is eSAGE, developed by Margulies and Innis (2000), which is also freely available for noncommercial use (the e-mail contact is ehm@umich.edu). This latter software uses the Windows 95/98 or NT version 4.0 operating system, and also requires the installation of Microsoft Access.

108. Identify transcripts using the NCBI SAGE tag database: <http://www.ncbi.nlm.nih.gov/SAGE>.

PHENOL-CHLOROFORM (PC8) EXTRACTION

The application of Phase Lock Gels as part of the SAGE procedure, for purification of the DNA after phenol extraction, significantly increases the purity and recovery of the DNA. If using a 50-ml tube, a centrifuge with a swinging-bucket rotor is recommended.

Materials

Phase Lock Gel Light (PLG; Eppendorf; purchased in 2- or 50-ml tubes)
DNA solution to be extracted
PC8 solution (see recipe)

1. Precentrifuge Phase Lock Gel Light tube without added sample immediately prior to use. If using a 2-ml tube, microcentrifuge 20 sec at maximum speed, room temperature; if using a 50-ml tube, centrifuge 1 min at $1500 \times g$, room temperature.
2. Add the sample and an equal volume of PC8 solution to the tube.
3. Shake to mix; e.g., by inverting the tube seven times.
4. If using 2-ml tubes, microcentrifuge 5 min at maximum speed, room temperature; if using 50-ml tubes, centrifuge 5 min at $1500 \times g$, room temperature, in a swinging-bucket rotor.
5. Transfer aqueous (top) layer to a new microcentrifuge tube using a pipet.

KINASING OF LINKERS

To ligate DNA with oligonucleotide linker, 5' phosphorylation of the oligonucleotide linkers prior to ligation is necessary.

Materials

Linkers 1A, 1B, 2A, and 2B (see recipe)
LoTE buffer (see recipe)
10 \times kinase buffer (NEB)
10 mM ATP (NEB)
10 U/ μ l T4 polynucleotide kinase and 5 \times ligase buffer
6 \times Loading Dye Solution (Fermentas)
10-bp DNA ladder
12% polyacrylamide gel (see Support Protocol 5)
10 mg/ml ethidium bromide
1.5- to 1.7-ml RNase/DNase-free microcentrifuge tube (PGC Scientific or other supplier)
16 $^{\circ}$, 50 $^{\circ}$, and 65 $^{\circ}$ C water baths or heating blocks

**SUPPORT
PROTOCOL 2**

**SUPPORT
PROTOCOL 3**

**Whole Organism
and Tissue
Analysis**

19.3.19

Additional reagents and equipment for polyacrylamide gel electrophoresis (see Support Protocol 5)

1. Dilute linkers 1A, 1B, 2A, and 2B to 350 ng/μl with water.
2. Prepare the following reaction mixes in two separate ~1.5-ml RNase/DNase-free microcentrifuge tubes:

Tube 1:

9 μl 350 ng/μl linker 1B
6 μl LoTE buffer
2 μl 10× kinase buffer
2 μl 10 mM ATP
1 μl 10 U/μl T4 polynucleotide kinase.

Tube 2:

9 μl 350 ng/μl linker 2B
6 μl LoTE buffer
2 μl 10× kinase buffer
2 μl 10 mM ATP
1 μl 10 U/μl T4 polynucleotide kinase.

3. Incubate at 37°C for 30 min.
4. Heat-inactivate at 65°C for 10 min.
5. Mix 9 μl of 350 ng/μl linker 1A with 20 μl of the kinased linker 1B (i.e., from the reaction mix in tube 1).

The final concentration of both linker 1A and linker 1B should be 217.2 ng/μl.

6. Mix 9 μl of 350 ng/μl linker 2A with 20 μl of the kinased linker 2B (i.e., from the reaction mix in tube 2).

The final concentration of both linker 2A and linker 2B should be 217.2 ng/μl.

7. Anneal linkers by heating as follows:

95°C for 2 min
65°C for 10 min
37°C for 10 min
Room temperature for 20 min.

Store at -20°C.

The result of the kinasing reaction will be “annealed linker 1” and “annealed linker 2,” used in Basic Protocol 2.

8. Test effectiveness of the kinase reaction as follows.
 - a. Prepare the following mixture in a sterile microcentrifuge tube on ice to self-ligate each annealed linker pair:

1 μl 200 ng/μl linker 1 (annealed linker 1A and 1B, see step 5)
1 μl 200 ng/μl linker 2 (annealed linker 2A and 2B, see step 6)
5 μl LoTE buffer
2 μl 5× ligase buffer.
 - b. Complete the ligation reaction exactly as described in steps 14 to 16 of Basic Protocol 2.

- c. Mix 5 μl of reaction with 1 μl of 6 \times Loading Dye Solution (LDS from Fermentas) and load on a 12% PAGE gel. Use a 10-bp DNA ladder to provide markers. Perform gel electrophoresis at 150 V for 1.5 to 2 hr (see Support Protocol 5). Stop electrophoresis when the bromphenol blue marker is 1 cm from the bottom of the gel.
- d. Stain with ethidium bromide (use 1 $\mu\text{g}/\text{ml}$ prepared by mixing 5 μl of 10 mg/ml ethidium bromide with 50 ml water).
- e. Examine on a UV transilluminator for linker-linker dimers.

Kinased linkers should allow linker-linker dimers (80 to 100 bp) to form after ligation, while unkinased linkers will prevent self-ligation. Only linker pairs that self-ligate >70% should be used.

When linkers 1 and 2 are to be used at 15 ng/ μl in steps 12 and 13 of Basic Protocol 2, 15 μl of each linker (200 ng/ μl) are diluted with 185 μl of water.

GEL-SHIFT TEST FOR COMPLETE BIOTINYLATION OF BIOTIN-OLIGO dT

Biotinylated oligo(dT) primers synthesized by Integrated DNA Technology or by Invitrogen were used by the authors. Efficiency of binding to the streptavidin tube is an important factor for successfully constructing the T7-SAGE library and should be checked by investigators if they substitute another company's oligo(dT) primer.

Materials

- Biotinylated oligo(dT) primer (custom-synthesized, e.g., by Integrated DNA Technologies or core facility; gel-purified)
- 1 $\mu\text{g}/\mu\text{l}$ streptavidin
- 6 \times Loading Dye Solution (Fermentas)
- 100-bp DNA ladder
- 12% polyacrylamide gel (see Support Protocol 5)
- 10 mg/ml ethidium bromide

1. Prepare the following reaction mixes.

Tube 1:

- 2 μl 0.5 mg/ml biotinylated oligo(dT) primer
- 2 μl 1 $\mu\text{g}/\mu\text{l}$ streptavidin
- 6 μl H₂O.

Tube 2 (negative control, no streptavidin):

- 2 μl 0.5 mg/ml biotinylated oligo(dT) primer
- 8 μl H₂O.

Tube 3 (negative control, no primer):

- 2 μl 1 $\mu\text{g}/\mu\text{l}$ streptavidin
- 8 μl H₂O.

2. Incubate mixtures 15 min at room temperature.
3. Add 2 μl of 6 \times loading dye solution (LDS from Fermentas) to 5 μl of each reaction.
4. Load 6 μl of each reaction solution on a 12% PAGE gel. Use a 100-bp DNA ladder to provide markers. Perform gel electrophoresis at 150 V for 1.5 to 2 hr (see Support Protocol 5).
5. Stain with ethidium bromide (use 1 $\mu\text{g}/\text{ml}$ prepared by mixing 5 μl of 10 mg/ml ethidium bromide with 50 ml of water). Examine on a UV transilluminator.

SUPPORT PROTOCOL 4

If the oligo dT is well-biotinylated, the entire band should be shifted to a single band of higher molecular weight, and this gel shift should occur only in the lane containing the streptavidin.

**SUPPORT
PROTOCOL 5**

PREPARATION OF POLYACRYLAMIDE GELS USED IN SAGE ANALYSIS

Gels should be poured according to the following procedures before they are used in the respective protocols.

Materials

- 40% polyacrylamide (19:1 acrylamide:bis; Bio-Rad)
- 40% polyacrylamide (37.5:1 acrylamide:bis; Bio-Rad)
- 10× TAE buffer (APPENDIX 2A)
- 10% (w/v) ammonium persulfate (prepare fresh)
- TEMED
- XCELL II mini cell (Invitrogen)
- Gel electrophoresis cassettes with 1.5 mm spacers (Invitrogen)
- 10-well combs (Invitrogen)
- Power Pac 200 power source (Bio-Rad)

CAUTION: Refer to the manufacturer's instructions for electrophoresis conditions specific for the investigator's apparatus.

To prepare 12% PAGE gel (for purification of PCR products and ditags)

1a. Combine and gently mix (avoiding bubbles) the following reagents:

- 10.5 ml 40% polyacrylamide (19:1 acrylamide:bis)
- 20.7 ml H₂O
- 3.5 ml 10× TAE buffer
- 350 μl 10% ammonium persulfate
- 30 μl TEMED.

2a. Immediately pour into vertical gel apparatus (XCELL II mini cell) cassette with 1.5 mm spacers, or equivalent equipment. Add 10-well comb and allow gel to polymerize for at least 30 min.

To prepare 8% PAGE gel (for purification of concatemers)

1b. Combine and gently mix (avoiding bubbles) the following reagents:

- 7 ml 40% polyacrylamide (37.5:1 acrylamide:bis)
- 24.2 ml H₂O
- 3.5 ml 10× TAE buffer
- 350 μl 10% ammonium persulfate (make fresh)
- 30 μl TEMED.

2b. Immediately pour as described for 12% PAGE gel (step 2a).

DIRECT SEQUENCING USING A COMMERCIAL SEQUENCING KIT

If local arrangements can be made for direct sequencing of PCR products from the SAGE library after investigator preparation of samples for loading on an automated DNA sequencer, the following protocol using the ABI BigDye terminator sequencing system is recommended. This kit is suitable for performing fluorescence-based cycle sequencing reactions on PCR fragments using high-sensitivity dyes, e.g. 6-carboxyfluorescein (6-FAM).

Materials

ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit
(Applied Biosystems)

30 ng/μl PCR product to be sequenced (>600 bp; see Basic Protocol 2)

1 pmol/μl M13 forward primer

Low-speed centrifuge with 96-well plate holder

System for PCR using 96-well plates (e.g., GeneAmp PCR system 9700, Applied Biosystems)

Centri-Sep 96 plate (Princeton Separations)

Automated DNA sequencer (e.g., ABI Prism 3100 genetic analyzer system, Applied Biosystems)

1. For each reaction to be sequenced, combine the following reagents in individual wells of a 96-well PCR plate:

8 μl Terminator ready reaction mix from kit

4 μl 30 ng/μl PCR product

4 μl 1 pmol/μl M13 forward primer (1 pmol/μl)

4 μl H₂O.

2. Mix well and centrifuge 1 min at 3000 × g, 4°C. using a 96-well plate holder in a low-speed centrifuge.

3. Perform PCR using the following cycling parameters.

25 cycles: 10 sec 96°C (denaturation)

 5 sec 50°C (annealing)

 4 min 60°C (extension).

4. Perform a rapid thermal ramp down to 4°C and hold at that temperature until ready to purify.
5. Microcentrifuge briefly at maximum speed to bring down the contents of the tubes.
6. Transfer extension products to a Centri-Sep 96 plate using a multichannel pipet. Purify according to the manufacturer's instructions for the Centri-Sep plate.
7. For the library, cycle-sequence the concatemer inserts (PCR products) using an ABI Prism 3100 genetic analyzer system according to the manufacturer's instructions.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.

Linkers

Linker 1A:

5' TTT GGA TTT GCT GGT GCA GTA CAA CTA GGC TTA ATA GGG ACA
TG 3'

Linker 1B:

5' TCC CTA TTA AGC CTA GTT GTA CTG CAC CAG CAA ATC C [amino-
modified C7] 3'

Linker 2A:

5' TTT CTG CTC GAA TTC AAG CTT CTA ACG ATG TAC GGG GAC ATG 3'

Linker 2B:

5' TCC CCG TAC ATC GTT AGA AGC TTG AAT TCG AGC AG [amino-modi-
fied C7] 3'

When purchasing the above oligonucleotides from a company that custom synthesizes oligonucleotides (the authors recommend Integrated DNA Technologies), or when submitting requests to a DNA synthesis core facility, the modifications described above must be communicated along with the sequence: i.e., for linkers 1B and 2B, a 3' modification consisting of an amine moiety with a 7-carbon spacer. These oligonucleotides must be gel-purified; the investigator should request that this be done by the custom-synthesis company or core facility.

Amino-modified C7 is helpful to eliminate the native 3'-OH group from the oligo, which functionally blocks the oligo from participating as a primer in DNA synthesis, sequencing, or PCR (<http://www.idtdna.com/program/techbulletins/amino.asp>).

LoTE buffer

3 mM Tris·Cl, pH 7.5 (**APPENDIX 2A**)

0.2 mM EDTA, pH 7.5 (**APPENDIX 2A**)

Store at 4°C

PC8 solution

Combine the following in the order indicated:

480 ml phenol, prewarmed to 65°C

320 ml 0.5 M Tris·Cl, pH 8.0 (**APPENDIX 2A**)

640 ml chloroform

Shake and place at 4°C. After 2 to 3 hr, shake again. After another 2 to 3 hr, aspirate aqueous (top) layer. Divide into aliquots and store at -20°C.

PCR buffer, 10×

166 mM (NH₄)₂SO₄

670 mM Tris·Cl, pH 8.8 (**APPENDIX 2A**)

67 mM MgCl₂

100 mM 2-mercaptoethanol

Store at -20°C

SOC medium

0.5% yeast extract

2% tryptone

10 mM NaCl

2.5 mM KCl

10 mM MgCl₂

10 mM MgSO₄

20 mM glucose

Store at room temperature (stable for years)

Zeocin-containing low-salt LB plates

For 1 liter:

10 g tryptone

5 g yeast extract

5 g NaCl

Adjust the pH to 7.5 and add 15 g Bacto agar. Autoclave solution. Allow to cool to 50°C, then add Zeocin to 50 µg/ml and pour into 10-cm plates.

COMMENTARY

Background Information

In order to acquire reliable gene expression data from nanogram quantities of total RNA, this set of protocols optimizes amplification of low-abundance RNA samples by combining T7-based antisense RNA (aRNA) amplification (Van Gelder et al., 1990) with serial analysis of gene expression (SAGE). The T7 procedure yields up to 10⁶-fold linear amplification of high-fidelity aRNA from very small amounts of total RNA and is applicable regardless of whether total RNA or poly(A)⁺ RNA is used. The effectiveness and accuracy of T7 amplification has been confirmed in microarrays (Wang et al., 2000). Recently, this method has been applied to microarray analyses with laser-capture microdissected samples (Luo et al., 1999), generation of full-length cDNA libraries from a single prostate cancer-cell (Ying et al., 1999) and analysis of gene expression of single live neurons (Eberwine et al., 1992).

For T7 amplification, an RNA polymerase promoter is incorporated into each cDNA molecule by priming cDNA synthesis with a synthetic oligonucleotide containing the T7 RNA polymerase promoter. After synthesis of double-stranded cDNA (ds-cDNA), T7 RNA polymerase is added, and aRNA is transcribed from the cDNA template. The repetitive synthesis of multiple RNA molecules from a single cDNA template results in amplified aRNA, which serves as the starting material for synthesizing first-strand cDNA with reverse transcriptase using random primers.

This T7-SAGE protocol includes some modifications to increase the efficiency of the procedure. Biotinylated ds-cDNA (bds-cDNA) containing the T7 promoter, rather than plain ds-cDNA, is synthesized using a biotinylated primer in place of the usual T7 primer after two cycles of T7 amplification. As a result, bds-cDNA is synthesized from amplified aRNA, instead of poly(A)⁺ RNA, using a biotinylated primer. Consequently, the product can be added directly after silica-gel membrane column purification to a streptavidin-coated tube in order to

immobilize it for SAGE. This tube-based modification reduces losses of valuable material.

This technique synthesizes a broad range of sizes (~100 to 3000 bp) of double-stranded cDNA (bds-cDNA) of high purity. However, the *Nla*III enzyme used for subsequently making ditags is sensitive to different conditions. It has been observed that *Nla*III often digests DNA templates with low efficiency because of its short half-life, improper storage, or the sensitivity of this enzyme to the digestion conditions (Angelastro et al., 2000). In order to maximize the effectiveness of *Nla*III, the bds-cDNA for this protocol is purified by a silica-gel membrane column. The resulting 102-bp bands were clearer and easier to excise from the gel than in published microSAGE (Datson et al., 1999) and SAGE-Lite (Peters et al., 1999) procedures. In order to isolate and amplify very small quantities of samples in this SAGE procedure, the authors used the mRNA Capture Kit (Boehringer Mannheim) to carry out in single tubes the multiple steps of enzyme cleavage of cDNA, ligation of linkers to bound cDNA, and release of cDNA tags (Datson et al., 1999).

The use of tiny quantities of starting material demands an amplification step. The authors found that only two cycles of T7 amplification were necessary for 4 ng of starting material. In the original SAGE procedure, 25 to 28 cycles of PCR are performed to amplify the pool of ditags. Although this PCR step should be relatively free of bias because all ditags are of approximately equal length, preferential amplification of some ditags will occur and will increase the possibility of duplicate ditags (Datson et al., 1999). Because the possibility of duplicate ditags occurring by chance is generally very low, their presence is thought to be the result of nonrepresentative overamplification (van Kampen et al., 2000). The SAGE software counts each exclusive ditag combination only once, thereby removing duplicate ditags. However, the frequency of duplicate ditags, and of ditags that are too long or short, are good parameters for judging the accuracy of a SAGE

library (van Kampen et al., 2000). Two-step ditag PCR amplifications were found to yield large numbers of duplicate ditags (Peters et al., 1999) and the artifactual ditag 5'-TCCCCGTA-CANNNTTAATAGGGA-3' (Datson et al., 1999). In recent testing of the present protocol, the resultant T7-SAGE library contained only 0.8% repeated ditags (25 duplicated ditags out of 2986 tags), in contrast with a miniSAGE library containing 4% (160 duplicated ditags out of 3838 tags; Ye et al., 2000). The authors'

T7-SAGE results also compared favorably with previously reported results using SAGE-Lite and conventional SAGE: 8.6% (994 duplicated ditags out of 11,495 tags; Peters et al., 1999) and 8.3% (5726 duplicated ditags out of 68,691 tags; Velculescu et al., 1997), respectively. The reason that the frequency of duplicated ditags is lower with T7-SAGE and miniSAGE is most likely due to the elimination of additional PCR amplification. Moreover, besides having a lower rate of duplicate ditags, the T7-SAGE

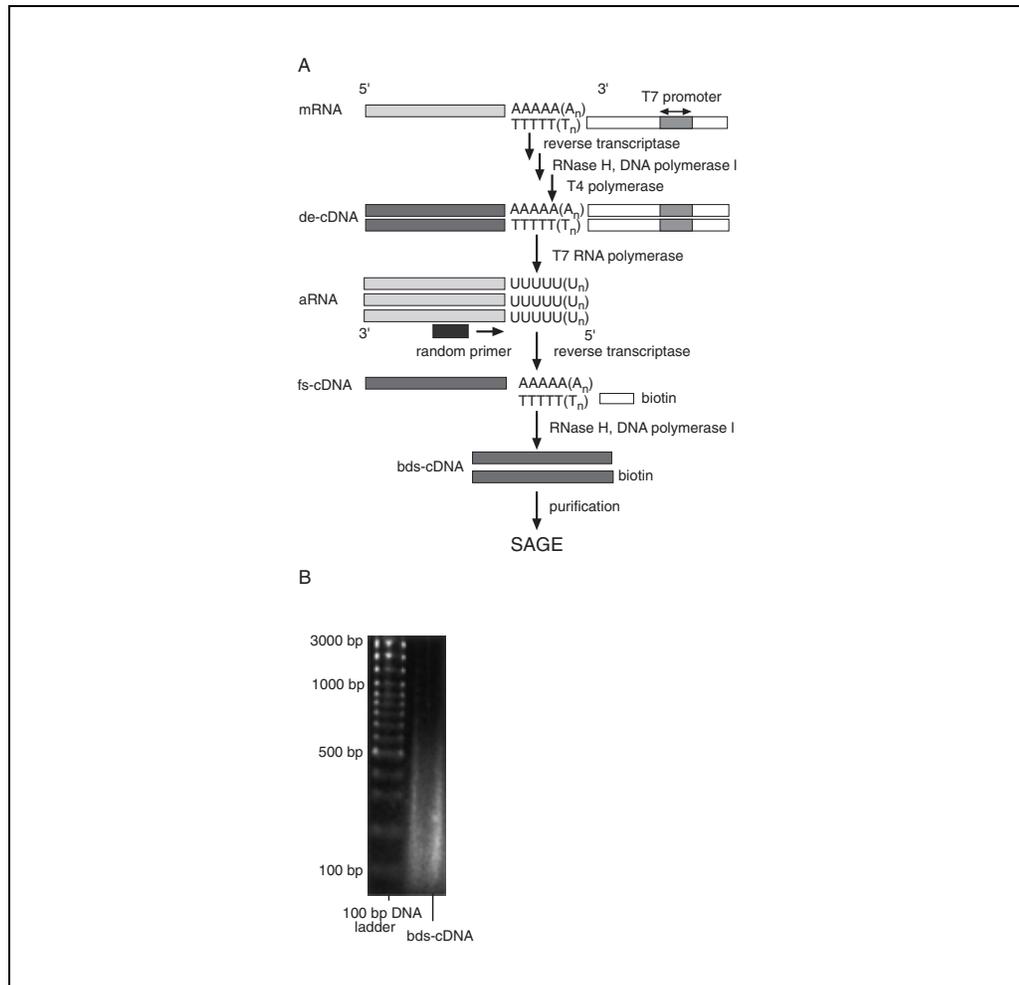


Figure 19.3.1 T7-based mRNA amplification and cDNA synthesis. **(A)** Schematic diagram of T7-based mRNA amplification. Modified from Van Gelder et al. (1990). Whole RNA is reverse-transcribed using a synthetic primer containing the T7 RNA polymerase binding site (sequences listed in Materials section of Basic Protocol 1). Second-strand cDNA synthesis to produce double-stranded (ds) cDNA is performed with *E. coli* DNA polymerase I and RNase H. After the cDNA is blunt-ended with T4 DNA polymerase, the cDNA is purified and transcribed with T7 RNA polymerase, yielding amplified antisense RNA (aRNA). These steps are repeated once to amplify the aRNA further. The amplified aRNA is reverse-transcribed using random primers to produce first-strand (fs) cDNA. Finally, biotinylated double-stranded (bds) cDNA is synthesized using a biotinylated oligo(dT) primer, *E. coli* DNA polymerase I, and RNase H, and then purified for SAGE. **(B)** Synthesis of biotinylated double-stranded (bds) cDNA from T7-amplified antisense RNA (aRNA). Input of total aRNA for the synthesis of bds cDNA is 4 nanograms, as indicated in Basic Protocol 1. This synthesis resulted in libraries of bds cDNA molecules that vary in length from ~100 to 3000 bp. Fragment sizes of the 100-bp DNA ladder are shown in bp.

library had no ditags that were too short, no ditags that were too long, and no other ditags indicative of artifacts.

Analysis of SAGE results is routinely performed using the NCBI SAGEtag database. Ongoing database additions continue to increase its value for the analysis of SAGE tags, and cDNA sequence data from RIKEN, NCI, and other sources should continue to promote SAGE research and to facilitate the identification of novel genes. Commercial databases using SAGE may also prove useful (e.g., Celera).

In conclusion, this T7-SAGE procedure, a new modification of SAGE, can be utilized in studies where mRNA is extremely limited. However, it is also effective for larger amounts of RNA, from which a 4- to 5-ng aliquot can be used. However, if large amounts of RNA are available, standard SAGE without amplification is recommended. Because studies of gene expression generally require confirmation of initial findings by independent methods such as RT-PCR and Northern blot analysis, any remaining RNA not used for this protocol can be saved for such additional analyses. Although

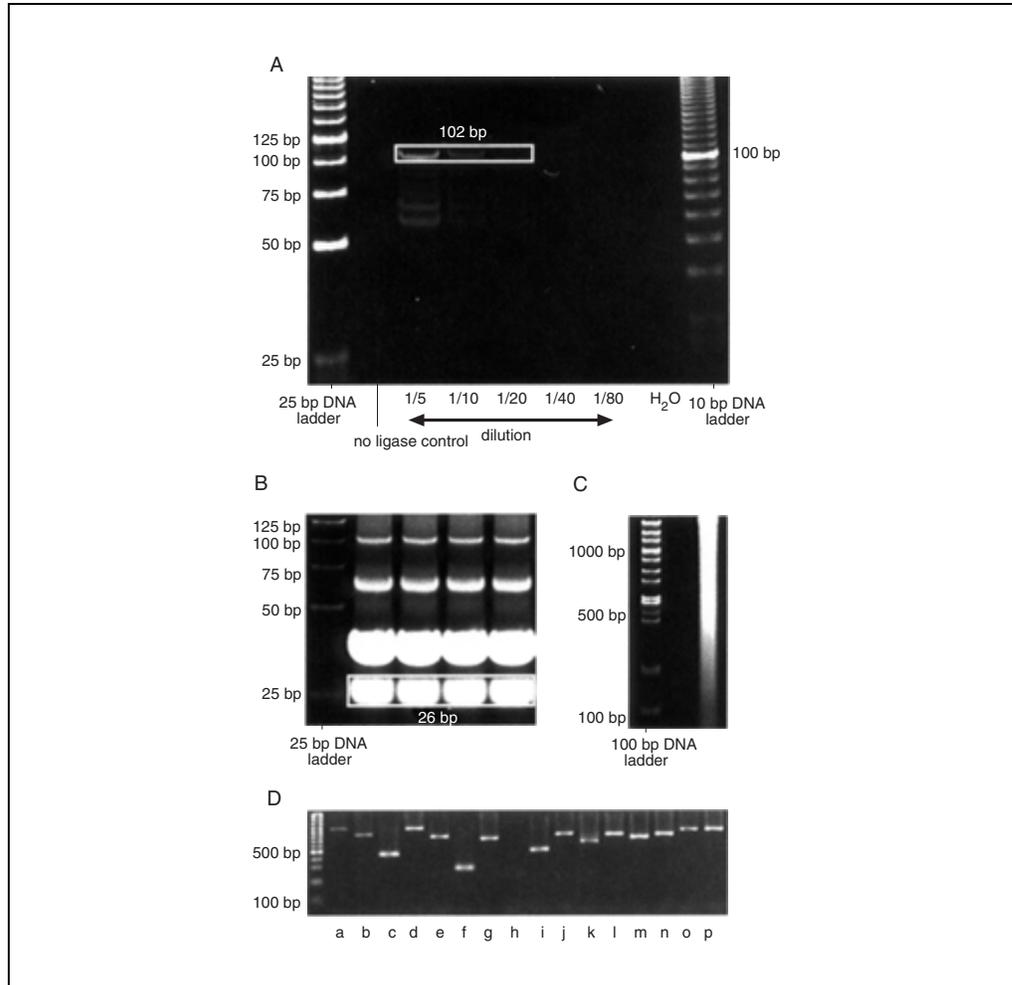


Figure 19.3.2 Selected steps of the SAGE procedure. **(A)** Linked SAGE ditags generated by T7-SAGE and separated on a 12% polyacrylamide gel. The 102-bp bands corresponding to amplified linked SAGE ditags are indicated by the rectangle. Shown after 28 PCR cycles are the no-ligase control, 1/5, 1/10, 1/20, 1/40 and 1/80 dilutions of the linked SAGE ditags, and the negative H₂O control (lanes 2-8), respectively. Lane 1, 25-bp DNA ladder. Lane 9, 10-bp DNA ladder. **(B)** SAGE ditags digested by *N/AIII* and separated on a 12% polyacrylamide gel. Clear 26-bp bands (SAGE ditags), 40-bp bands (linkers) and 60- to 102-bp bands (products with incomplete *N/AIII* digestion) should be observed. Fragment sizes of the 25-bp DNA ladder are shown in bp. **(C)** SAGE concatemer separated on an 8% polyacrylamide gel. A smear from ~100 bp to >1 kb should be observed. Fragment sizes of the 100-bp DNA ladder are shown in bp. **(D)** Example of sizes of inserts present in the T7-SAGE library. An insert of >300 bp (>500 bp band) will be contained in most of the clones (lanes a, b, d, e, g, and i to p). Lanes c and f contain small inserts. A 226-bp band indicates no insert (not shown). Fragment sizes of the 100-bp DNA ladder are shown in bp.

the authors have used T7-SAGE for global analysis of gene expression using only 4 ng of total RNA, they expect that the amount of starting material can be decreased further, perhaps down to a single cell. Microscale applications of SAGE such as this T7-SAGE method should help to broaden its future applications in studies of gene expression.

Critical Parameters and Troubleshooting

RNA preparation

It is essential to start with a clean preparation of RNA that is free of any salts or other substances that might inhibit reverse transcription. Successful RNA isolation from minute tissue samples depends on the complete elimination of ribonucleases. RNA and antisense RNA (aRNA) should be checked for intactness on a denaturing gel before cDNA synthesis if the investigator has sufficient RNA, or especially aRNA.

Purity of oligonucleotides

The quality of the primers is crucial to the success of the procedure. Where indicated (see materials lists of respective protocols), primers should be gel-purified, e.g., by the custom synthesis company from which they are purchased.

Production of 102-bp ditag-containing PCR products

Possible pitfalls in the synthesis of the 102-bp ditags include the following possible problems.

1. The cDNA was of poor quality or there were insufficient amounts. Check cDNA quality by gel electrophoresis and PCR. Check the extent of linker kinasing by self-ligation (see Support Protocol 3) and the extent of biotinylation of the oligo dT by a biotin gel shift test (see Support Protocol 4).

2. The protocols were not followed in exact detail. Because specific conditions in the protocol have been optimized, they should be followed as precisely as possible. For example, use the exact PCR sample sizes, buffers, and cycle temperatures described in the protocol.

3. Some reagent(s) were not of adequate quality. Obtain fresh reagents from the recommended companies.

4. The concentration of dNTPs was too high in the 102-bp PCR amplification. Although adding more dNTPs can increase amounts of PCR product, slightly exceeding a threshold can cause a paradoxical loss of the PCR product. Because of lot-to-lot variations in dNTP concentration, dNTPs should be titrated in a

preliminary test before doing the large-scale preparation, e.g., starting with the lowest dNTP concentration of 0.5 mM.

*Nla*III digestion of 102-bp ditag-containing PCR products

There are several reasons why the *Nla*III digestion may yield poor results.

1. The *Nla*III was not sufficiently active. *Nla*III should be stored in aliquots at -80°C , which helps to prolong its activity. Make sure the enzyme is fresh, since its half-life is only a few months even under optimal conditions. If the *Nla*III is less active and/or there are high yields of PCR product, more enzyme may be needed.

2. There was exonuclease contamination. The CATG cutting site of the PCR product may be missing because of exonuclease contamination or activity, e.g., from the DNA polymerase I. Phenol extractions and the use of Phase Lock Gel (PLG) is recommended to remove enzymes completely.

3. There were other interfering contaminants. The gel-purified 102-bp ditag preparation can be further purified by gel filtration before *Nla*III digestion as described by Angelastro et al. (2000).

Generation of large concatemers

Formation of sufficiently large concatemers depends on having sufficient amounts and purity of the ditags, as well as good ligation.

1. Problems can be caused by insufficient quantities of ditags present in the ligation reaction. The PCR protocol specified in the basic T7-SAGE protocol routinely uses 192 to 288 50- μl PCR reactions to ensure efficient production of 10 to 20 μg of 102-bp ditags. If a lower quantity of ditags is found, the safest course is to repeat the process to obtain at least 10 μg of ditags in order to ensure sufficient quantities for the *Nla*III digestion, so that enough 26-bp ditags are available for the ligation step to form concatemers.

2. The 26-bp ditags appear to degrade easily and should be kept at 4°C and in high-salt buffer at all times to prevent loss.

3. The 26-bp bands in gels are purified away from contaminating linkers by cutting them out with a razor blade. The cuts should be as close as possible to the bands to reduce contamination by materials of slightly different size, especially linkers, which interfere with the ligation reaction.

4. If linker contamination still appears to be causing poor concatemer formation, biotin-la-

beled primers can be substituted for primers 1 and 2 and then removed by binding to streptavidin beads (Powell, 1998).

Anticipated Results

Amplification of mRNA

The T7-based RNA amplification method (Fig. 19.3.1A) should be able to amplify the starting mRNA by ~1000-fold with one cycle (Luo et al., 1999). Theoretically, from 1 ng of total RNA containing roughly 1% mRNA (~10 pg), one mg of antisense RNA (aRNA) can be obtained after 2 cycles of T7 amplification. A clearly visible but smeared band (representing a range of RNA sizes) of amplified aRNA should be seen after running the sample on a gel.

Synthesis of biotinylated double-stranded (bds)-cDNA from aRNA

This synthesis should also result in a clearly visible but smeared zone of bds-cDNA molecules after running the sample on a gel; the bds-cDNA varies in length from ~100 to 3000 bp (Fig. 19.3.1B).

Synthesis of SAGE ditags (102 bp)

The 102-bp bands corresponding to amplified linked SAGE ditags should be clearly visible for excision and purification away from several other background bands (Fig. 19.3.2A). In the large-scale PCR amplification step using 96-well plates, 192 to 288 50- μ l PCR reactions are routinely performed. After pooling, this generates a total of 10 to 20 μ g ditags (see Basic Protocol 2, step 65).

NlaIII digestion of SAGE ditags

The 26-bp band corresponding to SAGE ditags without linker should be visible for purification away from other background bands (Fig. 19.3.2B). If the 26-bp band cannot be seen clearly, the investigator should confirm the efficiency of the *NlaIII* enzymatic reaction (see Troubleshooting above) and consider the possibility of degradation of ditags. The ditags occasionally melt in the presence of low salt, especially if the temperature rises above room temperature or if the DNA pellets are allowed to dry, although the ditags are stable in the presence of high salt. Therefore, subsequent procedures in Basic Protocol 2, such as, step 72, the overnight incubation in step 75, and then step 76, are performed under cold conditions, before the samples are placed in the high-salt TAE gel buffer. In addition, pellets are resuspended in TE instead of LoTE buffer.

Formation of SAGE concatemer

A clear high-molecular-weight smear above 400 bp should be visible in the gel (Fig. 19.3.2C). If the investigator cannot see evidence of these large concatemers, linker contamination or insufficient initial quantities of ditags are likely causes (see Critical Parameters and Troubleshooting).

The size of inserts present in the T7-SAGE library

An insert of >300 bp (>500-bp band) should be visible in most of the clones (Fig. 19.3.2D). A 226-bp band indicates the absence of any insert (negative control).

Time Considerations

In Basic Protocol 1, the entire procedure of T7-based RNA amplification, from steps 1 to 44, will require several days. In the Alternate Protocol, the procedure of T7-based RNA amplification using the RiboAmp kit from Arcturus will also require several days.

In Basic Protocol 2, the SAGE procedure from steps 1 to 105, can take several weeks, depending on the number of tags. Sequencing clones and analysis of sequence data, from steps 106 to 108, can require several weeks to months, depending on the number of tags and the efficiency of the sequencing facilities used.

In Support Protocol 1, RNA extraction takes about 1 hr, depending on the type of kit used. In Support Protocol 2, PC8 extraction requires about 30 min. In Support Protocol 3, the kinasing reaction for linkers takes about 4 hr. In Support Protocol 4, testing biotinylation of biotin-oligo dT requires about 3 hr. Support Protocol 5 takes 1 hr, and Support Protocol 6 takes 12 hr.

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SAGE Analysis from 1 μg of Total RNA

UNIT 19.4

Serial analysis of gene expression (SAGE; Velculescu et al., 1995; also see *UNIT 19.3*) is a powerful transcription-profiling method that allows simultaneous expression analysis of thousands of transcripts, provides absolute digital readout of the expression level, and identifies new genes. A disadvantage of SAGE is the relatively high amount of input RNA required. Consequently, several techniques have been developed to overcome this limitation, so that SAGE can be applied to very limited amounts of starting material, e.g., small biological samples such as tissue biopsies or microdissected materials. Here we describe a modified version of the original microSAGE protocol (Datson et al., 1999; also see <http://www.sagenet.org>), which requires only 1 to 2 μg total RNA. This method avoids PCR amplification of cDNA (Peters et al., 1999; Neilson et al., 2000) and reamplification of ditags (Datson et al., 1999; Virlon et al., 1999), procedures which potentially compromise the quantitative nature of this technique. Compared with conventional SAGE, this modified microSAGE protocol has several advantages: (1) reduction of material loss between steps with the use of a “single-tube” procedure for all steps from RNA isolation to tag release; (2) increase in the reaction efficiency with the use of Dynal magnetic beads instead of streptavidin-coated PCR tubes to ensure uniform surface contact; and (3) increase in the recovery and purity of the cDNA with the use of Phase Lock Gel for optimizing phase separation in the several steps that involve phenol extraction.

CONSTRUCTION OF SAGE LIBRARY FROM 1 μg OF TOTAL RNA

The protocol described here is suitable for creating a SAGE library from 1 to 2 μg of total RNA; for less starting material, refer to *UNIT 19.3*. After isolation of polyadenylated RNA using Dynabeads, the procedure includes the following operations: reverse transcription of mRNA into cDNA, digestion with *Nla*III to form one end of the tags, ligation with linkers 1 and 2, cleavage with *Bsm*FI, filling in 5' overhangs, and PCR amplification with ditag primers 1 and 2. To complete construction of the library, release of 26-bp ditags, ligation of ditags to form concatemers, cloning into pZERO vector, and sequencing are then performed as in *UNIT 19.3*, Basic Protocol 2.

Materials

- 1 to 2 μg of total RNA from tissues or cells
- DEPC-treated H₂O (*APPENDIX 2A*)
- Dynabeads mRNA Purification Kit (Dyna) including:
 - Oligo(dT)₂₅ beads
 - Binding buffer
 - Washing buffer B
- 1 \times first-strand/glycogen buffer (see recipe)
- Superscript Choice System for cDNA Synthesis kit (Invitrogen), including:
 - 5 \times first-strand reaction buffer
 - 0.1 M dithiothreitol (DTT)
 - 10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
 - SuperScript II reverse transcriptase
 - 40 U/ μl RNaseOUT recombinant ribonuclease inhibitor
 - 5 \times second-strand reaction buffer
 - 10 U/ μl *E. coli* DNA polymerase I
 - 10 U/ μl *E. coli* DNA ligase
 - 2 U/ μl *E. coli* RNase H
 - 1 U/ μl T4 DNA polymerase
 - 0.5 M EDTA, pH 7.5 (*APPENDIX 2A*)

**BASIC
PROTOCOL**

**Whole Organism
and Tissue
Analysis**

19.4.1

Contributed by Jerry Cai, David Ash, and Ethylin Wang Jabs

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Supplement 16

1× B&W/SDS/glycogen buffer (see recipe)
1× B&W/BSA buffer (see recipe)
1× NEBuffer 4/BSA solution (see recipe)
LoTE buffer (*UNIT 19.3*)
100× (10 µg/µl) BSA (New England Biolabs; supplied with corresponding restriction enzymes)
10× NEBuffer 4 (New England Biolabs; supplied with corresponding restriction enzymes)
10 U/µl restriction endonuclease *Nla*III (New England Biolabs)
5 U/µl T4 DNA ligase and 10× ligase buffer (Invitrogen)
Annealed linker 1 and annealed linker 2 (see *UNIT 19.3*, Support Protocol 3)
7.5 M ammonium acetate
20 µg/µl glycogen
70% and 100% ethanol
10 U/µl Klenow fragment of DNA polymerase (Amersham Pharmacia Biotech or USB)
Platinum *Taq* DNA polymerase (Invitrogen) and 10× PCR buffer
16°, 50°, 65°, and 75°C water baths or heating blocks
Safe-Lock tubes (Eppendorf) or microcentrifuge tubes
Magnetic Particle Collector (MPC; Dynal, cat. no. 120.20)
Sample mixer (Dynal, cat. no. 947.01)
8-well PCR strips, sterile (USA Scientific)
Thermal cycler
Additional reagents and equipment for PCR (*APPENDIX 3F*) and constructing a SAGE library (see *UNIT 19.3*, Basic Protocol 2)

Isolate polyadenylated RNA

1. Suspend 1 to 2 µg total RNA in a final volume of 100 µl DEPC-treated water in a Safe-Lock or microcentrifuge tube.
2. Heat 2 min at 65°C to disrupt secondary structure. Place on ice.
3. Resuspend Dynabeads oligo(dT)₂₅ beads provided in the mRNA Purification Kit by vortexing.
4. Add 100 µl of the resuspended oligo(dT)₂₅ beads to a new Safe-Lock or microcentrifuge tube. Put on magnetic particle collector (MPC) for 30 sec, then remove the supernatant solution.
5. Resuspend the beads in 100 µl binding buffer from the Dynabeads kit. Put on MPC for 30 sec, then remove the solution.
6. Add 100 µl binding buffer and the RNA solution from step 2 to the beads, for a total volume of 200 µl in the tube.
7. Rotate on the sample mixer for 3 to 5 min at room temperature to allow hybridization of poly(A)⁺ RNA to the oligo(dT)₂₅ which is immobilized on the beads. Put on MPC for 30 sec, then remove supernatant.
8. Gently wash beads twice, each time by adding 200 µl washing buffer B (provided with the kit) to the beads, then placing the tube on the MPC for 2 min and removing the solution.

Synthesize cDNA with SuperScript Choice kit

9. Wash beads four times with 100 μ l 1 \times first-strand/glycogen buffer using the technique described in step 8.
10. Add the following reagents (provided in the SuperScript kit) to the tube:
 - 8 μ l 5 \times first strand reaction buffer (directly from kit; no glycogen)
 - 4 μ l 0.1 mM DTT
 - 2 μ l 10 mM dNTP mix
 - 24 μ l DEPC-treated H₂O.
11. Mix the contents by gently vortexing and collect the reaction at the bottom of the tube by microcentrifuging for 3 sec at maximum speed. Incubate 2 min at 37°C.
12. Add 2 μ l SuperScript II reverse transcriptase. Incubate 1 hr at 37°C with gentle vortexing every 10 to 15 min, then place on ice to terminate the reaction.
13. Add the following reagents to the tubes:
 - 40 μ l first-strand reaction buffer
 - 60 μ l 5 \times second-strand reaction buffer
 - 182 μ l DEPC-treated H₂O
 - 6 μ l 10 mM dNTP mix
 - 2 μ l 10 U/ μ l *E.coli* DNA ligase
 - 8 μ l 10 U/ μ l *E.coli* DNA polymerase I
 - 2 μ l 2 U/ μ l *E.coli* RNase H.
14. Mix the contents of the tube by gently vortexing, then collect the reaction at the bottom of the tube by microcentrifuging briefly at maximum speed. Incubate at 16°C for 2 hr, vortexing gently every 10 to 15 min.
15. Add 4 μ l 1 U/ μ l T4 DNA polymerase. Incubate 5 min at 16°C.
16. Add 20 μ l 0.5 M EDTA, pH 7.5, to terminate the reaction.

Wash the beads

17. Add 300 μ l of 1 \times B&W/SDS/glycogen buffer, then heat at 75°C for 10 min.
18. Put on MPC for 30 sec, then remove the supernatant. Add 300 μ l of 1 \times B&W/SDS/glycogen buffer, resuspend by vortexing, then put on MPC for 30 sec. Remove the supernatant.
19. Wash beads four times with 300 μ l of 1 \times B&W/BSA buffer using the technique described in step 8.

Cleave the cDNA with anchoring enzyme NlaIII

20. Wash the beads with the bound ds cDNA in the Safe-Lock tube or microcentrifuge tube once with 200 μ l of NEBuffer 4/BSA solution using the technique described in step 8.
21. Add the following reagents to the tube:
 - 173 μ l LoTE buffer
 - 2 μ l 100 \times (10 μ g/ μ l) BSA
 - 20 μ l 10 \times NEBuffer 4
 - 5 μ l 10 U/ μ l *NlaIII*.

22. Mix by vortexing gently, making sure to avoid bubbles. Collect the reaction in the bottom of the tube by microcentrifuging briefly at maximum speed, then incubate at 37°C for 1 hr, vortexing gently every 10 to 15 min.
23. Inactivate *NlaIII* by washing the beads twice with 750 µl 1× B&W/SDS/glycogen buffer using the technique described in step 8. Wash an additional four times with 750 µl 1× B&W/BSA buffer in the same manner.
24. Add 150 µl of 1× T4 DNA ligase buffer and mix by vortexing. Put on MPC for 30 sec, then remove the supernatant.
25. Add 150 µl of 1× T4 DNA ligase buffer. Resuspend by vortexing. Split into two new Safe-Lock or microcentrifuge tubes, each of which should contain 75 µl solution with beads. Label these tube 1 and tube 2.

Ligate linkers to the bound cDNA

26. Put the two tubes prepared in step 25 on the MPC for 30 sec, then remove the supernatants.
27. Add the following to tube 1 and tube 2, respectively:

Tube 1:

13 µl LoTE buffer
 2 µl annealed linker 1
 4 µl 5× T4 DNA ligase buffer.

Tube 2:

13 µl LoTE buffer
 2 µl annealed linker 2
 4 µl 5× T4 DNA ligase buffer.

Mix each tube, then heat 2 min at 50°C, and let stand 15 min at room temperature.

28. Add 1 µl of 5 U/µl T4 DNA ligase to each of the tubes, and mix. Incubate 2 hr at 16°C, vortexing gently every 10 to 15 min.
29. Wash beads five times, each time with 50 µl of 1× B&W/BSA buffer, using the technique described in step 8.

Release cDNA with the tagging enzyme BsmFI

30. Wash each tube twice, each time with 200 µl of 1× NEBuffer 4/BSA solution, using the technique described in step 8.
31. Add the following to each of the tubes:

86 µl LoTE buffer
 10 µl 10× NEBuffer 4
 2 µl 100× (10 µg/µl) BSA
 2 µl 2 U/µl *BsmFI*.

Mix each of the reactions by vortexing gently and collect at the bottoms of the tubes by microcentrifuging briefly at maximum speed. Incubate at 65°C for 2 hr.

32. Put the tubes on the MPC for 2 min. Collect and retain the supernatants in separate tubes.
33. Wash beads in each tube twice with 50 µl LoTE buffer, using the technique described in step 8. Pool washings with the corresponding supernatants from step 32.

Each tube should now contain $100 + (2 \times 50) = 200$ µl of solution.

34. Perform phenol/chloroform/isoamyl alcohol extraction on supernatants from each tube using Phase Lock Gel Heavy (see Support Protocol).
35. Ethanol precipitate by adding the following:
 - 133 μ l 7.5 M ammonium acetate
 - 3 μ l 20 μ g/ μ l glycogen
 - 1000 μ l 100% ethanol.Mix well and place on dry ice for 10 to 20 min. Microcentrifuge 30 min at maximum speed, 4°C, then carefully remove and discard the supernatants without disturbing the pellets.
36. Wash each pellet twice, each time by adding 200 μ l of 70% ethanol down the wall of the tube with a micropipet, taking care to avoid disrupting the pellet, then decanting slowly. After the final wash, microcentrifuge briefly at maximum speed to collect residual ethanol. Remove all of the ethanol with a micropipet and air dry the pellets for 5 to 10 min. Resuspend each of the two pellets in 10 μ l LoTE buffer.

Blunt-end the linked SAGE tags

37. Add the following reagents to tubes 1 and 2 containing 10 μ l linked SAGE tags:

- 10 μ l 5 \times second-strand buffer (from SuperScript kit)
- 1 μ l 100 \times (10 μ g/ μ l) BSA
- 2.5 μ l 10 mM dNTP mix
- 23.5 μ l H₂O
- 3 μ l 10 U/ μ l Klenow fragment of DNA polymerase.

Incubate at 37°C for 30 min.

38. Adjust the volume in each tube to 200 μ l by adding LoTE buffer, then combine the solutions in both tubes (for a total of 400 μ l solution). Mix well and split into two new tubes labeled “+Ligase” and “–Ligase,” each containing 200 μ l solution.
39. Perform phenol/chloroform/isoamyl alcohol extraction on each of the solutions using 200 μ l Phase Lock Gel Heavy (see Support Protocol). Ethanol precipitate the DNA from each of the tubes as in steps 35 to 36, then resuspend each pellet in 4 μ l LoTE.

Ligate the linked SAGE tags to form linked ditags

40. Prepare the following two reaction mixes as follows:

+Ligase:

- 4 μ l resuspended DNA from “+Ligase” sample (step 39)
- 1.2 μ l 5 \times T4 DNA ligase buffer
- 0.8 μ l 5 U/ μ l T4 DNA ligase.

–Ligase:

- 4 μ l resuspended DNA from “–Ligase” sample (step 39)
- 1.2 μ l 5 \times T4 DNA ligase buffer
- 0.8 μ l H₂O.

Mix each of the reactions and incubate at 16°C overnight.

41. After ligation, add 14 μ l LoTE buffer to each of the reaction mixtures to raise the final volume to 20 μ l.

PCR amplify ditags

42. Dilute the following samples (from step 41) with sterile water as follows:

“-Ligase” (control) to 1/5 (1 μ l sample + 4 μ l H₂O).

“+Ligase” (ligation product) to 1/5, 1/20, 1/50, 1/100, 1/200, 1/400.

43. Set up 8 PCR reactions using a sterile 8-well PCR strip as follows:

Positions 1 to 6: Different dilutions of the template (“+ligase”; step 42)

Position 7: No-ligase (“-ligase”) control

Position 8: H₂O control.

44. To each well add the following:

5 μ l 10 \times PCR Buffer (supplied with Platinum *Taq* polymerase)

1 μ l 10 mM dNTP mix

1.5 μ l 50 mM MgCl₂

1 μ l 350 ng/ μ l primer 1 (UNIT 19.3)

1 μ l 350 ng/ μ l primer 2 (UNIT 19.3)

38.5 μ l H₂O

1 μ l Platinum *Taq* DNA polymerase

1 μ l ligation product (at various dilutions) or control (see step 43).

45. Mix and amplify using the following cycle conditions:

1 cycle:	4 min	95°C	(denaturation)
26-30 cycles:	30 sec	95°C	(denaturation)
	1 min	55°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	4 min	72°C	(final extension).

46. Pick the lowest dilution of template that gives a clear 102-bp PCR product.

The optimum PCR conditions are critical for isolating adequate DNA for SAGE. Using too few cycles or a substrate that is too diluted will result in a low yield, which may cause problems in the subsequent steps. Too many cycles or a substrate that is too concentrated will give erratic results. Therefore, it is very important to pick the right parameters to do large-scale PCR.

47. Using the PCR conditions in step 45 and the dilution identified in step 46, perform several hundred PCR reactions.

Usually 384 reactions are performed in four 96-well plates to generate 10 to 20 μ g DNA.

48. Complete the construction of the SAGE library continuing with step 43 of Basic Protocol 2 in UNIT 19.3 (“Isolating Linked Ditags”).

SUPPORT PROTOCOL

PCI EXTRACTION

Phase-Lock Gel will form a semisolid barrier, so one can extract the aqueous layer without contacting the organic phase. The advantage to using Phase-Lock Gel over the standard phenol/chloroform/isoamyl alcohol extraction is that it saves time while optimizing the recovery of DNA.

Materials

DNA solution to be extracted

25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol solution (PCI; Invitrogen)

Phase-Lock Gel Heavy in 2-ml tubes (Eppendorf)

1. Microcentrifuge the 2-ml Phase-Lock Gel Heavy tube for 1 min at maximum speed without added sample.
2. Add the sample and an equal volume of PCI solution to the tube.
3. Shake to mix.
4. Microcentrifuge 5 min at maximum speed.
5. Transfer the top aqueous phase to a new microcentrifuge tube using a pipet.

SUBSTITUTING *Hsp92II* FOR *NlaIII* AS THE ANCHORING ENZYME

The anchoring enzyme *NlaIII* is used, but it is very unstable, even at -80°C . *Hsp92II* has the same recognition and cut site, 5'-CATG-3', as *NlaIII*, so it can also be used as an anchoring enzyme.

Additional Materials (also see *Basic Protocol*)

10 U/ μl restriction endonuclease *Hsp92II* (Promega)
100 \times BSA and 10 \times buffer K (provided with *Hsp92II*)

1. Isolate polyadenylated RNA and synthesize cDNA with SuperScript system (see *Basic Protocol*, steps 1 to 19).
2. Wash the beads with the bound ds cDNA in the Safe-Lock or microcentrifuge tube from step 19 of the *Basic Protocol* once with 200 μl of 1 \times NEBuffer 4/BSA solution, using the washing technique described in step 8 of the *Basic Protocol*.
3. Add the following reagents to the tube:
 - 173 μl LoTE buffer
 - 2 μl 100 \times BSA
 - 20 μl 10 \times Buffer K
 - 5 μl 10 U/ μl *Hsp92II*.
4. Mix by vortexing gently, making sure to avoid bubbles. Collect the reaction in the bottom of the tube by microcentrifuging briefly at maximum speed, then incubate at 37°C for 1 hr, vortexing gently every 10 to 15 min.
5. Inactivate *Hsp92II* by washing twice with 750 μl 1 \times B&W/SDS/glycogen buffer using the washing technique described in step 8 of the *Basic Protocol*, then wash an additional four times with 750 μl 1 \times B&W/BSA buffer in the same manner.
6. Continue with the SAGE procedure (see *Basic Protocol*, steps 24 to 48).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

B&W/BSA buffer, 1 \times

To prepare 4 ml, mix the following:

2 ml 2 \times B&W buffer (see recipe)
40 μl 100 \times (10 $\mu\text{g}/\mu\text{l}$) BSA (New England Biolabs; provided with the restriction enzymes)
1.96 ml H_2O
Store up to 1 week at 4°C

ALTERNATE PROTOCOL

B&W buffer, 2×

To prepare 10 ml, mix the following:

100 μ l 1 M Tris-Cl, pH 7.5 (APPENDIX 2A)

20 μ l 0.5 M EDTA, pH 7.5 (APPENDIX 2A)

4 ml 5 M NaCl (APPENDIX 2A)

5.88 ml H₂O

Store up to 1 week at room temperature

B&W/SDS/glycogen buffer, 1×

To prepare 3 ml, mix the following:

1.5 ml 2× B&W buffer (see recipe)

30 μ l 1 μ g/ μ l glycogen

300 μ l 10% (w/v) SDS

1.17 ml H₂O

Prepare fresh just prior to use

First strand/glycogen buffer, 1×

To prepare 500 μ l, mix the following:

100 μ l 5× first-strand buffer (Invitrogen; provided with the SuperScript II kit)

5 μ l 1 μ g/ μ l glycogen (prepare with DEPC-treated H₂O; store at 4°C)

395 μ l DEPC-treated H₂O (APPENDIX 2A, or provided with SuperScript II kit)

Store up to 1 week at 4°C

NEBuffer 4/BSA solution, 1×

To prepare 2 ml, mix the following:

200 μ l 10× NEBuffer 4 (New England Biolabs)

20 μ l 100× (10 μ g/ μ l) BSA (New England Biolabs; provided with restriction enzymes)

1.78 ml H₂O

Store up to 1 week at 4°C

COMMENTARY**Background Information**

The Basic Protocol in this unit optimizes the construction of a SAGE library from 1 μ g of total RNA by combining Dynabeads mRNA isolation with SAGE. Preparation of high-quality and full-length mRNA is essential for gene-expression studies. Dynabeads Oligo(dT)₂₅ was chosen in the protocol to isolate poly(A)⁺ RNA from total RNA. The isolation protocol relies on the base pairing between the poly(A) residues at the 3' end of mRNA and the oligo (dT) residues covalently coupled to the surface of the Dynabeads. The unique paramagnetic properties of Dynabeads facilitate easy and efficient handling, while also reducing the requirement for centrifugation and precipitation steps, thereby significantly increasing yield. Using a solid phase enables all procedures to be conducted in a single tube and ensures that losses caused by multiple sample manipulations are minimized. This is particularly important in this protocol, since the starting total

RNA is only 1 to 2 μ g, and the detection of low-abundance transcripts is desired. The true spherical shape and the hydrophilic surface of the Dynabeads Oligo(dT)₂₅ also eliminates clumping and nonspecific binding associated with irregularly shaped particles, achieving uniformity of the reaction. The isolated mRNA can then be reverse transcribed to double-stranded cDNA and subjected to the standard SAGE procedure (also see <http://www.sagenet.org>).

It is well known that *Nla*III is very unstable even at -80°C. *Nla*III often digests DNA with low efficiency because it is very sensitive to digestion conditions. Recently the authors switched to *Hsp*92II, which is an isoschizomer of *Nla*III, but has a long half-life and is not very sensitive to digestion conditions. The resulting digestion (see Alternate Protocol) is improved and more consistent, giving a better yield of SAGE tags.

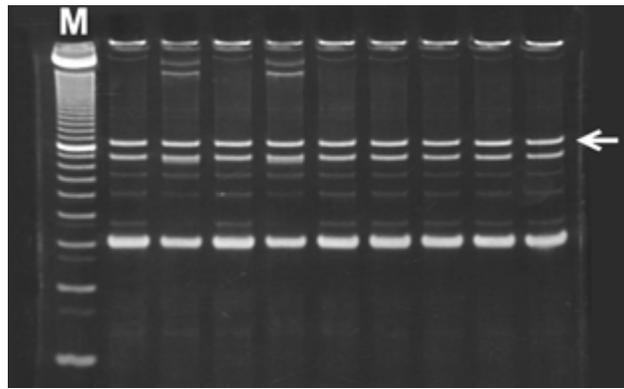


Figure 19.4.1 Linked SAGE ditags from large-scale PCR amplification are separated on a 12% polyacrylamide gel. The 102-bp bands corresponding to the linked SAGE ditags are indicated by an arrow. Lane M, 10-bp DNA ladder.

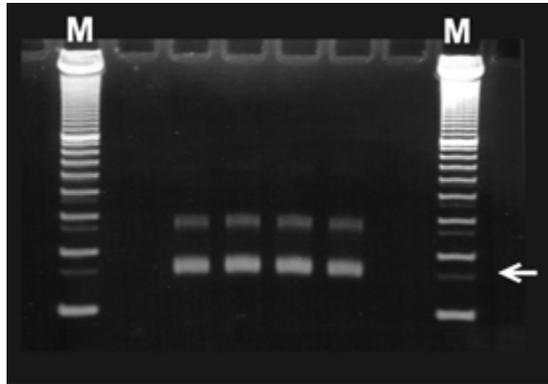


Figure 19.4.2 SAGE ditags digested by *NlaIII* are separated on a 12% polyacrylamide gel. The 26-bp bands corresponding to the SAGE ditags without linkers are indicated by an arrow. The other bands (around 40-bp) are linkers. Lane M, 10-bp DNA ladder.

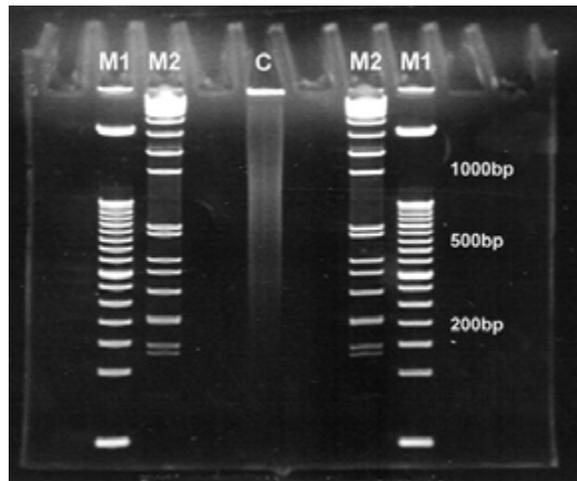


Figure 19.4.3 SAGE concatemers are separated on an 8% polyacrylamide gel. The concatemers appear as a smear in the lane indicated by C. Lane M1, 50-bp DNA ladder; lane M2, 1-kb DNA ladder.

Readers should also see the Commentary in *UNIT 19.3* to get more background information about SAGE.

Critical Parameters and Troubleshooting

RNA preparation

RNA quality is very critical to the success of the SAGE protocol. A high-quality RNA preparation without DNA contamination, salt, or other substances that might inhibit enzymatic activities is desired. If there is sufficient RNA, the integrity of the RNA should be checked on a denaturing RNA gel before the experiment.

mRNA isolation and cDNA synthesis

Isolation of mRNA is performed with the Dynabeads mRNA isolation kit. Since the beads tend to precipitate to the bottom, it is necessary to mix the solution from time to time during the incubation period by hand vortexing (flicking the bottom of the tube with one's finger) to ensure the uniformity of bead suspension.

Also see the Critical Parameters and Troubleshooting section in *UNIT 19.3* for more information.

Anticipated Results

Synthesis of 102-bp SAGE ditags

Figure 19.4.1 shows the typical result after large-scale PCR reaction. The 102-bp bands of linker SAGE ditags should be clearly visible for purification. This step usually generates 10- to 20- μ g ditags.

NlaIII digestion of SAGE tags

Figure 19.4.2 shows a typical result after releasing 26-bp ditags by *NlaIII* digestion. The 26-bp bands of SAGE ditags without linkers should be clearly visible from background bands. Since *NlaIII* is unstable even at -80°C , *Hsp92II* may be used instead (see Alternate Protocol).

Formation of SAGE concatemers

Figure 19.4.3 shows a typical result of formation of SAGE concatemers. A smear should be clearly visible.

Sequencing clones and analysis of sequence data

From 1 to 2 μ g total RNA, it is possible to generate 50,000 SAGE tags.

Time Considerations

In the Basic Protocol, steps 1 to 47 (including the large-scale PCR performed in step 47) take 1 week to complete. After large-scale PCR, step 48 in the Basic Protocol of this unit (which would comprise steps 43 to 106 in Basic Protocol 2 of *UNIT 19.3*) takes 2 weeks to complete.

The time requirement for sequencing clones and analysis of sequencing data depends on the number of tags and efficiency of the sequencing facilities used.

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The Chick Chorioallantoic Membrane as an In Vivo Angiogenesis Model

UNIT 19.5

**BASIC
PROTOCOL**

This unit presents the basic steps involved in the chick chorioallantoic membrane (CAM) assay, a model originally developed to study the angiogenic activity of tumor samples (Ausprunk et al., 1974; Auerbach et al., 1974). One of the major advantages of this method is that it is the simplest of the in vivo angiogenic models. In contrast to other in vivo models, the CAM assay does not require any major training or highly technical surgical skills. In addition, there is no need for sophisticated equipment, and it does not require an animal protocol. The uninitiated can quickly learn this simple method. Although the CAM assay is a simple procedure, it requires considerable “hands-on” effort for the following two reasons. First, the initial number of eggs needed per data point (~20) is large due to embryo mortality during the assay, and second, the background of positive responses is ~20%. Other disadvantages to this assay include the fact that it takes 10 days to complete and that the number of samples that can be tested is limited by the number of embryos required. Therefore, the CAM assay is not recommended as a screening technique, but rather as a procedure that can be used to confirm results from other experiments suggesting that a substance has a potential to be active in vivo. Overall, the advantages well outweigh the disadvantages, since it is an in vivo assay that can be readily performed in any laboratory setting.

Materials

3-day-old embryonated chicken eggs (~20 eggs/condition), maintained in 37°C incubator (an egg incubator is not necessary)

70% ethanol

Test substance(s)

Positive control: bFGF (PeproTech) or another angiogenic stimulus

Phosphate-buffered saline (PBS; APPENDIX 2A)

Heavy cream

Sterile dissecting implements:

Scalpel

Curved tweezers

Smooth-tipped (nonserrated) tweezers

Scissors

6-ml syringes, sterile

20-G, 1.5-in. (3.5-cm) needles, sterile

1-in. (2.5-cm) wide clear adhesive tape

Single-edged razor blades

13-mm-diameter Thermanox coverslips (Nunc), sterile

Stereoscopic microscope of variable magnification, equipped with camera and light source

NOTE: As an additional, optional sterility precaution, this protocol may be performed in a sterile laminar-flow hood (also see UNIT 1.3).

**Whole Organism
and Tissue
Analysis**

19.5.1

Contributed by M. Lourdes Ponce and Hynda K. Kleinman

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Supplement 18

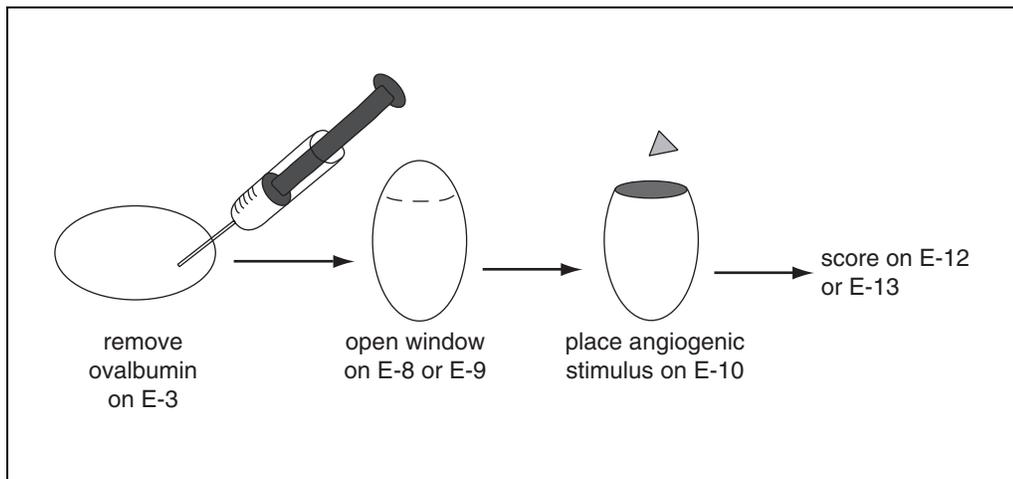


Figure 19.5.1 Schematic representation of the CAM procedure. Using a syringe and needle, the first step is to remove 3 to 4 ml of ovalbumin from 3 day-old embryonated eggs. The next step is to open a window on E-8 or E-9 after scoring the shell with a razor blade and pulling the egg shell out with a pair of tweezers. After the sample is dried out on the coverslip, the stimulus is placed on the CAM for 2 to 3 days. The eggs are then evaluated for angiogenesis under a stereoscopic microscope.

Prepare eggs

1. On embryonal day 3 (E-3), when the embryo is still small, prepare eggs to be used for the procedure by spraying them with 70% ethanol to minimize contamination. Make a small hole with a scalpel on the wide end of the egg, where the air sac is located (Fig. 19.5.1).

IMPORTANT NOTE: Also spray eggs with 70% alcohol before each subsequent step in which they are to be opened.

2. Lay the egg sideways (Fig. 19.5.1). Insert the sterile needle with syringe into the hole at an angle of $\sim 45^\circ$, without touching the far end of the egg shell, to a depth of ~ 1 to 1.25 in. (equivalent to 2.5 to 3.1 cm), and gently remove 3 to 4 ml of ovalbumin. Return the egg to its original position.

If yolk is being removed, withdraw the needle and reposition it to withdraw ovalbumin.

The ovalbumin ("egg white") is pale yellow in color. If any bright yellow-orange material is seen in the syringe, yolk is being aspirated and the viability of the embryo could be compromised. Ovalbumin is removed to lessen the volume of material in the egg so contents do not spill out when the window is opened.

If only a small amount of yolk is removed, continue to incubate the egg as the embryo may survive.

3. Cover the hole with a small piece of tape, 0.5 to 0.75 in. (1.25 to 1.9 cm) long. Proceed in a similar manner with the rest of the eggs, making sure to return them to the incubator within 45 min to avoid decreased viability. Continue incubation.
4. On E-9, using a single-edged razor blade, score a circle (~ 1 in., or 2.5 cm, in diameter) or a square (of ~ 1 in.², or 6.25 cm²) through the tape covering the egg (Fig. 19.5.1). Insert sterile curved tweezers through the needle hole created on E-3, puncturing the tape, and gently push up from inside the egg against the eggshell and all around the scored area until the shell comes out to produce an opening of the above dimensions in the eggshell. Remove any remaining membrane from the area immediately inside the window and close the opening with tape. Discard unviable embryos or unfertilized eggs.

If it is not possible to open the window on E-9, it can alternatively be done earlier on either E-7 or E-8, as necessary. Earlier times are not recommended since it increases the chances of contamination and decreases the number of viable chicks.

Unviable or dying embryos can be detected by observation. In these cases, vessels do not appear intact, the egg yolk looks discolored, and/or the embryo no longer moves.

Prepare the angiogenic stimuli

5. On E-10, examine embryos and determine the number of viable eggs; discard unviable or dying embryos (see annotation to step 4). Calculate the number of samples to be prepared for each condition.

It is important that there be at least 12 eggs for each sample since some of the embryos will continue to die.

6. Using sterile conditions and instruments, cut the coverslips into quarters with scissors while holding with sterile smooth-tipped tweezers in such a way as to avoid scratches.
7. Dissolve the test substance in distilled water and place a 5- μ l aliquot containing the desired amount to be tested in the center of one quarter of a coverslip. Prepare a minimum of 12 coverslips per sample (it is advisable to test at least two different concentrations of each test substance). Include controls that contain water only (negative control) or 50 ng of bFGF (positive control).

Samples should be dissolved in water. The presence of buffers or salts might create crystal deposits that can yield false positive results. Variations of the assay that allow the presence of salts, buffers, media or cells have been developed using small collagen gels (see Gho et al., 2001).

8. Allow the coverslips to air dry for 10 to 20 min. Open the window by cutting off the tape from each egg, then place the air-dried coverslip containing the corresponding sample on top of the CAM with the sample in direct contact with the CAM. Cover the eggs with tape, and return them to the incubator.

The area selected for placing the coverslip should be free of large vessels and away from any eggshell that might have fallen in on the CAM, since the shell can induce angiogenesis.

Score CAM for angiogenesis and photograph

Scoring of the CAM can take place on either day E-12 or E-13 depending on the degree of angiogenesis elicited by the test compound. Therefore, it is recommended that the CAM be examined on both days and that the best day for scoring be determined depending on the number of positive samples observed.

9. Remove the tape from the egg and using a stereoscopic microscope, look at the area where the coverslip was placed.
10. Score the CAMs by assessing the number of positive or negative responses or by assigning a score number varying from 0 to 3, in which 0 is no response and 3 is a strong response.

A strong positive angiogenic stimulus will result in vessels that radiate from the center of the coverslip simulating spokes from a wheel (Fig. 19.5.2). When the effect is not as strong, smaller and fewer vessels will be observed. In some instances, a very weak response can be detected in the form of small capillaries. Another way in which a very weak response can be seen is by observing the orientation of the major vessels near the area of the stimulus; the vessels may appear to make a turn towards this area, which is evidence of a very weak stimulus. These very weak responses are scored as 1 (see Fig. 19.5.2).

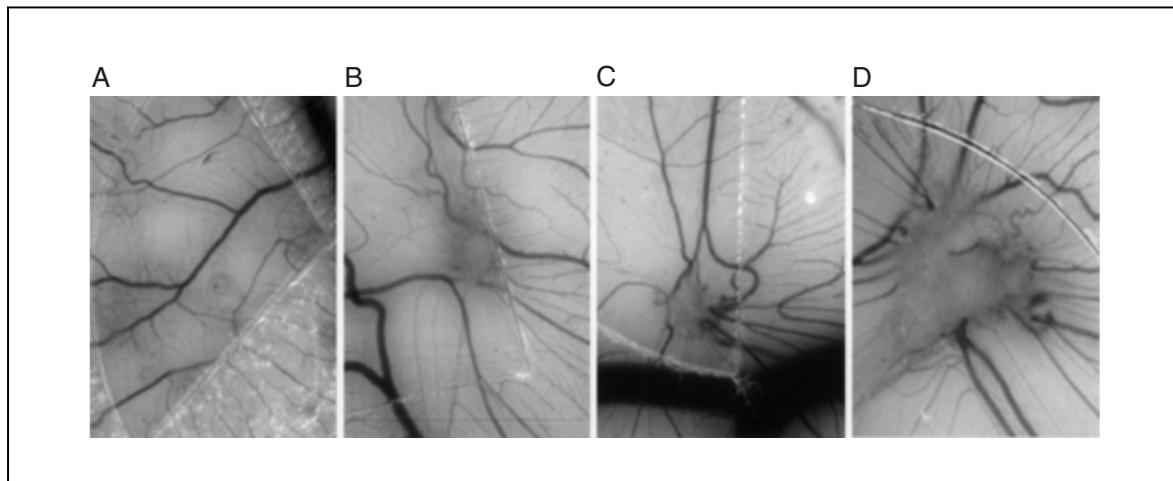


Figure 19.5.2 Positive responses and scoring. Examples of CAMs show different degrees of positive response. **(A)** A typical negative control showing normal CAM vessel levels but no induction of angiogenesis. Score = 0. **(B)** A slight response is observed in which some of the major vessels turn towards the stimuli. Smaller newly formed vessels are also observed. Score = 1. **(C)** A stronger angiogenic response is present. More, larger vessels are recruited to the area and a small “spoke wheel” begins to appear. Score = 2. **(D)** An intense angiogenic response is shown with large vessels and many smaller ones. The spoke wheel is present. Score = 3.

- 11a. *Photograph the CAM in situ:* Without dissecting, inject 3 to 4 ml of contrasting medium under the CAM consisting of a 1:1 mixture of PBS and heavy cream (or a similar substance with high buoyancy that would provide a contrasting background).
- 11b. *Photograph the CAM in dissected membrane:* Dissect out the area where the stimulus was placed using scissors and forceps, transfer it to a tissue culture dish, place the dish on a contrasting material, and photograph using the camera attached to the stereoscopic microscope.

COMMENTARY

Background Information

The formation of new blood vessels from pre-existing ones, i.e., angiogenesis, is important for physiological processes that occur during pregnancy, the menstrual cycle, and wound repair (Smith, 2001; Reynolds and Redmer, 2001; Sherer and Abulafia, 2001; Tonnesen et al., 2000). In addition, several pathological conditions have been associated with changes in the normal number of vessels present in various tissues. For instance, an increase in the level of the vascular supply has been linked to rheumatoid arthritis, psoriasis, corneal angiogenesis, and diabetic retinopathy, one of the leading causes of blindness in the U.S.A. (Arbiser, 1996; Yoshida et al., 1999; Campochiaro, 2000; Brenchley, 2001; Chang et al., 2001; Spanger and Pfeiffer, 2001). Vascular insufficiency, on the other hand, can also lead to serious conditions, such as myocardial ischemia, a form of heart disease, or ischemia of the limbs which can result, in extreme cases, in amputation (Majesky, 1996; Freedman and Is-

ner, 2001; Vale et al., 2001). In recent years, angiogenesis has been the focus of numerous studies that utilize antiangiogenic agents to inhibit cancer. The vascularization of tumors is essential for their growth and, in advanced stages, for metastasis. In essence, tumor growth and metastasis could be prevented by utilizing drugs that stop new vascularization. A number of laboratories have focused their efforts on the development of new antiangiogenic therapies that can affect tumor vessel formation. Several of these compounds are currently in clinical studies, including, but not limited to, endostatin, which is a collagen XVIII fragment, as well as thalidomide, interferon α , and antibodies to vascular endothelial growth factor and to integrin $\alpha v \beta 3$.

Angiogenesis is a complex multistep process that is usually initiated by a signal, an angiogenic stimulator, produced and/or released in the surrounding microenvironment. When the signal reaches the endothelium and binds to its receptor, the cell becomes activated

and the angiogenic cascade is initiated. This cascade includes degradation of the matrix, migration of endothelial cells, proliferation, establishment of a new matrix, and tubule formation and maturation. Due to the large number of conditions that are affected by angiogenesis and to the complexity of the process, it has been necessary to develop numerous methodologies for its study. Various *in vitro* assays have been used for this purpose and include migration, proliferation, formation of tubes on Matrigel or collagen gels, and aortic ring sprouting. *In vivo* studies include the rabbit corneal pocket assay, implanted sponges, subcutaneous Matrigel plugs, and the chick chorioallantoic membrane (CAM) assay, among others.

Angiogenesis in the chick CAM is a process that can be readily studied. During the development of the chick, the CAM undergoes rapid neovascularization—i.e., the formation of new vessels—which is complete by embryonal day 10 (E-10). When this neovascularization stops, the angiogenic process can be investigated. For this reason, the CAM assay is usually initiated on E-10, although some investigators have reported the application of the sample at earlier time points. It should also be noted that neovascularization can also be examined using the model described here, except that the sample is applied at an earlier stage (E-5 or E-6) when neovascularization is still taking place. In this case, the results are also observed 2 or 3 days later. In a similar manner, angiogenesis inhibitors can also be examined by mixing them together with an angiogenesis stimulator such as bFGF (Ponce et al., 2001).

Troubleshooting

As mentioned above, one of the caveats of the CAM assay is the high number of spontaneous positive responses obtained. In addition, false-positive responses can also be caused by the presence of salt crystals in the sample or pieces of eggshell that become lodged inside the egg. These responses are usually characterized by an inflammatory response that can cause indirect induction of angiogenesis.

Embryo viability, which can range between 30% and 70%, can also be seriously affected, in some cases, by the length of transportation between the farm and the laboratory, as well as the conditions of the trip, including bumpy rides and the time the eggs spend outside of the incubator. Although some of these issues can be addressed, often they are beyond the control of the investigator. Another serious problem that can reduce embryo viability and/or the

number of samples is contamination. This problem can be reduced by following more stringent sterile techniques including the use of sterile equipment, careful swiping of the eggs with betadine or 70% alcohol before opening, working in a sterile hood in extreme cases, or maintaining a low incubator humidity.

At a minimum an experiment requires 12 embryos per treatment group, 12 embryos for a negative control, and 12 embryos for a positive control. If at the end of an experiment the number of embryos is too low and no statistical differences are observed between the negative and positive controls, the experiment should be discarded.

Anticipated Results

Many angiogenic and anti-angiogenic compounds have been tested using this assay including growth factors, such as bFGF, VEGF, and ICAM, antibodies (anti-bFGF, VEGF, and integrins), peptides and protein fragments (laminin and fibronectin, thrombospondin and collagen-derived), and small molecules (Auerbach and Auerbach, 1994; Ribatti et al., 2001). The angiogenic response will vary according to the strength of the compound being tested. For example, bFGF will usually give a strong response in which a well-formed spoke-wheel can be observed in as many as 80% of the eggs tested. Less angiogenic substances may not form a spoke-wheel, but the number of positive CAMs and strength of the response will vary depending on their angiogenicity and/or concentration. This will also be the case for anti-angiogenic molecules, in which the most potent ones will greatly reduce the angiogenicity of the inducing agent at lower amounts, whereas, weaker ones could require much larger amounts to observe a decreased response. All these factors will ultimately have to be experimentally determined by the investigator.

Time Considerations

The CAM assay takes 9 to 10 days to complete. The amount required for each one of the steps is as follows. Removal of ovalbumin on the first experimental day, E-3, requires ~1 to 1.5 hr per every 100 eggs. Opening of the windows on E-9 will take a minimum of 3.5 hr per 100 eggs depending on experience. On E-10, allow 2 to 3 hr for sample preparation and placing the coverslips on the CAM. On E-12, allow 0.5 to 1 hr for checking the angiogenic response. On the final day, E-12 or E-13, allow 2 to 3 hr to score and photograph the CAMs.

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Experimental Metastasis Assays in the Chick Embryo

Tumor metastasis is the spread of cancer from a primary tumor to form secondary tumors in distant organs. This process consists of a series of steps, starting with the release of cancer cells from a primary tumor into the blood or lymphatic circulation (intravasation), the transport of cells to distant organs via the circulation, arrest in capillaries in these sites, exit of the cancer cells from the circulation into the tissues of the new organ (extravasation), and the growth of some of these cells to form metastatic tumors in the new organ. Experimental metastasis assays model the latter half of this process by injection of cancer cells into the circulation of an experimental animal and the detection of the formation of metastatic tumors. This is in contrast to spontaneous metastasis assays, in which a primary tumor is created by injection of cancer cells into an appropriate tissue of an experimental animal and the presence of metastatic tumors in distant organs is assessed, which will not be discussed here.

Mice are most commonly used for experimental metastasis assays; however, for a variety of reasons (see Background Information), there are some distinct advantages to using alternate assays. For example, the chick embryo can provide a convenient and cost-effective animal model in which to study the process of tumor metastasis. It is a naturally immune-deficient host and can thus be used to assess the metastatic potential of cancer cells from a variety of species, including human and mouse. Also, housing requirements are minimal and accessible to most laboratories. Chicken embryos are readily available and experimental metastasis assays in this system are straightforward. Experimental metastasis assays can be combined with *in vivo* videomicroscopy on readily accessible thin tissues, such as the chick embryo chorioallantoic membrane (CAM), in order to learn about individual steps in the metastatic process. Methods for using chick embryos for experimental metastasis assays (see Basic Protocol 1) and *in vivo* videomicroscopy (see Basic Protocol 2) are provided in this unit, as are the details of routine maintenance of the embryos (see Support Protocol 1). Additional protocols for labeling cells with fluorescent nanospheres (see Support Protocol 2) and for assessing cells in tissues using fluorospheres (see Support Protocol 3) are also included.

NOTE: All protocols using live animals must be first reviewed and approved by the appropriate Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of these animals.

NOTE: All solutions and equipment coming into contact with cells and tissues must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Do not use a culture incubator for eggs.

EXPERIMENTAL METASTASIS ASSAY IN THE CHICK EMBRYO

Experimental metastasis assays measure the ability of cells injected intravenously into the circulation to form metastatic tumors in distant organs (see Welch 1997 for general review of techniques commonly used). In this assay using the chick embryo, which was described in 1982 by Chambers et al. (see Chambers and Tuck, 1988 for review), cells are injected intravenously (*i.v.*) into chorioallantoic membrane (CAM) veins. An illustration of this experimental approach is presented in Figure 19.6.1, which diagrams the injection of cells into a CAM vein of an 11-day-old chick embryo. Cells injected by this

BASIC PROTOCOL 1

Whole Organism and Tissue Analyses

19.6.1



Figure 19.6.1 Diagram of experimental metastasis assay in chick embryos. Cancer cells are injected via a chorioallantoic membrane (CAM) vein, through a window opened in the shell of 11-day-old chick embryos. Cells injected via this route travel via the circulation first to the embryonic liver. After incubation for up to 7 days, metastatic cells in an embryonic organ such as liver are quantified using the ouabain plating assay, or (for rapidly growing cell lines) macroscopic metastases can be quantified. Republished with permission of Anticancer Research from Chambers and Tuck (1988).

route are taken by the circulation first to the embryonic liver. Some cell lines may grow rapidly *in vivo* in the chick, such that macroscopic metastatic tumors are visible in the liver and other internal organs at 7 days postinjection (Chambers and Ling, 1984; Chambers and Wilson, 1988); however, many cell lines, even highly metastatic ones, may not grow visible tumors in the time period available before embryo hatching. For these cell lines, the ouabain plating assay was developed (Chambers et al., 1982). This assay permits the quantification of numbers of viable rodent cells in chick organs at various times after injection.

The ouabain plating procedure is based on natural interspecies differences in sensitivity to the cytotoxic effects of the drug ouabain: rodent cells are inherently considerably more resistant to ouabain than chick cells, while human cells are more sensitive than chick cells (Chambers et al., 1982; Chambers and Ling, 1984). Due to this latter sensitivity, this assay cannot be used for human cells in chick tissues. However, this approach can be adapted for use with human cells that carry a selectable drug resistance marker, such as neo/G418 (Chambers and Wilson, 1988). Because the assay requires adherent colonies to form, it is best suited for rodent cells that grow as adherent, monolayer cultures, rather than for cells that grow as suspension cultures.

A suspension of chick organ cells, in which there are unknown numbers of rodent cells, is plated in a concentration of ouabain sufficient to kill the chick cells while allowing the rodent cells to grow and form clonal colonies *in vitro*. At the end of the assay (the timing of which will depend on the growth rate of the cancer cells *in vitro*, in order to form clonal colonies of countable sizes), the rodent cell colonies that grow out from the chick liver suspension can be stained with methylene blue and counted to determine the number of viable (rodent) cells per chick embryo liver. Alternatively, viable cells can be recovered from some plates by standard trypsinization procedures to study the properties of cells that have been selected *in vivo* in the chick embryo (Chambers and Ling, 1984; Bondy et al., 1985; Chambers and Wilson, 1985; Hill et al., 1988).

Materials

Eggs containing 11-day-old chick embryos (see Support Protocol 1)
Adherent rodent tumor cells, optionally labeled with nanospheres (see Support Protocol 2)
Medium and serum used to grow tumor cells
Paraplast wax
Paraffin oil
70% ethanol
Sterile calcium- and magnesium-free PBS, 4°C (see recipe)
Hank's balanced salt solution (HBSS), 4°C (see recipe)
0.3% (w/v) collagenase/0.02% (w/v) trypsin (see recipe)
DNase I (see recipe)
 2×10^{-3} M ouabain (see recipe)
Detergent or other decontaminating solution
Methylene blue stain (see recipe)
Egg candler (local farm supply store)
Pencil
Enclosed still hood with glass front, interior light, and electrical outlet
Dremel Moto tool with polishing wheel, $\frac{6}{8} \times \frac{1}{8}$ -in. thick (available at local hobby shop)
38.5°C (99° to 100°F) automatic rotating egg incubator with 80% to 82% humidity (e.g., March Farms Roll-X incubators, Lyon Electric; available at farm supply stores)
Hemocytometer (*UNIT 1.1*) or Coulter counter
Adjustable gooseneck light source and fiber optic unit (e.g., Schott Glas Fiber Optics unit, Carl Zeiss unit) or focused intense light source
Egg support stand (i.e., three rubber corks glued on a plastic petri dish; Figs. 19.6.3 and 19.6.4)
1-cc syringes
30-G $\frac{1}{2}$ -in. needles
Cotton swab
Disposable underpad
1-liter plastic beaker lined with a 12-lb. plastic bag
Small plastic beaker
Dissecting instruments, autoclaved:
 Straight medium-point dissecting forceps, 115 mm in length
 Straight narrow-blades dissecting scissors with fine points, 110 mm in length
 Two straight fine-point dissecting forceps, 110 mm in length
 Two scalpel handles with no. 10 disposable blades
150 × 20-mm plastic dishes
100 × 15-mm nontissue and tissue culture dishes
24-well tissue culture plates
100-mm glass dishes (autoclave in a canister)
5-ml pipet
17 × 100-mm polystyrene test tubes
500-ml wash bottle
Funnel
Plastic wash basin with drainage holes drilled half-way up each end
Additional reagents and equipment for trypsinization (*UNIT 1.1*), adding accounting spheres (see Support Protocol 3), and inspecting for gross surface tumors (Chambers et al., 1982; Chambers and Wilson, 1988)

NOTE: During dissection, all instruments should be rinsed in 70% alcohol, wiped clean with a tissue, and flame sterilized between each step

Prepare egg windows

1. In a dark room, hold an egg candler directly against the shell on the air sac (blunt end) of an egg, which has been incubated for 11 days.

The 11-day-old embryo and the CAM veins should be clearly visible under the shell. Dead or deformed embryos can be readily identified when candled, as they will have either no veins present or black, darkened areas. These eggs should be disposed of carefully without cracking, as they may have a strong sulfur odor.

2. Gently rotate the egg in hand to identify a large dark vein branched in a Y shape near the air sac. With a pencil (not pen or marker), mark a 10×10 -mm square on the shell over this vein indicating the area to be drilled.

Do not select veins that move freely when the egg is rotated with quick turns, as these cannot be injected. Drilling a square that is too small will make injection difficult, while if the square is too large, the window will cave in after drilling.

3. In an enclosed still hood with glass front, interior light, and electrical outlet, drill all four sides of the marked square using a Dremel Moto tool with a $\frac{6}{8} \times \frac{1}{8}$ -in. polishing wheel using the following method (Fig. 19.6.2):
 - a. Hold the egg with two fingers and the thumb of the left hand (assuming that the researcher is right-handed) so that the egg is at a slight angle, with the air sac end up, and the square marked for drilling facing up and toward the experimenter.
 - b. Hold the Dremel drill in the right hand as if it were a large pencil.
 - c. Bring the drill bit at right angles to the marked square on the shell. Use only gentle pressure to start cutting since the drill operates at high speed.



Figure 19.6.2 The drilling of the marked square or window on the egg over the selected vein area with a Dremel Moto tool is done in an enclosed still hood. To minimize breathing of eggshell dust, hang a cloth from the bottom of the glass front (or alternatively wear a mask and protective eyewear). Drill all four sides of the window, with the egg at a slight angle and the air sac end (blunt end) up and the marked window facing toward the person drilling. The bit of the Dremel tool is brought at right angles to the surface of the shell. Gentle pressure is sufficient for cutting due to the high speed of the drill. Only the shell is cut while avoiding the underlying smoother papery-white membrane or the underlying CAM.

- d. Cut *only* the shell (which looks pebbly), and not the underlying papery-white shell membrane or the viable CAM tissue under this membrane. Do not remove the resulting window.

CAUTION: For safety, minimize inhalation of egg shell dust by hanging a cloth from the bottom of the glass front on the enclosed still hood. Alternatively, or additionally, a mask and protective goggles can be worn.

This step requires practice and a steady hand. If the CAM is nicked or bleeds, the egg should be discarded.

4. *Optional:* After drilling, return the eggs to a nonrotating egg incubator (i.e., with rotation turned off) for up to 1 hr before injection.

This step is useful when large numbers of eggs are to be injected in a single experiment; it helps to keep the embryos healthy.

Prepare tumor cells

5. Prepare a single-cell suspension of adherent rodent tumor cells from an 80% confluent culture, optionally with nanospheres, using standard laboratory procedures for trypsinization (*UNIT 1.1*). Count the cells using either a hemacytometer (*UNIT 1.1*) or Coulter counter.
6. Using the same medium and serum used to grow the tumor cells, adjust the volume to give the desired number of cells per embryo in a total injection volume of 0.1 ml per embryo. If desired, add accounting spheres (see Support Protocol 3).

The number of cells to inject into each chick depends on the metastatic properties of the cells. The number can range from $1-5 \times 10^6$ cells per embryo for poorly and nonmetastatic cells (Hill et al. 1988), down to 5×10^3 cells per embryo for highly metastatic cell lines (Chambers and Wilson, 1988).

The prepared cell suspension can be briefly (i.e., up to 2 hr) stored at 4°C until the eggs are ready for injection.

Set up injection equipment and area

7. In a small glass beaker melt Paraplast wax on a hot plate set at 60°C.

CAUTION: Paraplast is flammable and should be carefully watched during this procedure; do not allow it to get hot enough to smoke.

8. Set up the equipment needed for injection in the following manner:
 - a. Open the front of a tissue culture hood and place a fiber optic unit or focused intense light source near the back of the hood with the gooseneck light source extended to the front.
 - b. In front of this light source, position the egg support stand so that the egg will sit parallel to it.
 - c. Place empty 1-cc syringes, 30-G ½-in. needles, Kimwipes tissues, and a 1-cc syringe without needle half filled with paraffin oil into the hood.
 - d. When all the equipment is ready, place the eggs (windows cut but not removed, step 3 or 4) on their sides in an egg tray with the windows up and bring them into the tissue culture hood.
 - e. Once the Paraplast has melted, allow it to cool, but still remain liquid, and place it in the hood.

The arrangement of these components in preparation for egg injection is illustrated in Figure 19.6.3.

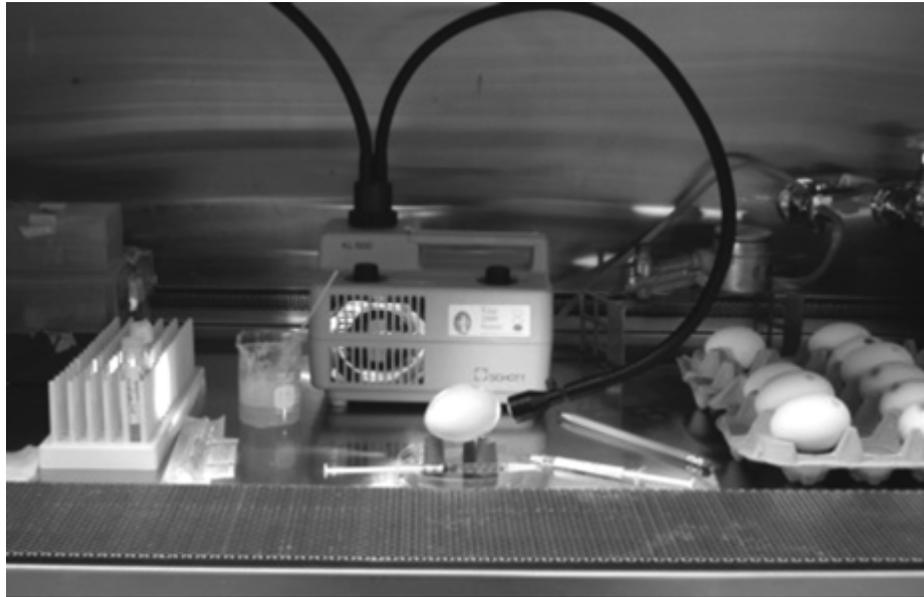


Figure 19.6.3 The arrangement of equipment inside the tissue culture hood just before injections proceed. The front of the hood is opened for easier access during the procedure with adequate sterility still being maintained. The gooseneck arm of the fiber optics light is illuminating the air sac end of an egg positioned on the egg stand (made by gluing three rubber corks onto a plastic petri dish) ready for injection. The remaining eggs have had the shell of the drilled window carefully removed over the vein area and are placed in a tray with the windows facing upwards awaiting injection. The cell suspension has been prepared at a predetermined concentration ready to be drawn up into the 1-cc syringe following gentle vortexing. The other 1-cc syringe contains the paraffin oil of which a drop will be placed on the exposed window area prior to injection. The melted Paraplast in the glass beaker (left) is cooling in preparation for sealing of the entire window area following injection.

Inject eggs

9. Gently remove the cut square of the shell from each egg with a pair of fine forceps. If the shell membrane or the underlying CAM become nicked or damaged, the egg cannot be used.

This step also requires practice and a steady hand.

10. Set one of the eggs on the support stand with the air sac end facing the fiber optics so the veins inside the egg are illuminated. Add a drop of the paraffin oil from the 1-cc syringe to the surface of the shell membrane to further enhance visualization of the vein.
11. Gently vortex the suspension of cells to be injected (step 6). Draw the cell suspension up into a 1-cc syringe, attach the 30-G needle, and remove air plus any excess liquid with a Kimwipe.

As a control, to determine the effect of passage of the cell suspension through a 30-G needle, it is recommended to perform an in vitro plating efficiency assay. For the cell lines used by the authors, passage of cells through a 30-G needle has not led to reduced plating efficiency of the cells.

12. Inject the CAM vein with tumor cells as follows (Fig. 19.6.4):

- a. With the beveled tip of the needle facing upwards and in line with the numbers on the barrel of the syringe (i.e., so both numbers and bevel face the experimenter), gently insert the needle into the CAM vein in the direction of blood flow.

The paraffin oil enhances the visibility of the vein and thereby allows the quality of the injection to be monitored. When the CAM window is illuminated, vessels appear bright red and the egg contents appear yellow.

- b. Inject 0.1 ml quickly and evenly into the vein.
- c. Withdraw the needle carefully and smoothly from the vein to prevent bleeding.
- d. If any bleeding occurs or a clot forms at the injection site, discard the embryo.
- e. Return the injected egg to the egg tray in the hood, placing it on its side with the window facing upwards.

To determine blood flow direction, follow the vein to where it branches; blood will generally travel along the two arms toward the single arm (Y). Areas of the vein near the branch seem to withstand injection the best. If a proper injection is done, the clear inoculum entering the vein will be visible as blood flow is temporarily blocked as will the pulsing of the blood flow beyond the end of the needle. See inset of Figure 19.6.4.



Figure 19.6.4 Injection of cells into a chick embryo CAM vein. Position the egg on the egg stand, with the fiber optics light illuminating the air sac end for visualization of the large Y shaped vein. The addition of a small drop of paraffin oil onto the injection site enhances the clarity of the vein. The beveled tip of the needle is facing upwards for gentle insertion into the branch area of the Y (see inset) just until the beveled end is completely into the vein. The needle should be at the angle seen in the picture above to prevent damage to the vein. If a proper injection is performed, the vein will temporarily become clear as the 0.1-ml inoculum is smoothly injected, and there will be no blood seeping from the injection point either during or after the injection.

13. Repeat steps 10 to 12 for each egg to be injected.

When injecting several eggs from the same syringe of cells, be sure to agitate the syringe gently between injection, to avoid settling of the cells. This will ensure that all eggs receive the same number of cells.

14. Seal the exposed windows with cooled, but still melted Paraplast using a cotton swab. Cover the entire window area including at least 5 mm of shell on each side. After the Paraplast has hardened, apply a second coat to ensure a complete seal.
15. With a pencil, label the side of each egg with the date, cell line, and other important details.

Allow tumor cells to metastasize

16. Return the eggs, on their sides, to a nonrotating egg incubator (i.e., turn off rotator) for further incubation, up to a maximum of 7 days (hatching occurs on day 19 to 21). Use the same conditions of humidity and temperature as during the initial incubation (see Support Protocol 1)

The incubation time depends on the type of assay, either having a single 7-day end point or, if determination of the growth kinetics of the cell is desired, a time course of intervals from 0 to 7 days from the time of injection.

The temperature may be altered at this point if experiments are being performed to test the effects of temperature-sensitive mutants (Chambers and Wilson, 1985).

*Much information can be obtained from kinetics studies on the growth of injected cells in embryonic organs (Chambers et al., 1982; Chambers and Ling, 1984; Chambers and Wilson, 1985). Figure 19.6.5 shows an example of kinetic data, quantifying the in vivo increases in numbers of tumor cells (ouabain-resistant; i.e., rodent) in chick liver over time after injection. Figure 19.6.5B also illustrates how the chick embryo assay can be used to assess the in vivo effects of temperature-sensitive (*ts*) genes, by maintaining the embryos at different temperatures. In the example shown, the LA23-NRK cells carry a *ts-src* oncogene, which is active at 36°C but inactive at 38°C, resulting in dramatic in vivo growth abilities of the cells shown in Figure 19.6.5B (Chambers and Wilson, 1985).*

17. Check the viability of the embryos to be dissected by candling (step 1) in a darkened room.

The embryo should be clearly visible and veins still present. If there are no veins or the eggs contain discolored interiors, the embryos have died and should be carefully discarded.

Set up dissection equipment and area

18. On a bench top near the tissue culture hood spread a disposable underpad. On the underpad, place a 1-liter plastic beaker lined with a 12-lb. plastic bag for disposal of the egg shells and other waste.
19. Place 115-mm, straight, medium-point dissecting forceps on the underpad outside of the hood. Place the remaining autoclave-sterilized dissecting instruments into a small plastic beaker containing 70% ethanol. Transfer the beaker with ethanol into the tissue culture hood.

Isolate chick embryo

20. Remove the embryo from the shell on the bench over the disposable underpad, as follows:
 - a. Hold the egg with the air-sac end up. Break the shell at the air-sac end of the egg with a sharp tap from the handle of the 115-mm forceps.

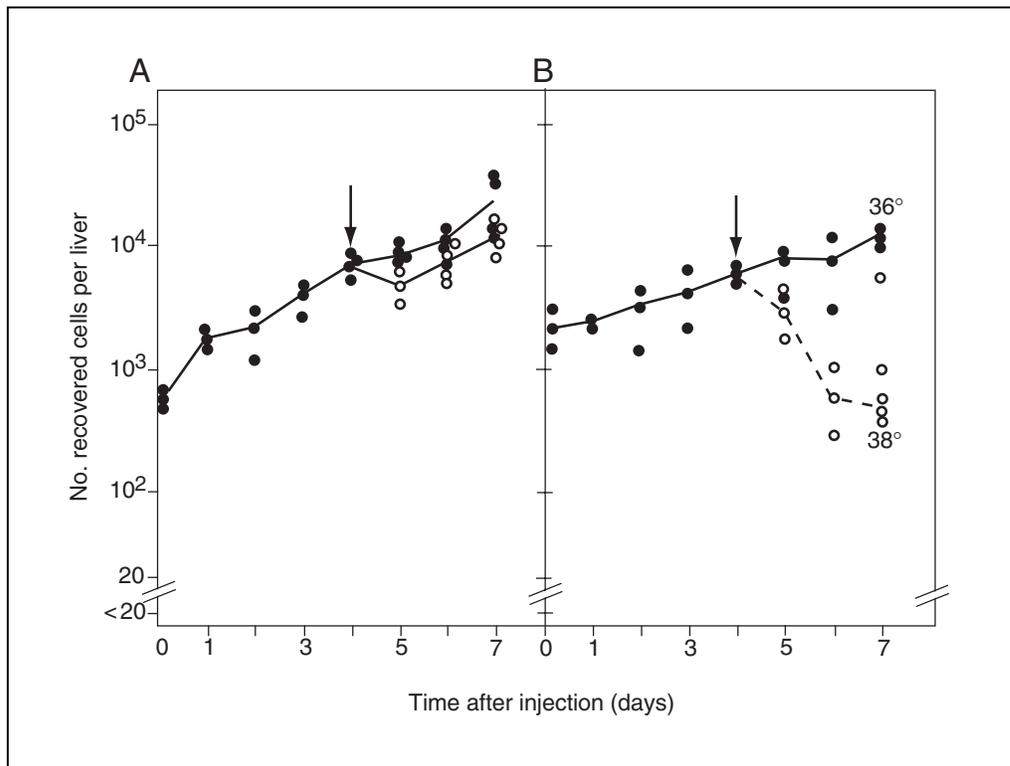


Figure 19.6.5 Example of in vivo growth kinetics results that can be obtained with the chick embryo experimental metastasis assay and the ouabain plating assay to recover rodent (and thus, ouabain-resistant) cells at various times after injecting cells via a CAM vein. **(A)** B77-NRK (normal rat kidney) cells transformed with the src oncogene, or **(B)** LA23-NRK cells (NRK cells carrying a temperature-sensitive src oncogene, which is active at 36° and inactive at 38°C) were injected into CAM veins at 5×10^4 cells per embryo. Closed circles: embryos maintained at 36°C; Open circles: embryos transferred to 38°C at day 4. Each point represents the number of viable ouabain-resistant cells present in the liver of one embryo; lines connect median points. Republished with permission of American Society of Microbiologists from Chambers and Wilson (1985).

- b. Over the 1-liter plastic-lined beaker, break through the air sac membrane towards the embryo.
- c. Position the 115-mm forceps around the neck of the embryo and extract it from the shell.

Gloves should be worn for these steps.

21. Use the remaining shell to pull away any residual yolk or other tissue to completely free the embryo; discard the shell into the plastic-lined beaker.

The CAM lies directly under the shell membrane, and usually remains attached to the inside of the shell. It can be peeled from the inside of the shell and examined for tumor growth—e.g., if melanoma cells have been injected that produce easily visible tumors (Chambers et al., 1982)—or it can be used for histological assessment.

22. Use a 150 × 20–mm plastic dish to transfer the embryo to the tissue culture hood. Place each embryo into a 100 × 15–mm nontissue culture dish and decapitate the embryo with 110-mm, straight, narrow-blades, dissecting scissors with fine points. Cover with the lid.

The embryos can be left in the dishes inside the tissue culture hood until all the embryos have been extracted from the eggs.

Expose liver

23. Start dissecting by turning the embryo onto its back with the breast facing upwards. Just below the pectoral (breast) muscle, gently lift the skin with a pair of 110-mm, straight, fine-point dissecting forceps.
24. With sterile scissors, cut the skin above the breast all the way to the neck. Pull away the skin to the sides to expose the pectoral muscle, as well part of the liver (gold in color) and the gut.
25. Expose the entire liver and the heart as follows:
 - a. Lift the area below the pectoral muscle at the point where a thin white membrane forms a V.
 - b. Cut upwards through the middle of the pectoral muscle towards the neck, slightly lifting the muscle during cutting to avoid damaging the liver underneath.
 - c. At the neck, make a transverse cut, then pull back the pectoral muscle to each side to expose the heart and entire liver.

Isolate liver

26. With sterile forceps, carefully detach the three areas of attachment of the liver to the other organs: two on the left side under one lobe and one under the right lobe.
27. Slip sterile opened forceps around the liver and move towards the heart which is the last point of attachment. Pinch the forceps together and lift out the liver.
28. In the tissue culture hood, transfer the liver to a well of a 24-well tissue culture plate containing 4°C sterile calcium- and magnesium-free PBS.

The liver can remain up to 2 hr at 4°C.

During this procedure (steps 25 to 27), do not puncture the gut while removing the areas of attachment under the liver. The gall bladder, situated directly under the liver, is usually dissected out at the same time as the liver. Avoid puncturing it if possible, but if this does occur, it will only discolor the PBS and not affect viability of cells present in the liver.

Dispose of waste and visually inspect livers for tumors

29. Incubate any undissected but viable eggs at 4°C for at least 1 hr prior to disposal to avoid hatching. Wrap the dissected embryos and shells in plastic bags and dispose by incineration.
30. Inspect the livers (and other organs) for gross surface tumors (Chambers et al., 1982; Chambers and Wilson, 1988) or abnormalities before dissociation, using proper sterile technique.

Alternatively, organs may be fixed for histological examination and identification of internal micrometastases (Chambers and Ling, 1984).

Wash liver

31. Rinse each liver in a series of three sterile PBS washes in a 24-well plate by transferring the liver with sterile forceps from one well to the next.

This allows six livers to be washed per 24-well dish.
32. Place the rinsed liver into a sterile 100-mm glass dish. Mince the liver into tiny pieces with crossed sterile scalpel blades.

One scalpel can hold the tissue in place while the other cuts, then use both blades to finely mince the tissue. Avoid spreading the tissue over a large area to minimize drying.

Homogenize liver

33. Add 5 ml HBSS. Resuspend the minced liver tissue by pipetting up and down using a 5-ml pipet.
34. Dispense this suspension into a 17 × 100-mm polystyrene test tube (one tube per liver). Rinse the glass dish with another 5 ml HBSS to ensure all the tissue is removed from the petri dish. Pool with the 5 ml tissue suspension already in the test tube.
35. Centrifuge the liver suspension 10 min at 3000 × g, room temperature. Gently remove and discard the supernatant, leaving the liver pellet.

Prepare single-cell suspension

36. Add 3 ml freshly prepared 0.3% collagenase/0.02% trypsin and 0.5 ml DNase I into the tube and vortex.
37. Incubate ~1 hr in a 37°C heat block or water bath. Vortex occasionally during the incubation until a single-cell suspension is produced and there are no visible tissue fragments.

The length of time necessary for complete dissociation of the liver tissue is dependent upon the age of the embryo—i.e., 18-day-old embryos require 1 hr, while 11-day-old embryos may require only 20 to 30 min. Other organs can also be dissociated; times will vary according to type of tissue and age of the embryo.

38. Centrifuge the single-cell suspension 10 min at 3000 × g, room temperature. Gently remove and discard the supernatant, leaving the liver cell pellet.

Select tumor cells with ouabain

39. Add 10 ml appropriate medium with serum for the cells injected with a final concentration of 2×10^{-5} M ouabain. Vortex the suspension to thoroughly mix.
40. Plate two dilutions of each liver suspension in 100 × 15-mm labeled tissue culture dishes containing 15 ml medium plus serum with 2×10^{-5} M ouabain. Use two to three plates per dilution.

The total amount of liver cells plated should not exceed 1/20 of an 18-day old embryo liver per 100-mm dish, to avoid background problems due to large numbers of dying liver cells. All of the cells from younger (e.g., 11-day) livers can be plated in one to three dishes, without background problems. The fraction of the liver plated should reflect the expected number of rodent cells present in the liver and should be adjusted to give numbers of ouabain-resistant rodent colonies per dish that can be counted (optimal being 10 to 250 colonies per dish). Some suggestions of appropriate dilutions are 1/20 and 1/100 per 100-mm dish of a liver suspension for potentially low metastatic cell lines, and up to 1/100 and 1/2000 of liver suspension for a highly metastatic one.

41. Gently swirl each dish three times in both directions to ensure proper distribution of the cells over the bottom of the dish. Incubate the dishes undisturbed for 10 to 21 days, depending upon the doubling time of the injected cells.

During the incubation of the diluted cell suspensions, the dishes should not be moved or small satellite colonies will be seeded that will make counting colonies difficult. Periodically, checking one plate during the incubation will assist in determining when to recover the cells from the colonies (following counting) for culturing or to stain for counting. Note which plate is used for these observations.

Prior knowledge of the plating efficiency of the cell line injected will assist in determining the length of the incubation time.

Stain colonies and dry

42. Gently pour off the medium from the dishes into a beaker containing detergent or other decontaminating solution.
43. Add 5 ml methylene blue stain, from a 500-ml wash bottle, by allowing it to run down the side of the dish onto the bottom without disturbing the colonies. Make sure the bottom of the dish is completely covered with the stain. Allow to sit at least 15 min or until the colonies are a dark blue.

The glutaraldehyde and alcohol in the stain will fix the cells to the dish.

44. Pour the stain off the colonies, collecting it into a 500-ml wash bottle using a funnel.

This stain can be collected back into the 500-ml wash and reused.

45. Place a plastic wash basin with drainage holes drilled half-way up into a sink. Gently run tap water continuously into the basin. Wash each dish individually by gently moving it slowly through the water. Do not allow direct water flow to dislodge colonies from the dish.

The holes in the basin will allow the stained water to go down the sink while fresh water is poured in from the tap.

Determine number of rodent cells in each chick liver

46. Allow washed dishes to dry inverted, using the lids as a support to assist drying. Count colonies on the dried dishes any time, with the stained colonies being a permanent record.
47. Calculate the number of rodent cells present in each chick liver based on the fraction of liver plated in each plate.

For example, calculate the mean of the total stained colonies counted per dish at a particular liver dilution multiplied by the dilution factor of the liver—e.g., $(45 + 54 + 48)/3 \times 20$ (1/20 of liver plated per dish) = 980 cells per liver. For most dissociations, two separate liver dilutions are performed. In this case, do the above calculation for the second set of plates from the same liver—e.g., $(9 + 11 + 10)/3 \times 100$ (1/100 of liver plated per dish) = 1000 cells per liver. To calculate the total number of cells per liver, take the two values obtained from each dilution and average: $(980 + 1000)/2 = 990$ cells per liver. The previously known plating efficiency in vitro of the cell line injected must be factored into this averaged value. If the cell line has a plating efficiency of 90% then calculate as follows: $990/0.9 = 1100$. Therefore, the mean final number of viable cells present in the chick livers would be 1100 cells. Standard deviations can be calculated from the variations in original cell counts per plate.

SUPPORT PROTOCOL 1

ROUTINE MAINTENANCE OF EGGS

Fertilized chick eggs (outbred White Leghorn) can be delivered fresh from a local hatchery on a regular schedule depending on experimental need (e.g., once a week). Larger eggs tend to be stronger and thus lead to more successful injections. During transport from the local hatchery, the eggs should not be subjected to extreme temperature changes and should be transported to the laboratory promptly. An insulated carrier may be necessary to keep them cool. When the eggs arrive in the laboratory, they should immediately be placed at 4°C, where they can be kept for up to one week before starting incubation. A schedule should be determined to provide embryos that have been incubated for exactly 11 days on the day on which the experiment is to be performed.

Materials

Fresh fertilized eggs (standard outbred White Leghorn), 4°C

Pencil

38.5°C (99° to 100°F) automatic rotating egg incubator with 80% to 82% humidity (e.g., March Farms Roll-X incubators, Lyon Electric; available at farm supply stores)

Hygrometer to routinely monitor egg incubator humidity

1. To start the incubation, remove fresh fertilized eggs from 4°C storage. On the blunt, rounded end of the egg, where the air sac is located, write the date when the incubation is begun in pencil.

Do not use pen or marker, as these may be toxic to the embryos.

2. Place the eggs in a 38.5°C automatic rotating egg incubator with 80% to 82% humidity, with the air sac end up and the date clearly visible.

The rotating incubator will prevent deformities from occurring in the embryos during incubation, by rotating the eggs at proper time intervals (New, 1966; Sturkie, 1976).

Do not use a CO₂ incubator designed for tissue culture, as this will kill the embryos. The temperature is critical since a slightly lower incubator temperature will result in developmentally younger embryos that will not survive injection at day 11.

3. Continue incubating until eggs are the appropriate age. Record daily temperature readings to note fluctuations in temperature. Maintain the humidity by daily checking with a hygrometer and addition of either tap or distilled water into the interior egg incubator reservoir according to manufacturer's instructions.

The incubators should be connected to an emergency back-up supply to prevent embryo death in case of a power failure.

INTRAVITAL VIDEOMICROSCOPY OF THE CHICK EMBRYO CAM

The technique of intravital videomicroscopy (IVVM) uses a videocamera attached to an inverted microscope to visualize, record, and quantify the movement of blood through microcirculatory pathways of intact organs in living animals. General information about this procedure can be found in MacDonald et al. (1992, 1998, 2002), and Chambers et al. (2002). Here the applications of this approach to studying the interactions of injected cancer cells with the chick embryo chorioallantoic membrane (CAM) will be discussed.

IVVM uses a fiber optic light source to supply oblique illumination of an organ of an experimental animal (e.g., chick embryo, mouse) placed on the stage of an inverted microscope. This approach yields high-resolution, three-dimensional images of the microcirculation. IVVM thus allows for the real-time observation of the hematogenous metastasis process involving the initial arrest of cancer cells, as well as their extravasation, post-extravasation migration, and replication to form tumors. This technique is able to visualize these events in thick tissues, such as mouse liver, to a depth of 50 to 100 μm. It is also very easy to visualize these events in thin tissues, such as mouse or rat mesentery, or chick CAM. Interactions between tumor cells and the host microvasculature and/or the surrounding tissues of the CAM of the chick embryo have been reported (e.g., Chambers et al., 1992; MacDonald et al., 1992; Koop et al., 1994, 1996; Morris et al., 1997).

The capillary network of the CAM closely resembles that of the lung (Sethi and Brookes, 1971), and at the embryonic stage it has a capillary plexus with a complete basement membrane, which is fully lined with endothelial cells (Rizzo and Defouw, 1993). This is an appropriate model for studying various aspects of metastasis, in that tumor cells in the

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circulation are required to cross the endothelium and basement membrane barrier in order to form metastases. The CAM also provides an immune-deficient model easily accessible for observation without the need for surgery.

Labeled cells are injected intravascularly into the CAM vein of 11-day-old chick embryos (see Basic Protocol 1). Examination by IVVM of individual eggs can be done immediately (up to 4 hr) or at various times post-injection, up to 7 days. This provides a visual record of the interactions of the cancer cells with the CAM vasculature, which provides valuable data that can complement results from other procedures.

Materials

Eggs containing 11-day-old chick embryos (see Support Protocol 1)
Adherent tumor cells with or without nanospheres (see Support Protocol 2)
Medium and serum used to grow tumor cells
Citrate saline (see recipe)
Paraffin oil
Plasticine

Egg candler (local farm supply store)
Pencil
Enclosed still hood with glass front, interior light, and electrical outlet
Dremel Moto tool with polishing wheel, $\frac{9}{8} \times \frac{1}{8}$ -in. thick (available at local hobby shop)
38.5°C (99° to 100°F) automatic rotating egg incubator with 80% to 82% humidity (e.g., March Farms Roll-X incubators, Lyon Electric; available at farm supply stores)
30-G $\frac{1}{2}$ -in. needle attached to a PE-10 cannula and 1-cc syringe
Hemocytometer (*UNIT 1.1*) or Coulter counter
Vinyl tape
No. 1 coverslip, 45 × 50-mm
180 × 130 × 3-mm acrylic viewing center with 40 × 40-mm hole cut in the center
Masking tape
Inverted microscope (e.g., Nikon Diaphot TMD) with 10× to 60× or 100× objectives and mercury arc lamp with B2-A filter block (570-nm dichroic mirror and 520-nm barrier filter; 450- to 490-nm excitation wavelength; Nikon)
Infrared heat lamp
Fiber optic light source with 150 W halogen bulb
Newvicon tube video camera with extended red sensitivity (Panasonic WV1550 or Hamamatsu C2400)

Additional reagents and equipment for preparing windows in eggs (see Basic Protocol 1, steps 1 to 6 and step 9) and injecting tumor cells into eggs (see Basic Protocol 1, steps 10 to 13 and steps 15 to 16)

Prepare eggs for injection

1. Prepare 11-day-old embryos for injection with tumor cells (see Basic Protocol 1, steps 1 to 6, and 9).

Tumor cells can be labeled with fluorescent nanospheres (Support Protocol 2) to aid in their detection. Accounting spheres (Support Protocol 3) can be used to monitor blood flow and delivery of cells to the tissue.

2. Prepare to observe the initial interactions between injected cells and CAM microcirculation:
 - a. Carefully place a 30-G ½-in. needle attached to a PE-10 cannula and 1-cc syringe containing warmed citrate saline rinse into the large CAM vein. Keep in place with vinyl tape.
 - b. Inject 0.25 ml citrate saline, keeping the cannula filled with solution, to avoid vessel blockage by air bubbles.

Prepare window for IVVM

3. Remove the shell and outer shell membrane from the air sac region of the egg by gently using forceps to open an observation area ~3 cm in diameter and leaving the CAM at the same level as the border of the shell.
4. Apply paraffin oil to the inner shell membrane, covering the entire air sac area of the CAM to be observed so it becomes transparent.
5. Secure a no. 1 coverslip on the opening of the acrylic viewing platform, using masking tape, so that the CAM comes into direct contact, forming a window. Place the windowed egg on the platform, so that the CAM covered in paraffin oil comes in direct contact with the coverslip, thus creating a window to directly view the capillary plexus of the CAM.

Set up microscopic viewing system

6. Carefully place the acrylic platform and egg setup on the stage of an inverted microscope equipped with 10× to 60× or 100× objective, mercury arc lamp, and Nikon B2-A filter block.

Refer to UNIT 4.2 for a discussion of fluorescence microscopy.

7. Keep the egg in place using vinyl tape on the shell and microscope stage and building a protective wall around the shell on the platform with Plasticine where the egg meets the acrylic platform, to provide stability and prevent cracking of the egg shell during the experiment. Maintain the temperature of the egg using an infrared heat lamp.

The arrangement of the egg and CAM tissue on the stage of the inverted microscope is illustrated in Figure 19.6.6.

With an inverted microscope, the thin CAM tissue rests on a stationary coverslip, minimizing the effects of embryonic and respiratory movement. By focusing up and down, the CAM tissue can be optically sliced throughout its depth.

8. Use the lateral window prepared for cell injection for oblique illumination using a fiber optic light source with 150 W halogen lamp. Direct the light at an angle of ~45° to the optical axis.

This oblique angle will assist in obtaining high-contrast views of the microcirculation. This type of illumination refracts more light from one side of the cells than the other, producing a shadowy effect that gives a three-dimensional quality to the image. Fluorescent labeling of the cells aids in identifying them in vivo. To avoid quenching, however, the fluorescence illumination should only be used periodically (i.e., for positive identification of cells). Transillumination using the fiber optic light source should be used otherwise. The video camera with the extended red sensitivity avoids the problem of having a monochromatic red image due to the high blood volume in the light path.

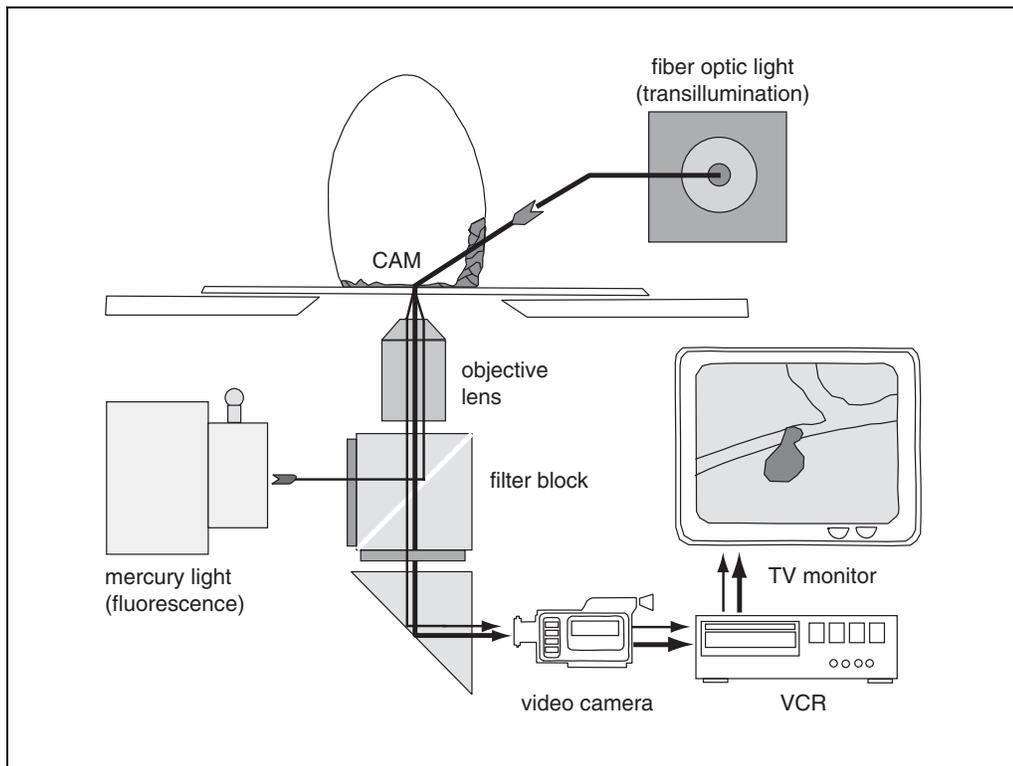


Figure 19.6.6 Schematic of the intravital videomicroscopy technique, as used for real-time observations of the interactions of injected cancer cells with the CAM microcirculation. The CAM is exposed and positioned on a plastic platform with a coverslip window above the objective lens of inverted microscope. For visualization of fluorescently labeled cancer cells, oblique transillumination is provided by the fiber optic light and epifluorescence through the objective aids in positive identification of the cancer cells. Images are collected through the video camera and saved to SVHS tape or digitally on a computer. Diagram by S. Koop and I.C. MacDonald, republished with permission of Plenum Publishers from Chambers et al. (1995).

Perform IVVM

9. Capture the microcirculation images using a Newvicon tube video camera with extended red sensitivity and appropriate capture device.

These black-and-white images can be viewed directly on a video monitor, recorded on a SVHS videocassette recorder, or captured by computer programs such as Optimas Image Analysis (Media Cybernetics) or Northern Eclipse (Empix Imaging). A 35-mm camera can also be attached to the microscope for color photographs of the CAM. A character generator adds time, date, and stopwatch information to the video signal. A stage micrometer can be used to calibrate the magnification of the video image for each objective lens. Images can be obtained using either transillumination or epifluorescence alone, or a combination of both.

10. When the entire system is set up for viewing the CAM, inject 0.1 ml tumor cell suspension through the 30-G needle attached to the cannula (step 2).
11. View the cells at various magnifications starting with 10× to identify the cells. Carry out detailed examination of these cells and their immediate environment at higher magnification. Use video replay to measure cell and vessel dimensions, locations, and other parameters.

Refer to Chambers et al. (1992), MacDonald et al. (1992, 1998), and Koop et al. (1994, 1995, 1996) for further discussion.

IVVM observations can be carried out for up to 4 hr post-injection without visible changes in blood flow or embryo movement. After the observations, the CAM tissue can be fixed in neutral-buffered formalin for later histopathological analysis.

The calibration scale on the fine-focus knob of the microscope permits the measurement of the cancer cell depth below the capillary plexus of the CAM. As a reference point for these measurements, the red blood cells within the plexus are clearly imaged, then the distance below this point, where the fluorescence image of the cancer cells are in sharpest focus, is determined using the fine-focus knob of the microscope.

Perform real-time in vivo kinetic analyses

12. Observe later stages of the interactions between tumor cells and CAM microcirculation by injecting as described (see Basic Protocol 1, steps 10 to 13 and 15 to 16). At specific time intervals (1 to 7 days postinjection) remove individual eggs from the incubator and observe as described above.

Since the injection window is sealed, a new small window for observation with oblique illumination must be prepared (see Basic Protocol 1, step 3). The observation procedures are then carried out as described above.

LABELING CELLS WITH FLUORESCENT NANOSPHERES

In order to allow positive identification of cancer cells, human or rodent, within the CAM (or in mouse tissues), it is necessary that the cells be labeled in some way. Some lines, such as melanoma cells (e.g., B16F1), express melanin, which can be detected by eye (Chambers et al., 1982, 1992; Koop et al., 1994). Exogenous labels such as Calcein-AM (Chambers et al., 1992; Morris et al., 1994) or fluorescent nanospheres (~0.05- μm labeled polystyrene microspheres; Morris et al., 1994; Naumov et al., 2002) have been used to label the cytoplasm of cells to be injected without any effect on membrane integrity or growth potential of these cells. Alternatively, the gene for green fluorescence protein (GFP) can be transfected into cell lines, providing a heritable label that does not dilute with cell division (Naumov et al., 1999, 2002). Clones of GFP-transfected cells must be tested for brightness for use with IVVM as well as maintaining genotypic and phenotypic properties of the parental cell line (Chambers and Wilson, 1988; Naumov et al., 1999). Most cell lines readily take up the fluorescent nanospheres when incubated with them in vitro, prior to injection.

Materials

- 0.05- μm -diameter, fluorescent, carboxylated P(S/V-COOH), dragon-green (480/520 nm) nanospheres (Bangs Laboratories) for cell labeling; store at 4°C in the dark
- OptiMEM serum-reduced medium (Invitrogen)
- Cells to be labeled
- Sonicator
- 50-ml conical polystyrene centrifuge tubes
- 0.2- μm syringe filter and 10-ml syringe
- Aluminum foil
- 75-cm² tissue culture flask
- 150 × 15-mm tissue culture dishes
- Tissue culture incubator, standard, 37°C, 5% CO₂
- Additional reagents and solutions for culturing, trypsinizing, and counting cells (UNIT 1.1)

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Prepare fluorescent nanospheres

1. Sonicate a 10-ml vial of 0.05- μ m-diameter, fluorescent, carboxylated P(S/V-COOH), dragon-green nanospheres 15 min.
2. In a tissue culture hood, add 75 μ l nanospheres to 10 ml OptiMEM serum-reduced medium or 375 μ l nanospheres to 50 ml OptiMEM medium in a 50-ml conical polystyrene centrifuge tube.
3. Sterilize the nanosphere suspension by passing through a 0.2- μ m syringe filter, attached to a 10-ml syringe, into a clean, sterile 50-ml conical centrifuge tube. Wrap the tube in aluminum foil and store undisturbed at 4°C overnight.

Prepare cells to be labeled

4. The next day, centrifuge the nanospheres 10 min at 3000 \times g, room temperature. Carefully remove the upper 2.5 ml for a 10-ml suspension or 30 ml for a 50-ml suspension. Avoid disrupting the sediment at the bottom of the tube. Vortex the suspension.
5. Cells to be labeled to 80% confluency by standard laboratory methods (*UNIT 1.1*) in either a 75-cm² tissue culture flask or 150 \times 15-mm tissue culture dish.
6. Wash the cells twice with 10 ml OptiMEM medium.

Allow cells to take up nanospheres

7. Completely remove the last wash and add either 7.5 ml filtered nanospheres for a 75-cm² tissue culture flask, or 20 ml for a 150 \times 15-mm tissue culture dish.
8. Incubate 2 hr at 37°C in CO₂ incubator, swirling occasionally to distribute the nanospheres evenly.
9. Following the incubation, wash cells twice with 10 ml OptiMEM to remove excess nanospheres.

Prepare cells for injection

10. Trypsinize the cells by standard laboratory procedures (*UNIT 1.1*).
11. Perform a cell count with a hemacytometer to determine cell number (*UNIT 1.1*).
12. Using the medium and serum in which the cells are normally grown, resuspend and adjust the volume of the cell suspension to give the desired number of cells per 0.1 ml injection volume. Store the cells at 4°C for up to 2 hr prior to injection (see Basic Protocol 1, step 5).

In general, fluorescent labeling of cells should be carried out using cytoplasmic rather than nuclear markers. Cells labeled with the nuclear stain acridine orange were shown to be prone to lysis, which markedly affects their metastatic properties in vivo (Morris et al., 1994). Alternatively, cells can be detected using stably transfected fluorescent markers such as green fluorescent protein (GFP; Naumov et al., 1999).

13. Determine if the uptake of the nanospheres affects the viability or growth of the cells by plating labeled and unlabelled cells in six 60-mm dishes at 100 cells per dish. Incubate undisturbed for 11 to 14 days. Stain colonies, count, and determine plating efficiencies:

Plating efficiency = (no. colonies/no. cells plated) \times 100%.

CELL ACCOUNTING IN TISSUES

This experimental procedure enables one to quantify the survival of cells injected into the CAM. To do this, the number of cells actually observed in a sampled volume of tissue at any time must be expressed relative to the number of cells that originally entered that volume. Based on the standard method of measuring blood flow, inert plastic microspheres (10 μm) are added to the cell suspension, prior to injection, at a known ratio (e.g., 1 microsphere:10 cells). These microspheres become trapped by size restriction in the blood capillaries where they remain indefinitely. Thus, the total number of microspheres in a particular volume of tissue provides a reference marker for the total number of cells that originally entered this area, and they can be assessed by either IVVM (see Basic Protocol 2) or histology on thick sections (30 to 50 μm). General information about this procedure can be found in Koop et al. (1995), Morris et al. (1997), and MacDonald et al. (1998).

Materials

Tumor cell suspension, with or without fluorescent nanospheres (see Support Protocol 2)

10- μm -diameter, yellow/green (505/515 nm) fluorescent, plastic microspheres (fluorospheres; Molecular Probes)

60-mm tissue culture dishes

Additional reagents and equipment for fluorescent microscopy (UNIT 4.2)

1. Prepare a tumor cell suspension labeled with fluorescent nanospheres if desired (see Support Protocol 2).
2. Calculate the final injection volume and adjust the tumor cell concentration in a smaller volume so the same number of cells are being injected, compensating for the additional volume of the predetermined amount of accounting spheres that will be needed to give the final volume of 0.1 ml per embryo.

For example, the injection suspension is calculated as follows to compensate for the additional volume of the accounting sphere suspension: 1 ml (the final volume that will contain the predetermined number of cells and accounting spheres) minus the volume containing this number of accounting spheres (e.g., $\frac{1}{10}$ the number of tumor cells) = the volume of growth medium in which to resuspend the tumor cells.

3. Add the appropriate amount of 10- μm -diameter, yellow/green, fluorescent, plastic microspheres to the cell suspension.

The fluorospheres, also known as accounting spheres, are at a known concentration and are already sterile. A recommended ratio is 1 accounting sphere:10 cells.

4. Confirm the intended calculated ratio by putting a drop of the suspension to be injected onto a microscope slide, and counting cells and beads in a given area using a standard light microscope (UNIT 4.1).

The prepared suspension of cells with accounting spheres are now ready for injection and can be maintained at 4°C until injection into the CAM.

5. Inject the cell/microsphere suspension into the CAM.
6. Examine the embryonic tissue for the presence of accounting microspheres and fluorescently labeled cells (nanospheres). Use the numbers to calculate injected cell survival.

The ratio in the tissues at various times after injection, divided by the ratio in the syringe before injection, is referred to as the cell survival (CS) index (Koop et al., 1995; Morris et al.; 1997; MacDonald et al., 1998). Thus, a CS index of 1.0 (i.e., 10 cells observed in vivo for every microsphere in a given tissue volume) would imply 100% survival, whereas a decrease in CS index would imply <100% survival (i.e., if only five cells were observed for every microsphere in a given tissue volume at some time after injection, 50% of the cells could be accounted for, and 50% of the cells would have been lost, either by cell death or by passage through the organ). This assay allows for cell accounting relative to the total number of injected cells rather than the percentage of observed cells remaining in vivo at times after injection.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Citrate saline

4.4 g/liter sodium citrate
10.0 g/liter KCl
Sterilize by autoclaving 20 min
Store up to 3 months at 4°C

No pH adjustment is necessary.

Collagenase, 0.3%/trypsin, 0.02%

Prepare a trypsin stock by adding a 10-ml bottle of trypsin to 100 ml sterile HBSS (see recipe) using proper sterile technique. Store up to 2 weeks at 4°C.

Prepare a fresh collagenase stock by combining 300 mg collagenase IV with 80 ml HBSS. Stir 1 hr and filter through a 0.8- μ m filter, then through 0.2- μ m filter.

Prepare the collagenase/trypsin mix by adding 20 ml trypsin stock to 80 ml collagenase stock. Mix well and store up to 2 weeks at 4°C.

DNase I

Add 1 mg DNase I per milliliter HBSS (see recipe). Sterilize by passing through a 0.2- μ m filter. Store up to 2 weeks at 4°C.

HBSS

Autoclave 450 ml water in a 500-ml bottle, then allow to cool. Under sterile conditions, add 50 ml of 10 \times HBSS solution (InVitrogen) and 1.5 ml of 7.5% sodium bicarbonate (InVitrogen), and adjust to pH 7.0. Store up to 3 months at 4°C.

Methylene blue stain

Prepare a 1% (w/v) solution of methylene blue in 70% ethanol. Stir for a few hours until the crystals are completely dissolved. Add glutaraldehyde to a final concentration of 1%. Store in a 500-ml plastic wash bottle up to 2 months at room temperature. Supplement with glutaraldehyde every third staining. If a precipitate forms, filter through chromatography paper.

Ouabain, 1 $\times 10^{-3}$ and 2 $\times 10^{-5}$ M

Prepare a 10⁻³ M stock by dissolving 72.86 mg ouabain in 100 ml tissue culture medium with serum for the cell line used. Filter sterilize by passing through a 0.2- μ m filter. Store up to 2 weeks at 4°C.

Prepare the 2 $\times 10^{-5}$ M working solution by adding 10 ml of 10⁻³ M stock to a 500-ml bottle of tissue culture medium with serum. Store up to 2 weeks at 4°C.

PBS, calcium and magnesium free

8.0 g NaCl

0.2 g KCl

0.2 g KH₂PO₄

1.15 g Na₂HPO₄

Adjust to 1 liter with water

Autoclave for 20 min

Store up to 3 months at 4°C.

This buffer is calcium and magnesium free.

Trypsin, 2.5% (w/v), in citrate saline

Add one 10-ml vial of Difco Bacto-Trypsin to a 500-ml bottle of sterile citrate saline (see recipe). Store up to 3 months at 4°C. Warm in a 37°C water bath before trypsinization of cells.

COMMENTARY

Background Information

The experimental metastasis assay in the chick embryo chorioallantoic membrane (CAM) allows for two standard types of experiments. First, single time-point experiments are generally terminated 7 days after injection. These assays give a measure of metastatic growth ability of injected cancer cells in the chick liver, which is the first capillary bed reached by cells injected via CAM veins. These assays are useful for quickly comparing the metastatic properties of different cell lines or treatments. This end point would be analogous to single-time-point experiments in mice, in which the numbers and sizes of metastatic foci are determined at some time after i.v. injection. For quickly growing tumor cell lines, this end point (macroscopic tumors detected at the liver surface) can also be used in the chick embryo. In contrast, in the ouabain plating assay, the numbers of tumor (rodent) cells are counted. This number will be related to the number of metastases that grow, their growth rate, and the numbers of cells that remain without division.

Kinetic experiments can also easily be performed with the chick assay, by measuring the numbers of viable rodent cells in chick organs at various times after injection, as illustrated in Figure 19.6.5. See Chambers et al. (1982), Chambers and Ling (1984), and Chambers and Wilson (1985), for examples of the results that can be obtained with this assay. The fate of injected cells over time after arrival in the target organ determines the growth or death rates of cells in vivo. This information is generally not obtained from experimental metastasis assays in mice, because information on the cells is not obtained until metastases are large enough to be detected by the eye, and then only from a

single end point. It should be noted that due to the short experimental time of this assay, genetically-manipulated cells which may have unstable genotypes should be considered for this assay rather than long-term tumor formation assays. The ability to easily perform growth kinetics, from immediately after injection to 7 days post-injection, is a strength of the chick embryo experimental metastasis assay.

The ability of the chick to develop over a range of temperatures can be a powerful genetic tool when applied to the study of metastatic properties of cancer cells having temperature-sensitive mutations. The experimental metastasis assay is also very sensitive to small changes in growth ability, to the point of being able to detect a response within a day of a change in growth conditions, as described for temperature-sensitive *src*-transformed cells shown in Figure 19.6.5B (see Chambers and Wilson, 1985).

The laboratory mouse is the most commonly used animal for in vivo metastasis assays. Many of these assays require the use of an immune-deficient host, such as athymic nude or SCID mice, which are expensive and require special care and housing. The authors have previously shown that the cost of assessing the metastatic properties of cell lines in the experimental metastatic assay using the chick embryo is approximately one-quarter the cost of testing the same cells in nude mice, and approximately one-fifth the cost of testing in nude mice and plating mouse livers to recover viable cells (Chambers et al., 1990). It should be noted that these calculations did not include the start-up cost for expensive facilities to house the immune-deficient mice. In contrast, the equipment for maintaining the chick embryo is inex-

pensive and readily available (Chambers et al., 1990). The experimental metastasis assay in the chick embryo is thus a cost-effective method that can be useful for prescreening cells, which can subsequently be tested in nude or SCID mice, using considerably smaller numbers of mice. The authors have shown that results from the two assays can give comparable results for individual cell lines (Chambers et al., 1990). In addition, in some institutions, use of chicken embryos may be subjected to less complex animal care and use regulations than those applied to the use of mice in tumor studies. Overall, the use of chick embryos to study metastasis provides a useful alternative to metastasis assays in mice and may offer advantages for many researchers.

Critical Parameters and Troubleshooting

Many of the critical parameters have been outlined above in the appropriate protocol sections. In general, several steps require a steady hand, such as the drilling of windows in the egg shells, lifting the shell to form the injection window, and injection of cells into CAM veins. These steps are not hard but do require practice. A good approach to developing injection skills is to carry out experiments in which a melanotic cell line, such as murine B16F1 or B16F10, are injected into CAM veins. Success of the injections can be determined 7 days later, as these cell lines form macroscopic tumors in the CAM tissue and liver; these can be readily detected upon dissection of the embryo and inspection by eye.

The fertility rate of chick embryos may vary seasonally and with geography and weather conditions; however, with experience, this rate can be predicted for the local situation. Extra eggs should be ordered until this fertility rate is known.

Anticipated Results

In Basic Protocol 1, some rapidly growing cancer cell lines (such as B16F1 and B16F10 melanoma) form metastases detectable by eye during a 7-day incubation period following injection into chick CAM veins (e.g. Chambers and Wilson, 1988, Koop et al., 1994). Detection of these may be aided by production of melanin by some cell types. Growth of many other cancer cell types can be detected using the ouabain recovery procedure, and these have been compared with results from experiments in mouse (Chambers et al., 1990). The *in vivo* growth kinetic analysis (Fig. 19.6.5). offers an

especially powerful approach to characterizing *in vivo* behavior of cancer cell growth in metastatic sites and permits subtle changes in growth to be detected (e.g., due to a temperature shift, affecting a temperature-sensitive oncogene in the example shown; Chambers and Wilson, 1985).

Further information on the behavior of cancer cells *in vivo* can be obtained by direct observations, as permitted by the use of *in vivo* videomicroscopy (Basic Protocol 2). For example, use of this procedure permitted the *in vivo* effects of over-expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in B16F10 cells to be determined (Koop et al., 1994). While it had been expected that TIMP-1 would inhibit escape of cancer cells from the microcirculation, that study found instead that the TIMP-1 overexpressing cells extravasated well, but had a defect in micrometastatic growth. Because the CAM is a thin structure, it is relatively easy to determine if cells are intravascular or extravascular, permitting kinetics and efficiency of extravasation to be easily quantified (e.g., Koop et al., 1995, 1996). These studies showed that the large majority of cells injected into the CAM circulation successfully extravasate, a finding also seen in mice (Chambers et al., 1995, 2002). The chick embryo thus can provide a low-cost, simple complement to more expensive and complex experiments in mice.

Time Considerations

The experimental metastasis assay (see Basic Protocol 1) involves injecting 11-day-old chick embryos and then collecting data at time intervals from time 0 to 7 days post-injection. Experiments must be planned ahead, in order to have embryos of the desired age on the appropriate day. The set-up time required for injection of the embryos is ~2 hr including the preparation of the cells for injection. The injection time is dependent on the number of embryos being injected. The dissection of the chick embryos, dissociation of the livers, and setting up of the ouabain plating assay usually require 3 hr, again depending on the number of embryos in the experiment. The *in vitro* part of this assay requires 10 to 14 days for formation of colonies, depending on the growth rate of the cells *in vitro* to form colonies.

IVVM of chick embryo CAMs (see Basic Protocol 2) can generate data in as short a time as 2 min post-injection and up to 4 hr. The embryos can be used from time 0 to 7 days post-injection. The set-up time for the embryo before visualization is ~2 hr including the cell

suspension preparation. IVVM, although a powerful observational tool, is generally a labor-intensive procedure, as information can be obtained in real-time for only one embryo at a time.

For Support Protocol 1, daily monitoring of the eggs in the incubator and addition of water takes ~15 min per day. For Support Protocol 2, labeling of cells with nanospheres, preparation of the nanospheres the day before the experiments requires 0.5 to 1 hr. Labeling of the cells and preparation of the cells on the day of the experiment takes ~4 hr. Addition of accounting spheres in Support Protocol 3 will require ~1 hr of preparation time.

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Figure 19.6.1 Diagram of experimental metastasis assay in chick embryos. Cancer cells are injected via a chorioallantoic membrane (CAM) vein, through a window opened in the shell of 11-day-old chick embryos. Cells injected via this route travel via the circulation first to the embryonic liver. After incubation for up to 7 days, metastatic cells in an embryonic organ such as liver are quantified using the ouabain plating assay, or (for rapidly growing cell lines) macroscopic metastases can be quantified. Republished with permission of Anticancer Research from Chambers and Tuck (1988).

Figure 19.6.2 The drilling of the marked square or window on the egg over the selected vein area with a Dremel Moto tool is done in an enclosed still hood. To minimize breathing of eggshell dust, hang a cloth from the bottom of the glass front (or alternatively wear a mask and protective eyewear). Drill all four sides of the window, with the egg at a slight angle and the air sac end (blunt end) up and the marked window facing toward the person drilling. The bit of the Dremel tool is brought at right angles to the surface of the shell. Gentle pressure is sufficient for cutting due to the high speed of the drill. Only the shell is cut while avoiding the underlying smoother papery-white membrane or the underlying CAM.

Figure 19.6.3 The arrangement of equipment inside the tissue culture hood just before injections proceed. The front of the hood is opened for easier access during the procedure with adequate sterility still being maintained. The gooseneck arm of the fiber optics light is illuminating the air sac end of an egg positioned on the egg stand (made by gluing three rubber corks onto a plastic petri dish) ready for injection. The remaining eggs have had the shell of the drilled window carefully removed over the vein area and are placed in a tray with the windows facing upwards awaiting injection. The cell suspension has been prepared at a predetermined concentration ready to be drawn up into the 1-cc syringe following gentle vortexing. The other 1-cc syringe contains the paraffin oil of which a drop will be placed on the exposed window area prior to injection. The melted Paraplast in the glass beaker (left) is cooling in preparation for sealing of the entire window area following injection.

Figure 19.6.4 Injection of cells into a chick embryo CAM vein. Position the egg on the egg stand, with the fiber optics light illuminating the air sac end for visualization of the large Y shaped vein. The addition of a small drop of paraffin oil onto the injection site enhances the clarity of the vein. The beveled tip of the needle is facing upwards for gentle insertion into the branch area of the Y (see inset) just until the beveled end is completely into the vein. The needle should be at the angle seen in the picture above to prevent damage to the vein. If a proper injection is performed, the vein will temporarily become clear as the 0.1-ml inoculum is smoothly injected, and there will be no blood seeping from the injection point either during or after the injection.

Figure 19.6.5 Example of in vivo growth kinetics results that can be obtained with the chick embryo experimental metastasis assay and the ouabain plating assay to recover rodent (and thus, ouabain-resistant) cells at various times after injecting cells via a CAM vein. (A) B77-NRK (normal rat kidney) cells transformed with the src oncogene, or (B) LA23-NRK cells (NRK cells carrying a temperature-sensitive src oncogene, which is active at 36° and inactive at 38°C) were injected into CAM veins at 5×10^4 cells per embryo. Closed circles: embryos maintained at 36°C; Open circles: embryos transferred to 38°C at day 4. Each point represents the number of viable ouabain-resistant cells present in the liver of one embryo; lines connect median points. Republished with permission of American Society of Microbiologists from Chambers and Wilson (1985).

Figure 19.6.6 Schematic of the intravital videomicroscopy technique, as used for real-time observations of the interactions of injected cancer cells with the CAM microcirculation. The CAM is exposed and positioned on a plastic platform with a coverslip window above the objective lens of inverted microscope. For visualization of fluorescently labeled cancer cells, oblique transillumination is provided by the fiber optic light and epifluorescence through the objective aids in positive identification of the cancer cells. Images are collected through the video camera and saved to SVHS tape or digitally on a computer. Diagram by S. Koop and I.C. MacDonald, republished with permission of Plenum Publishers from Chambers et al. (1995).

CHAPTER 20

Expression and Introduction of Macromolecules into Cells

INTRODUCTION

Cells are fundamentally insular things isolated and protected from their surroundings by their plasma membranes. However, it is frequently necessary for cell biologist to overcome the isolation of the cell's interior in order to probe its workings at a molecular level. As a result, many experimental strategies employed by cell biologists rely upon the introduction or expression of particular macromolecules within cells of interest. The goals of such techniques include the expression or introduction of fluorescently-tagged proteins, allowing the examination of the subcellular localization and behavior of the protein within living cells. Similarly, these techniques can be used to perturb the function of particular proteins through the addition of antibodies, inhibitors or mutant forms of the protein or through inappropriate expression with altered abundance or timing of protein accumulation. Additionally, techniques for introduction of macromolecules into cells are frequently utilized to load cells with impermeant dyes that can be used to monitor various aspects of cellular physiology, including changes in ion concentrations. This chapter will cover methods that have been developed for such experimental strategies. The first two units of this chapter discuss simple and inexpensive methods for the introduction of a variety of macromolecules (*UNIT 20.1*) or specific fusion proteins (*UNIT 20.2*) into cells. *UNITS 20.3, 20.4, 20.5, 20.6 & 20.7* discuss the introduction of nucleic acids into cells for the purpose of altering gene expression.

UNIT 20.1 provides a variety of flexible protocols that can be inexpensively used to load a broad range of macromolecules into cells. Methods covered in *UNIT 20.1* include scrape loading, scratch loading, bead loading and syringe loading. These techniques all rely upon the physical disruption of the plasma membrane to promote the uptake of target molecules, and upon the capacity of cell to re-seal the plasma membrane after damage. The techniques discussed in *UNIT 20.1* have a number of advantages, including the fact that their implementation does not require specialized equipment and that they can be utilized to simultaneously load a large number of cells. They are also useful because a large range of macromolecules can be introduced in this manner. These techniques would not be preferred under conditions where the macromolecule being loaded is particularly precious, since each of the techniques require a relatively large amount of such material.

UNIT 20.2 describes the introduction of proteins into cells using an eleven amino acid sequence from the HIV-TAT transduction domain. This amino acid sequence has the remarkable property of allowing the HIV-TAT protein to pass through the intact plasma membrane and enter the cytoplasm of cells. It has also been shown to confer this property to a number of other proteins when expressed as a fusion moiety. Thus, investigators can express this sequence as a tag to their proteins of interest in bacteria, purify the resultant fusion peptide using standard biochemical means, and introduce the fusion protein into cells by simply introducing the fusion protein to the cell culture medium. Like the methods discussed in *UNIT 20.1*, these techniques have considerable advantages because of their simplicity and because of the fact that they are economical and have minimal requirements for specialized equipment.

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Expression and
Introduction of
Macromolecules
into Cells

20.0.1

Supplement 21

While *UNITS 20.1 & 20.2* offer the possibility of direct introduction of proteins into cells, expression of cloned genes from transfected plasmids is more commonly used to probe protein function. *UNITS 20.3, 20.4, 20.5, 20.6 & 20.7* discuss transfection protocols to introduce DNA into cells for this purpose. These protocols include calcium phosphate transfection (*UNIT 20.3*), DEAE dextran transfection (*UNIT 20.4*), electroporation (*UNIT 20.5*) and lipid-mediated transfection (*UNIT 20.6*). Calcium phosphate (*UNIT 20.3*) and DEAE dextran (*UNIT 20.4*) chemically induce the association of plasmid DNA to the cell surface, resulting in its uptake through endocytosis. Cationic lipids spontaneously associate with nucleic acids through charge interactions. In the case of plasmids, the resulting DNA-lipid structures are also capable of transducing genes into cells through endocytosis (*UNIT 20.6*). In all cases, the pathway through which DNA eventually enters the nucleus after endocytosis is poorly defined. By contrast, electroporation uses an electric field to open pores in the cell to allow the entry of DNA through diffusion. Electroporation is less dependent upon special characteristics of the cell than other transfection techniques and can therefore be used for introduction of plasmid DNA into a very broad spectrum of cell types. Calcium phosphate, DEAE dextran, and lipid-mediated transfection are typically employed in the transfection of adherent cell lines. Lipid-mediated transfection and electroporation are typically used for non-adherent cells. Since the successful use of all transfection methods depends heavily on the cell line under study and conditions of the particular experiment, *UNIT 20.7* discusses strategies for optimization of transfection efficiency using reporter systems.

Together, these techniques comprise some of the most basic tools available to cell biologists. In each case, they are designed to overcome the challenges posed by the insular nature of cells and to help us to manipulate and understand the machinery of the intact cell.

Mary Dasso

Direct Introduction of Molecules into Cells

UNIT 20.1

Techniques for introducing normally impermeant macromolecules into the living cell—referred to as “cell-loading techniques”—open up many possibilities for the cell biologist. Examples of the investigations that can be carried out using such methods include measuring cytosolic ion concentrations accurately by means of a large, highly membrane-impermeant fluorescent probe; defining the native location of a protein (fluorescently tagged) dynamically in time and space; perturbing the normal functioning of a cellular protein (for example, by introducing antibodies or other specific inhibitors); or altering the genome (by introducing antisense or expression-vector nucleotide sequences). Microinjection is probably the most commonly used technique for introducing fluorescent probes, fluorescently tagged proteins, and antibodies into living cells for short-term studies of cell physiology and protein location and function. It is, however, not the only technique available, nor the easiest or least expensive to implement. Among the alternatives are several closely related techniques that, like microinjection, rely on the cell’s ability to reseal a mechanically induced plasma membrane disruption (see McNeil, 2002, for a review) created in order to gain temporary access to cell cytosol. Four such techniques are described here: scrape loading (see Basic Protocol), scratch loading (see Alternate Protocol 1), bead loading (see Alternate Protocol 2), and syringe loading (see Alternate Protocol 3). Although these techniques may not be competitive with microinjection in terms of economy of use of the macromolecule to be loaded, or as efficient at loading very large macromolecules (>100,000 mol. wt.), their implementation does not require the acquisition of a specialized skill or expensive equipment. Additionally, unlike microinjection, they allow one to rapidly load (in a matter of minutes) thousands or even many millions of many types of mammalian cells with normally impermeant molecules, and so to facilitate quantitative analyses of the effect of loading (Doberstein et al., 1993).

STRATEGIC PLANNING

An excellent probe for testing the effectiveness of each of the loading techniques below, and for working out the critical parameter of imposed mechanical load (see Critical Parameters), is fluorescein-labeled dextran (available from Sigma or Molecular Probes). It is inexpensive and can be purchased in a range of molecular weights (from $\sim 3 \times 10^3$ to 5×10^6), allowing one to approximately match it in terms of size to the protein or other probe ultimately to be loaded. Fluorescein-labeled dextran is available in a fixable form (conjugated with lysine residues), allowing it to be used initially with fixed rather than living cell specimens.

Because the loading techniques described in this unit, like microinjection, damage cells, it is important to compare control populations of loaded cells, such as those loaded with fluorescein dextran only, with experimental cells loaded with both fluorescein dextran and the molecule of interest, before concluding that an effect of loading is specific to the molecule loaded. Mixing the molecule of interest with fluorescein dextran creates a “loading solution” that will provide this experimental population. It is important also to compare the relevant behavior of the fluorescein dextran-loaded “control” population with that of undisturbed (nonfluorescent) cells, which are always present in the populations generated by these techniques. In this way, those effects on cell behavior that are caused by the loading procedure alone can be detected. In this regard, the authors of this unit have noticed that cells suffering plasma membrane disruptions often contain more intracellular vesicles than undisturbed neighbors (this is probably related to the membrane-membrane fusion process that mediates resealing; Terasaki et al., 1997), but, in the cell types that have been studied in the authors’ laboratory, there has been no evidence

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that apoptosis is induced among loaded cells. Indeed, one can observe fibroblasts that have been scratch-loaded with fluorescein dextran locomoting into adjacent denuded zones of the coverslip within 60 min post-wounding, and then undergoing cell division there 12 to 24 hr later (Swanson and McNeil, 1987). Cytosolic levels of Ca^{2+} are rapidly (within seconds to minutes) restored to normal levels after wounding (by microinjection or other, more radical means of making cell-surface disruptions)—another indication of the remarkable, and biologically essential, capacity of cells to rapidly reseal and hence survive plasma membrane disruptions (McNeil, 2002).

One or more of these techniques should be applicable to any type of mammalian cell, and also to other eukaryotic cells that lack an external cell wall. For cells that must be loaded or are most conveniently loaded as a suspension, syringe loading is the technique of choice. For adherent cells, any of the additional techniques described below could be used.

A final question is what quantity of one's, often precious molecule is needed? The answer to this question will depend, of course, on the experimenter's goal. If it is simply to produce a fluorescent signal readily visualized or measured microscopically or via a flow cytometer, then initial tests with a fluorescent dextran of appropriate size and concentration (e.g., the size and concentration of the molecule of interest) should provide a useful preliminary answer. The authors find that 0.5 to 1.0 mg/ml solutions of fluorescein dextran (mol. wt., 10 to 70 kDa) result in a readily assayed or measured fluorescent signal from cells loaded by any of these techniques. Sample volume is another important issue. Some of these techniques require that only a very small volume of loading solution be employed. For example, using syringe loading, an ultramicropipettor (1- to 10- μl range), and ultramicro pipet tips, no more than 1 μl is needed. In general, the minimum usable volume is that which prevents the cells from being damaged by drying during the ~1 to 2 min required to execute the crucial plasma membrane-disrupting step of each of these techniques.

BASIC PROTOCOL

SCRAPE LOADING

Transient, survivable plasma membrane disruptions are produced in the presence of the molecule to be loaded by tearing the cells off of their culture substratum (McNeil et al., 1984).

Materials

Adherent cells of interest, growing in tissue culture (also see *UNIT 1.1*)
Dulbecco's phosphate-buffered saline (DPBS; *APPENDIX 2A*) or equivalent
physiological saline containing 1 to 1.5 mM CaCl_2 at physiological temperature
(37°C for mammalian cells)

Loading solution: DPBS (with 1 to 1.5 mM calcium) containing molecule to be
loaded, at physiological temperature (37°C for mammalian cells)

Rubber policeman

Circular tissue culture dishes

Additional reagents and equipment for cell culture (*UNIT 1.1*)

1. Culture cells on a substratum to which they adhere strongly and which allows an unobstructed approach with a rubber policeman (see step 4).

Circular-profile tissue-culture-grade dishes are usually a good choice for mammalian cells, as unimpeded access is available after the lid has been removed.

Basic techniques for culturing mammalian cells are presented in UNIT 1.1.

2. Wash the cells twice with 37°C DPBS (or other physiological saline compatible with the macromolecule to be loaded and with cell viability).

This and all saline solutions used in subsequent steps should be maintained as closely as possible to 37°C, the optimum resealing temperature.

3. Remove the second DPBS wash. Add the loading solution and swirl to mix it thoroughly with any plain saline that might still be present on top of the cell layer.

The minimal volume required is that which will prevent cell-drying damage during the minute or two of the scraping procedure.

4. Scrape the cells off of their substratum using a rubber policeman. Leave the cells 5 to 10 min in the loading solution before proceeding with the next step. Check for completeness of cell removal by examining with a phase-contrast microscope.
5. Mix the now suspended and loaded cell population with a 10-fold or larger volume of plain DPBS, and wash the cells several times by centrifuging for 10 to 20 min at $5000 \times g$, at a temperature appropriate for the cell type, removing the supernatant, resuspending the cells in ≥ 10 volumes of DPBS, and repeating the centrifugation.
6. Replate the cells and return them to normal culturing conditions if the goal is to study adherent, loaded cells, e.g., for microscopic analysis, or use them immediately after washing if the goal is to study suspended, loaded cells, e.g., for flow cytometric analysis.

SCRATCH LOADING

Partial, rather than total, cell removal (as in Basic Protocol), as well as severing of cell processes, is used to create plasma disruptions (Swanson and McNeil, 1987).

Additional Materials (also see Basic Protocol)

30-G syringe needle or similar sharp implement (e.g., Fisher)
Glass coverslips

1. Culture cells on any substratum to which they adhere strongly and which allows approach with a syringe needle tip (see step 4).

A glass coverslip is a good choice, especially if the goal is to observe loaded cells under the microscope. Basic techniques for culturing mammalian cells are presented in UNIT 1.1.

2. Wash the cells twice with DPBS (or other physiological saline compatible with the macromolecule to be loaded and with cell viability).

If using a coverslip, the washings are easily accomplished by grasping the coverslip with forceps and then transferring it from one beaker containing saline wash to another.

This and all saline solutions used in subsequent steps should be maintained as closely as possible to 37°C , the optimum resealing temperature.

3. Cover the monolayer with loading solution and mix well to ensure that the loading solution combines thoroughly with any plain saline still present on top of the layer.

If a coverslip is being used, pipetting the loading solution onto and off of the cells several times will accomplish this.

4. Scratch the monolayer surface one or more times with a 30-G syringe needle.

This will denude small (two- to four-cell-wide) strips of the monolayer. Loaded cells will be present along these denuded zones but not elsewhere in the culture. Increasing the number of scratches will increase the proportion of loaded cells.

5. Wash the monolayer three to four times thoroughly with plain DPBS or other appropriate physiological saline.

6. Examine or experiment with the cells.

The cells are ready for immediate microscopic analysis. Many of the successfully loaded cells along the denudation sites will have morphologies quite normal in appearance; others will be slightly rounded. Within ~ 30 min, loaded cells will display obvious signs of viability—locomotion into the denuded zone and division there ~ 12 to 24 hr later.

**ALTERNATE
PROTOCOL 1**

**Expression and
Introduction of
Macromolecules
into Cells**

20.1.3

BEAD LOADING

Survivable plasma membrane disruptions are produced in the presence of the molecule to be loaded by the impact of glass beads falling onto and rolling across cells, which remain adherent to the culturing substratum (McNeil and Warder, 1987).

Additional Materials (also see Basic Protocol and Alternate Protocol 1)

Glass beads, 50- to 500- μ M diameter (Sigma)

Glass coverslips

1. Culture cells on any substratum to which they adhere strongly and which will allow glass beads to be added and removed (see steps 3 and 4).

A glass coverslip is convenient for this purpose. Basic techniques for culturing mammalian cells are presented in UNIT 1.1.

2. Wash cells twice with DPBS and immerse them in loading solution (see Alternate Protocol 1, steps 2 and 3). Mix the loading solution well with any residual DPBS.

3. Sprinkle glass beads (50- to 500- μ m diameter) onto the cell monolayer evenly, until ~75% of the monolayer surface appears, by eye, to be covered by them.

To increase the frequency of loading, cause the beads to roll around on the monolayer by rocking it to and fro several times. The glass beads can be used "off the shelf," or sterilized by autoclaving if desired in experiments where sterility must be maintained.

4. Wash monolayer thoroughly with plain DPBS to remove beads and loading solution (see Alternate Protocol 1 for technique).

5. Examine or experiment with cells.

As with Alternate Protocol 1, many cells will have apparently normal morphologies immediately after this loading procedure.

SYRINGE LOADING

Survivable plasma membrane disruptions are created in the presence of the molecule to be loaded by shear forces generated by aspirating and expelling cells repeatedly through a narrow orifice, such as that of a syringe needle or micropipettor tip (Clarke and McNeil, 1992).

Additional Materials (also see Basic Protocol)

Adherent *or* suspension cells of interest, growing in tissue culture (UNIT 1.1)

30-G syringe needle and 1-ml syringe *or* micro- or ultramicropipettor (1- to 10- μ l range) and appropriate pipet tips (e.g., Fisher)

1. Trypsinize or otherwise harvest cells (UNIT 1.1), and wash twice, each time by centrifuging for 10 to 20 min at 5000 \times g, at a temperature appropriate for the cells, removing the supernatant, resuspending the cells in ≥ 10 volumes of PBS, and repeating the centrifugation.

Basic techniques for mammalian cell culture, including trypsinization, are presented in UNIT 1.1.

2. Resuspend the pellet from the final wash in the loading solution.

3. Draw the cell suspension up into a 1-ml syringe fitted with a 30-G needle, or into a micro- or ultramicropipet tip and then expel it (five to ten times is a good starting point). Repeat this maneuver as many times as necessary in order to achieve the desired compromise of loading level in comparison to cell viability (see Critical Parameters).

4. Wash the cells (see Basic Protocol, step 5).
5. Replate the cells and return them to normal culturing conditions if the goal is to study adherent, loaded cells, e.g., for microscopic analysis, or use them immediately after washing if the goal is to study suspended, loaded cells, e.g., for flow cytometric analysis.

COMMENTARY

Background Information

The capacity to seal a plasma membrane disruption is critical to the survival of many cells (McNeil, 2002). This is because many normal, mechanically active cell environments, including many tissues of the body, promote membrane disruption injury. Resealing, in other words, is not merely Nature's gift to the experimental biologist. Knowledge of this capacity did, however, lead to the development of each of the techniques described in this unit. Conditions that promote resealing—physiological temperature and calcium levels—must be maintained during each procedure.

How does one choose among the various methods presented—scrape loading, scratch loading, bead loading, and syringe loading? If analysis post-loading is best done on a suspension of cells, e.g., flow cytometry, then scrape or syringe loading should be used; if it is best done on adherent cells, e.g., microscopy, then scratch or bead loading should be used. However, scrape, scratch, and bead loading require adherent cells as starting material.

Microinjection is the most obvious alternative method. In cases where there is a minimal, limited amount of reagent for loading, microinjection is unrivaled in its economy of reagent: one needs only enough of the reagent to load a microneedle, e.g., less than a microliter. However, microinjection requires special equipment and is labor intensive. The loading techniques described in this unit are by comparison, far less costly to implement and easier to learn. Moreover, they can produce virtually unlimited numbers of loaded cells in minutes.

Critical Parameters

The common feature of all of these techniques is that they bring mechanical force to bear on cells in order to create transient plasma membrane disruptions. Normally impermeant molecules can then enter into the cytosol, until resealing prevents further access. Therefore, to increase loading efficiency by these mechanically based techniques, one simply increases the force applied and hence the number and size of the disruptions. On the other hand, as the extent of

plasma membrane disruption increases, viability decreases. Hence, when first attempting to load a particular cell by these techniques, it will usually be advisable to vary the mechanical force imposed over a wide range, and then to select the loading conditions that provide an acceptable level of both loading and viability.

The following provides some guidelines for manipulating the level of mechanical force imposed by each technique.

In scrape loading (see Basic Protocol), the extent of plasma membrane disruption is mainly a function of the strength of cell-substratum attachment. Agents such as poly-L-lysine can, for many cells, be used to increase adhesion and therefore loading efficiency. Conversely, a wash or two with Ca^{2+} -free saline decreases adhesion for many cultured cells, and therefore decreases the level of loading. These same considerations apply also to the closely related technique of scratch loading (Alternative Protocol 1).

In bead loading (see Alternate Protocol 2), the extent of plasma membrane disruption depends on the size of the beads employed (the authors have used beads ranging from 50 to 500 μm in diameter), the number of beads sprinkled onto the cells, and the degree to which the beads are caused to roll around on the monolayer.

In syringe loading (see Alternate Protocol 3), the size of the orifice and probably other hydrodynamic factors related to barrel length and shape are important. Certainly, smaller orifices, which produce greater shear forces, result in increased loading. Additionally, higher ejection pressures increase loading efficiency. The authors of this unit have described an automated device that allows one to precisely control pressure, but this is not necessary unless one desires a high level of reproducibility between one loading procedure and the next (Clarke and McNeil, 1994). Any narrow-bore orifice can be used, and the authors often find it convenient in minimizing loading solution volume to employ a micropipettor and pipet tips instead of a syringe and needle.

Resealing does not occur in the cold or in the absence of Ca^{2+} , so these conditions must be avoided during the step when plasma mem-

brane disruptions are being created, and for ~1 min thereafter (McNeil and Steinhardt, 1997).

Troubleshooting

Successful loading by the techniques described in this unit requires: (1) that sufficient mechanical force be brought to bear on the cell plasma membrane for tearing or disrupting it; and (2) that the cell then be able to reseal the disruption thus created. Poor efficiency in loading can be explained by a problem in either or both of these two areas.

One or more, though not necessarily all, of the techniques described above will result in the imposition of sufficient force for the loading of most mammalian cells. For example, cells smaller than a typical cultured mammalian cell kept in suspension (~10 μm diameter or greater) may not be amenable to syringe loading with a 30-G needle; a needle of this bore may not impose sufficient shear stress, and another technique must be utilized. As a case in point, the authors have found that mammalian red blood cells (~5 μm diameter) are not susceptible to wounding by syringe loading (P. McNeil, unpub. observ.). Red blood cells can, however, be wounded by scraping after they have been stuck to a plastic substratum coated with poly-L-lysine.

On the other hand, too much force can create disruptions too large or too numerous to be resealed. Low (<50%) recovery of viable cells is a key indicator of this problem, which can be solved by reducing the amount of mechanical force applied. For example, this can be accomplished by using smaller beads in the bead-loading technique, by using a larger-gauge needle or fewer intake and expulsion strokes in the syringe-loading technique, or by treatments that loosen cell substratum adherence (such as prescraping rinses with low-calcium medium for mammalian cells) in the scrape-loading technique. Moreover, it is essential for cell viability when applying these membrane-disrupting techniques that resealing occur. For this to happen, cells need physiological levels of extracellular Ca^{2+} and a near-physiological temperature. Thus, if loading fails, with a heavy loss of cell viability, one should check to ensure that these two requirements have been met—both during the loading procedure, when disruptions are being created, and for the 1- to 5-min period after membrane disruption is initiated, when resealing is taking place.

A few cell types, notably echinoderm eggs, reseal extremely rapidly (even with disruptions >1000 μm^2 in extent), and so provide the ex-

perimeter much less temporal access to cytosol. Indeed, it is the authors' experience that these cells are difficult to load by the techniques described, but easy to microinject since they are rarely killed by this membrane-disrupting technique. In theory, this rapid resealing capacity could be countered by reducing extracellular Ca^{2+} below physiological levels or by chilling the cells that rapidly reseal.

Anticipated Results

The authors and others have successfully loaded fibroblasts, endothelial cells, smooth muscle cells, epithelial cell lines, neurons, and free-living amoebas with these techniques. It is expected that they will work on almost any cell type lacking a cell wall.

The extent of loading is a direct function of the concentration of the macromolecule and an inverse exponential function of its molecular weight. Both of these observations are similar to what would be predicted for a process dependent on diffusion down a concentration gradient through a hole in an otherwise impermeable barrier. Therefore smaller molecules are more effectively loaded than larger ones, and the highest possible concentration of the molecule to be loaded should be employed in the loading solution. One can expect from all of these techniques that the extent of loading will vary over a large range (three-log scale as assessed by flow cytometry). This can be of advantage if one wishes to conduct, for example, a dose-response type of experiment. If, on the other hand, a homogeneous population of, say, heavily loaded cells is desired, then some selection process must be employed, such as flow sorting or microscopic discrimination, based on whole-cell fluorescence derived from the macromolecule loaded.

Time Considerations

These techniques are very rapid, generally taking <30 min.

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Protein Transduction: Generation of Full-Length Transducible Proteins Using the TAT System

Described here is the technology that allows an investigator to transduce full-length proteins by utilizing a minimal, eleven–amino acid, HIV-TAT transduction domain that can be fused to a protein of choice using the pTAT or pTAT-HA protein expression plasmids. Bacterial expression (see Basic Protocol 1), followed by solubilization of protein aggregates with a denaturing agent, affords high yields of transducible fusion protein. The fusion protein, once added to the culture medium, can cross the cell membrane and then be degraded or refolded by the cellular machinery. Correct targeting and function of the fusion protein can be easily examined by fluorescent microscopy or immunohistochemistry.

This strategy was established and improved to its current state by the purification and transduction of a multitude of fusion proteins. Because the pool of fusion proteins span many different functions including sequestering proteins (i.e., p16, p27, and CDK2DN), proenzymes (caspase-3), viral proteins (HPV E6, E7, and E1A), enzymes (HIV protease, β -galactosidase), GTPases (rac, rho and cdc-42), and transcriptional regulators (E2F-1-5, pRb), the protocols cover a wide variety of commonly used protein isolation and characterization methods. Table 20.2.1 lists a few examples of some of TAT fusions and details the size, optimal isolation method, dose required to yield a phenotypic result, biological result obtained, and time in which the result was observed.

No special equipment is necessary to generate or transduce fusion proteins, although Basic Protocol 2 does recommend the use of fast protein liquid chromatography (FPLC) to reproducibly bind and elute denatured fusion proteins. FPLC, although recommended, is not required. Bulk ion-exchange resins are available and have been successfully used in place of the Mono Q/Mono S resins that Basic Protocol 2 describes. Another frequently used column is the PD-10 column (Amersham Pharmacia Biotech). This is a disposable column, packed with a gel-filtration resin, which is ideal for the removal of small molecules such as salt, urea, or unconjugated fluorescent molecules.

The unit illustrates the steps of the basic procedure with various fusions, to give the investigator a broader base of information upon which to begin specific isolations.

CAUTION: TAT-protein fusions have been shown to cross most lipid bilayers, including all tissues in a mouse. Therefore, when designing and using TAT-fusion proteins, precautions regarding safe handling and disposal are necessary. It is very important to analyze the health effects of each fusion protein individually and to observe appropriate biosafety procedures for disposal and decontamination. The authors suggest using a 0.1% (w/v) trypsin solution to decontaminate any large spills of fusion proteins. NIH Biosafety Level 2/3 containment should be used at all times. Also, refer to Backus et al. (2001), which provides further guidelines for the safe handling of TAT-transducing proteins.

STRATEGIC PLANNING

This unit is broken down into two sections: (1) isolation, optimization, and large-scale production of the fusion protein (see Basic Protocols 1 and 2 and Alternate Protocols 1 to 4) and (2) analysis of the transduction of the fusion proteins into target cells (see Basic Protocol 3 and Alternate Protocol 5). This unit contains a compilation of different

Table 20.2.1 Detailed Description of Selected TAT-Fusions

Fusion protein	Apparent size (kDa)	Isolation method	In vitro dose (nM)	Biological effect	Time biological effect was examined	References
TAT-p16 WT	24	Denaturing/ PD-10	200-1000	Inhibitor of CDK4/6, and induces G1 arrest	30 hr after addition, to G1-arrested, synchronized cells	Ezhevsky et al., 1997
TAT-HA-p27 WT	35	Denaturing/ Mono S ion-exchange	100-200	Inhibitor of CDK2/4/6 complexes, and induces cell scattering	30 min	Nagahara et al., 1998
TAT-E1A WT	60	Denaturing/ rapid dialysis	100	Sequesters pRb	15 min	Unpub. observ.
TAT-HA-E7 Wt	20	Denaturing/ PD-10	100	Sequesters pRb	3 hr	Lissy et al., 1998
TAT-HA-CDK2 DN	40	Soluble/ PD-10	200	Inactivates cyclin E:CDK2 complexes, resulting in G1 arrest	30 hr after addition to synchronized cells	Nagahara et al., 1998
TAT-HA-Caspase 3 WT	39	Denaturing/ Mono Q ion-exchange	100	Processed form induces apoptosis	1-6 hr	Vocero-Akbani et al., 1999
TAT-HA-HIV protease	20	Denaturing/ Mono S ion-exchange	100	Cleaves HIV protease Recognition sequence	1-6 hr	Vocero-Akbani et al., 1999
TAT-HA- β -galactosidase	120	Soluble/ PD-10	100	Cleaves ONPG and Xgal	30 min	Schwarze et al., 1999
TAT-HA-cdc42	21	Denaturing/ PD-10	25	Filopodia formation	5 min	Becker-Hapak et al., 2001

techniques that have been used to successfully isolate many different fusion proteins. One rule of thumb that must be remembered is that every TAT fusion protein is unique, and, while the method described in this unit can cover many fusions, every fusion is different. Therefore the following describes a starting point to begin one's isolations. Figure 20.2.1, outlines the overall strategy.

Isolation and Purification of Fusion Protein

In general, first identify bacterial clones expressing the cDNA of interest using the pTAT or pTAT-HA expression vector (Fig. 20.2.2). The plasmid containing the appropriate insert is then transformed into an *E. coli* strain that is specifically designed for the expression of recombinant proteins. Then, clones expressing the desired fusion may need to be boosted with inducer molecules such as IPTG to yield sufficient quantities of fusion protein for the desired study. The pTAT/TAT-HA vector utilizes a 6-His domain for the convenient isolation of recombinant protein by Ni-NTA chromatography resin. The 6-His domain can lead to fusions that are generally insoluble and compartmentalized into inclusion bodies within *E. coli*. Therefore, buffered urea is routinely used as a denaturing agent to obtain large quantities of unfolded recombinant protein that can bind to the nickel affinity resin. Once the protein is bound and the resin washed, imidazole is used as a competitor to elute the fusion protein from the nickel resin.

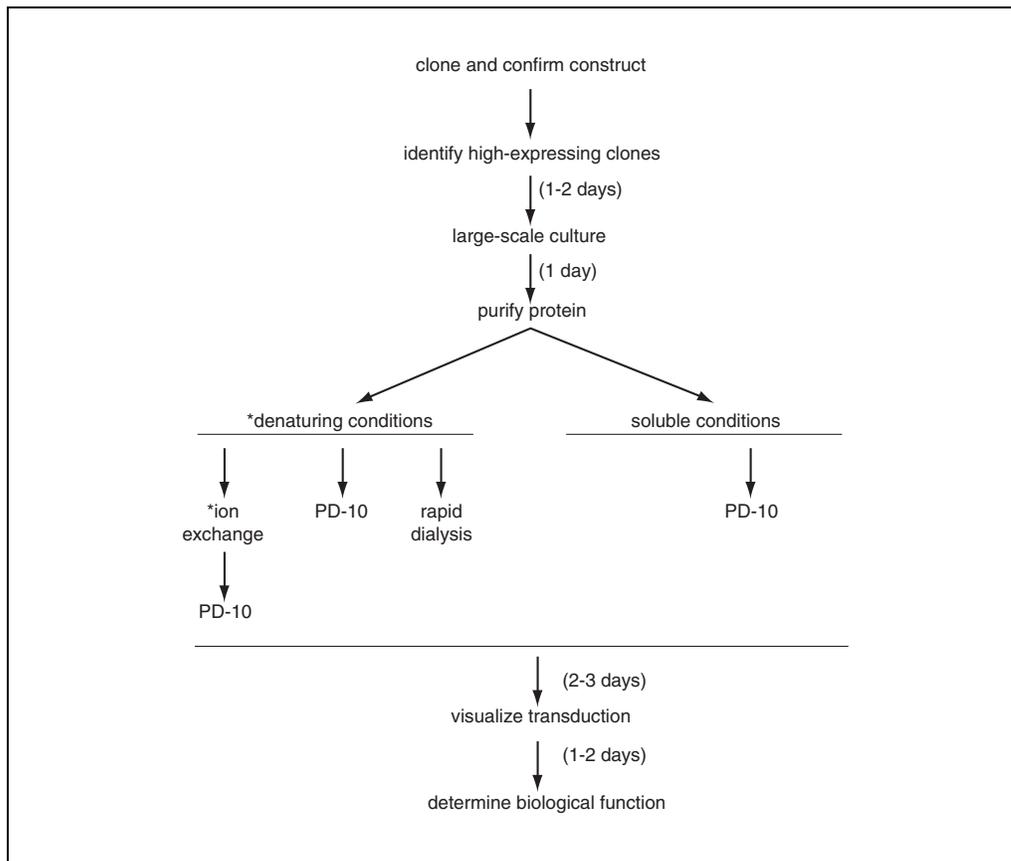


Figure 20.2.1 Flow diagram outlining the overall strategy and time frame required to perform protein transduction using the TAT system. Asterisks (*) denote the preferred methods that consistently lead to fusions that are biologically active.

To remove the urea from the peak nickel affinity protein fraction, one of three different approaches can be used: ion-exchange, gel filtration, or rapid dialysis. The most reliable method for producing transducible recombinant proteins from inclusion bodies is through the use of strong ion-exchange resins to capture the unfolded protein by its ionic charge on an anion (Mono Q) or cation (Mono S) exchange resin using an FPLC platform. Once the unfolded protein is captured, the environment in the column is immediately changed to an aqueous one. The protein is quickly released from the resin using a salt bump, which theoretically leads to a pool of correctly folded and misfolded proteins. The pool of proteins is then desalted and ready for use or storage. The routine method of urea removal from bacterially expressed proteins involves the removal of the denaturant by slow dialysis. While this method works when preparing small quantities of soluble, properly folded proteins, dialysis of high concentrations of the TAT-fusion proteins usually leads to dramatic protein precipitation. Another method of urea removal utilizes a disposable gel-filtration column (PD-10) to exchange the buffer environment around the protein. This procedure has afforded somewhat better success than dialysis, but it is not routinely advisable. Note that the PD-10 column is used in this unit for more than one purpose. While it is not recommended to routinely remove urea from the nickel chromatography purification portion of the procedure, it is ideal for buffer exchange and removal of small molecules such as unconjugated FITC.

In some cases, the 6-His TAT-fusion proteins are maintained in the bacterium in a soluble conformation. In these rarer cases, the fusion protein can be isolated after simply suspending the bacteria in an aqueous buffer, sonicating then clarifying the suspension, and finally performing nickel affinity chromatography.

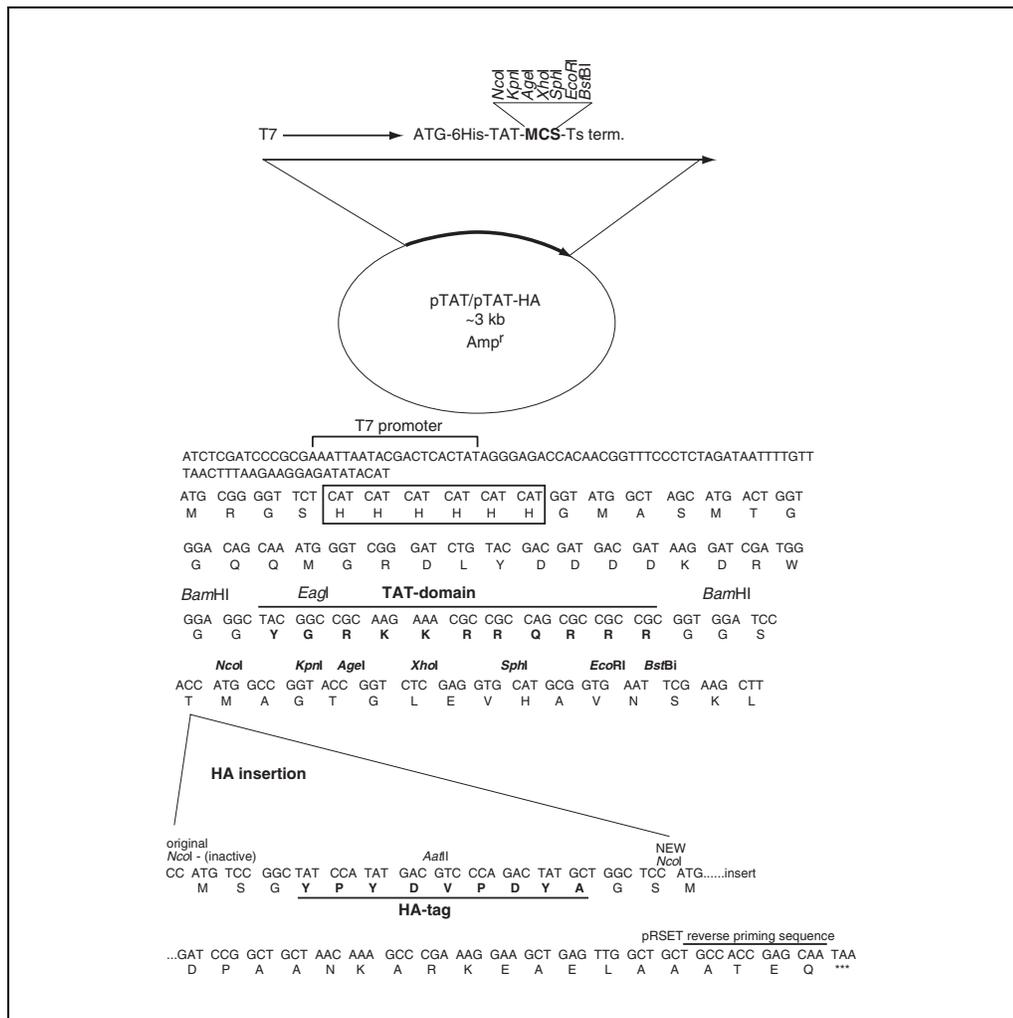


Figure 20.2.2 Vector map of pTAT/pTAT-HA. The functional domains are in boldface. Convenient forward and reverse priming sequences are noted, as well as the peptide sequence of the TAT-HA leader. The pTAT vector does not contain the HA insert. Insertion of desired cDNA using any of the restriction enzymes noted in the multiple cloning site (MCS) will yield an in-frame fusion.

Transduction and Detection

Two general methods are given for monitoring of full-length TAT-fusion transduction into target cells. The first method detects the intracellular location of the fusion protein, by fluorescently labeling the protein using fluorescein isothiocyanate (FITC), adding the labeled protein to the target cells, washing, fixing, and then observing the resultant transduction by fluorescence microscopy. This standard method for protein labeling covalently attaches the fluorescein molecule to basic residues such as lysine and arginine. Eight out of the eleven amino acids in the TAT-transduction domain are comprised of these basic residues and over-labeling in this functional domain can lead to artificial inhibition of transduction.

The second method given to detect protein transduction is indirect immunofluorescence. This method uses commercially available, fluorescent antibodies to detect transduced protein within cells that have been subjected to the fusion protein over various amounts of time, washed, and then fixed. It is the method of choice when detecting transduction on adherent cells.

Both of these methods focus solely on detection of the fusion protein inside the cell and provide no evidence of fusion protein function. Phenotypic results of fusion-targeted

events are clearly the ultimate detection of protein transduction however, these methods are fusion specific and will not be covered in this unit.

Controls

Depending on the goal of the study, one may choose to use one of two negative controls. The first control to consider is the preparation of a 6-His-fusion of the protein of interest without the transduction domain. This control would be advantageous in cases where proof of the transduction is required. If this is necessary, simply digest the pTAT/pTAT-HA vector containing the cDNA of interest with *Bam*HI, purify, and re-ligate. This will “pop out” the TAT domain of the protein while maintaining the expression of the 6-His-HA tagged protein of interest. Removal of the TAT transducing domain can be verified by digestion of the re-ligated plasmid with the restriction enzyme *Eag*I (Fig. 20.2.2). If the transducing domain has been removed, it will no longer be possible to linearize the plasmid with this restriction enzyme. The purification of this species is generally the same as for the TAT-fused protein.

The second control to consider is the creation of a site-specific mutant within the protein of interest. These fusions are highly recommended when doing in vitro studies in order to prove the specificity of the TAT-fusion protein. Generally, no major deviations from the already optimized protocol for the wild-type fusion will be necessary.

EXPRESSION, VERIFICATION, AND YIELD OPTIMIZATION OF TAT-FUSION PROTEINS

This protocol assumes that the investigator has already confirmed the insertion of the cDNA of interest into the TAT expression vector, as well as the identity of the cDNA. It is important that the DNA sequence be confirmed and that the possibility of frame shifts or point mutations be eliminated. The verified plasmid should be stored as a glycerol stock in *E. coli*, DH5 α . Storage of the plasmid in bacteria used for protein expression is not generally recommended because of possible plasmid instability. If the investigator needs further background into creating fusion cDNA, see *APPENDIX 3A*, which provides references for procedures that are not described in detail in this unit.

Materials

Pure pTAT/pTAT-HA expression vectors (Nagahara et al., 1998) with and without the gene of interest inserted (available from Dr. S. Dowdy, sdowdy@ucsd.edu)
E. coli strains BL-2 (DE3) pLysS (Novagen) and DH5 α (Invitrogen, Life Technologies)

LB medium and plates both containing 50 μ g/ml ampicillin (see recipe)

2 \times SDS-PAGE sample buffer (*APPENDIX 2A*)

Antibody specific for target protein *or* anti-HA mAb (Berkeley Antibody Company) if using pTAT-HA vector

Glycerol, ultrapure, 50% (v/v), sterile filtered

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*), immunoblotting (*UNIT 6.2*), Coomassie blue staining (*UNIT 6.6*), and basic molecular biology procedures (including transformation of bacteria and IPTG induction; see *APPENDIX 3A*)

1. Transform the verified, pure plasmid, into competent *E. coli* strain BL-21(DE3)pLysS (*APPENDIX 3A*). Select transformants on LB plates containing 50 μ g/ml ampicillin. Also transform the original vector (without the gene of interest) in the same *E. coli* strain for later use as a whole bacterial protein control (empty-vector control).

This bacteria will not produce any detectable 6-His fusion protein if using the pTAT vector, or will express the hemagglutinin antigen (HA-tag) if using the pTAT-HA vector (6 to 9 kDa).

BASIC PROTOCOL 1

Expression and Introduction of Macromolecules in Cells

20.2.5

2. Incubate the agar plate overnight. Pick 6 to 10 colonies from the fusion-positive plate and grow in 3 ml of liquid LB medium containing 50 µg/ml ampicillin, overnight at 37°C with shaking at 220 rpm. Pick one colony from the empty-vector control plate and grow in the same fashion.
3. Vortex the overnight cultures. Remove 50 µl of each culture and place into an individual microcentrifuge tube containing 50 µl of 2× SDS-PAGE sample buffer.
4. Vortex briefly and heat samples 5 min in a boiling water bath.
5. Microcentrifuge the lysate 3 min at maximum speed, room temperature, to bring down any particulates.
6. Load 20 µl of each supernatant from step 5 (including that from the negative control) on two separate SDS-PAGE gels of the appropriate percentage (UNIT 6.1).

One gel will be stained with Coomassie Brilliant Blue (UNIT 6.6) and the other will be transferred to nitrocellulose for immunoblotting (UNIT 6.2).

7. Place one of the two gels in Coomassie Brilliant Blue staining solution for 1 hr with gentle agitation, then remove staining solution and replace with destain solution. Change the destaining solution every 15 min until desired background is achieved.

The procedures and solutions used here are described in UNIT 6.6.

8. In the lanes containing the clones of interest, determine if there is an overexpressed band at the appropriate size when compared to the lane loaded with lysed bacteria transformed with pTAT or pTAT-HA alone (the empty-vector control). If no clear differences are evident, rely on the immunoblot for confirmation of the expression of the fusion protein.
9. Perform immunoblotting and detection procedures (UNIT 6.2). Use the anti-HA mAb at a 1:5000 dilution for the primary antibody if using the HA tag as a marker to follow fusion protein expression. For an antibody against a specific protein, use the dilution recommended by the manufacturer. Use the secondary antibody (UNIT 6.2) at a concentration of 1:1000.

The authors typically use extended-life chemiluminescent development reagents such as SuperSignal (Pierce).

10. Using the results from the immunoblot, determine the size of the fusion protein.

The TAT/TAT-HA fusion proteins run 6 to 9 kDa larger than the untagged gene of interest.

11. *Optional:* If a sufficient level of overexpression of the desired fusion is not observed by immunoblotting, induce the system using IPTG (APPENDIX 3A).

See Troubleshooting for other possible solutions to poor fusion expression.

12. Determine the clone or clones that express the protein of interest at the highest levels. Prepare glycerol stocks by adding 700 µl of overnight culture into 300 µl of sterile 50% glycerol, and freeze at -80°C.

As mentioned earlier, not all fusions are stable in BL-21(DE3)pLysS bacteria. If this is discovered, transform into the expression bacteria, let the cells recover for 1 hr without antibiotic selection, and then prepare a 100-ml overnight inoculum in LB medium containing 5 µg/ml ampicillin. Use this culture to inoculate a large-scale culture as described in the next section.

Also, for permanent storage of the unstable plasmid, transform the plasmid into DH5α and store as glycerol stock at -80°C as mentioned earlier.

LARGE-SCALE ISOLATION OF THE TAT-FUSION PROTEIN

This protocol describes the large-scale isolation of the TAT-fusion protein from the high-expressing clones identified in the previous steps. See Key References for more information on these approaches. To perform ion exchange, it is necessary first to determine the isoelectric point (pI) of the purified protein. This can be done easily on various molecular biology Web sites (see Internet Resources for one of these). It is then necessary to determine which ion-exchange resin will best suit the protein. The pI of the fusion protein will, in large measure, determine whether to use a Mono Q column (for basic proteins) or a Mono S column (for acidic proteins). Although the TAT leader is a basic entity (8 of 11 residues are basic), it has been the experience of the authors that ~50% of all TAT fusion proteins will bind to the Mono S resin regardless of pI predictions. Following the successful elution of the protein, pool the appropriate fractions and exchange the buffer using a PD-10 column. The columns are provided as prepacked disposable columns of 30 per box and should be stored at 4°C.

In general, when isolating proteins from crude extracts, the preparation should be kept in the cold or on ice. However, in this procedure, it is not necessary to keep the preparation on ice until the urea has been removed from the sample, except when sonicating the bacteria. In fact, if the Ni-NTA purified fractions are kept on ice after elution from the column, crystallization of the eluate will be observed. Therefore, during this phase, avoid cold conditions unless it is necessary to freeze the partially purified protein for storage and later purification.

Materials

- LB medium containing 50 µg/ml ampicillin (see recipe)
- Glycerol stock of clone with high expression of TAT fusion protein (see Basic Protocol 1)
- Phosphate-buffered saline (PBS; *APPENDIX 2A*)
- Buffer Z (see recipe) containing 1× protease inhibitors (see recipe)
- 5 M (340 g/liter) imidazole (store in foil-wrapped bottle at 4°C)
- 50% (w/v) stock suspension of Ni-NTA agarose (Qiagen)
- 100 mM, 250 mM, 500 mM, and 1 M imidazole in buffer Z (see recipe for buffer Z), prepared fresh daily
- PBS (*APPENDIX 2A*) containing 0.1% (w/v) sodium azide
- 20 mM HEPES, pH 8 (for Mono Q resin) or pH 6.5 (for Mono S resin)
- Buffer A (binding): 20 mM HEPES, pH 8.0, for Mono Q; pH 6.5 for Mono S
- Buffer B (elution): 20 mM HEPES/1 M NaCl, pH 8.0, for Mono Q; pH 6.5 for Mono S
- PBS (*APPENDIX 2A*) containing 1× protease inhibitors (see recipe)
- Bovine serum albumin (BSA)
- Glycerol (ultrapure), 50% (v/v) sterile filtered
- Sorvall refrigerated centrifuge with GSA rotor, or equivalent
- Sonicator with microprobe (Branson)
- Disposable 50-ml Econo columns (Bio-Rad)
- Mono Q or Mono S 5/5 or 10/10 ion-exchange FPLC columns *or* bulk resin (Resource Q or S), all products of Amersham Pharmacia Biotech
- FPLC apparatus
- PD-10 gel filtration columns (Amersham Pharmacia Biotech)
- Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and Coomassie blue staining (*UNIT 6.6*)

Prepare fusion-protein-containing lysate

1. Inoculate 200 ml of LB medium containing 50 µg/ml ampicillin with a sterile loop or scraping of the high-expressing clone glycerol stock (see Basic Protocol 1). Incubate overnight at 37°C with shaking at 220 rpm.
2. Pour the entire contents of the overnight culture into 1 liter of LB medium containing 50 µg/ml ampicillin. Incubate 5 to 6 hr at 37°C with shaking at 220 rpm.

*Protein production usually decreases after stationary phase has begun in *E. coli*.*

If it has been determined that IPTG is required to obtain large quantities of the protein, be sure to add it at this step.

3. Centrifuge the cell suspension 5 min at 5000 × g, 4°C, in a Sorvall GSA or equivalent rotor. Discard supernatant.
4. Resuspend pellet in PBS. Centrifuge the suspension again as in step 3.

The washed pellet can be stored at -20°C for one month if necessary.

5. Decant supernatant and add 10 ml buffer Z with protease inhibitors to the pellet. Make a homogenous suspension by pipetting up and down using a wide-bore pipet, or by vortexing.

A homogenous suspension is critical for efficient lysis of the bacteria by sonication in the next step.

6. Sonicate the suspension using four 15-sec on/off cycles at 60% (microtip limit), at 4°C or on ice.

The cold temperatures are required to keep the proteins from being irreversibly denatured by the heat generated during the sonication process.

7. Centrifuge the suspension 10 min at 12,000 × g, 4°C. Carefully decant the supernatant into a clean tube and measure its volume, then add sufficient 5 M imidazole to a final concentration of 20 mM imidazole.

The concentration of imidazole to add at this point must be determined experimentally. In most cases the fusion protein binds specifically at 20 mM imidazole; however, some proteins will require lower concentrations (from 5 to 15 mM). At lower imidazole concentrations, the background (non-6-His labeled proteins) can also bind to the nickel resin. Usually, this is not a problem because the desired protein is in vast excess with respect to the contaminating proteins. Additionally, the contaminating bacterial proteins do not contain the transduction domain and therefore will not transduce.

Purify fusion protein on Ni-NTA agarose

8. Prepare a 5-ml bed volume Ni-NTA affinity column by adding 10 ml of the 50% stock suspension of Ni-NTA agarose to a 50-ml Bio-Rad Econo-Column with the Luer lock in place to control the flow of buffers or extract to be added.
9. Wash the resin twice, each time with 10 bed volumes (50 ml) of Milli-Q water, to remove the resin storage buffer.
10. Equilibrate the resin with 10 bed volumes of buffer Z containing 20 mM imidazole (or whatever concentration of imidazole was added as in step 7).

Remember to save a small portion of each purification step so that it will be possible to follow the protein purification by SDS-PAGE.

11. Apply the clarified sonicated bacterial lysate (from step 7) to the resin.

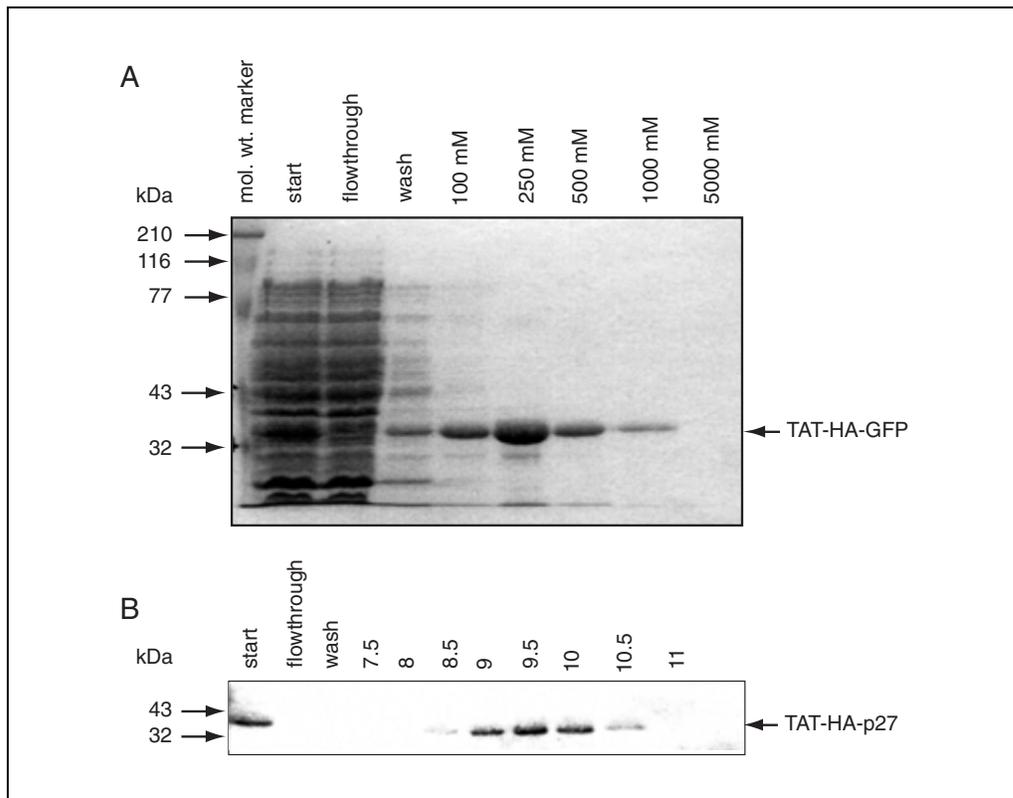


Figure 20.2.3 (A) Typical elution profile of TAT-fusion protein (TAT-HA-GFP) from a Ni-NTA agarose resin. BL-21(DE3)pLysS bacteria were transformed with pTAT-HA-GFP plasmid and cultured in 200 ml of LB medium containing 50 μ g/ml ampicillin, overnight. This inoculum was then added to 1 liter of LB-ampicillin and cultured for another 5 hr with shaking at 220 rpm, 37°C. The bacteria were lysed in buffer Z with protease inhibitor cocktail (see Reagents and Solutions), clarified, and the imidazole concentration was brought up to 20 mM. The crude lysate was applied to a 5-ml-bed-volume Ni-NTA column and washed with 50 ml of buffer Z containing 20 mM imidazole. The fusion protein was then eluted with 5 ml aliquots of buffer Z containing 100, 250, 500, 1000, and 5000 mM imidazole. The 12.5% SDS-PAGE gel was loaded with 5 μ l of each fraction noted. The gel was stained for 60 min with Coomassie Blue and destained as necessary. (B) Ion-exchange profile of TAT-p27 WT on a Mono S (5/5) column, equilibrated in buffer A and loaded with a 1:1 dilution of a Ni-NTA elution fraction. The column was washed with 40 ml of buffer A. TAT-HA-p27 WT fusion protein consistently eluted after 8 through 11 ml of buffer B. 10 μ l of each elution fraction was separated on a 15% SDS-PAGE gel and stained with Coomassie Blue for 1 hr, then destained as necessary. The numbers above each band denote the volume of elution buffer (buffer B) that had passed through the Mono S (5/5) column.

Some lysates can be very viscous. If this is observed, dilute the lysate with more buffer Z or apply the lysate directly and use slight pressure on the column to gently force the lysate through the resin. If the lysate is not very viscous and clears the resin too quickly, reduce the flow rate and apply the lysate over the resin again.

Remember to maintain the imidazole concentration (20 mM or other concentration added at step 7) throughout the column application and wash steps.

12. Wash the resin with 10 ml of buffer Z containing 20 mM imidazole, then with an additional 40 ml of buffer Z containing 20 mM imidazole (ten bed volumes total).

The first 10 ml of wash will contain flow through proteins. The subsequent 40 ml is the wash that removes weakly bound proteins.

13. Elute the protein stepwise by sequential addition of 5 ml each of 100 mM, 250 mM, 500 mM, and 1 M imidazole in buffer Z, and collect fractions. Finally, strip the resin with 5 ml of 5 M imidazole.

14. Wash the resin with 20 bed volumes of PBS. Store the column at 4°C in PBS containing 0.1% sodium azide.
15. Load 10 µl of each purification fraction (from step 13) on an appropriate-percentage SDS-PAGE gel (UNIT 6.1). Perform electrophoresis, then stain the gel with Coomassie Brilliant Blue for 1 hr and destain appropriately (UNIT 6.6).

Note that purified protein can be readily observed as early as 2 min after placing the gel in the staining solution.

16. Determine the fraction(s) that contain the desired protein.

See Fig. 20.2.3A for an example of a typical urea elution profile.

The procedure can be suspended at this step if necessary. The imidazole-eluted fractions may be stored at -20°C until the SDS-PAGE is completed and one is ready to do the urea-removal steps (no more than one month).

Purify by ion-exchange chromatography

17. Pool the Ni-NTA fractions containing the peak protein concentration determined by SDS-PAGE.

18. Dilute the pooled fractions 1:1 with 20 mM HEPES, pH 8.0 (for Mono Q), or pH 6.5 (for Mono S).

This dilutes the protein pool to a low-enough salt and denaturant concentration to allow binding to the ion-exchange resin.

19. Inject the sample into a 10/10 (preferably) Mono Q or S column attached to an FPLC apparatus and equilibrated in buffer A.

A gravity column may be used in place of FPLC (see Alternate Protocol 1).

20. Wash with ~50 ml buffer A and elute with 10 ml buffer B, collecting 0.5-ml fractions.

Switching from the non-urea-containing buffer A to buffer B results in elution via a single 1 M NaCl step.

21. Analyze the fractions by SDS-PAGE (UNIT 6.1) and stain with Coomassie Brilliant Blue (UNIT 6.6).

Figure 20.2.3B shows an example of an elution profile from ion-exchange chromatography. The sample is ready at this point for desalting on a PD-10 column equilibrated in PBS, followed by collection and final analysis by SDS-PAGE (steps 22 to 26). The only decision that must be made by the investigator at this point is the equilibration buffer to be used. The authors recommend desalting into PBS and adding sterile glycerol to a minimum concentration of 10% (v/v). For further discussion on the use of this column see Becker-Hapak (2001).

IMPORTANT NOTE: *If the protein fails to bind to the predicted resin, or weak binding of protein is observed, try the other column type (Mono Q or Mono S) regardless of predicted pI. If strong binding is observed with weak elution, decrease (for Q resin) or increase (for S resin) the pH of buffer A by steps of 0.5 pH units, until a small amount of protein is detected in the flowthrough fraction. Also see Troubleshooting for problems associated with the ion-exchange step.*

Perform buffer exchange using PD-10 column

22. Drain the storage buffer from the PD-10 column and equilibrate the column with 25 ml of PBS with 1× protease inhibitors.

Culture medium such as RPMI-1640 (e.g., Life Technologies), without serum and antibiotics, but containing 1× protease inhibitors, can be used in place of the PBS.

23. Apply the pooled protein fraction to the column (do not exceed 2.4 ml), and allow the solution to absorb into the resin. Apply 4 ml PBS with 1× protease inhibitors to the column and collect fourteen 0.5-ml fractions.

The protein will begin eluting in the sixth or seventh fraction. If 2.4 ml of protein was applied to the resin, then the protein will stop eluting in the thirteenth or fourteenth fraction.

The column can be reused once by applying another 25 ml of buffer with protease inhibitors onto the column; however, it is not wise to reuse the column if working with different fusion proteins, because it is always possible to carry over contaminants from a previous preparation.

24. Analyze the fractions by SDS-PAGE (UNIT 6.1) using BSA as a standard. Load 0.1 to 2 µg protein standard per lane and a known volume of the purified fusion protein on the same gel.
25. Stain the gel with Coomassie Blue and destain as desired (UNIT 6.6).
26. Pool the appropriate fractions and add glycerol to a final concentration of 10% (v/v). Divide into 0.25-ml aliquots and flash freeze on dry ice. Store fractions at -80°C.

Fusions have been stored for over 2 years in this manner and still maintained activity.

USE OF ION-EXCHANGE GRAVITY COLUMNS INSTEAD OF FPLC

If an FPLC system is not available, bulk ion-exchange resin can be used to pack a gravity-flow column.

Additional Materials (also see Basic Protocol 2)

30-µm Resource Q or S ion-exchange resin (Amersham Pharmacia Biotech; see Basic Protocol 2 for choice of resin)

50-ml Econo columns (Bio-Rad)

NOTE: Perform all steps at 4°C and add 1× protease inhibitors (see recipe) to all solutions.

Replace steps 19 to 21 of Basic Protocol 2 with the following:

1. Pack a 10-ml bed-volume ion exchange column using 30-µm Resource Q or S resin.
2. Wash the resin with 20 bed volumes of Milli-Q water to remove the storage buffer and rehydrate the resin.
3. Wash the column with 10 bed volumes of buffer A.
4. Apply the diluted sample (see Basic Protocol 2, step 18) onto the resin and allow the protein to enter the gel bed slowly.
5. When the protein has completely entered the gel, wash with 10 bed volumes of buffer A.
6. Elute with two bed volumes of ml buffer B, collecting 0.5-ml fractions. Analyze the fractions by SDS-PAGE (UNIT 6.1) and pool the fractions containing the protein of interest.

IMPORTANT NOTE: At this point the fusion protein is very pure. It is absolutely necessary to maintain the protein on ice from this point on. Failure to do so will yield degraded protein.

7. Perform buffer exchange and analyze the final fractions (see Basic Protocol 2, steps 22 to 26).

ALTERNATE PROTOCOL 1

DIRECT BUFFER EXCHANGE OF UREA-DENATURED PROTEIN

In theory, rapid desalting of a denatured protein from 8 M urea through an aqueous interface of PBS or culture medium without serum forces the protein to rapidly hide its hydrophobic residues and become soluble in an aqueous environment. The PD-10 desalting column has a 1:1.4 dilution factor; therefore denatured proteins are rapidly separated from each other, helping to avoid aggregation of the proteins and subsequent precipitation on the column. In this procedure, 1 to 1.5 ml of the Ni-NTA affinity-purified TAT fusion protein in 8 M urea (i.e., in buffer Z) is applied to the PD-10 column equilibrated in PBS/HEPES buffer or serum-free culture medium. Column fractions of 0.5 ml are isolated and analyzed by SDS-PAGE as in Basic Protocol 2. Reasonable success has been achieved by this rapid and inexpensive procedure, however, use of this method routinely leads to dramatic protein precipitation. While some soluble protein can be obtained using this protocol, more soluble (and therefore, transducible) protein can be obtained by working out the ion-exchange conditions.

Additional Materials (also see Basic Protocol 2)

Serum-free culture medium (e.g., RPMI-1640) without antibiotics, containing 1× protease inhibitor cocktail (see recipe) *or* PBS plus 1× protease inhibitor cocktail

Replace steps 17 to 26 of Basic Protocol 2 with the following:

1. Equilibrate PD-10 column with 25 ml of culture medium without serum or antibiotics but with 1× protease inhibitors.

Alternatively, the fusion can be buffer exchanged into PBS plus 1× protease inhibitor cocktail.

2. Load a maximum of 1.5 ml of the peak protein fraction from the Ni-NTA affinity column (see Basic Protocol 2, steps 16 and 17) on to the gel bed.
3. After the sample has completely entered the gel bed, apply more of the culture medium with protease inhibitors to the column and collect 0.5-ml fractions.

Protein can precipitate as it is eluted from this column. Microcentrifuging the fractions immediately for 5 min at maximum speed, 4°C, can minimize this precipitation. Transfer the supernatant fractions in separate microcentrifuge tubes.

4. Keeping the fractions on ice at all times, determine which fractions contain the desired protein by SDS-PAGE (UNIT 6.1).
5. Pool the fractions containing the protein of interest and add glycerol to a final concentration of 10% (v/v). Store the protein in 0.2-ml aliquots at –80°C.

Some proteins may require the additional 0.1% (w/v) BSA to stabilize the pure protein. This must be determined experimentally. In general, storing the protein in the 10% glycerol will be sufficient. Also, keep in mind that if the fusion protein is an enzyme, it may not be active if frozen at any point.

DIALYSIS OF THE UREA-DENATURED PROTEIN

One may choose to rapidly dialyze the Ni-NTA affinity-purified protein into the desired buffer, replacing the ion-exchange and gel-filtration steps (Basic Protocol 2, steps 17 to 23). However, 6-His fusion proteins are highly susceptible to precipitation when using this method to remove urea. Also, some proteins are not biologically active after dialysis, whereas ion-exchange chromatography will routinely yield proteins with higher biological specific activity. For example, TAT-p27 WT, if prepared by rapid dialysis, will not

cause G1 arrest, whereas if it is subjected to ion-exchange chromatography, it does (Nagahara et al., 1998). Conversely, other proteins such as TAT-E1A and TAT-E7 are biologically active when desalted by rapid dialysis (Beker-Hapak, unpub. observ.).

Additional Materials (also see Basic Protocol 2)

Slide-A-Lyzer dialysis cassettes (Pierce) with membrane of MWCO appropriate for protein of interest

Replace steps 17 to 23 of Basic Protocol 2 with the following:

1. If the protein in the purified fraction is $> 5 \mu\text{g}/\mu\text{l}$, dilute at least 1:1 in buffer Z.
2. Apply the sample to the dialysis cassette. Be sure to remove all air bubbles.

In the authors' laboratory, use of the Slide-A-Lyzer cassettes with large surface-to-volume ratio provided by Pierce has proven to yield more soluble protein than regular dialysis tubing.

3. Dialyze the protein at 4°C against 4 liters of pre-chilled buffer appropriate for the downstream application. Change the buffer after 1 hr, then again 2 hr later, and let stir overnight. After the overnight incubation period, remove the protein from the dialysis cassette and remove any solids by centrifuging 10 min at $5000 \times g$, 4°C , prior to use or storage.

For example, if using the protein for orthophosphate labeling, use 20 mM HEPES/137 mM NaCl, pH 7.2, but if simply adding the protein to tissue culture cells, use $1\times$ PBS at 4°C .

4. Analyze the protein concentration with BSA standards on a SDS-PAGE gel and store the protein in aliquots (see Basic Protocol 2, steps 24 to 26).

ISOLATION OF SOLUBLE TAT-FUSION PROTEINS

Some proteins can or must be isolated under soluble (nondenaturing) conditions. For example TAT- β -galactosidase is not active if purified in the presence of any urea. Use of nondenaturing conditions can reduce yield as well as transduction efficiency; therefore, use of this protocol is not generally recommended. However, several reports successfully utilized the isolation of TAT fusions under nondenaturing conditions. According to the authors' experience, the yield can be much lower than proteins prepared under denaturing conditions and the transduction efficiency may be lower. Exact effects must be determined experimentally.

For materials, see Basic Protocol 2.

1. Prepare pellet of cells from 5- to 6-hr culture (see Basic Protocol 2, steps 1 to 4).
2. Resuspend the pellet fraction in 10 ml of PBS containing $1\times$ protease inhibitors. Perform Ni-NTA purification (see Basic Protocol 2, steps 5 to 14), replacing the buffer Z with PBS with protease inhibitors. Identify the fractions containing the protein of interest by SDS-PAGE (see Basic Protocol 2, steps 15 and 16).

Sonication of cells in PBS is more difficult than when done in buffer Z. One may need to modify the sonication procedure to optimally lyse the bacteria without damaging the fusion protein.

The ion-exchange steps (Basic Protocol 2, steps 17 to 21) are not performed.

3. After Ni-NTA chromatography, remove the imidazole and exchange the buffer using PD-10 column, and store the protein in 10% (v/v) glycerol at -80°C (see Basic Protocol 2, steps 22 to 26).

ALTERNATE PROTOCOL 4

**Expression and
Introduction of
Macromolecules
in Cells**

20.2.13

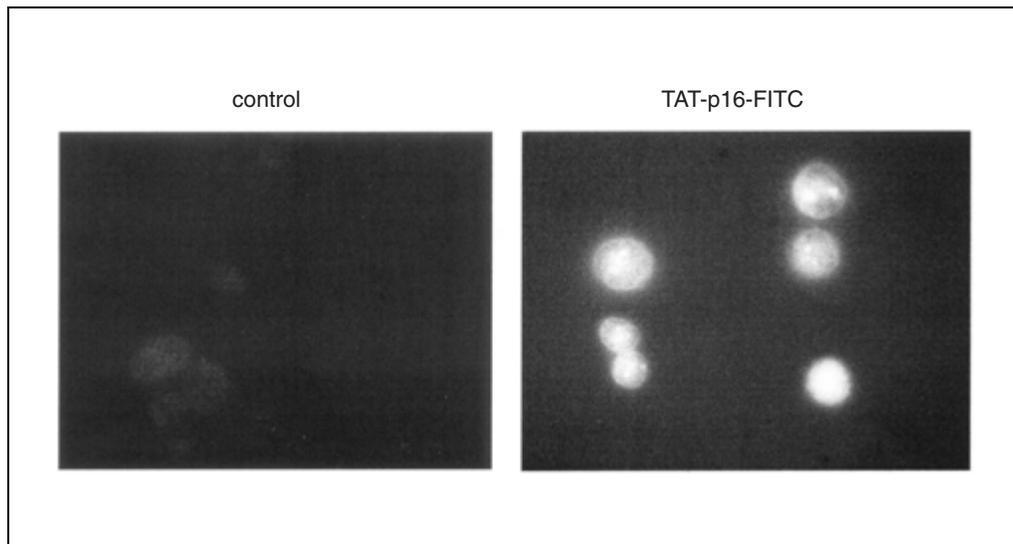


Figure 20.2.4 Confocal microscopy analysis of Jurkat T cells transduced with p16 WT-FITC, control (left panel) or TAT-p16WT-FITC protein (right panel). Jurkat T cells were transduced for 1 hr with the FITC-labeled pure protein. Cells were washed in PBS and fixed with 4% paraformaldehyde, washed again, and then mounted on slides with antifade mounting medium. Note the generalized fluorescence of TAT-p16WT-FITC protein in the cell. Higher-intensity staining can be observed in the nucleoli, typical of nuclear targeted TAT-fusions. The cells treated with non-TAT-fused FITC-labeled p16 show little to no fluorescence.

**BASIC
PROTOCOL 3**

**TRANSDUCTION AND DETECTION WITH FLUOROPHORE-LABELED
FUSION PROTEIN**

TAT-mediated protein transduction occurs without the use of specialty reagents or instrumentation. A TAT-fusion protein can be simply added to cultured cells along with the culture medium. The process is concentration dependent but seemingly temperature independent (see Commentary for a detailed discussion of all of the parameters affecting transduction). This unit will not detail a regimen for transduction because the procedure will be different for every fusion, cell type, and cell culture system. To optimize, the researcher should consider trying several different doses of the transducing protein (10 to 200 nM) in culture medium, varying incubation times with the target cell population to achieve the lowest concentration of protein in shortest time frame required to achieve the phenotypic effect. Suspension (e.g., Jurkat T-cell) and adherent (e.g., NIH 3T3) cell lines, are used in this section to illustrate two different and routinely used methods for visualizing transduced proteins in tissue culture cells. This protocol describes transduction of a fusion protein labeled with fluorescein. Alternatively, other fluorescent molecules such as Alexa (Molecular Probes) can be used to label the fusion. These molecules are reported to have a higher half-life when compared to FITC. The authors recommend using the manufacturer's labeling protocol whenever using an alternative fluorophore.

Materials

- Fluorescein isothiocyanate (FITC; Molecular Probes)
- DMSO
- Purified fusion protein (see Basic Protocol 2 and Alternate Protocols 1 to 4)
- 10× FITC conjugation buffer (see recipe)
- PBS (*APPENDIX 2A*) containing 1× protease inhibitors (see recipe)
- Glycerol, ultrapure
- Cell line of interest for transduction or Jurkat T cell culture
- Paraformaldehyde fix solution (see recipe)
- Antifade mounting medium (Molecular Probes)

**TAT-mediated
Protein
Transduction**

20.2.14

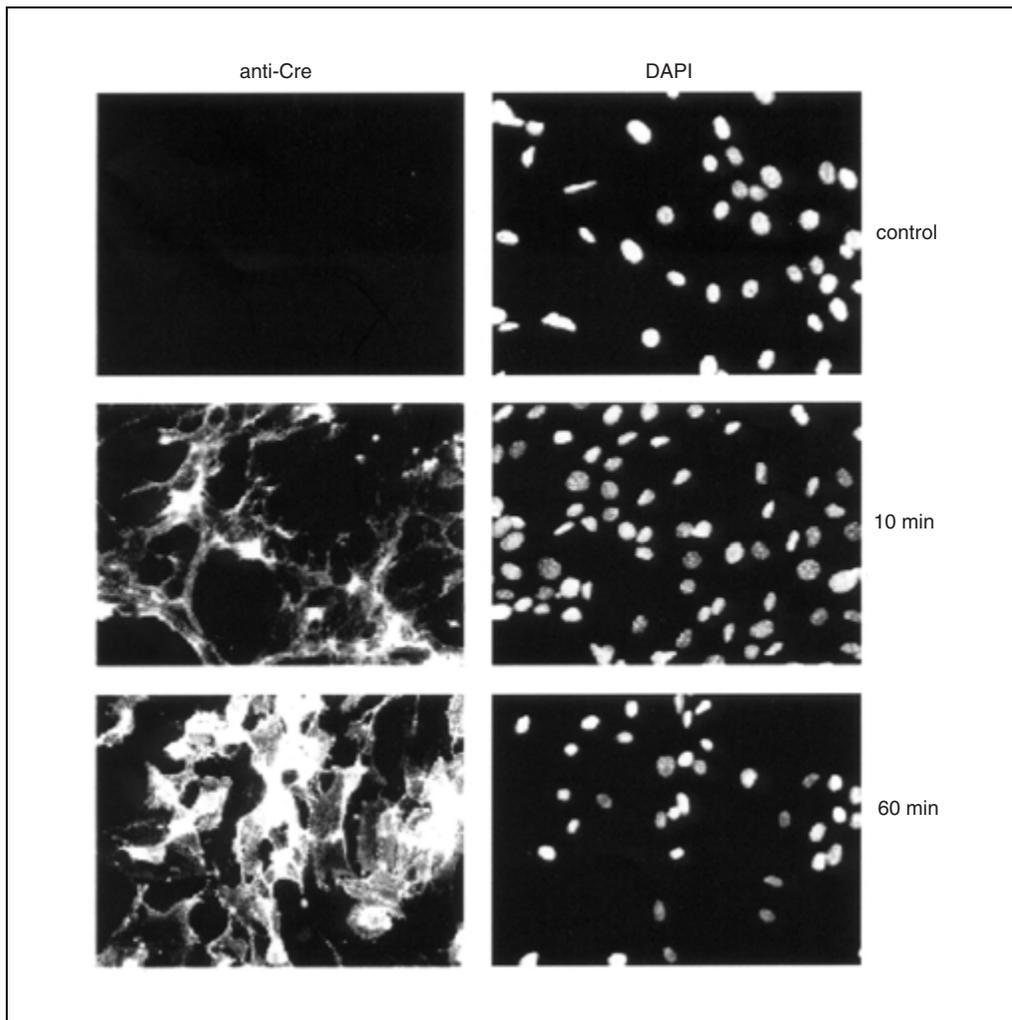


Figure 20.2.5 Detection of TAT-HA-Cre transduced into NIH-3T3 cells containing a phloxed β -galactosidase gene. Cells were transduced for 0 (no TAT-HA-Cre), 10, and 60 min in 8-chamber Lab-Tek slides. The cells were washed and fixed as described in Alternate Protocol 5. Rabbit anti-Cre polyclonal antibody was used at a 1:3000 dilution and a TRITC-labeled goat anti-rabbit secondary antibody was used at a 1:1000 dilution. TAT-HA-Cre is detected as early as 10 min after introduction of the protein into the cells. The panel showing the cells treated for 10 min clearly shows that many of the cells have detectable protein in the cytoplasm. Accumulation of the fusion protein in most cell nuclei is evident at 60 min.

Clear nail polish

PD-10 gel-filtration columns (Amersham Pharmacia Biotech)

Microscope slides and coverslips

Label TAT fusion protein

1. Prepare a FITC stock solution by dissolving 1 mg FITC per 0.5 ml DMSO. Keep in the dark.

The FITC stock solution should be prepared fresh daily.

2. Prepare the labeling reaction by combining:

540 μ l (0.1 to 0.5 μ g) purified fusion protein

60 μ l 10 \times conjugation buffer

1 μ l FITC stock solution (see step 1).

It is wise to set up at least three separate labeling reactions of high, medium, and low protein concentration to be conjugated to FITC. Overlabeling can cause inhibition of the transduction, presumably due to blockage of the basic groups in the transduction domain. Therefore, it is a good idea to label and purify the reactions prepared at all three protein concentrations; one of them will provide the best visualization of the transduction.

3. Incubate at room temperature in the dark for 2 hr.

Purify the fluorophore-labeled fusion protein

4. Equilibrate a PD-10 column with 25 ml of PBS with 1× protease inhibitors.
5. Apply the entire labeling reaction from step 3 to the column.
6. After the volume enters the gel bed, apply more PBS with protease inhibitors and collect twelve 0.5-ml fractions.

The unconjugated FITC will remain in the gel bead because of its small size. Do not reuse the column.

7. Pool fractions 6 to 8 containing the labeled protein (which will be slightly yellow), add glycerol to 10% (v/v), and store at -80°C .

Transduce labeled fusion protein

8. Incubate various volumes of FITC-labeled TAT fusion protein with 5×10^5 suspension cells (e.g., Jurkat T cells) in 200 μl culture medium (i.e., RPMI/10% FBS) for 30 min at 37°C .

Equilibration is reached in as little as 5 to 15 min.

9. Microcentrifuge 5 min at 5000 rpm, 4°C . Remove the supernatant, add 0.5 ml of ice-cold PBS and immediately microcentrifuge again. Remove the supernatant to complete the wash.

Fix and visualize cells

10. Resuspend the pellet in 500 μl paraformaldehyde fix solution and incubate cells for 15 min at room temperature on an end-over-end rotator.
11. Gently pellet the cells by microcentrifuging 5 min at $5000 \times g$. Resuspend the cell pellet in 200 μl of PBS and mount onto slides (using antifade mounting medium if slides are to be stored overnight prior to examination). Seal the coverslips with clear nail polish.
12. Examine slides by fluorescence microscopy using excitation and emission wavelengths appropriate to the fluorophore (APPENDIX 1E).

See Figure 20.2.4 for an example of FITC-labeled TAT-p16 WT transduced into Jurkat T cells.

ALTERNATE PROTOCOL 5

TRANSDUCTION AND DETECTION BY INDIRECT IMMUNOFLUORESCENCE

When the necessity of colocalization of transduced protein to cellular organelles or substructure is required, use of indirect immunofluorescence is highly desirable. The method described below will allow the researcher to make direct observations of where the TAT fusion protein is located within an individual cell. Use of the Lab-Tek 8-chamber slides makes it easy to examine and manipulate many conditions (i.e., antibody concentration) at the same time. Be sure to include a secondary antibody control to ensure low nonspecific binding of the fluorescent secondary antibody. An example of immunofluorescence completed on NIH 3T3 cells transduced with TAT-Cre is shown in Figure 20.2.5.

TAT-mediated Protein Transduction

20.2.16

Materials

Adherent cells of interest for transduction, e.g. NIH 3T3 cells
Culture medium for NIH 3T3 cells (i.e., DMEM/10% FBS)
Purified fusion protein (see Basic Protocol 2 and Alternate Protocols 1 to 4)
Phosphate-buffered saline (PBS; *APPENDIX 2A*), ice-cold and room temperature
Paraformaldehyde fix solution (see recipe)
100% ethanol, ice-cold
1% and 0.1% (w/v) bovine serum albumin (BSA) in PBS (prepare from 10% w/v BSA stock)
Primary antibody: antibody of choice to fusion protein or mAb to the HA epitope (Berkeley Antibody Company)
TRITC- or PE-labeled secondary antibody
0.2 $\mu\text{g/ml}$ DAPI (prepare fresh from 1 mg/ml DAPI stock; store stock in dark at 4°C)
Slowfade mounting medium (Molecular Probes)
Clear nail polish
Lab-Tek 8-chamber glass slides with lids (Nalge Nunc International)
40°C heat block
50 \times 24-mm coverslips (Fisher)

Prepare cells in chamber slides

1. Culture 1000 NIH 3T3 cells/chamber on a Lab-Tek 8-chamber slide overnight in a minimum volume of 0.5 ml of culture medium.
2. Wash the cells by flooding the chambers with fresh medium followed by removal of the medium by gentle aspiration.

It is a good idea to aspirate from the same position in the well for each wash to minimize loss of cells during the wash steps.

Transduce fusion protein

3. Transduce fusion protein into cells as desired for specific time points.

The useful concentration of the protein will vary from 10 to 200 nM and the times for transduction will also vary depending on the investigator. The minimum volume to cover the cells is 200 μl per chamber. Transduction can be detected in as early as 5 min and usually maximizes between 2 and 6 hr after protein addition (this is fusion dependent).

4. Remove medium and wash once with ice-cold PBS using the technique described in step 2.
5. Fix cells by adding 400 μl of ice-cold paraformaldehyde fix solution per chamber and incubating on ice for 15 min.

It is critical that the fixation buffer be prepared fresh daily; its pH must be verified prior to use.

Expose transduced cells to antibody reagents

6. Wash three times with ice-cold PBS using the technique described in step 2.
7. Permeabilize the cells by adding 400 μl of ice-cold 100% ethanol and incubating on ice for 10 min.
8. Wash three times with ice-cold with PBS using the technique described in step 2.
9. Block with 1% BSA and incubate 10 min on top of a 40°C heat block.
10. Wash five times with PBS at room temperature using the technique described in step 2.

11. Dilute the primary antibody in PBS containing 0.1% BSA according to the manufacturer's recommendation. Add 400 μ l of the diluted antibody to each chamber and incubate 15 min at 40°C.

For anti-HA, a 1000- to 3000-fold dilution is optimal.

12. Wash five times with with PBS at room temperature.
13. Dilute TRITC- or PE-labeled secondary antibody 1000- to 5000-fold in PBS containing 0.1% BSA. Add 400 μ l of the diluted antibody to each chamber and incubate 15 min at 40°C.

If the background (i.e., cells stained with secondary antibody alone) is high, increase the dilution of this antibody.

14. Wash five times with PBS at room temperature.

Counterstain cells with DAPI

15. Counterstain with DAPI by adding 200 μ l of 0.2 μ g/ml DAPI to each chamber and incubating 15 min at room temperature.
16. Rinse chambers with water, remove the attached chambers and seal, then dry in the dark.

Visualize the results

17. After drying is complete, add 5 drops of Slowfade to mount, add 50 \times 24-mm coverslip then seal coverslip with nail polish. Keep in dark until ready to view.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Buffer Z

480.0 g urea
8.0 g NaCl
4.8 g HEPES
Adjust the pH to 8.0 with NaOH
Add H₂O to 1 liter
Store up to 1 month at room temperature

FITC conjugation buffer, 10 \times

Dissolve 0.84 g NaHCO₃ in 9 ml of H₂O. Adjust to pH 9.0 with NaOH, then add H₂O to a final volume of 10 ml. Store at room temperature; prepare fresh solution every week.

LB medium and plates

LB liquid medium: Add 25.0 g LB broth mix (Sigma) per liter of water. Autoclave for 30 min on liquid cycle. Let cool to 50°C and add ampicillin to final concentration of 50 μ g/ml.

LB plates: Prepare as above, but include 5 g/liter agar and pour plates.

Paraformaldehyde fix solution 4% (w/v)

4 g paraformaldehyde
100 ml of PBS (without calcium or magnesium; APPENDIX 2A)
Heat gently with constant stirring (do not boil).
Adjust pH to exactly 7.2 after solution has cooled to room temperature
Chill on ice prior to use
Store up to one week at 4°C

Protease inhibitor stock solutions

Prepare stocks:

1 mg/ml aprotinin in H₂O (1000× stock; store up to 1 year at 4°C)

1 mg/ml leupeptin in H₂O (1000× stock; store up to 1 year at 4°C)

10 mg/ml phenylmethylsulfonyl fluoride (PMSF) in 100% ethanol (200× stock; store up to 1 year at –20°C)

Add each inhibitor to desired solution for a final concentration of 1×

COMMENTARY

Background Information

There are many methods that can be used to introduce biologically interesting molecules into live cells. Most of these methods such as electroporation, microinjection, and lipofection can be inefficient or laborious. Other methods, such as the use of small peptides, which can essentially diffuse into a live cell, are not desirable because of the high concentrations of the peptides required to achieve the biological effect. Therefore, a method which can deliver full-length, target-specific, concentration-dependent, and biologically active molecules into a live cell in the absence of harsh conditions or mechanical disruption would be highly advantageous.

Domains that have the ability to transport (transduce) heterologous molecules have been identified in many different biological systems (Wadia and Dowdy, 2002). Briefly, four different biological systems have been exploited as tools for the exogenous delivery of heterologous proteins: the Antennapedia protein from *Drosophila* (Derossi et al., 1998), VP22 protein from HSV (Elliot and O'Hare, 1997), MPG domain (synthetic fusion peptide of HIV-1 gp41 and the NLS of SV40 large T-antigen; Morris et al., 1997), and TAT from HIV (Fawell et al., 1994). The focus of the method described here, is the transduction domain that originated from the 87 amino acid HIV-Tat protein. The transduction phenomenon was first identified in 1988 when full-length, exogenously added HIV-Tat protein was shown to trans-activate a reporter gene when added to cultured cells at concentrations as low as 1 nM. Fawell et al. (1994) expanded on this observation by demonstrating that proteins chemically cross-linked to a 36 amino acid peptide (residues 37-72) of HIV-Tat were able to transduce into cells. Vives et al. (1997) using a FITC-labeled Tat-peptide showed that residues 37-60 were important for transduction ability. This region was shown to adopt an α -helical structure with amphipathic characteristics from residues 38-49 but a cluster of basic amino acids from

residues 49-58 appeared to be unstructured due to charge repulsions (Loret et al., 1991). The eleven amino acids used in the pTAT/pTAT-HA vectors encompass residues 47-57 of the HIV-Tat molecule. Residues 53-57 (QRRR) seem to be essential for the transduction since FITC-labeled peptides devoid of this region failed to transduce into HeLa cells (Vives et al., 1997). Various mutations in this minimal domain have been completed and certain substitutions have been shown to enhance transduction of peptides (Ho et al., 2001). Translation of these transduction domains (PTDs) into expression vectors is the subject of ongoing investigation, and use of these domains could provide even greater transduction potential (S.F. Dowdy, unpub. observ.).

To date, the exact mechanism of HIV Tat-mediated transduction across cellular membranes remains unclear. An attempt at defining this mechanism was reported in 1997 (Vives et al., 1997) using various versions of residues 37-72. They determined that internalization of TAT peptides did not involve endocytosis (temperature sensitive), potocytosis (caveolae or non-coated plasmalemmal vesical-mediated), or membrane destabilization. Another proposed mechanism is analogous to the Penetratin-1 transduction system. In this case, internalization could be caused by local reorganization of the lipid bilayer, resulting in inverted micelles and eventual delivery of the protein in the cytoplasm (Vives et al., 1997). Alternatively, the Tat transduction domain could utilize heparan sulfate proteoglycans as a means of gaining entry into the cell (Tyangi et al., 2001). Clearly differences in mechanisms of transduction between peptides using protein transduction domains (PTDs) and full-length fusions could vary, and this may explain the differences observed in these studies. Although the mechanism by which full-length TAT-fusion proteins gain entry into cells is not concrete, phenotypic effects of these proteins are being observed in many biological systems (Table 20.2.2), and

Table 20.2.2 Selected TAT-Fusions and the Various Cell Types Transduced

TAT fusion	Function	Cell type/line transduced	Reference
Filamin A	Actin binding Scaffolding protein of caveoli MAPK signaling cascade	HEK-293 Parathyroid cells	Hjalm et al., 2001
Ovalbumin	Used to sensitize dendritic cells (DC) in a solid tumor model	EL-4 thymoma cells Mouse dendritic cells	Shibagaki and Udey, 2002
HPC-1/syntaxin 1A	Binds to SNAP-25 or VAMP2 forming stable SNARE complexes	PC-12 neuronal Cells	Fujiwara et al., 2002
Rac/Rho	GTPases	Eosinophils Leukocytes	Alblas et al., 2001
Pur alpha ^a	Multifunctional DNA and RNA binding protein	T98G (human glioblastoma) U-87 MG (human astrocytoma) J3671 and Daoy (human medulloblastoma)	Darbinian et al., 2001
Merlin/NF2	Neurofibromatosis Type 2 tumor suppressor	Schwannoma tumors Normal Schwann cells	Bashour et al., 2002
ApolipoproteinB	Lipid metabolism	Rat primary hepatocytes McArdle RH7777	Yang et al., 2001
Bcl X _L PEA-15	Apoptosis inhibitors	BTC-3 (insulinoma cells) Rat islet cells	Embury et al., 2001
eGFP	Fluorescent protein	NIH 3T3 Myoblasts	Caron et al., 2001
CRIB	Inhibitor of Cdc42-GTP	Peripheral blood mononuclear cells	Haddad et al., 2001
C3 (exoenzyme of <i>C. botulinum</i>)	Inhibitor of Rho	Rat vascular smooth muscle cells	Sauzeau et al., 2001

^aA C-terminal TAT-fusion molecule.

new reports of novel applications of this technique are rapidly growing.

A TAT fusion protein can be easily constructed using the pTAT/pTAT-HA vectors. The pTAT-HA vector has several features that make it ideal for use as an expression vector. The HA tag can be used to follow the fusion protein throughout purification and transduction in a specific system. A 6-His domain in the N-terminus of the fusion protein affords a simple method of affinity purifying the protein on a nickel affinity resin pre- or post-transduction. The vector contains a multiple cloning region that yields an in-frame fusion protein. A 3' transcriptional terminator is also present if truncations of the fusion proteins are desired. Other fusion vectors have recently been described in which the HIV-Tat transduction domain has been used for C-terminal fusions (Darbinian et al., 2001) instead of N-terminal fusions that pTAT/pTAT-HA would yield (see Table 20.2.2).

Many bacterially expressed recombinant proteins are stored as insoluble inclusion bodies because the bacteria lack the machinery to cor-

rectly fold the eukaryotic protein they are forced to produce. The bodies are insoluble aggregates that cannot be resolubilized without the use of detergents or denaturing agents such as guanidine-HCl or urea. The system described in this unit makes use of 8 M urea to denature the aggregates, allowing binding to an ion-exchange resin, and immediate exchange of the protein into an aqueous environment. This yields protein that is stable enough to be added to tissue culture media where it can be correctly refolded or degraded once inside the cell. Fluorescently labeled fusion proteins have been shown to distribute uniformly in the nucleus and cytoplasm of all cells tested, including: peripheral blood lymphocytes (PBLs), diploid human fibroblasts, keratinocytes, bone marrow stem cells, osteoclasts, fibrosarcoma cells, leukemic T cells, osteosarcoma, glioma, hepatocellular carcinoma, renal carcinoma, NIH 3T3 cells, and all cells present in whole blood, in a concentration-dependent manner (Nagahara et al., 1998). A TAT-β-galactosidase fusion has been shown to penetrate every cell type in an intact live mouse and remain enzymati-

cally active after traversing all of the cellular layers in various organs, including the brain (Schwarze et al., 1999). Fusion proteins of up to 120 kDa have been successfully transduced and shown to be biologically active; therefore the size of the fusion protein does not appear to retard transduction or function, unlike other transduction domains (Wadia et al., 2002). The concentration of the transduced protein required to achieve a biological function is very low when compared to peptide mimetics. This feature of TAT transduction technology makes it ideal when targeting therapeutic strategies.

The method has already been applied to many biological questions. For example, fusions of TAT-p16 were used to define the phosphorylation events of the retinoblastoma protein (pRb) in cell cycle progression (Ezhevsky et al., 1997); TAT-E7 has been shown to sequester pRb and rescue T-cells from T-cell Receptor-Activated Induced Cell Death (TCR-AID; Lissy et al., 1998); and the role of p27 in inducing hepatocellular carcinoma cell scattering was elucidated using TAT-p27 (Nagahara et al., 1998). This technology has been exploited to “trick” an HIV-infected cell into committing suicide by inducing caspase-3 cleavage (Vocero-Akbani et al., 1999). The implications of this idea are broadly applicable to any disease that depends upon protease-specific cleavage of pro-molecules. Many more applications have been and continue to be reported (Table 20.2.2).

The limitations for the use of this strategy seem to be three-fold. First, mammalian systems have evolved to defend against foreign proteins. Clearly, proteolytic degradation of exogenously added fusion proteins found in serum reduces the effective half-life of the protein *in vitro* and can be amplified *in vivo*. Similarly, inhibitors within serum can interfere with function of transduced molecules. For example, TAT-thymidine kinase prepared by the soluble method described in this unit (see Alternate Protocol 4) is fully functional and rapidly transduces into cells; however, the fusion protein showed no enzymatic activity after lysis of the transduced cells (S.R. Schwarze, unpub. observ.) because of a serum-derived enzymatic inhibitor. Culture systems can minimize the serum effects by using protease inhibitors or by increasing the dosage of the fusion proteins. Additionally, one can use agents such as TNF/cyclohexamide *in vitro* (Embary et al., 2001) to slow the cellular machinery and allow the cell to refold the transduced proteins more rapidly. The second limitation to this technology may be easily addressed. Since some eukaryotic proteins are

modified post-translationally (i.e., phosphorylation and glycosylation), and bacteria do not have the ability to make such modifications, a eukaryotic fusion protein expression system must be designed in order to achieve fully functional protein. Lastly, localization of membrane fusion proteins is probably impossible. Studies with fluorescinated TAT-PTEN (which contains a C2 domain that is known to bind to phospholipid membranes; Lee et al., 1999) showed aggregation of the fusion protein at LnCaP cell membranes (S.R. Schwarze, unpub. observ.).

Critical Parameters

Maintaining protein solubility

Since many TAT-fusion proteins will be isolated in 8 M buffered urea and then quickly exchanged into an aqueous buffer, it is important to determine if the protein maintains stability after freeze/thaws and addition into the culture medium. Centrifuging the proteins at $5000 \times g$ for 10 min at 4°C will ensure that only soluble protein will be added to the cells. If a pellet is evident after centrifugation, another SDS-PAGE gel comparing protein concentrations should be completed.

If precipitation of the fusion protein is noticed on the cells during the transduction procedure, the buffer in which the fusion is stored should be changed. Use of the PD-10 column to exchange the buffer for cell-specific culture media without serum or any other additives usually solves the problem. If the protein is stored in the culture medium, the authors suggest using 0.1% (w/v) BSA to stabilize the protein. Also, when freezing the protein, always add at least 10% (v/v) glycerol, prior to flash freezing and storage at -80°C.

When thawing the proteins, quick thawing at 37°C is recommended to decrease the chances of precipitation and shearing from ice crystals. Immediately after the quick thaw, keep the proteins on ice until they are added to the medium. The thawed protein can be kept on ice at 4°C for up to 1 week with no significant loss of transducibility. Continual storage of the protein at 4°C will eventually result in protein precipitation, and is therefore not recommended for long-term storage.

Addition of the fusion proteins to tissue culture system

Because of the efficiency of this transduction system, it may be important to add the fusion protein to the culture medium prior to

addition of the medium to the cells. Therefore, after plating the cells at the appropriate density for the experimental procedure, wash the cells once in fresh medium. Add the appropriate volume of fusion protein to the required volume of tissue culture medium—i.e., 0.2 ml to 1.8 ml of DMEM containing 10% fetal bovine serum and 1× penicillin/streptomycin per well of 6-well dish—and then add this volume to the cells. It is important that the volume of fusion protein not exceed 10% of the tissue culture volume. Toxic effects and slowing of the cell cycle have been observed in some cases when greater volumes were used.

Dose and toxicity of the fusion proteins

It is virtually impossible to predict possible toxicity of the fusion protein in certain cell culture systems. If toxicity is noticed, several options are available. First, one can decrease the molar concentration of the fusion protein to the cells. In general, concentrations of >200 nM fusion protein are not necessary. The working range of these proteins vary from 50 to 200 nM. In a rare case, 1 μM was necessary (Ezhevsky et al., 1997). Therefore, the range in which a protein can be used must be decided on a fusion-to-fusion basis. Second, changing the buffer in which the protein is stored may be necessary (see Alternate Protocols 2 and 3).

The final concentration of cells used in a transduction experiment is critical. The concentration of transducing protein that is required to observe an effect is dependent upon the starting cell population. The smaller the target cell population, the greater the intracellular concentration will be. Studies using FITC-labeled TAT-p16 in normal diploid fibroblasts have showed greater fluorescence intensity of the fusion when fewer cells were plated (M. Becker-Hapak, unpub. observ.). Therefore, in suspension cultures or adherent cells, it is generally recommended that the lowest possible concentration of cells be used when fusion protein concentrations are limited.

Half-life of fusion proteins

The authors have observed dramatic differences in the half-lives of fusion proteins. Degradation of the fusion protein can be determined by immunoblotting extracts of the trypsinized and washed cells. Generally, if the transducing protein is to be incubated for longer than 24 hr, addition of supplemental protein is suggested. The necessity for supplementary additions of any protein of interest must also be experimentally determined.

Troubleshooting

Established clone is no longer expressing fusion protein

In some cases, the bacteria may stop expressing the fusion protein. If this occurs, simply go to another one of the backup clones or retransform the plasmid into fresh BL-21 (DE3)pLysS and rescreen for high-expressing clones.

Poor expression of the fusion protein

It may be necessary to induce the fusion protein of interest. BL-21 cells, carry an IPTG-inducible, T7 polymerase (DE3) and a T7 polymerase specific protease (pLysS) under a chloramphenicol resistance marker. The pLysS keeps the T7 polymerase at a negligible concentration until the organism goes into log phase (during IPTG induction). If the expression of the specific fusion protein is low, induce the T7 polymerase by adding 2 μg/ml IPTG when the 200 ml overnight culture is added to 1 liter of LB-ampicillin medium. Culture as described in the protocols above.

Another factor affecting the protein expression levels is the choice of supplier for the LB medium. In the authors' experience, LB medium purchased from certain vendors have produced more recombinant protein than others, so it is recommended that various vendors and lots be tested for maximal yield of any protein of interest. One may, at the same time, culture the fusion in a richer medium such as Terrific Broth (TB). However, the routine use of this medium is not recommended because some fusions have proven to express more robustly in LB than TB.

Many T7 promotor-driven fusions can be expressed in a multitude of BL-21(DE3) derivatives. The authors have found a single source of many of these hosts. Novagen provides a reasonably priced set of competent DE3 derivatives (cat. no. 71032-3) which are very helpful in expression of various eukaryotic proteins that cannot be efficiently expressed in BL-21 (DE3) pLysS. Transformation of the pure plasmid into a panel of these hosts and side-by-side detection via immunoblotting can show dramatic differences in host-to-host differences in fusion expression.

Ion-exchange chromatography

Difficulties encountered during any standard ion-exchange procedure are also observed with TAT-fusion proteins. The authors highly recommend the batch test tube procedure outlined in Amersham Pharmacia Biotech's ion-exchange

handbook (Amersham Biosciences, 2002) to rapidly identify binding and elution protocols.

Protein seems to be degrading during the purification procedure

Be sure to use the protease inhibitor cocktail described in Reagents and Solutions. This cocktail should be added at every step of the purification when a new buffer is used. If the protein was sonicated in PBS maintain the sonicate at 4°C at all times and perform the chromatographic steps in the cold.

Protein precipitates after dialysis

The concentration of the protein may be too high. Dilute the protein in buffer Z to a concentration of <1 mg/ml before applying it to the dialysis cassette.

Try using the PD-10 column to remove the urea. However, the authors have noticed that if the protein precipitates during dialysis, it is likely to precipitate during the PD-10 step as well. Therefore, the only way to ensure soluble protein is to use the ion-exchange method of achieving soluble proteins.

No biological effect with wild-type protein

The transduced protein, may require a concentration higher than 200 nM (working concentrations with in cell culture varies from 50 to 200 nM, but can require upwards of 1 μ M) to obtain the specific effect. For example, the authors have observed that in wild-type cells (i.e., SiFTs) that have an already high concentration of p16 (later passage), less TAT-p16 wt is required to achieve a G1 arrest, than in those expressing low concentrations of the protein.

Another factor that can affect function of the TAT-fusion protein is the position of the TAT-leader. The authors have observed that the TAT-leader inhibits the ability of p16 to bind to TAT-CDK4, but the leader has no effect on TAT-CDK2. In such cases, C ϵ terminal fusions should help.

Check if the protein is active after freeze-thaw. Some proteins cannot be frozen at any step of the purification from bacterial harvesting to storage. If this is the case, store the protein on ice at 4°C. BSA may also be required to help keep the protein from precipitating or degrading.

Unknown serum factors can inhibit, inactivate, or degrade exogenously added fusion proteins causing negative results. To examine if this is occurring with the fusion, simply dilute the pure protein into cell-free culture medium (usually in the presence of 10% v/v fetal bovine serum) at 37°C for varying amounts of time, then immunoblot for the pure protein. Nonspe-

cific proteolytic degradation can be minimized by the addition of specific protease inhibitors. However, solutions to these types of problems are most likely found by the preparation of point-specific mutants.

Choice of tags

While the pTAT-HA vector utilizes the hemagglutinin tag to allow one to detect the fusion, other popular tags used concomitantly can inhibit transduction of the purified protein. It is presumed that the very acidic nature of tags such as FLAG can associate with the TAT domain and very effectively negate the transducibility of the fusion (M. Becker-Hapak, unpub. observ.).

Anticipated Results

Protein yield

The quantity of protein yielded by a 1-liter LB-ampicillin culture will vary depending upon the construct. For example, TAT- β -galactosidase will yield 4 to 6 mg of total protein, whereas TAT-caspase 3 only yields 500 μ g. In general non-cytotoxic constructs, such as TAT-p27, yield 1 to 2 mg of protein per liter of LB-ampicillin medium.

Fluorescein-labeled fusion proteins

The efficiency of fluorescein labeling will vary from day to day and protein to protein. One can quantitate the fluorescence intensity using a fluorometer prior to addition of the label to the cell culture. When comparing fluorescently labeled TAT-fusion versus the nontransducing pure protein by confocal microscopy, TAT-fusion proteins will show an even fluorescence intensity throughout the cell, with some punctate staining in the nucleoli (Fig. 20.2.4). The nontransducing protein will show a lower intensity “rim” fluorescence due to nonspecific sticking of the labeled protein to the cell membrane. If examining transduction by immunohistochemistry, typical results show detection of the transducing protein after as little as 5 min and peaking between 4 and 6 hr after transduction, when incubated at 37°C. Early time points will show the protein nonspecifically distributed throughout the whole cell, while the later time points will show a more specific staining pattern. The putative nuclear localization sequence will not prevent the fusion from going in and out of the nucleus. For example, TAT-p27 WT shows a ubiquitous distribution when transduced into HepG2 cells in the first 12 hr, then concentrates in the nucleus 24 hr after introduction of the fusion protein. However, if HepG2 cells are

cultured in the presence of HGF, TAT-p27 is shuttled out of the nucleus and into the cytoplasm over a 24 hr period (S.S. McAllister and S.F. Dowdy, unpub. observ.).

Biological activities of transduced

TAT-fusion proteins

The activities expected of the TAT-fusion proteins vary with application. Consequently, phenotypic readouts vary as well. In general, the time frame in which the phenotype can be recorded can vary from 5 min (enzymatic assays) to days (morphological changes). Table 20.2.1 shows some results obtained from various classes of TAT-fusion molecules. The table details the dose of fusion that was required, biological effect observed, and time frame in which the result was measured. To expand on examples of the utilization of TAT-full length fusion technology, examples of selected fusions are shown in Table 20.2.2. The table not only lists the TAT-fusion created and the function, but it also summarizes some other cell types that were not used in transduction experiments listed in Table 20.2.1.

Time Considerations

Total time required

Identification of protein and determining necessity of IPTG induction requires 2 days

Isolation of protein requires 1 to 2 days.

Detection of protein transduction requires 1 to 2 days.

For biological readouts the time required is pathway/protein-dependent.

Stopping points

In general, one should consider any protein left at room temperature as being susceptible to degradation. Therefore, if one wishes to stop at any point during the protein purification, one should consider freezing the preparation if the duration will be longer than overnight. Alternatively, store the protein at 4°C on ice overnight, if the protein of interest is sensitive to freeze/thaw. For example; TAT-β-gal, whose enzymatic activity is abolished if frozen at any point but it retains transducibility, must be stored at 4°C in the presence of 0.1% BSA to remain active for up to one month on ice at 4°C.

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Calcium Phosphate Transfection

UNIT 20.3

This unit presents two methods of calcium phosphate–based eukaryotic cell transfection that can be used for both transient and stable transfections. In these protocols, plasmid DNA is introduced to monolayer cell cultures via a precipitate that adheres to the cell surface. A HEPES-buffered solution is used to form a calcium phosphate precipitate that is directly layered onto the cells (see Basic Protocol). For some cells, shocking the cells with glycerol or DMSO (see Support Protocol) improves transfection efficiency. In the alternate high-efficiency method, a BES-buffered system is used that allows the precipitate to form gradually in the medium and then drop onto the cells (see Alternate Protocol). The alternate method is particularly efficient for stable transformation of cells with circular plasmid DNA. For transformation with linear plasmid or genomic DNA, or for transient expression, however, the Alternate Protocol is comparable to the Basic Protocol. Both methods of transfection require very high-quality plasmid DNA. Factors that can be optimized for calcium phosphate transfections are presented in *UNIT 20.7*. Additional details of mammalian cell culture are given in *UNIT 1.1*.

TRANSFECTION USING CALCIUM PHOSPHATE–DNA PRECIPITATE FORMED IN HEPES

**BASIC
PROTOCOL**

A precipitate containing calcium phosphate and DNA is formed by slowly mixing a HEPES-buffered saline solution with a solution containing calcium chloride and DNA. This precipitate adheres to the surface of cells and should be visible in the phase contrast microscope the day after transfection. Depending on the cell type, up to 10% of the cells on a dish will take up the DNA precipitate through an as yet undetermined mechanism. Glycerol or dimethyl sulfoxide shock increases the amount of DNA absorbed in some cell types (see Support Protocol).

Materials

Exponentially growing eukaryotic cells (e.g., HeLa, BALB/c 3T3, NIH 3T3, CHO, or rat embryo fibroblasts)

Complete medium (depending on cell line used)

CsCl-purified plasmid DNA (10 to 50 μg per transfection)

2.5 M CaCl_2 (see recipe)

2 \times HEPES-buffered saline (HeBS; see recipe)

PBS (*APPENDIX 2A*)

10-cm tissue culture dishes

15-ml conical tube

Additional reagents and equipment for ethanol precipitation (*APPENDIX 3A*) and mammalian cell tissue culture (*UNIT 1.1*)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

1. Split exponentially growing eukaryotic cells into 10-cm tissue culture dishes the day before transfection. Feed cells with 9.0 ml complete medium 2 to 4 hr prior to precipitation.

When transfecting adherent cells that double every 18 to 24 hr, a 1:15 split from a confluent dish generally works well. On the day of the transfection, it is important that cells are thoroughly separated on the dish, as the ability to take up DNA is related to the surface

**Introduction and
Expression of
Foreign
Macromolecules
in Cells**

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20.3.1

Supplement 19

area of the cell exposed to the medium. Cells should be split in a manner that accomplishes this.

The desired density of cells on dishes to be transfected will vary with cell type and the reason for doing the transfection. The optimal density is that which produces a near confluent dish when the cells are harvested or split into selective medium.

2. Ethanol precipitate the DNA to be transfected and air dry the pellet by inverting the microcentrifuge tube on a fresh Kimwipe inside a tissue culture hood. Resuspend the pellet in 450 μ l sterile water and add 50 μ l of 2.5 M CaCl_2 .

The amount of DNA that is optimal for transfection varies from 10 to 50 μ g per 10-cm plate, depending on the cell line to be transfected.

DNA to be transfected should be purified twice by CsCl gradient centrifugation. DNA can also be prepared using column procedures. Some column procedures produce DNA that does not transfect well, so column-purified DNA should be tested and compared to CsCl -purified DNA for transfection efficiency. Supercoiled DNA works well in transfections. Impurities in the DNA preparation can be deleterious to transfection efficiency. A description of how to optimize the amount of DNA to transfect and other parameters of calcium phosphate-mediated transfection is provided in the discussion of optimization of transfection (UNIT 20.7).

Ethanol precipitation sterilizes the DNA to be transfected. For transfections that will be harvested within 3 to 4 days (transient analysis), this is not necessary. For transient experiments, many researchers make a 450- μ l aqueous solution containing the DNA directly, without ethanol precipitation. If this is done, care should be taken to keep the amount of Tris in the solution to a minimum, as Tris may alter the pH of the precipitate and therefore reduce transfection efficiency.

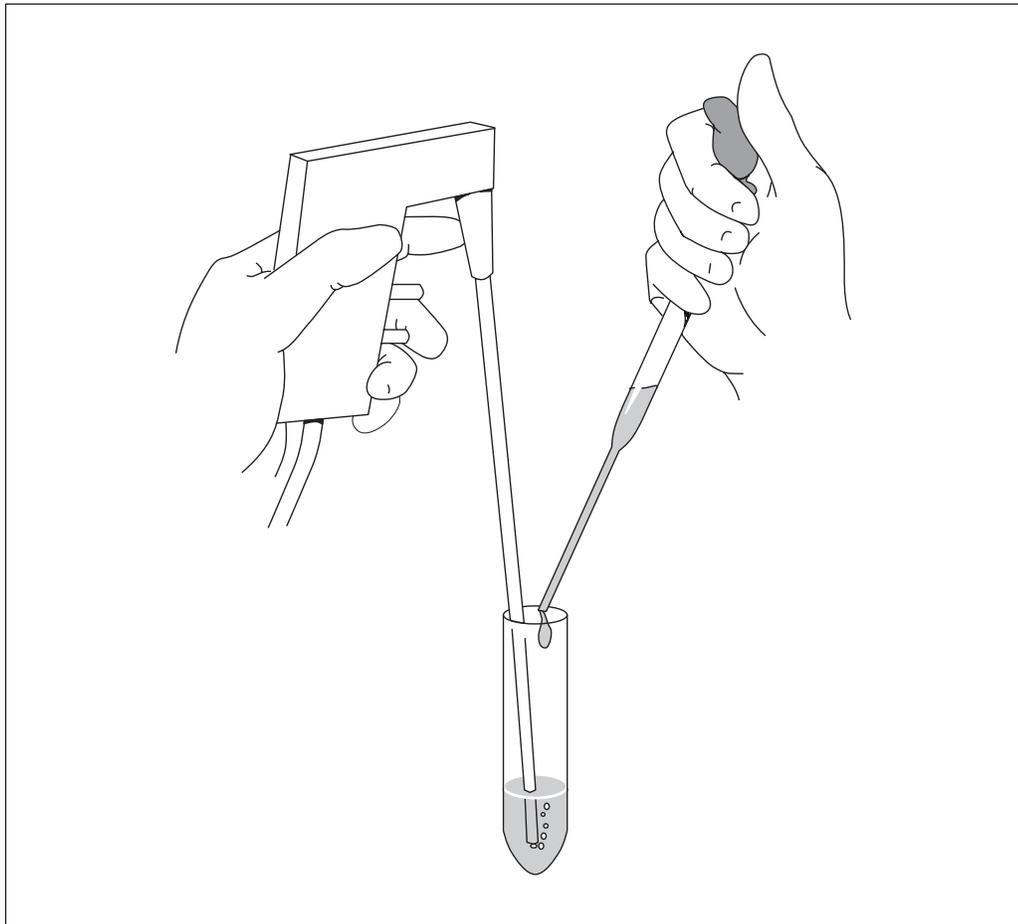


Figure 20.3.1 Formation of calcium phosphate precipitate.

3. Place 500 μ l of 2 \times HeBS in a sterile 15-ml conical tube. Use a mechanical pipettor attached to a plugged 1- or 2-ml pipet to bubble the 2 \times HeBS and add the DNA/CaCl₂ solution dropwise with a Pasteur pipet (see Fig. 20.3.1). Immediately vortex the solution for 5 sec.

If no mechanical pipettor is available, the solution can be bubbled by blowing through rubber tubing that is attached to a pipet via a filter. The filter is necessary to maintain sterility. This does not give as reproducible results as the mechanical pipettor.

4. Allow precipitate to sit 20 min at room temperature.
5. Use a Pasteur pipet to distribute the precipitate evenly over a 10-cm plate of cells and gently agitate to mix precipitate and medium.
6. Incubate the cells 4 to 16 hr under standard growth conditions. Remove the medium. Wash cells twice with 5 ml PBS and feed cells with 10 ml complete medium.

The amount of time that the precipitate should be left on the cells will vary with cell type. For hardy cells such as HeLa, NIH 3T3, and BALB/c 3T3, the precipitate can be left on for 16 hr. Other cell types will not survive this amount of exposure to the precipitate. See discussion of optimization of transfection (UNIT 20.7) for optimization of this step as well as for a discussion of how to determine whether glycerol shock is useful.

7. For transient analysis, harvest the cells at the desired time point. For stable transformation, allow the cells to double twice before plating in selective medium.

GLYCEROL/DMSO SHOCK OF MAMMALIAN CELLS

The Basic Protocol works well for cell lines such as HeLa, BALB/c 3T3, NIH 3T3, and rat embryo fibroblasts. Transfection efficiency in some cell lines, such as CHO DUKX, is dramatically increased by “shocking” the cells with either glycerol or DMSO. Precipitates are left on the cell for only 4 to 6 hr, and the cells are shocked immediately after removal of the precipitate.

Additional Materials (also see Basic Protocol)

10% (v/v) glycerol solution or DMSO in complete medium, sterile
PBS (APPENDIX 2A), sterile

Replace step 6 of the Basic Protocol with the following:

- 6a. Incubate the cells 4 to 6 hr and remove the medium. Add 2.0 ml of a sterile 10% glycerol solution. Let the cells sit 3 min at room temperature.

Alternatively, 10% or 20% DMSO can be used. DMSO tends to be somewhat less harmful to the cells, but also may not work as well.

- 6b. Add 5 ml of PBS to the glycerol solution on the cells, agitate to mix, and remove the solution. Wash twice with 5 ml of PBS. Feed the cells with complete medium.

It is important to dilute the glycerol solution on the cells with PBS before removing the glycerol solution so the cells do not stay in glycerol too long. Excessive exposure to glycerol will kill cells.

SUPPORT PROTOCOL

Introduction and Expression of Foreign Macromolecules in Cells

20.3.3

HIGH-EFFICIENCY TRANSFECTION USING CALCIUM PHOSPHATE–DNA PRECIPITATE FORMED IN BES

A solution of calcium chloride, plasmid DNA, and *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer, pH 6.95, is added to a plate of cells containing culture medium. The plates are incubated overnight while a calcium phosphate–DNA complex forms gradually in the medium under an atmosphere of 3% CO₂. With this method, 10% to 50% of the cells on a plate stably integrate and express the transfected DNA. Transient expression under these conditions is comparable to that obtained with the Basic Protocol. Glycerol or DMSO shock does not increase the number of cells transformed.

Materials

Exponentially growing mammalian cells (see Critical Parameters)
Complete medium: Dulbecco modified Eagle medium containing 10% (v/v) fetal bovine serum (FBS)
CsCl-purified plasmid DNA
TE buffer, pH 7.4 (APPENDIX 2A)
2.5 M CaCl₂ (see recipe)
2× BES-buffered solution (BBS; see recipe)
PBS (APPENDIX 2A)
Selection medium (APPENDIX 3A; optional)

10-cm tissue culture dishes
35°C, 3% CO₂ humidified incubator
35° to 37°C, 5% CO₂ humidified incubator
Fyrite gas analyzer (optional; Fisher Scientific or Curtin Matheson)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

1. Seed exponentially growing mammalian cells at 5×10^5 cells/10-cm tissue culture dish in 10 ml complete medium the day prior to transfection.

There should be $<10^6$ cells/dish just prior to infection. Enough surface area should remain on the plate for at least two more doublings.

2. Dilute CsCl-purified plasmid DNA with TE buffer to 1 µg/µl. Store the DNA solution at 4°C.

Purity of the plasmid DNA is critical.

The optimum amount of plasmid to use can be determined by transfecting three dishes of cells with 10, 20, and 30 µg of plasmid DNA and incubating overnight. The dishes should then be examined with a microscope at 100×. A coarse, clumpy precipitate will form at DNA concentrations that are too low, a fine (almost invisible) precipitate will form at concentrations that are higher than optimal, and an even, granular precipitate will form with optimal DNA concentrations.

3. Prepare 0.25 M CaCl₂ from 2.5 M stock. Mix 20 to 30 µg plasmid DNA with 500 µl of 0.25 M CaCl₂. Add 500 µl of 2× BBS, mix well, and incubate 10 to 20 min at room temperature.
4. Add the calcium phosphate–DNA solution dropwise onto the medium-containing plate while swirling the plate. Incubate 15 to 24 hr in a 35°C, 3% CO₂ incubator.

Level of carbon dioxide is critical. Use a Fyrite gas analyzer to measure percent CO₂ prior to incubation.

5. Wash the cells twice with 5 ml PBS, and add 10 ml complete medium. For stable transformation, incubate overnight in a 35° to 37°C, 5% CO₂ incubator. For studies involving transient expression, incubate the cells for 48 to 72 hr after adding the DNA.
6. Split the cells 1:10 to 1:30, depending on the growth rate of the host cell, before beginning to select stable transformants. Incubate overnight in a 35° to 37°C, 5% CO₂ incubator.
7. Start selection by changing the medium to selection medium or by incubating cells under appropriate selection conditions.

REAGENTS AND SOLUTIONS

Use deionized, distilled water or equivalent for all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.

BES-buffered solution (BBS), 2×

50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES;
Calbiochem-Novabiochem)
280 mM NaCl
1.5 mM Na₂HPO₄, pH 6.95
800 ml H₂O
Adjust to pH 6.95 with 1 N NaOH, room temperature
Add H₂O to 1 liter
Filter sterilize through a 0.45- μ m nitrocellulose filter (Nalgene)
Store in aliquots at -20°C

As discussed in the Commentary, the pH of this solution is critical (pH 6.95 to 6.98). When a new batch of 2× BES buffer is prepared, its pH should be checked against a reference stock prepared (and tested) earlier.

This solution can be frozen and thawed repeatedly.

CaCl₂, 2.5 M

183.7 g CaCl₂·2H₂O (Sigma; tissue culture grade)
H₂O to 500 ml
Filter sterilize through a 0.45- μ m nitrocellulose filter (Nalgene)
Store at -20°C in 10-ml aliquots

This solution can be frozen and thawed repeatedly.

HEPES-buffered saline (HeBS) solution, 2×

16.4 g NaCl (0.28 M final)
11.9 g HEPES (N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; 0.05 M final)
0.21 g Na₂HPO₄ (1.5 mM final)
800 ml H₂O
Titrate to pH 7.05 with 5 N NaOH
Add H₂O to 1 liter
Filter sterilize through a 0.45- μ m nitrocellulose filter
Test for transfection efficiency
Store at -20°C in 50-ml aliquots

An exact pH is extremely important for efficient transfection. The optimal pH range is 7.05 to 7.12.

There can be wide variability in the efficiency of transfection obtained between batches of 2× HeBS. Efficiency should be checked with each new batch. The 2× HeBS solution can be rapidly tested by mixing 0.5 ml of 2× HeBS with 0.5 ml of 250 mM CaCl₂ and vortexing. A fine precipitate should develop that is readily visible in the microscope. Transfection efficiency must still be confirmed, but if the solution does not form a precipitate in this test, there is something wrong.

COMMENTARY

Background Information

Calcium phosphate transfection was first used to introduce adenovirus DNA into mammalian cells by Graham and van der Eb (1973). It was later found to be possible to integrate exogenous DNA into mammalian chromosomes using this technique (Wigler et al., 1978). The protocol described here has evolved from these methods; in particular, the order of addition of the HEPES-buffered saline solutions (HeBS) to calcium chloride is different. In addition, many investigators have found that it is not necessary to add chromosomal DNA as carrier when doing a transfection. Transfections work well if only plasmid DNA, such as pUC vectors, is used.

This HEPES-based approach has been used to analyze replication and promoter function using “transient” protocols, in which cells are harvested 48 to 60 hr after the transfection is started. It also is presently the most widely used technique for producing cell lines in which transfected DNA is stably integrated into the chromosome, although the BES-based technique (see Alternate Protocol) is gaining widespread use for production of stable transformants. Calcium phosphate-mediated transfection tends to work much better than DEAE-dextran-mediated transfection (*UNIT 20.4*) in formation of stable cell lines. It is believed that this is because cells in a calcium phosphate transfection pick up more DNA than DEAE-dextran-transfected cells. Electroporation (*UNIT 20.5*) and liposome-mediated transfection (*UNIT 20.6*) can also be successfully used to produce stable cell lines.

Another major strength of calcium phosphate transfection is that transfected cells generally contain a representative sampling of the various plasmids in the precipitate. Hence, one can prepare a 10:1 ratio of two plasmids and expect that the plasmids will be present in that ratio in the transfected cells.

The BES-based high-efficiency calcium phosphate transfection (see Alternate Protocol) is a modification of the standard calcium phosphate method that employs a buffer system originally developed for phage particle-mediated gene transfer (Ishiura et al., 1982). With this buffer system the calcium phosphate–DNA precipitate forms gradually in the culture medium, dropping gently onto the cells over a 15- to 24-hr period. This method can stably transform most common mammalian fibroblast and epithelial cell lines 10- to 100-fold more effi-

ciently than other methods. For transient expression, it is no better than the standard method.

Critical Parameters and Troubleshooting

Calcium phosphate transfections are finicky—they are not difficult to do, they just do not always work—even in the hands of people who routinely do them. In the Basic Protocol, the most common reason for failure is a 2× HeBS solution that is no longer at the appropriate pH. The optimum pH range for transfection is extremely narrow (between 7.05 and 7.12; Graham and van der Eb, 1973). The pH of the solution can change during storage and an old 2× HeBS solution may not work well. Some investigators also have noticed that the 2.5 M CaCl₂ solution can go bad over time. Both solutions should be made fresh if transfections have stopped working well.

A second problem is that the pH of the medium can turn acidic while the transfection is in progress. This results in an extremely heavy precipitate (making the medium resemble orange juice) and generally results in cell death. Care should be taken to maintain a pH of 7.2 to 7.4 and CO₂ concentrations in the incubator as listed in the protocols. Incubator and medium conditions that are fine for routinely growing cells may not suffice for calcium phosphate transfection.

Many factors can influence the efficiency of HEPES-buffered calcium phosphate-mediated transfection. A description of experiments that can be done to optimize transfection efficiency using both this procedure and the BES procedure can be found in the discussion of optimization of transfection (*UNIT 20.7*).

Several parameters are crucial to achieve high efficiency with the Alternate Protocol: pH of the 2× BES-buffered saline (BBS), percentage of carbon dioxide in the incubator during formation of precipitate, and form and amount of DNA used.

A pH curve of the 2× BBS buffer should be made because minor variations in pH can have substantial effects on transfection efficiency. Perform pilot experiments with buffers of varying pH. The optimal pH is within a very narrow range (6.95 to 6.98). Once the optimal buffer is found, use it as a reference to prepare buffer stocks. If no precipitate forms, the concentration of calcium chloride or 2× BES solution (BBS) may be wrong. Be sure to mix reagents

thoroughly before adding them to the DNA. Crystal formation upon the addition of calcium chloride indicates incorrect calcium chloride concentration, and the transfection must be repeated.

The first overnight incubation should be at 3% CO₂, but a variation of 0.5% may be acceptable. After overnight incubation at 3% CO₂ the culture medium should be alkaline (pH 7.6). Measure the CO₂ levels of the incubator before using. A Fyrite device is recommended for this.

Only plasmid DNA gives high efficiencies of gene transfer with the Alternate Protocol. Efficiency is also dependent on the purity and concentration of the DNA. Toxicity that often occurs with common fibroblasts and epithelial cell lines is usually caused by impure DNA, not calcium phosphate. DNA prepared by cesium chloride gradient centrifugation is rarely toxic to cells. The optimal DNA concentration varies among plasmid preparations as well as with different cells and media. Each new plasmid preparation and each new cell line being transfected should be tested for optimum DNA concentration.

If it is suspected that a particular plasmid preparation is toxic, use a control plasmid—one known not to be toxic to these cells—to test for toxicity. If the plasmid DNA is toxic, prepare new DNA.

Cotransfection efficiency is 10- to 20-fold better with the BES method (see Alternate Protocol) than with the Basic Protocol, although efficiencies vary with the plasmid and marker gene used. A 1:10 ratio of selectable marker to nonselected gene is recommended, but the efficiency of transfection will depend on optimum DNA dose. Glycerol shock or DMSO treatment will not increase the number of cells transfected with this method.

The BES protocol (see Alternate Protocol) has been optimized for use with cells that grow in Dulbecco modified Eagle medium containing 10% (v/v) fetal bovine serum (FBS). RPMI and minimal essential medium (MEM α) have also been demonstrated to give good results under the stated conditions. The FBS must be tested before use by examining the growth, plating efficiency, and transformation efficiency of at least two cell lines. (Serum is tested without heat inactivation.) Because FBS is very costly, 10% (v/v) newborn calf serum and 5% (v/v) FBS can be added to the medium (a total of 75 ml serum/500 ml medium) instead of 10% FBS. This gives equivalent efficiencies of stable transformation. The lots of new-

born calf serum also must be tested for growth and plating efficiencies, as these have been found to vary. If horse or other serum or medium is required, optimum conditions may need to be rechecked.

The Alternate Protocol works well for most established cell lines that grow as monolayers, including mouse, rat, hamster, monkey, and human. It does not seem to work well for neuronal lines. This may be due to the deleterious effect of the calcium on these cells. Cells that grow in suspension are transfected rather poorly by this method, but their stable transformation frequencies seem to be better than those obtained by the Basic Protocol and almost comparable to those obtained by electroporation.

Anticipated Results

The efficiency that can be obtained with calcium phosphate-mediated transfection varies with cell type and other parameters as described. Methods for optimizing these parameters are presented in the discussion of optimization of transfection (UNIT 20.7). Up to 10³ colonies can be obtained by transfecting 1 μ g of a plasmid containing a dominant selectable marker into 10⁶ cells by the HEPES-based protocol. Efficiencies for stable transformants are generally >10- to 100-fold higher when the BES-based protocol is used, with 10% to 50% of the cells on a plate stably transformed (Chen and Okayama, 1987). Transient expression is comparable in the Basic and Alternate Protocols (Chen and Okayama, 1988).

Time Considerations

For the Basic Protocol, preparation of twelve DNA precipitates and addition of the precipitates to the cells takes 1 to 2 hr. Without the ethanol precipitation step, the procedure can be done in 1 hr. With practice, the actual mixing of the CaCl₂ and 2 \times HeBS solutions will take ~1 min. This means that up to eighteen precipitates can be made before the first precipitate is ready to apply to the cells.

For the Alternate Protocol, no ethanol precipitation step has been necessary for either transient or stable transfections. It takes slightly less time than the Basic Protocol because the 2 \times BBS does not need to be added dropwise to the calcium chloride-DNA solution.

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Ishiura et al., 1982. See above.

Provides the basis for BES-mediated transfection.

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Transfection Using DEAE-Dextran

UNIT 20.4

Transfection of cultured mammalian cells using diethylaminoethyl (DEAE)-dextran/DNA can be an attractive alternative to other transfection methods in many circumstances. The major advantages of the technique are its relative simplicity and speed, limited expense, and remarkably reproducible interexperimental and intraexperimental transfection efficiency. Disadvantages include inhibition of cell growth and induction of heterogeneous morphological changes in cells. Furthermore, the concentration of serum in the culture medium must be transiently reduced during the transfection. Any of these factors may adversely affect or be incompatible with some bioassays or experimental goals. In addition, for nonstandard cell types there may be a requirement for extensive preliminary investigation of optimal transfection conditions. Together, these factors influence the suitability of this technique to specific purposes. In general, DEAE-dextran DNA transfection is ideal for transient transfections with promoter/reporter plasmids in analyses of promoter and enhancer functions, and is suitable for overexpression of recombinant protein in transient transfections or for generation of stable cell lines using vectors designed to exist in the cell as episomes. The procedure may also be used for expression cloning (Aruffo and Seed, 1987; Kluxen and Lubbert, 1993; Levesque et al., 1991), although electroporation is usually preferred for this purpose (Puchalski and Fahl, 1992).

This unit presents a general description of DEAE-dextran transfection (see Basic Protocol) as well as two more specific protocols for typical experimental applications (see Alternate Protocols 1 and 2).

The Basic Protocol is suitable for transfection of anchorage-dependent (attached) cells. For cells that grow in suspension, electroporation (UNIT 20.5) or lipofection (UNIT 20.6) is usually preferred, although DEAE-dextran-mediated transfection can be used (Fregeau and Bleackley, 1991). For suspension cells, the transfection step should be performed on collected cells that have been resuspended at 10^7 cells/ml in transfection medium, using reagents and conditions that are otherwise similar to those of the Basic Protocol.

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be followed accordingly.

NOTE: All culture incubations are performed in a 37°C, 5% CO₂ incubator unless otherwise specified.

GENERAL PROCEDURE FOR DEAE-DEXTRAN TRANSFECTION

Cultured cells are incubated in medium containing plasmid DNA and DEAE-dextran, which form complexes that are taken up by cells via endocytosis. Chloroquine can be included to inhibit degradation of plasmid DNA. Cells are exposed transiently to DMSO or another permeabilizing agent to increase DNA uptake (DMSO “shock”). Important variables include the concentration of DEAE-dextran, the ratio of DNA to DEAE-dextran, the duration of transfection, and the presence and timing of chloroquine exposure (see Critical Parameters). This protocol is suitable for transfection of COS and CV1 cells; Alternate Protocols 1 and 2 describe two examples of transfection experiments.

Materials

- Cells to be transfected and appropriate culture medium (e.g., complete DMEM; UNIT 1.1) with and without 10% FBS
- 100 mM (1000×) chloroquine diphosphate in PBS, filter-sterilized (store at 4°C)
- Plasmid DNA(s), prepared by CsCl density-gradient centrifugation or affinity chromatography
- TE buffer (APPENDIX 2A)

BASIC PROTOCOL

Introduction and
Expression of
Foreign
Macromolecules
in Cells

20.4.1

Contributed by Tod Gulick

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Supplement 19

10 mg/ml DEAE-dextran stock solution (see recipe)
 10% (v/v) dimethyl sulfoxide (DMSO) in PBS, filter-sterilized (store up to 1 month at room temperature)
 Phosphate-buffered saline (PBS; *APPENDIX 2A*)
 Appropriate-sized tissue culture vessels (Table 20.4.1)
 Inverted microscope
 Additional reagents and equipment for mammalian cell culture (*UNIT 1.1*)

1. Plate cells at a density that will achieve 50% to 75% confluence on the target day for transfection. For COS or CV1 cells, perform a 1:10 split 2 days prior to transfection.

The surface area of various cell culture vessels given in Table 20.4.1 can be used to determine how to split cells to the desired density.

Some cell types including many primary cells show particular sensitivity to the toxicity of DEAE-dextran. These cells should be plated at higher density or transfected after reaching near-confluence.

2. Determine the total volume of medium to be used in the transfection based on the number of culture vessels containing cells to be transfected and the volume per vessel shown in Table 20.4.1. Make up this amount of medium (plus some excess) to contain 2.5% FBS by combining 1 part medium containing 10% FBS with 3 parts serum-free medium.

DEAE-dextran can precipitate in the presence of high medium protein, necessitating use of a low FCS concentration. Alternatively, NuSerum (Collaborative Research), which contains only ~30% serum, can be used at a final concentration of 10%.

3. Add 100 mM (1000×) chloroquine diphosphate stock solution to the 2.5%-FBS-containing transfection medium prepared in step 2 to achieve a final concentration of 100 μM. Warm transfection medium to 37°C.

Chloroquine is toxic to all cells, so exposure time should be limited to <4 hr. If longer transfection times are required for optimal transfection of a particular cell type, chloroquine should be added during the final hours of the transfection.

Table 20.4.1 Surface Areas of Commonly Used Tissue Culture Vessels and Corresponding Appropriate DEAE-Dextran Transfection Medium Volumes

Vessel	Area (cm ²)	Appropriate vol. DEAE-dextran medium ^a (ml)
T175 flask	175	
T150 flask	150	
T75 flask	75	
T25 flask	25	
150-mm dish	148 ^b	10
100-mm dish	55 ^b	4
60-mm dish	21 ^b	2
35-mm dish	8 ^b	1
6-well plate (35-mm wells)	9.4 ^b	1
12-well plate (22-mm wells)	3.8 ^b	0.5
24-well plate (15.5-mm wells)	1.9 ^b	0.25

^aThese volumes are roughly a linear function of vessel surface area. To ensure that cells are completely covered by medium during the transfection, small wells require proportionately larger volumes due to annular sequestration of medium because of surface tension at the periphery.

^bCostar; other manufacturer products may deviate slightly.

4. Dilute plasmid DNA in TE buffer or distilled water to between 1.0 and 0.1 $\mu\text{g}/\mu\text{l}$, depending on the quantity to be transfected. Add the DNA solution directly to the warmed transfection medium to a final concentration of 1.0 $\mu\text{g}/\text{ml}$.

DNA solution(s) should comprise <1% of the total volume of transfection medium, so that the concentration of medium components is not significantly altered. Optimal DNA concentration in the transfection medium may have to be determined experimentally.

Maintaining dilute stock DNA solutions for dedicated use in transfections reduces interexperimental variation as well as the time required to set up transfection experiments.

5. Warm the 10 mg/ml DEAE-dextran stock solution to 37°C and mix thoroughly by inversion. Add to the DNA-supplemented transfection medium to a final concentration of 100 $\mu\text{g}/\text{ml}$ DEAE-dextran and mix by inversion.

The order of addition to the transfection medium is critical. Adding plasmid DNA to medium that has already been supplemented with DEAE-dextran can result in precipitation, seen as a rosy white glob. Optimal DEAE-dextran concentration in the transfection may have to be determined experimentally.

6. Aspirate medium from the 50% to 70% confluent cell cultures (see step 1) and replace with the appropriate volume of 37°C DEAE-dextran/DNA-supplemented transfection medium (see Table 20.4.1). Incubate 4 hr.

Uniformity of transfection efficiency may be improved by placing culture vessels on a rocker platform within the incubator during the transfection to ensure even exposure of cells to DEAE-dextran/DNA in the medium and to avoid dessication of cells in the center of the vessel. Optimal transfection time may have to be determined experimentally.

7. Examine cells with an inverted microscope.

Cells may appear granular, some cell nuclei may appear pyknotic, and some cell borders may be somewhat ragged. An efficient DEAE-dextran transfection is usually associated with 25% to 75% cell death.

8. Warm the 10% DMSO/PBS to 37°C. Aspirate the transfection medium, note the volume, and replace with 2 to 3 volumes of 37°C DMSO/PBS. Incubate at room temperature for >2 but <10 min. Aspirate the DMSO/PBS and wash the cell layer with a volume of PBS equal to the amount of DMSO/PBS removed. Aspirate and replace with a standard amount of complete medium containing 10% FBS.

Loss of firm cellular anchorage to the culture vessel may occur. Medium exchange and cell washing should therefore involve careful aspiration and pipetting, perhaps by holding the tip of the pipet against a wall of the culture dish or well. It is sometimes advisable to omit the PBS wash (as in the experiment described in Alternate Protocol 1, step 8) and simply add the complete medium, then change the medium a second time several hours after the DMSO shock when cells have recovered and are more firmly adherent.

9. Continue incubating the cells and analyze at times appropriate to the bioassay or intended purpose of the experiment.

The onset and duration of expression of the transfected gene varies from one cell type to another, and especially with the expression vector used. It is advisable to perform a parallel transfection with a readily assayable reporter gene in the identical vector to assess the temporal features of expression. A reporter that is secreted by the cell into the culture medium, such as human growth hormone or secreted alkaline phosphatase, is ideal for this purpose, since aliquots of medium from a single transfection sample can be collected at serial time points. This parallel transfection can also be used in preliminary experiments to optimize transfection conditions.

**SAMPLE EXPERIMENT: TRANSFECTION TO TEST PROMOTER
FUNCTION**

In this protocol for a typical application of DEAE-dextran transfection, a thyroid hormone–response element in a hormone-responsive gene promoter will be mapped. Thyroid hormone (T_3) modulates transcription by activating the thyroid hormone receptor (TR), a transcription factor that binds to specific response elements in target-gene promoters as a heterodimer with the retinoid X receptor (RXR). Expression of TR, but not RXR, is minimal and limiting in fibroblasts, such that overexpression of TR using an expression vector significantly increases transcriptional response to T_3 in these cells. In this example, four promoter/reporter constructs will be tested, representing a promoter 5' deletion series. The hormones, retinoids, and fatty acids in fetal bovine serum (FBS) can interfere with or cause high background in transcription assays in transfected cells. These moieties can be removed from FBS by charcoal treatment (see Support Protocol).

This protocol corresponds step-for-step with Basic Protocol; variations from the original procedure and reagents specific to this particular experiment are noted.

Additional Materials (also see Basic Protocol)

- CV-1 cells (ATCC #CCL 70) growing in 100-mm dish
- Complete DMEM medium (UNIT 1.2) with and without 10% FBS
- Complete DMEM medium (UNIT 1.2) with 10% charcoal-treated FBS (see Support Protocol)
- Plasmid DNAs:
 - Control reporter plasmid (e.g., β -galactosidase, secreted alkaline phosphatase, or growth hormone, driven by a viral promoter)
 - Four test promoter constructs (promoter/CAT or promoter/luciferase)
 - Expression plasmid with TR gene insert (pTR)
 - No-insert expression plasmid (p[–])
- Complete DMEM medium (UNIT 1.2) with 10% charcoal-treated FBS (see Support Protocol), supplemented with 10 nm thyroid hormone (T_3)
- 12-well tissue culture plates
- 100-ml tissue culture dishes
- Additional reagents and equipment for trypsinizing and subculturing monolayer cells (UNIT 1.1) and analyzing reporter gene activity (APPENDIX 3A)

1. Two days prior to the transfection, trypsinize and suspend CV1 cells from a confluent 100-mm dish in 36 ml complete DMEM medium/10% FBS. Place 1 ml complete DMEM/10% FBS in each well of four 12-well plates, then add 250 μ l of the cell suspension to each of these wells. Plate the residual cells in 100-mm dishes for later use, or discard. 12 to 24 hr before the transfection, change medium to complete DMEM/10% charcoal-treated FBS.

The sensitivity of most reporter assays permits use of many fewer cells/transfection conditions than are generally used, with consequent cost savings. CV1 cells in a well of a 12-well plate will provide sufficient reporter-gene activity for most promoter/reporters. In this protocol, triplicate wells for each condition will be analyzed. Four promoter/CAT or promoter/luciferase reporters will be tested. Cells will include or exclude pTR cotransfection. Transfected cells will be incubated in the presence or absence of T_3 . Thus, four 12-well plates are required for the experiment—i.e., 3 wells (triplicates) \times 4 wells (four reporters) \times 2 wells (with and without pTR cotransfection) \times 2 wells (with and without T_3) = 48 wells. These 48 wells include \sim 180 cm^2 (see Table 20.4.1) such that approximately one-third of the cells on one 100-mm (55 cm^2) dish will be used in order to achieve a 1:10 split.

Addition of suspended cells to empty tissue culture plates or wells results in an uneven distribution of adherent cells, which can introduce undesirable intersample variability; hence medium is added to the wells first.

Preincubation in medium supplemented with charcoal-stripped FBS (see Support Protocol) ensures that any hormones of interest are absent from medium bathing the control cells during subsequent experiments.

2. Add 7 ml DMEM/10% charcoal-treated FBS to 21 ml serum-free DMEM to make 28 ml of DMEM/2.5 % FBS.

In this experiment, the FBS in the DMEM/10 has been stripped of low molecular weight hydrophobic moieties, including T_3 , using charcoal.

The amount here was calculated as 48 wells multiplied by 0.5 ml/well, and a small excess was added. In experiments in which the transfection medium will be divided into multiple aliquots carrying different plasmids or plasmid combinations, it is useful to carry a volume excess throughout the preparation of the separate transfection media to adjust for pipet-calibration errors.

3. Add 28 μ l 100 mM chloroquine diphosphate to the medium and place the tube in a 37°C water bath.
4. Dilute the reporter plasmids (control and test promoter constructs) in TE buffer to 1 μ g/ μ l; dilute the TR (pTR) and no-insert (p[-]) expression plasmids in TE buffer to 0.2 μ g/ μ l. Add 14 μ l of the diluted reporter plasmid to the transfection medium (final concentration, 0.5 μ g/ml). Divide this medium into four equal 6.6-ml aliquots and add 6.6 μ l of a test promoter construct to each separate tube (final concentration, 1 μ g/ml). Divide each of these into two tubes, each containing 3.2 ml. Add 8 μ l pTR to one set of four tubes and 8 μ l p[-] to the other set of four tubes (final concentration, 0.5 μ g/ μ l). Use each of these eight transfection medium samples to transfect cells within six wells in step 6, below.

All cells will be transfected with a control reporter—e.g., β -galactosidase, secreted alkaline phosphatase, or growth hormone—driven by a viral promoter. Each of the four test promoter/reporters will be transfected into cells within 12 wells. A TR expression vector will be transfected into 6 of each of these sets of 12 wells. Cells in triplicate wells in each of these conditions will be cultured in medium supplemented with T_3 , while those in the other triplicate wells will be cultured in medium devoid of T_3 .

Plasmids that are included in more than one transfection condition are added prior to division of medium into separate aliquots to ensure that these samples receive equivalent amounts of plasmid DNA. The final concentration of DNA in each transfection medium sample should be equivalent. For this and other reasons, an “empty vector” should be used as described for p[-] above.

5. Warm the stock DEAE-dextran to 37°C, mix by inversion, and add 32 μ l to each of the eight tubes containing DNA-supplemented transfection medium (final DEAE-dextran concentration, 100 μ g/ml). Mix by gentle inversion.
6. Aspirate medium from six wells on one plate, and replace with one of the eight transfection-medium samples prepared in step 4 at 500 μ l per well. Repeat for each of the different transfection-medium samples. Incubate 4 hr.
7. Examine cells with inverted microscope.
8. Aspirate medium from wells in one plate and add 1 ml DMSO/PBS per well. Repeat for each plate. Return to the first plate, aspirate the DMSO/PBS and replace with 1 ml/well complete DMEM/10% charcoal-treated FBS. Incubate cells for 4 to 12 hr. Aspirate medium from wells and replace the medium from one set of triplicate wells for each transfection condition with 1 ml/well T_3 -supplemented DMEM/10% char-

coal-treated FBS and the other set of triplicate wells for each transfection condition with (T_3 -unsupplemented) DMEM/10% charcoal-treated FBS.

9. Incubate cells 24 to 48 hr. Aspirate 500 μ l of medium from each separate well and save for control reporter activity determinations in order to normalize test reporter activities. Wash wells with PBS and harvest cells for CAT or luciferase activity measurements.

Reporter activity is used to confirm and quantitate promoter T_3 responsiveness ($+T_3$ / $-T_3$), to verify a direct transcriptional response mediated by the TR (augmentation of response to T_3 in pTR-cotransfected cells), and to map the T_3 -responsive region of the promoter (region present in a T_3 -responsive promoter/reporter and absent in an unresponsive one).

The use of a control reporter permits normalization for transfection efficiency and for nonspecific-stimulus effects on gene expression. In many cases, when using this transfection technique, adequate and informative preliminary experiments can be conducted without this control (since transfection efficiency is so uniform), thereby saving time and expense. This is seldom possible when using other transient-transfection techniques, especially calcium phosphate coprecipitation (UNIT 20.3), where transfection efficiency varies markedly within an experiment. Of course, initial experiments should be performed to exclude nonspecific stimulus effects.

ALTERNATE PROTOCOL 2

SAMPLE EXPERIMENT: TRANSFECTION TO TEST ENZYME STRUCTURE/ACTIVITY RELATIONSHIPS

In this experimental application of DEAE-dextran transfection, an enzyme and several enzyme mutants are overexpressed in COS cells to provide material for kinetic analyses in a structure/activity analysis. A vector designed for high-level expression that replicates in SV40-transformed cells (e.g., CDM8) will be used.

This protocol corresponds step-for-step with Basic Protocol; variations from the original procedure and reagents specific to this particular experiment are noted.

Additional Materials (also see Basic Protocol)

- COS cells (ATCC #1650) growing in 100-mm dish
- Complete DMEM medium (UNIT 1.2) with and without 10% FBS
- Control plasmid containing reporter gene (e.g., luciferase, CAT, or secreted alkaline phosphatase)
- CDM8 vectors containing gene for wild-type enzyme and genes for four mutant enzymes
- 100-ml tissue culture dishes
- Additional reagents and equipment for analyzing reporter gene activity (APPENDIX 3A) and analysis of recombinant proteins (APPENDIX 3A)

1. Two days prior to the transfection, split five 100-mm dishes of confluent COS cells into fifty 100-mm dishes.

Ten 100-mm dishes of COS cells will provide sufficient recombinant enzyme activity for kinetic analyses. Recombinant wild-type enzyme and four mutant enzymes will be overexpressed. Thus, fifty dishes are required for this experiment.

2. Add 50 ml DMEM/10% FBS to 150 ml serum-free DMEM to make 200 ml DMEM/2.5% FBS.

This was calculated as fifty dishes at 4 ml/100-mm dish (see Table 20.4.1).

3. Warm the transfection medium in a 37°C water bath. Do not add chloroquine diphosphate.

Transfection Using DEAE-Dextran

20.4.6

Chloroquine treatment increases DEAE-dextran transfection efficiency, but may reduce the amount of recombinant protein produced by the transfected cells. It is advisable to test this in early pilot experiments.

4. Dilute the reporter gene plasmid and each of the CDM8/enzyme expression vectors to 1 µg/µl in TE buffer. Add 20 µl reporter plasmid to the transfection medium and mix (final concentration, 0.1 µg/ml). Divide the medium into five aliquots of 40 ml. Add 160 µl of one CDM8/enzyme expression plasmid to one aliquot (final concentration, 4 µg/ml) and repeat this for each of the CDM8/enzyme expression plasmids.

All cells will be transfected with a control plasmid containing a reporter gene such as luciferase, CAT, or secreted alkaline phosphatase to evaluate transfection efficiency. Each of the five enzyme expression vectors will be used to transfect cells in ten dishes.

If the COS cells have an endogenous activity identical or similar to the activity of the recombinant protein to be overexpressed, it may be prudent to include cells that are transfected with “empty vector” in experiments of this type to permit parallel assays of endogenous COS cell activities for “background” subtraction.

Plasmids may compete for replication and/or transcription factors. The control reporter plasmid can be included at low concentration because the reporter has a very sensitive assay.

5. Warm the stock DEAE-dextran to 37°C, mix by inversion, and add 800 µl to each of the tubes containing DNA-supplemented transfection medium (final DEAE-dextran concentration, 200 µg/ml). Mix by gentle inversion.
6. Aspirate medium from ten dishes and replace with 4 ml of the appropriate DEAE-dextran/DNA-supplemented transfection medium. Repeat for each set of ten dishes. Incubate 3 hr.
7. Examine cells with inverted microscope. Continue the transfection until some cells appear slightly granular.
8. Aspirate transfection medium from one of the sets of ten dishes and replace with 10 ml/dish DMSO/PBS. After 2 min, aspirate the DMSO/PBS and wash gently with 10 ml PBS. Aspirate PBS and add 10 ml DMEM/10 FBS. Repeat for each set of ten dishes.
9. Incubate cells 48 to 96 hr. Harvest cells as appropriate for recombinant protein assays. Save aliquots of culture medium (for secreted control reporter) or cell extract (intracellular reporter) to determined transfection efficiency.

Normalization for transfection efficiency may not be necessary, particularly in circumstances where an independent assay for recombinant protein production is available, as might be provided by a specific antibody.

CHARCOAL STRIPPING OF FETAL BOVINE SERUM

Activated charcoal is used to remove low-molecular-weight lipophilic compounds from serum including hormones, retinoids, and fatty acid ligands of nuclear receptor transcription factors.

Materials

Fetal bovine serum (FBS), heat-inactivated (*UNIT 1.1*)

Activated charcoal, acid-washed (Sigma)

Ultracentrifuge with Beckman SW 28, JA-20.1, or equivalent swinging-bucket rotor

SUPPORT PROTOCOL

Introduction and Expression of Foreign Macromolecules in Cells

20.4.7

1. Add 2 g activated charcoal per 100 ml heat-inactivated FBS. Add a stir-bar and place on a stir plate in a 4°C cold room or refrigerator. Stir for 2 hr.

Although it is difficult to maintain aseptic conditions during charcoal stripping, care should be taken to avoid flagrant contamination and to keep the serum at 4°C.

2. Collect serum in centrifuge tubes and centrifuge 30 min at $72,000 \times g$ (20,000 rpm in SW 28 rotor) or 60 min at $51,500 \times g$ (20,000 rpm in JA-20.1), 4°C.
3. Gently pour off serum from each centrifuge tube into a sterile beaker, divide into aliquots in sterile conical tubes, and store frozen at -20°C .

Some residual charcoal may be present but the serum should be only lightly peppered with charcoal after centrifugation.

4. Prior to use, thaw a tube of charcoal-stripped medium and use immediately to supplement complete medium. Filter sterilize the medium using a 0.22- μm bottle-top filter and collect in a sterile bottle. Store at 4°C.

The serum is filtered after it is added to the medium because the undiluted serum is too viscous to filter readily.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

DEAE-dextran stock solution, 10 mg/ml (100 \times)

Make a 10 mg/ml stock solution of diethylaminoethyl (DEAE)-dextran (mol. wt. \sim 500,000 Da; Sigma) in tissue culture-quality PBS (APPENDIX 2A). Mix well, filter sterilize using an 0.22- μm filter, mix again, divide into aliquots, and store up to 3 months at 4°C.

Warm to 37°C and mix well by inversion immediately before each use.

COMMENTARY

Background Information

The mechanism by which cells take up DNA in DEAE-dextran-mediated transfection appears to involve endocytosis after adsorption of DNA/DEAE-dextran complexes onto cells. The advantages of the technique relate largely to the relative simplicity, limited expense, and lack of interexperiment and intraexperiment variability in transfection efficiency.

Critical Parameters

There are several crucial parameters in the transfection procedure, and the weight of importance of each differs for different cell types. Furthermore, maximizing efficiency of a transfection (percent of cells transfected) does not necessarily correspond with optimizing the desired goal of the transfection, such as production of recombinant protein (Kluxen and Lubbert, 1993) or maintenance of colony-forming potential of cells containing transfected DNA (Puchalski and Fahl, 1992). Systematic analyses of variables in DEAE-dextran/DNA trans-

fection for particular cell types have been reported (Fregeau and Bleackley, 1991; Puchalski and Fahl, 1992; Kluxen and Lubbert, 1993; Yang and Yang, 1997), and these studies provide useful information. However, the existence of numerous variables and their mutual codependence makes interpolation, extrapolation, or guessing transfection conditions toward any specific end for any particular cell type difficult to extract from literature reports. Thus, the investigator should carry out a modest set of pilot experiments to optimize conditions.

The major variables that influence DEAE-dextran/DNA transfection include: (1) DEAE-dextran concentration; (2) DNA concentration and the ratio of DNA concentration to DEAE-dextran concentration; (3) duration of transfection; (4) use of chloroquine; (5) use of permeabilizing agents; and (6) serum concentration. The influence of each of these factors on the various goals of DNA transfection will be addressed.

The concentration of DEAE-dextran used in transfections varies from 50 to 500 $\mu\text{g/ml}$. There is an inverse relationship between the concentration used and the duration of exposure prior to onset of cytotoxicity for all cell types. In initial experiments, it may be best to use a low concentration (100 or 200 $\mu\text{g/ml}$) to provide an adequate temporal window to evaluate cytotoxicity microscopically. Lethal cytotoxicity can precede microscopic evidence of the same, such that transfections with high concentrations of DEAE-dextran can result in near-complete cell loss even when most cells appear healthy at the end of the transfection period.

The DNA concentration used depends in part on the vector, cell type, and the purpose of the transfection. For maximal cell adsorption of complexes and maximal transfection efficiency, the ratio of transfection-medium DEAE-dextran concentration to DNA concentration should be 40:1 to 50:1, perhaps because of the existence of an optimal electrostatic cell membrane/DEAE-dextran/DNA-complex interaction (Yang and Yang, 1997). Thus, at a DEAE-dextran concentration of 200 $\mu\text{g/ml}$, plasmid DNA should be included at 4 to 5 $\mu\text{g/ml}$. Since this ratio influences transfection efficiency, "empty vector" should be included in controls at the same concentration as test vectors. This ideal [DEAE-dextran]/[DNA] ratio may depend on transfection-medium serum concentration.

The amounts of DNA to be used in cotransfection experiments again depend on the intended purpose of each component transfection. When a control reporter plasmid is used to simply normalize for transfection efficiency, it is often possible and desirable to include a low concentration of the plasmid, since reporter enzyme assays are generally exquisitely sensitive. In experiments in which simultaneous cotransfection of single cells with two plasmids is a goal, it is appropriate to add the plasmids at the same or similar concentrations. Furthermore, transfection efficiency is of paramount importance in this circumstance, since the percentage of cells that take up both plasmids is a fraction of those that take up each alone. This is particularly important in a case like that presented in Alternate Protocol 1, where coexistence of the transcription-factor expression vector and the promoter/reporter plasmid in a single cell is important.

DEAE-dextran-mediated cytotoxicity is a function of exposure time. In general, efficient transfections can be achieved with a 4-hr incu-

bation if other parameters are adjusted appropriately. This is a convenient transfection duration since it corresponds to the maximal allowable period of cell exposure to chloroquine. Shorter transfection times may be appropriate for some cell types.

The utility of chloroquine in increasing the efficiency of DEAE-dextran transfection is well documented. However, there may be an attenuation in the amount of transfected gene expression (Kluxen and Lubbert, 1993) and increased cell loss and disruption of cellular morphology (Puchalski and Fahl, 1992) when this reagent is included. Thus, use of chloroquine is appropriate for purposes where transfection efficiency is a dominant priority. In other cases, preliminary experiments should evaluate the impact of this agent on the bioactivity desired. While some investigators add chloroquine in the final stage of (or after) a longer transfection, it is generally easier to increase the DEAE-dextran concentration in this circumstance to permit a single 4-hr transfection in medium containing all components.

Using a final cell "shock" with a permeabilizing agent markedly increases DEAE-dextran transfection efficiency without additional cytotoxicity (Lopata et al., 1984) and should be used universally, unless this manipulation somehow interferes with the desired bioactivity. DMSO at a concentration of 10% is generally used, although 15% glycerol may be more effective for some cell types. Enhancement of transfection efficiency increases as a function of permeabilizing-agent exposure time up to 2 min, after which there is no additional impact (Sussman and Milman, 1984). Since longer exposure, up to 5 to 10 min, produces no negative effect, the "shock" should be for >2 min, but need not be rigorously timed or reproduced, even between transfected samples within an experiment.

Use of transfection medium supplemented with 10% FBS results in formation of macroscopic protein/DEAE-dextran/DNA complexes that are not compatible with efficient transfection. Serum-free medium can be used during the transfection, as can medium supplemented with either 2.5% FBS or 10% NuSerum. The concentration of serum in the transfection medium affects the extent of DEAE-dextran-mediated and chloroquine-mediated cytotoxicity, with a protective effect provided by the serum. FBS concentration may also influence the optimal ratio of [DEAE-dextran]/[DNA].

Anticipated Results

The efficiency of DEAE-dextran-mediated transfection varies considerably among cell types. One can expect to achieve 20% to 60% transfection efficiency with many cells using this procedure if proper attention is paid to optimization of transfection conditions. Following transfection using this technique, there are generally significant morphological changes in cells, and some of these may weigh against the use of this technique for certain purposes.

Time Considerations

Splitting cells into the required number of plates several days prior to the transfection may take ~30 min. Depending on the complexity of the transfection, preparing transfection media may take 5 min to 1 hr. If stock solutions of DNA, DEAE-dextran, chloroquine, and DMSO/PBS are prepared in advance, 30 min should be adequate for this phase, even in a moderately complex transfection experiment like that presented in Alternate Protocol 1. During the subsequent transfection, cells should be monitored using a microscope periodically after 3 hr. The DMSO or glycerol shock, cell wash, and medium replacement requires only ~10 min. Thus, with minimal but careful planning of experimental design, a transfection can readily be completed in 5 hr.

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Transfection by Electroporation

UNIT 20.5

Electroporation—the use of high-voltage electric shocks to introduce DNA into cells—is a procedure that is gaining in popularity. It can be used with most cell types, yields a high frequency of both stable transformation and transient gene expression, and, because it requires fewer steps, can be easier than alternate techniques (UNITS 20.3, 20.4 & 20.6).

The Basic Protocol describes the electroporation of mammalian cells. The alternate protocol outlines modifications for preparation and transfection of plant protoplasts.

ELECTROPORATION INTO MAMMALIAN CELLS

**BASIC
PROTOCOL**

Electroporation can be used for both transient and stable transfection of mammalian cells. Cells are placed in suspension in an appropriate electroporation buffer and put into an electroporation cuvette. DNA is added, the cuvette is connected to a power supply, and the cells are subjected to a high-voltage electrical pulse of defined magnitude and length. The cells are then allowed to recover briefly before they are placed in normal growth medium. Factors that can be varied to optimize electroporation effectiveness are discussed in UNIT 20.7.

Materials

- Mammalian cells to be transfected
- Complete medium (UNIT 1.2) without and with appropriate selective agents (APPENDIX 3A)
- Electroporation buffer, ice-cold (see recipe)
- Linear or supercoiled, purified DNA preparation (see step 7)
- Beckman JS-4.2 rotor or equivalent
- Electroporation cuvettes (Bio-Rad #165-2088) and power source
- Additional reagents and equipment for stable transformation in selective medium and for harvesting transfected cells (APPENDIX 3A)

Prepare the cells for electroporation

1. Grow cells to be transfected to late-log phase in complete medium.

Each permanent transfection will usually require 5×10^6 cells to yield a reasonable number of transfectants. Each transient expression may require $1-4 \times 10^7$ cells, depending on the promoter.

2. Harvest cells by centrifuging 5 min at $640 \times g$ (1500 rpm in a JS-4.2 rotor), 4°C .

Adherent cells are first trypsinized (UNIT 1.1) and the trypsin inactivated with serum.

3. Resuspend cell pellet in half its original volume of ice-cold electroporation buffer.

The choice of electroporation buffer may depend on the cell line used. See Critical Parameters for a complete discussion.

4. Harvest cells by centrifuging 5 min as in step 2.

5. Resuspend cells at $1 \times 10^7/\text{ml}$ in electroporation buffer at 0°C for permanent transfection. Higher concentrations of cells (up to 8×10^7) may be used for transient expression.

6. Transfer 0.5-ml aliquots of the cell suspension into desired number of electroporation cuvettes set on ice.

**Introduction and
Expression of
Foreign
Macromolecules
into Cells**

Contributed by **Huntington Potter**

Current Protocols in Cell Biology (2003) 20.5.1-20.5.6

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20.5.1

Supplement 19

Add DNA and electroporate the cells

7. Add DNA to cell suspension in the cuvettes on ice.

For stable transformation, DNA should be linearized by cleavage with a restriction enzyme that cuts in a nonessential region and purified by phenol extraction and ethanol precipitation. For transient expression, the DNA may be left supercoiled. In either case, the DNA should have been purified through two preparative CsCl/ethidium bromide equilibrium gradients followed by phenol extraction and ethanol precipitation. The DNA stock may be sterilized by one ether extraction; the (top) ether phase is removed and the DNA solution allowed to dry for a few minutes to evaporate any remaining ether. See APPENDIX 3A for cross-references to these procedures.

For transient expression, 10 to 40 μg is optimal. For stable transformation, 1 to 10 μg is sufficient. Cotransfection, although not recommended because of the work required to select and test transformants, can be done with 1 μg of a selectable marker containing DNA and 10 μg of the DNA containing the gene of interest.

8. Mix DNA/cell suspension by holding the cuvette on the two “window sides” and flicking the bottom. Incubate 5 min on ice.
9. Place cuvette in the holder in the electroporation apparatus (at room temperature) and shock one or more times at the desired voltage and capacitance settings.

The number of shocks and the voltage and capacitance settings will vary depending on the cell type and should be optimized (Critical Parameters; see also UNIT 20.7).

10. After electroporation, return cuvette containing cells and DNA to ice for 10 min.

Culture and harvest the transfected cells

11. Dilute transfected cells 20-fold in nonselective complete medium and rinse cuvette with this same medium to remove all transfected cells.

- 12a. *For stable transformation:* Grow cells 48 hr (about two generations) in nonselective medium, then transfer to antibiotic-containing medium.

Selection conditions will vary with cell type. For example, neo selection generally requires ~400 $\mu\text{g}/\text{ml}$ G418 in the medium. XGPRT selection requires 1 $\mu\text{g}/\text{ml}$ mycophenolic acid, 250 $\mu\text{g}/\text{ml}$ xanthine, and 15 $\mu\text{g}/\text{ml}$ hypoxanthine in the medium.

It is often convenient to plate adherent cells at limiting dilution immediately following the shock, or suspension cells at the time of antibiotic addition.

- 12b. *For transient expression:* Incubate cells 50 to 60 hr, then harvest cells for transient expression assays.

Transfected cells can be visualized by standard transient expression assays.

ALTERNATE PROTOCOL

ELECTROPORATION INTO PLANT PROTOPLASTS

This is a modification of the Basic Protocol that is intended for use with plant cells. Plant cells are stripped of their cell walls and DNA is introduced into the resulting protoplasts.

Additional Materials (also see Basic Protocol)

5-mm strips (1 g dry weight) sterile plant material
Protoplast solution (see recipe)
Plant electroporation buffer (see recipe)

80- μm -mesh nylon screen
Sterile 15-ml conical centrifuge tube

Additional reagents and equipment for plant RNA preparation (APPENDIX 3A)

1. Obtain protoplasts from carefully sliced 5-mm strips of sterile plant material by incubating in 8 ml protoplast solution for 3 to 6 hr at 30°C on a rotary shaker.
2. Remove debris by filtration through an 80- μ m-mesh nylon screen.
3. Rinse screen with 4 ml plant electroporation buffer. Combine protoplasts in a sterile 15-ml conical centrifuge tube.
4. Centrifuge 5 min at $300 \times g$ (1000 rpm in a JS-4.2 rotor). Discard supernatant, add 5 ml plant electroporation buffer, and repeat wash step. Resuspend in plant electroporation buffer at $1.5\text{--}2 \times 10^6$ protoplasts/ml.

Protoplasts can be counted with a hemacytometer (UNIT 1.1).

5. Carry out electroporation as described for mammalian cells (steps 6 to 11 of the Basic Protocol). Use one or several shocks at 1 to 2 kV with a 3- to 25- μ F capacitance as a starting point for optimizing the system.

Alternatively, use 200 to 300 V with 500 to 1000 μ F capacitance if the phosphate in the electroporation buffer is reduced to 10 mM final.

6. Harvest cells after 48 hr growth and isolate RNA, assay for transient gene expression, or select for stable transformants.

Protoplasts can also be selected and grown into full transgenic plants (Rhodes et al., 1988).

REAGENTS AND SOLUTIONS

Use deionized distilled water for all recipes and protocol steps. For common solutions, see APPENDIX 2A; for suppliers see SUPPLIERS APPENDIX.

Electroporation buffers

Choice of electroporation buffer depends on the cells being used in the experiment (see Critical Parameters). The following buffers (stored at 4°C) can be used:

1. PBS (APPENDIX 2A) without Ca^{2+} or Mg^{2+}
2. HEPES-buffered saline (HeBS; UNIT 20.3)
3. Tissue culture medium without FBS (UNIT 1.1)
4. Phosphate-buffered sucrose: 272 mM sucrose/7 mM K_2HPO_4 (adjusted to pH 7.4 with phosphoric acid)/1 mM MgCl_2

Plant electroporation buffer

Prepare in PBS (APPENDIX 2A):

0.4 M mannitol

5 mM CaCl_2

Store at 4°C

Protoplast solution

2% (w/v) cellulase (Yakult Honsha)

1% (w/v) macerozyme (Yakult Honsha)

0.01% (w/v) pectylase

0.4 M mannitol

40 mM CaCl_2

10 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), pH 5.5

Prepare fresh before use

COMMENTARY

Background Information

DNA transfection by electroporation is a technique that is applicable to perhaps all cell types. It yields a high frequency of stable transformants and has a high efficiency of transient gene expression. Electroporation makes use of the fact that the cell membrane acts as an electrical capacitor that is unable to pass current (except through ion channels). Subjecting membranes to a high-voltage electric field results in their temporary breakdown and the formation of pores that are large enough to allow macromolecules (as well as smaller molecules such as ATP) to enter or leave the cell. The reclosing of the membrane pores is a natural decay process that is delayed at 0°C.

During the time that the pores are open, nucleic acid can enter the cell and ultimately the nucleus. Linear DNA with free ends is more recombinogenic and more likely to be integrated into the host chromosome to yield stable transformants. Supercoiled DNA is more easily packaged into chromatin and is generally more effective for transient gene expression.

The use of high-voltage electric shocks to introduce DNA into cells was first performed by Wong and Neumann using fibroblasts (Neumann et al., 1982; Wong and Neumann, 1982). The technique was then generalized (Potter et al., 1984) to all cell types—even those such as lymphocytes that, unlike fibroblasts, cannot be transfected with other procedures (e.g., calcium phosphate or DEAE-dextran DNA coprecipitates). Although whole plants or leaf tissue have been reported to be transfectable by electroporation, plant cells must generally be made into protoplasts before DNA can be easily introduced into them (Alternate Protocol; Fromm et al., 1985; Ou-Lee et al., 1986). Like mammalian cells, plant protoplasts may be electroporated under a variety of electrical conditions (Critical Parameters). Both high voltage with low capacitance (short pulse duration) or low voltage with high capacitance (long pulse duration) have been used to achieve successful gene transfer (Chu et al., 1987).

The wide use of electroporation has been made possible in large part by the availability of commercial apparatuses that are safe and easy to use and that give extremely reproducible results. Designs of these machines vary substantially, but fall into two basic categories that use different means of controlling pulse duration and voltage (the two electrical parameters that govern pore formation). One kind

uses a capacitor discharge system to generate an exponentially decaying current pulse, and the other generates a true square wave (or an approximation thereof). The capacitor discharge instruments charge their internal capacitor to a certain voltage and then discharge it through the cell-DNA suspension. Both the size of the capacitor and the voltage can be varied. Because the current pulse is an exponentially decaying function of (1) the initial voltage, (2) the capacitance setting of the instrument, and (3) the resistance of the circuit (including the sample), changing the capacitor size to allow more (or less) charge to be stored at the voltage will result in longer (or shorter) decay times and hence a different effective pulse duration. In contrast, square wave generators control both the voltage and pulse duration with solid-state switching devices. They also can produce rapidly repeating pulses.

Most of our electroporation experiments have used the Bio-Rad Gene Pulser, a capacitor discharge device, but are directly applicable to other capacitor discharge devices, and with some adjustment to square wave generators. Capacitor discharge devices are also available from Life Technologies, BTX, Hoefer Scientific, and International Biotechnologies (see *SUPPLIERS APPENDIX* for suppliers' addresses). These machines, either in a single unit or through add-on components, can deliver a variety of electroporation conditions suitable for most applications. Square wave generators are available from BTX or Baekon and offer great control over pulse width, allow multiple, rapid pulses, and can be more effective for cells that are very sensitive or otherwise difficult to transfect. These machines are generally more expensive. It has become apparent that alternating current pulses at ~100 kHz may be the most effective wave form for electroporation and possibly electrofusion (Chang, 1989). However, dedicated electroporation devices utilizing such waves are not yet commercially available and must be constructed from components. For a complete discussion of electroporation instruments, see Potter (1988).

Electroporation can be easier to carry out than alternative techniques, which is why it is becoming popular. Its drawback for use with transient analysis is that almost five-fold more cells and DNA are needed than with either calcium phosphate- or DEAE-dextran-mediated transfection (*UNITS 20.3 & 20.4*). The main difference between electroporation and cal-

cium phosphate coprecipitation procedures is the state of the integrated DNA after selection in appropriate antibiotic media. In the case of calcium phosphate, the amount of DNA taken up and integrated into the genome of each transfected cell is in the range of 3×10^6 bp. As a result, the transfected DNA often integrates as large tandem arrays containing many copies of the transfected DNA. This would be an advantage when transfection of genomic DNA into recipient cells and selection for some phenotypic change such as malignant transformation is desired; here a large amount of DNA integrated per recipient cell is essential. In contrast, electroporation can be adjusted to result in one to many copies of inserted DNA per recipient cell. This would be an advantage for gene expression studies, as the identity of the particular copy responsible for the gene expression can be controlled.

Critical Parameters

As discussed above, the two parameters that are critical for successful electroporation are the maximum voltage of the shock and the duration of the current pulse (see also *UNIT 20.7*). The voltage and capacitance settings must be optimized for each cell type, with the resistance of the electroporation buffer being critical for choosing the initial instrument settings. The guidelines presented in this unit are meant to be adapted according to the manufacturers' instructions and the individual investigator's needs. Optimal stable and transient transformations occur at about the same instrument settings, so transient expression can be used to optimize conditions for a new cell type.

For low-resistance (high-salt) buffers such as PBS, HeBS, or tissue culture medium, start with a capacitor setting of 25 μ F and a voltage of 1200 V for 0.4-cm cuvettes, then increase or decrease the voltage until optimal transfection is obtained (usually at ~40% to 70% cell viability as detected by trypan blue exclusion; *UNIT 1.1*). For many cell types, the choice between PBS, HeBS, and tissue culture medium is arbitrary. However, some cells (especially primary cells) are very easily killed and thus electroporate poorly at the high voltages needed for PBS or HeBS electroporation buffers. Particularly sensitive cells seem to prefer tissue culture medium, though it has been shown that the calcium and magnesium ions in the medium lower the electroporation efficiency (Neumann et al., 1982). Phosphate-buffered sucrose has the advantage that it can be optimized at voltages several hundred volts below those used

with PBS or HeBS. Alternatively, Chu et al. (1987) found many sensitive cells were electroporated more effectively in HeBS with a low voltage/high capacitance setting that resulted in at least 10-fold longer pulse duration. For these conditions, start at 250 V/960 μ F and change the voltage up to 350 V or down to 100 V in steps to determine optimal settings.

Keeping cells on ice (at 0°C) often improves cell viability and thus results in higher effective transfection frequency, especially at high power which can lead to heating (Potter et al., 1984). However, Chu et al. (1987) found that under low voltage/high capacitance conditions, some cell lines electroporate with higher efficiency at room temperature. Therefore, steps 6 to 10 of the Basic Protocol should be carried out separately at both temperatures to determine the optimum conditions for a new cell line.

Another factor contributing to cell death appears to be the pH change that results from electrolysis at the electrodes. This problem can be reduced by replacing some of the ionic strength of the PBS with extra buffer (e.g., 20 mM HEPES, pH 7.5).

Optimal parameters for plant electroporation differ depending on whether tissue culture cells or various parts of the whole plant are used as a source of protoplasts. In particular, the high salt in PBS can be damaging to protoplasts freshly produced from plant tissue. Replacing the NaCl in PBS with 135 mM LiCl may increase CAT transient gene expression in electroporated plant protoplasts 4- to 70-fold (Saunders et al., 1989). Alternatively, an electroporation buffer of 0.6 M mannitol/25 mM KCl for leaf cells, or 0.7 M mannitol/40 mM KCl/4 mM MES (pH 5.7)/1 mM 2-ME added for root and stem cells, is recommended (Sheen, 1990). In addition, 0.1% BSA/15 mM 2-ME/1 mM MgCl₂ can be added to either protoplast isolation buffer and the CaCl₂ reduced to 1 mM final. A low salt concentration in the electroporation buffer reduces the optimal capacitance setting to 200 μ F.

Anticipated Results

The efficiency of transfection by electroporation is dependent upon cell type. For fibroblasts, which are easily transfected by calcium phosphate or DEAE-dextran coprecipitation (*UNITS 20.3 & 20.4*), electroporation gives a stable transformation frequency of 1 in $\sim 10^3$ to 10^4 live cells—approximately that obtainable by the above traditional procedures. For cells refractory to traditional methods, electropora-

tion gives a stable transformation frequency between 1 in 10^4 to 10^5 for most cell types. Occasionally a cell line (e.g., some T lymphocytes) will transfect poorly under our standard conditions (1 in 10^6), and even this frequency is sufficient to obtain significant numbers of transfectants. In general, cells that transfect efficiently for stable transformants also do so for transient gene expression. Increasing the number of cells and the amount of DNA used in the electroporation for studying transient gene expression can circumvent problems of low transfection efficiency and low promoter/enhancer efficiency.

For plant protoplast electroporation, the frequency of stable transformants is between 1 in 10^2 and 1 in 10^3 dividing cells.

Time Considerations

The entire process of electroporation of mammalian cells will take <1 hr. Electroporation of plant cells requires ≤ 6 hr to prepare the protoplasts and <1 hr for the actual electroporation process. As with other transfection procedures, the experiment should be planned to allow for harvest or splitting of the cells 1 to 2 days after transfection.

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Key Reference

Potter et al., 1984. See above.

The original paper from which the Basic Protocol is adapted.

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Transfection of Cultured Eukaryotic Cells Using Cationic Lipid Reagents

The development of high-efficiency methods for the introduction of functional genetic material into eukaryotic cells using cationic lipid-based transfection reagents has accelerated biology research in the studies of gene expression, control of cell growth, and cell lineage. Cationic lipid-mediated transfection techniques are commonly used in industrial protein production as well as in some clinical gene therapy protocols.

Most natural lipids are either neutral or negatively charged (anionic). Positively charged (cationic) lipids were first introduced in 1987 (Felgner et al., 1987). The cationic lipids function by spontaneous electrostatic interaction of their positive charges with the negative charges in the backbone of DNA, RNA, or oligonucleotides, condensing the extended macromolecules to a compact structure. The cationic charges and the lipophilic nature of the cationic lipids then allow the condensed aggregates to interact with and cross the negatively charged and hydrophobic cell membrane and enter the target cells. It is also possible to deliver some proteins into cells using cationic lipids (Sells et al., 1995). Most (but not all) cationic lipid reagents used for transfection consist of mixtures of cationic and neutral lipids (e.g., DOPE, cholesterol) that are formulated in water to yield noncovalent structures called liposomes—hollow spheres with aqueous cores and a diameter of 100 to 400 nm (Felgner et al., 1987). Some are dissolved in ethanol and form micelles (Behr et al., 1989). There are many cationic lipid reagents available (see Background Information and Table 20.6.1). They perform with various efficiencies in different applications and target cells.

This unit describes cationic lipid-mediated transfection of a variety of cell types. DNA transfection is presented for adherent mammalian cells (cell lines as well as primary cultures; see Basic Protocol 1), along with a modified protocol for enhanced transfection (see Alternate Protocol). DNA transfection of suspension mammalian cells (lymphoid, myeloid, and leukemic-derived cells) is also presented (see Basic Protocol 2). For adherent mammalian cells, RNA transfection is also covered (see Basic Protocol 3). Finally, DNA transfection is presented for insect cells (see Basic Protocol 4). To determine

Table 20.6.1 Partial Listing of Commercially Available Cationic Lipid Transfection Reagents

Supplier	Reagent	Uses
Invitrogen	PerFect Lipid Transfection Kit (8 lipids)	Test for different cell types
Life Technologies	LipofectAmine 2000	General, rapid
	LipofectAmine Plus	Difficult-to-transfect adherent cells
	LipofectAmine	General
	Lipofectin	Liver and endothelial cells, oligonucleotides
	DMRIE-C	Suspension cells, RNA
Promega	CellFectin	Insect cells
	Transfectam	General
	Tfx Transfection Trio	Test for different cell types
Qiagen	Effectene	General
Roche/BMB	DOTAP	General
	DOSPER	General
	FuGENE 6	General

Contributed by Pamela Hawley-Nelson and Valentina Ciccarone

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the best transfection conditions for a particular reagent, use the optimization/fine-tuning procedure (see Support Protocol).

NOTE: There are minor differences among the recommended protocols for the various commercially available cationic lipid reagents. The appropriate product profile sheets should be consulted before beginning an experiment.

NOTE: All culture incubations for mammalian cells should be performed in a humidified 37°C, 5% CO₂ incubator. Some media may require altered levels of CO₂ (e.g., for growth of CHO cells in suspension, 8% CO₂ is preferable) to maintain pH 7.4. Insect cells are cultured at 27°C.

**BASIC
PROTOCOL 1**

**CATIONIC LIPID-MEDIATED TRANSFECTION OF ADHERENT
MAMMALIAN CELLS WITH DNA**

This protocol describes the procedure for transfection of DNA into most adherent mammalian cell lines or cultures of primary cells grown attached to culture vessels (Fig. 20.6.1). DNA and lipid reagent are diluted into separate aliquots of serum-free medium, and are then mixed together and allowed to form complexes. Complexes and transfection medium (which may contain serum) are added to the cells in one of two ways. Either the complexes are diluted with transfection medium and this mixture is added to cells that have been rinsed and aspirated (as shown in Fig. 20.6.1), or complexes are added directly to transfection medium that has already been added to the cells (with or without washing). The cells and complexes are incubated together for several hours. After transfection, the volume of the medium is increased if necessary to prevent drying, serum is added if the transfection was serum free, and cells are incubated an additional day or two to allow expression of the transgene.

It is advisable to optimize the conditions for transfection using the Support Protocol. Suggested starting ranges for optimizing the various components of these mixtures are given in Table 20.6.2 for six different popular culture vessel sizes. Transfections can be scaled up to other vessel sizes by increasing the amounts of lipid, DNA, and medium in

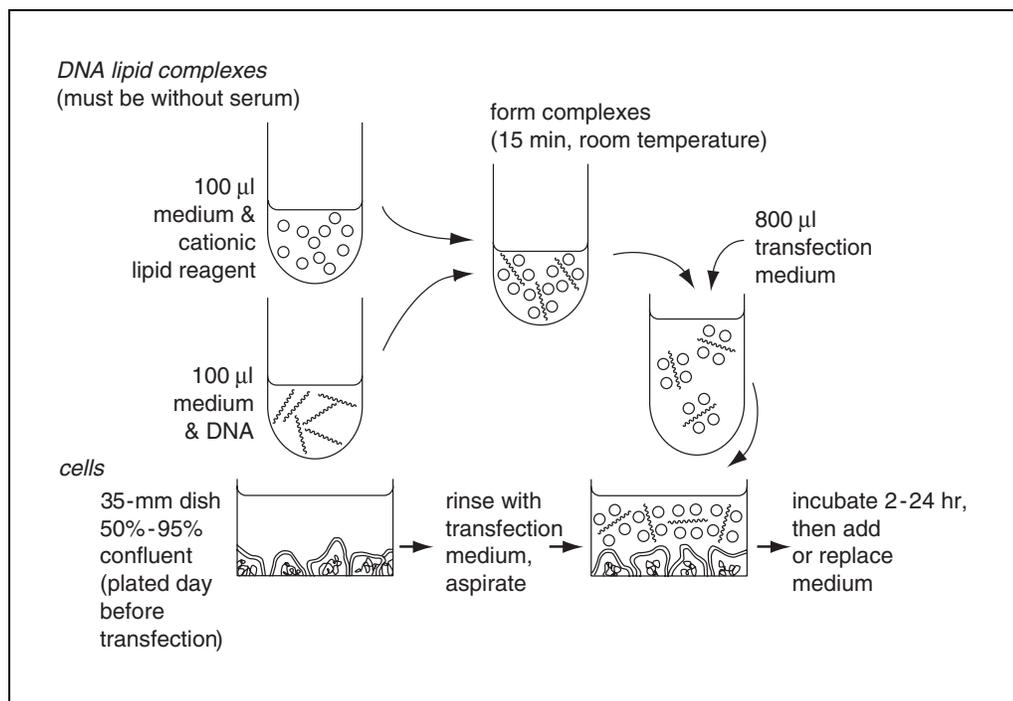


Figure 20.6.1 Diagram of cationic lipid-mediated transfection procedure.

proportion to the difference in surface area. Table 20.6.3 shows the surface areas of several popular culture vessels.

Materials

Adherent cells

Cell culture medium with serum (e.g., complete DMEM, *UNIT 1.2*)

Dilution medium: serum-free cell culture medium or specialized medium for lipid-mediated transfection (e.g., Opti-MEM I, Life Technologies)

Plasmid DNA, purified by anion-exchange chromatography (e.g., Concert High Purity columns, Life Technologies; or see Goldsborough et al., 1998), cesium chloride density gradient, or alkaline lysis (*UNIT 1.6*)

Cationic lipid reagent (see Table 20.6.1)

Polystyrene or polypropylene tubes

Additional reagents and equipment for trypsinization and counting of cells (*UNIT 1.1*), Xgal staining, and selection of stable transformants (*APPENDIX 3A*)

1. The day before transfection, trypsinize and count adherent cells (*UNIT 1.1*). Plate cells in cell culture medium with serum so that they are 50% to 95% confluent on the day of transfection. Avoid antibiotics at the time of plating and during transfection.

The single most important factor in reproducible, high-efficiency transfection is a consistent number of healthy, proliferating cells. Transfection is most efficient when the cells are maintained in mid-log growth. Because transfection efficiency is sensitive to culture

Table 20.6.2 Suggested Starting Ranges of Reagents for Lipid-Mediated Transfection^a

Culture vessel	DNA (μg)	Dilution medium (μl)	Cationic lipid reagent (μl)	Transfection medium (ml)	Transfection volume (ml)
	Step 2	Steps 2 & 3	Step 3	Step 5	Step 6
96-well	0.05-0.4	10-25	0.075-1.5	0.08-0.1	0.1-0.15
24-well	0.2-1.6	25-50	0.5-10	0.2-0.5	0.25-0.6
12-well	0.4-3.2	50-100	1-20	0.4-1	0.5-1.2
6-well	1-8	100-250	2.5-50	0.8-2.5	1-3
60-mm	2-16	250-500	5-100	2-5	2.5-6
100-mm	6-48	750-1500	15-300	5-15	6.5-18

^aStep numbers are indicated from Basic Protocol 1. Volumes have been optimized for LipofectAmine 2000. See Support Protocol for additional optimization strategies.

Table 20.6.3 Surface Areas of Commonly Used Culture Vessels

Culture vessel	Surface area (cm ²)
96-well	0.3
24-well	2
12-well	4
6-well	10
35-mm	8
60-mm	20
100-mm	60
150-mm	140
T25	25
T75	75
T150	150

confluency, cultures should be maintained carefully and passaged frequently, and a standard seeding protocol should be followed from one experiment to the next. Antibiotics may cause some toxicity if present during transfection. For some transfection reagents, the higher cell density is recommended (90% to 95%).

Multiwell cell culture dishes are the easiest to use. Some cells are weakly adherent. If necessary, increase adherence by plating cells on polylysine-coated wells (0.1 mg/ml poly-L-lysine, Sigma; poly-D-lysine-precoated plates, Becton Dickinson).

This protocol is written without specific amounts of reagents. Table 20.6.2 gives the recommended starting amounts for several popular vessels. See Support Protocol for fine tuning the reagent volumes for highest transfection efficiency.

2. On the day of transfection, dilute plasmid DNA into dilution medium in a polystyrene or polypropylene tube and mix. Prepare in bulk for multiple transfections.

Although DNA prepared by anion-exchange chromatography or CsCl gradients yield the best results, DNA prepared by alkaline lysis (miniprep) will work with lower efficiency.

Commercial medium that is specialized for lipid-mediated transfection (e.g., Opti-MEM I) yields the highest efficiency, but other serum-free media may be used.

Polystyrene or polypropylene tubes work well for dilutions. For small-scale transfections, round-bottom 96-well plates with covers are suitable.

3. Dilute cationic lipid reagent into dilution medium in a second tube and mix. Prepare in bulk for multiple transfections.

See Table 20.6.1 for a partial listing of commercially available reagents. A preparation of the cationic lipid DDAB with the neutral lipid DOPE can be made in the laboratory (Rose et al., 1991), although efficiency may be lower than with some commercial preparations.

If using Lipofectin, dilute into Opti-MEM I and allow to incubate at room temperature for 30 to 45 min (Ciccarone and Hawley-Nelson, 1995). If using LipofectAmine 2000, dilute into Opti-MEM I and allow to incubate no more than 30 min (Ciccarone et al., 1999).

4. Combine diluted DNA and diluted cationic lipid reagent, mix, and incubate for 15 min at room temperature.

Incubation times >15 min (up to 6 hr for some reagents) work just as well.

5. While complexes are forming, replace medium on the cells with the appropriate volume of fresh transfection medium.

This step is not necessary if the complexes are diluted with transfection medium or if using LipofectAmine 2000 (see step 6).

This medium can be the same as the dilution medium. It is possible to use serum in the transfection medium at this step.

6. Add DNA-lipid complexes to each well containing cells. Mix complexes into the medium gently, holding the plate at a slant. Incubate 5 hr at 37°C in 5% CO₂.

Adding transfection medium directly to the cells (as described in the steps) helps prevent cells in multiwell plates from drying out, as transfection medium can be added to multiple wells rapidly. Alternatively, complexes can be diluted with the appropriate volume of fresh transfection medium and added to cells from which the cell culture medium has been aspirated (with or without washing, see Fig. 20.6.1). If using LipofectAmine 2000, fresh transfection medium is not required. Undiluted complexes can be added directly to the cell culture medium.

The exposure time may be >5 hr (up to overnight). Be sure that there is sufficient medium to prevent the cells from drying out (it is not necessary to increase other components if this is done). If necessary to maximize cell growth, replace the medium containing complexes with fresh complete medium after 5 hr incubation.

7. After 5 hr incubation, add cell culture medium to reach normal volume and add serum to bring the final concentration to that of normal cell culture medium.

This step is omitted in some protocols, especially when serum is present during transfection and volumes are adequate to prevent drying.

- 8a. *For transient expression analysis:* Assay cell extracts or stain cells in situ 24 to 48 hr after the start of transfection, depending on cell type and promoter activity.
- 8b. *For stable expression analysis:* Passage cells into fresh culture medium 1 day after the start of transfection. At 2 days posttransfection, add the appropriate antibiotic to select for expression of the transfected antibiotic-resistance gene.

Several days or weeks of selection are required for stable expression.

ENHANCED CATIONIC LIPID-MEDIATED TRANSFECTION OF ADHERENT MAMMALIAN CELLS WITH DNA

This is an efficient and reproducible protocol for transfection of DNA into most adherent mammalian cell lines or cultures of primary cells grown attached to culture vessels (Dube, 1997; Shih et al., 1997; Fig. 20.6.2). The procedure is essentially as described in Basic Protocol 1, except that DNA is diluted into serum-free medium along with a proprietary enhancing reagent and incubated for 15 min, allowing the formation of precomplexed DNA before addition to the diluted cationic lipid reagent.

The protocol that follows was developed for LipofectAmine Plus, which is composed of two reagents: LipofectAmine (the cationic lipid reagent) and Plus reagent (the enhancer). One of the main advantages of the Plus enhancer is a high plateau of transfection activity

ALTERNATE PROTOCOL

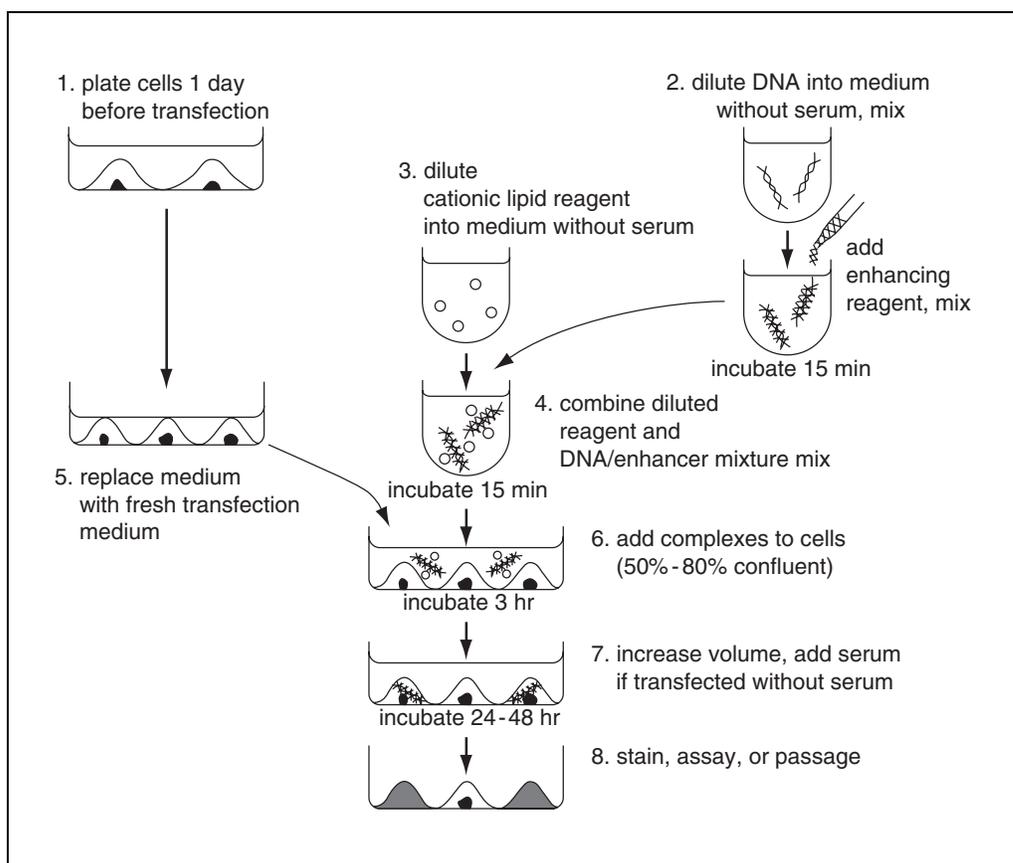


Figure 20.6.2 Diagram of enhanced cationic lipid-mediated transfection procedure.

Introduction and Expression of Foreign Macromolecules into Cells

20.6.5

Table 20.6.4 Suggested Starting Amounts of Reagents for Transfection with LipofectAmine Plus^a

Culture vessel	DNA (μg)	Plus reagent (μl)	Dilution medium (μl)	LipofectAmine (μl)	Transfection medium (ml)	Transfection volume (ml)
	Step 2	Step 2	Steps 2 & 3	Step 3	Step 5	Step 6
96-well	0.1	1	10	0.5	0.08	0.1
24-well	0.4	4	25	1	0.2	0.250
12-well	0.7	5	50	2	0.4	0.5
6-well	1	6	100	4	0.8	1.0
60-mm	2	8	250	12	2	2.5
100-mm	4	20	750	30	5	6.5

^aStep numbers indicated from Alternate Protocol. It is possible to fine tune transfections by testing a range of lipid and DNA concentrations to obtain optimal efficiency; however, the peak activity is usually a broad plateau with this reagent.

across a broad range of lipid and DNA concentrations. This decreases the necessity for optimization and allows the recommendation of specific starting conditions (Table 20.6.4). Transfections can be scaled up to other vessel sizes by increasing the amounts of lipid, DNA, enhancer, and medium in proportion to the difference in surface area (see Table 20.6.3).

The other commercially available enhanced cationic lipid reagent is Effectene (Qiagen, see Table 20.6.1). If using this product, consult the appropriate product profile sheets before beginning the experiment.

NOTE: Consult annotations of the standard transfection procedure (see Basic Protocol 1) for additional details, which also apply to this protocol.

Additional Materials (also see Basic Protocol 1)

Cationic lipid reagent and enhancer (e.g., LipofectAmine Plus, including LipofectAmine and Plus reagents; Life Technologies)

1. The day before transfection, trypsinize and count adherent cells (*UNIT 1.1*). Plate cells in cell culture medium with serum so that they are 50% to 80% confluent on the day of transfection. Avoid antibiotics at the time of plating and during transfection.

For this protocol, refer to Table 20.6.4 for recommended amounts of reagents in different vessels.

2. On the day of transfection, dilute plasmid DNA into dilution medium in a polystyrene or polypropylene tube and mix well. Add enhancer, mix, and incubate 15 min at room temperature.

DMEM is preferred over Opti-MEM 1 in this protocol. It is important to add the DNA first and mix well before adding the Plus reagent to avoid precipitation of the DNA. Incubation times >15 min (up to an hour) work just as well.

3. Dilute cationic lipid reagent into dilution medium in a second tube and mix.
4. Combine precomplexed DNA and diluted cationic lipid reagent, mix, and incubate for 15 min at room temperature.

Incubation times >15 min (up to an hour) work just as well when LipofectAmine is the cationic lipid and Plus reagent is the enhancer.

5. While complexes are forming, replace medium on the cells with the appropriate volume of fresh transfection medium.

The medium can be the same as the dilution medium. It is possible to use serum in the transfection medium at this step. In some cells (e.g., HeLa and NIH 3T3), transfection in medium containing serum is as efficient or more efficient than in medium without serum.

6. Add DNA-enhancer-lipid complexes to each well containing cells. Mix complexes into the medium gently, holding the plate at a slant. Incubate at 37°C in 5% CO₂ for several hours.

The exposure time with LipofectAmine Plus may be as short as 3 hr or up to overnight. Be sure there is sufficient medium to prevent the cells from drying out (it is not necessary to increase other components if this is done).

7. After incubation, add cell culture medium to reach normal volume and add serum to bring the final concentration to that of normal cell culture medium.
8. Perform transient or stable expression analysis (see Basic Protocol 1, steps 8a and 8b).

CATIONIC LIPID-MEDIATED TRANSFECTION OF SUSPENSION CELLS WITH DNA

**BASIC
PROTOCOL 2**

This protocol is essentially the same as for adherent cells (see Basic Protocol 1) in that lipid and DNA are diluted separately into dilution medium, mixed, and allowed to form complexes before exposing to cells. However, complexes are formed in the wells of multiwell culture plates, and cells are then distributed into the wells containing complexes and allowed to transfect.

Materials

Dilution medium: cell culture medium without serum or specialized medium for transfection (e.g., Opti-MEM I, Life Technologies)

Cationic liposome reagent (e.g., DMRIE-C or LipofectAmine 2000, Life Technologies; also see Table 20.6.1)

Plasmid DNA, purified by anion-exchange chromatography or Goldsborough et al., 1998), cesium chloride density gradient, or alkaline lysis

Cell suspension: 1 × 10⁷ cells/ml in normal cell culture medium without serum or antibiotics

Cell culture medium (e.g., complete DMEM; UNIT 1.2)

Serum

6-well tissue culture plates

1. To each well of a 6-well tissue culture plate add 0.5 ml dilution medium.

Commercial medium that is specialized for lipid-mediated transfection (e.g., Opti-MEM I), without serum or antibiotics, gives the best results. However, other serum-free media may also be used.

When transfecting in different-sized culture plates, change the amounts of DNA, cationic lipid reagent, and medium in proportion to the difference in surface area (see Table 20.6.3).

2. Add 0, 2, 4, 6, 8, or 12 μl cationic lipid reagent to each well and mix gently by swirling the plate.

DMRIE-C was found to give high efficiency transfection of DNA in Jurkat (human T cell lymphoma), K562 and KG-1 (human myelogenous leukemia), and MOLT-4 (human lymphoblastic leukemia) cell lines. It is a lipid suspension that may settle with time. To ensure that a homogenous sample is taken, mix thoroughly by inverting the tube 5 to 10 times before removing a sample for transfection.

3. Add 0.5 ml dilution medium containing 4 μg plasmid DNA to each well. Mix by swirling plate.

The amount of DNA should be optimized for each cell line.

4. Incubate at room temperature for 15 to 45 min to allow formation of lipid-DNA complexes.

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5. Add 0.2 ml cell suspension (2×10^6 cells) to each well and mix gently.

The single most important factor in reproducible, high-efficiency transfection is a consistent number of healthy, proliferating cells. Transfection is most efficient when the cells are maintained in mid-log growth.

6. Incubate several hours at 37°C in a 5% CO₂ incubator.

A 4-hr incubation is adequate for DMRIE-C transfections.

7. To each well add 2 ml cell culture medium containing 1.5× the usual amount of serum.

For Jurkat and MOLT-4 cells, addition of 1 µg/ml phytohemagglutinin (PHA) and 50 ng/ml phorbol myristate acetate (PMA) enhances promoter activity and gene expression. For K562 and KG-1 cells, PMA alone enhances promoter activity.

8. Assay the cells at 24 or 48 hr post-transfection for transient or stable expression (see Basic Protocol 1, step 8a or 8b).

BASIC PROTOCOL 3

CATIONIC LIPID-MEDIATED TRANSFECTION OF ADHERENT CELLS WITH RNA

In this protocol, lipid is first diluted into dilution medium and mixed. RNA is then mixed directly into the diluted lipid and immediately added to cells (which have been rinsed with serum-free medium), and cells are incubated for transfection.

Materials

Adherent cells

Cell culture medium with serum (e.g., complete DMEM; UNIT 1.2)

Dilution medium: serum-free cell culture medium or specialized medium for transfection (e.g., Opti-MEM I, Life Technologies)

Cationic lipid reagent (e.g., DMRIE-C, Life Technologies; also see Table 20.6.1)
mRNA (see APPENDIX 3A)

6-well or 35-mm tissue culture plate

12 × 75-mm polystyrene tubes

Additional reagents and equipment for trypsinizing, counting, and plating cells
(UNIT 1.1)

1. The day before transfection, trypsinize and count adherent cells (UNIT 1.1). In each well of a 6-well tissue culture plate, or in six 35-mm tissue culture plates, seed $\sim 2\text{--}3 \times 10^5$ cells in 2 ml of the appropriate cell culture medium supplemented with serum.

Transfection is most efficient when the cells are growing rapidly. Cultures should be maintained carefully and passaged frequently. As transfection efficiency may be sensitive to culture confluency, it is important to maintain a standard seeding protocol from experiment to experiment.

2. Incubate at 37°C in a 5% CO₂ incubator until the cells are $\sim 80\%$ confluent.

This will usually take 18 to 24 hr, but the time will vary among cell types.

3. On the day of transfection, wash the cells in each well with 2 ml dilution medium at room temperature.

Commercial medium that is specialized for lipid-mediated transfection (e.g., Opti-MEM I), without serum or antibiotics, gives the best results. However, other serum-free media may also be used.

4. Add 1.0 ml dilution medium to each of six 12 × 75-mm polystyrene tubes.

5. Add 0, 2, 4, 6, 8, or 12 μl cationic lipid reagent to each tube and mix or vortex briefly.

DMRIE-C was found to give high-efficiency transfection of RNA in adherent cell lines (Ciccarone et al., 1995). It is a lipid suspension that may settle with time. To ensure that a homogenous sample is taken, mix thoroughly by inverting the tube 5 to 10 times before removing a sample for transfection.

6. Add 2.5 to 5.0 μg RNA to each tube and vortex briefly.

mRNA that is capped and polyadenylated is translated more efficiently and is more stable within the cell.

7. Immediately add lipid-RNA complexes to washed cells and incubate 4 hr at 37°C in a 5% CO₂ incubator.

The time of exposure of cells to lipid-RNA complexes, as well as the amount of RNA added to the cells, should be adjusted for each cell type.

8. Replace transfection medium with cell culture medium containing serum.
9. Allow cells to express the RNA for 16 to 24 hr and analyze them for expression of the transfected RNA as appropriate for the transgene used.

CATIONIC LIPID-MEDIATED TRANSFECTION OF ADHERENT Sf9 AND Sf21 INSECT CELLS WITH BACULOVIRUS DNA

**BASIC
PROTOCOL 4**

As for transfecting mammalian cells (see Basic Protocol 1), cationic lipid reagent and nucleic acid are diluted separately into serum-free medium and then mixed and allowed to form complexes. Complexes are then diluted with fresh transfection medium and added to the cells for transfection. After the cells are fed and incubated, budded virus can be isolated from the medium.

Materials

Insect cells: Sf9 or Sf21 cells

Insect medium (e.g., Sf-900 II SFM, Life Technologies) with and without serum and antibiotics

Baculovirus DNA: purified DNA or bacmid DNA miniprep (Anderson et al., 1995)

Cationic lipid reagent (Table 20.6.1)

6-well tissue culture plate

27°C incubator

12 \times 75-mm polystyrene tubes, sterile

Additional reagents and equipment for culturing insect cells and harvesting baculovirus from cell supernatants (APPENDIX 3A)

1. In each well of a 6-well tissue culture plate, seed $\sim 9 \times 10^5$ insect cells in 2 ml insect medium without serum or antibiotics.

Insect cells must be plated when they are in mid-log growth phase. Cells that have reached stationary phase transfect and infect at very low efficiency. Therefore, it is advisable to maintain a standard cell passage protocol that keeps the cells in log growth. For Sf9 or Sf21 cells adapted in Sf-900 II SFM, cells are passaged twice weekly to a density of 3×10^5 cells/ml in suspension, and plated for transfection on the third day postseeding, when they are in mid-log phase. For other cell culture media and growth conditions, adjust conditions to maintain similar growth characteristics.

For culture of insect cells, use 50 units/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin (half the usual final concentration). For transfection, it is preferable to omit antibiotics from the medium to avoid toxicity.

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2. Allow cells to attach at 27°C for ≥1 hr.
3. For each transfection, dilute 1 to 2 µg baculovirus DNA into 100 µl insect medium without serum or antibiotics in a 12 × 75–mm polystyrene tube.
4. For each transfection, dilute 1.5 to 9 µl cationic lipid reagent into 100 µl insect medium without serum or antibiotics in a separate 12 × 75–mm polystyrene tube.

The suggested amount is 6 µl, but this should be optimized for each system.

CellFectin gives high-efficiency transfection of DNA in insect cell lines (Anderson et al., 1995). It is a lipid suspension that may settle with time. To ensure that a homogenous sample is taken, mix thoroughly by inverting the tube 5 to 10 times before removing a sample for transfection.

5. Combine the two solutions, mix gently, and incubate at room temperature for 15 to 45 min to form lipid-DNA complexes.
6. For each transfection, add 0.8 ml insect medium without serum or antibiotics to each tube containing lipid-DNA complexes and mix gently.
7. Aspirate medium from cells and overlay diluted lipid-DNA complexes onto the washed cells.

Alternatively, the medium on the cells can be replaced with 0.8 ml fresh insect medium and the undiluted complexes can be added directly to the fresh medium on the cells.

8. Incubate cells for 5 hr in a 27°C incubator. Protect plates from evaporation by putting them in a humidified container.
9. Remove transfection mixture and add 2 ml insect medium containing antibiotics and serum, if desired. Incubate cells in a 27°C incubator for 72 hr.
10. Harvest baculovirus from cell supernatants.

Gene expression may also be evaluated in the cells after removal of virus-containing medium.

**SUPPORT
PROTOCOL**

**FINE TUNING OR OPTIMIZING CONDITIONS FOR CATIONIC LIPID
REAGENT TRANSFECTIONS**

This protocol provides an example of a simple one-step procedure for determining conditions conducive to high-efficiency transfections using cationic lipid reagents in any target cell type. A matrix of DNA and lipid reagent concentrations is used on transfections performed in a multiwell plate (Fig. 20.6.3). Once the best conditions have been determined, the transfections may be scaled up to larger vessels using the relative surface area (see Table 20.6.3) to increase the amounts of all reagents proportionately. This protocol can be modified for use with any transfection protocol.

Additional Materials (also see Basic Protocol 1 and Alternate Protocol)

- 24-well tissue culture plates
- 96-well round-bottom plates (sterile, with lid)

1. The day before transfection, trypsinize and count cells (*UNIT 1.1*). Plate cells in each well of a 24-well tissue culture plate using normal cell culture medium with serum, so that they are 50% to 95% confluent on the day of transfection. Avoid antibiotics at the time of plating and during transfection.

The single most important factor in high-efficiency transfection is healthy, proliferating cell cultures. Antibiotics may cause some toxicity if present during transfection.

In a 24-well plate, seeding 4×10^4 to 2×10^5 cells per well will usually give good plating density. Any type of plate may be used by scaling the reagent and cell amounts in proportion to the relative surface area (see Table 20.6.3).

2. Dilute DNA into dilution medium (appropriate for the lipid being optimized) without serum or antibiotics in four microcentrifuge tubes. Use a range of DNA concentrations, and use a volume of dilution medium that is 7× the appropriate protocol volume (see Basic Protocol 1, step 2, and Table 20.6.1). Mix gently after each addition.

This makes enough DNA per tube for seven wells on a 24-well plate. Good ranges include 0.2 to 1.6 μg DNA per well.

If the Plus enhancer is being used, include it in the diluted DNA tubes, using 10 μl Plus reagent per μg DNA. Add the Plus reagent to the diluted DNA after mixing well. If the Plus reagent is added first, precipitation may occur.

3. Dilute cationic lipid reagent into dilution medium without serum or antibiotics in six microcentrifuge tubes. Use a range of DNA concentrations, and use a volume of dilution medium that is 5× the appropriate protocol volume (see Basic Protocol 1, step 3, and Table 20.6.1). Mix gently after each addition.

Be sure to observe timing that works best for the cationic lipid reagent being used.

This makes enough diluted lipid per tube for five wells on a 24-well plate. Good ranges include 0.5 to 5 μl lipid reagent per well.

4. Pipet equal per-well volumes of diluted DNA and diluted cationic lipid reagent into the wells of a 96-well plate in a matrix corresponding to the wells on the 24-well plate (Fig. 20.6.3). Mix the complexes with the pipet tip by triturating. Cover the plate and incubate for 15 min at room temperature.

Incubation times >15 min (up to an hour) work just as well, but be sure the complexes do not dry by covering them well.

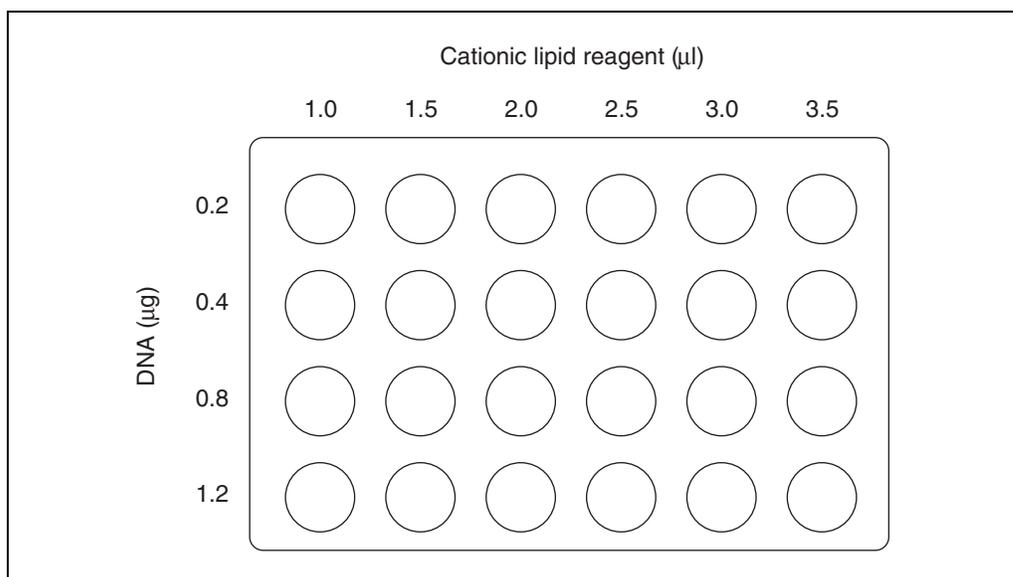


Figure 20.6.3 A sample matrix for fine tuning (optimizing) transfection reagent efficiencies using cationic lipid reagents.

5. While complexes are forming, replace medium on the cells with fresh transfection medium.

See Basic Protocol 1, steps 5 and 6, for alternate procedures for combining complexes, medium, and cells. The medium can be the same as the serum-free dilution medium. It is possible to use serum in the transfection medium at this step. It is also possible to omit this step when using LipofectAmine 2000.

6. Add aliquots of DNA-lipid complexes (total volume from wells in step 4) to each well containing cells with fresh transfection medium. Mix complexes into the medium gently, holding the plate at a slant. Incubate at 37°C in 5% CO₂ for 5 hr.

The exposure time may be >5 hr (up to overnight). Be sure there is sufficient medium to prevent the cells from drying out (it is not necessary to increase other components if this is done). If using the Plus enhancer, a 3-hr exposure is sufficient.

7. After 5 hr incubation, add cell culture medium to reach normal volume and add serum to bring the final concentration to that of normal cell culture medium.

If necessary to maximize cell growth, replace the medium containing the complexes with fresh complete medium after 5 hr incubation. This step may be omitted entirely for some protocols.

8. Check expression as described (see Basic Protocol 1, steps 8a and 8b).

If peak activity is found to occur on the edge of the matrix of concentrations tested, adjust the concentrations to include the observed peak at the center of a new matrix and repeat the transfection.

COMMENTARY

Background Information

There are currently at least eight companies that market cationic lipid-based transfection reagents. A partial listing of companies and products may be seen in Table 20.6.1. Many companies offer more than one type of reagent. Among the more popular ones are LipofectAmine 2000 and LipofectAmine Plus (Life Technologies), DOTAP and FuGENE 6 (Roche), and Effectene (Qiagen). Some of the structures are proprietary. The structures that are published can be classified into two general categories based on the number of positive charges in the lipid headgroup. The first cationic lipid (DOTMA) has a single positive charge per molecule and is used in Lipofectin (Life Technologies; Felgner et al., 1987). Several other cationic lipid-based transfection reagents such as DOTAP liposomal transfection reagent (Roche) and DMRIE-C (Life Technologies) also make use of singly charged cationic lipid molecules. Increasing the number of positive charges per cationic lipid molecule to as many as five improved transfection efficiency dramatically in most cell types. This can be seen in the examples of DOGS, the cationic lipid in Transfectam (Promega; Behr et al., 1989); DOSPA in LipofectAmine (Life Technologies;

Hawley-Nelson et al., 1993); and TMTPS in CellFectin (Life Technologies; Anderson et al., 1995). Further increase in transfection efficiency can sometimes be achieved by precomplexing DNA with a proprietary enhancer. Two commercially available transfection kits with enhancers are LipofectAmine Plus (Life Technologies; Shih et al., 1997) and Effectene (Qiagen).

Life Technologies has designed cationic lipid reagents with specialized applications such as high-efficiency transfection of insect cells (see Basic Protocol 4) or delivery of RNA (see Basic Protocol 3). Lipofectin has high activity for endothelial cell transfection (Tilkins et al., 1994).

Basic Protocol 1 and the Alternate Protocol described in this unit are the procedures with the highest potential for efficient DNA transfection of adherent mammalian cells (Shih et al., 1997; Ciccarone et al., 1999). LipofectAmine 2000 has a simple protocol that yields the highest transfection efficiencies in many cell types. Using the enhancer reagent results in more reproducible transfections without extensive optimization because of the overall high activity. Prior to the availability of enhanced cationic liposome transfections (e.g., using

LipofectAmine 2000 and Effectene), the most effective procedure for transfection of adherent mammalian cells with DNA was with other polycationic reagents (e.g., LipofectAmine; Hawley-Nelson et al., 1993) following Basic Protocol 1. In order to achieve high-efficiency transfections with Basic Protocol 1, it is necessary to optimize lipid and DNA concentrations with the target cells at the desired plating density using a procedure similar to that described in the Support Protocol. Many cationic lipid reagents, as well as transfection reagents based on other chemistries, are available that can be used in Basic Protocol 1 for adherent mammalian cell DNA transfection, but they may yield lower efficiencies than the Alternate Protocol with the enhancer. Optimization using the Support Protocol is highly recommended when not using the enhancer, and the protocol can be modified for use with any cationic lipid reagent.

Critical Parameters

The most critical parameter for successful transfection is cell health. Cells should be proliferating as rapidly as possible at the time they are plated for transfection. On the day of transfection, mitoses should be abundant in healthy cultures. Fresh cultures with a finite life span should be used at the earliest possible passage.

For reproducible transfection results, it is critical to plate the same number of healthy cells for each transfection. Cells should always be counted, preferably using a hemacytometer and trypan blue (*UNIT 1.1*).

Although optimization is not required for high-efficiency transfection when using an enhancer (see Alternate Protocol), it is essential for success without the enhancer, and may improve efficiency even with the enhanced method.

The medium used to dilute and form complexes between the cationic liposomes and the DNA must not contain serum. Serum contains sulfated proteoglycans and other proteins, which compete with the DNA for binding to the cationic lipids. The medium should also not contain antibiotics. There is toxicity to the cells when cationic lipid reagents are used in the presence of antibiotics.

The dilution medium/plating medium for the cells may have some influence on transfection efficiency. Some proprietary serum-free media contain components that inhibit transfection and should be replaced with Opti-MEM I, DMEM, or other media without serum during transfection (Hawley-Nelson and Ciccarone, 1996).

Serum may be present in the medium on the cells during transfection. For most cationic lipid reagents, on most cell types, transfection activity is not inhibited in the presence of serum provided the complexes were formed in serum-free medium (Brunette et al., 1992; Ciccarone et al., 1993, 1999; Shih et al., 1997).

The specific serum-free medium used to dilute the lipid and DNA can have a slight effect on the efficiency of transfection. For the enhanced protocol (Alternate Protocol), normal culture medium such as DMEM is recommended. For the standard procedure (Basic Protocols 1, 2, and 3), Opti-MEM I medium works best. The improvements resulting from using the recommended media are less than two fold. When using Lipofectin, dilution in Opti-MEM I followed by a 30- to 45-min incubation is recommended (Ciccarone and Hawley-Nelson, 1995). With LipofectAmine 2000, the reverse is true: extended incubation (>30 min) of LipofectAmine 2000 in Opti-MEM prior to addition of DNA results in lower transfection activity (Ciccarone et al., 1999).

High-purity DNA will increase transfection efficiency. Miniprep DNA does work, however, when efficiency is not critical. A wide range of sizes of polynucleotides may be transfected, from 18-mer single-stranded oligonucleotides (Chiang et al., 1991; Bennett et al., 1992; Yeoman et al., 1992; Wagner et al., 1993) to 400-kb YAC DNAs (Lamb et al., 1993). Excess vortexing of complexes or DNA solutions may result in some shearing, especially with larger molecules. The concentration of EDTA in the diluted DNA should not exceed 0.3 mM.

Transgene expression may be increased in some cell types by inducing the promoter. This is observed in Jurkat cells when phytohemagglutinin and phorbol myristate acetate are added following transfection to activate the cytomegalovirus promoter (Schifferli and Ciccarone, 1996).

Troubleshooting

The most common complaints surrounding transfections include decreased transfection efficiency and low cell yield. Decreases in efficiency often result from changes in the target cell line. Cultured cell lines are usually aneuploid and often consist of a mixture of genotypes and phenotypes that can be subject to selection in the laboratory environment. Primary cultures, although usually genotypically uniform, often consist of a mixture of phenotypes from different tissues and can change their population characteristics in response to

their environment. Whenever a decrease in transfection efficiency is observed, the first thing to try is to work with a freshly thawed culture or isolate (Hawley-Nelson and Shih, 1995). Be sure the same number of cells is plated in each experiment, since plating density affects efficiency and peak position (Hawley-Nelson et al., 1993).

Low cell yield often results from the use of too much DNA or cationic lipid reagent. Use lower concentrations of these two components and examine the results for transfection efficiency as well as cell yield. Acceptable efficiency can usually be obtained with higher cell yield by using lower concentrations of lipid and

DNA (Hawley-Nelson et al., 1993; Life Technologies, 1999).

Cell yield can also be improved in several other ways. (1) Increasing the plating density. This usually requires adjustment of lipid and DNA amounts, but often the transfection efficiency as well as the cell yield increases with higher plating input (Life Technologies, 1999). (2) Decreasing time of exposure of the cells to cationic lipid–DNA complexes. This can be done by increasing volume and adding back serum at earlier times or by removing the complexes from the cells at the end of transfection. (3) Performing the transfection in the presence of serum. Most cationic lipid reagents work

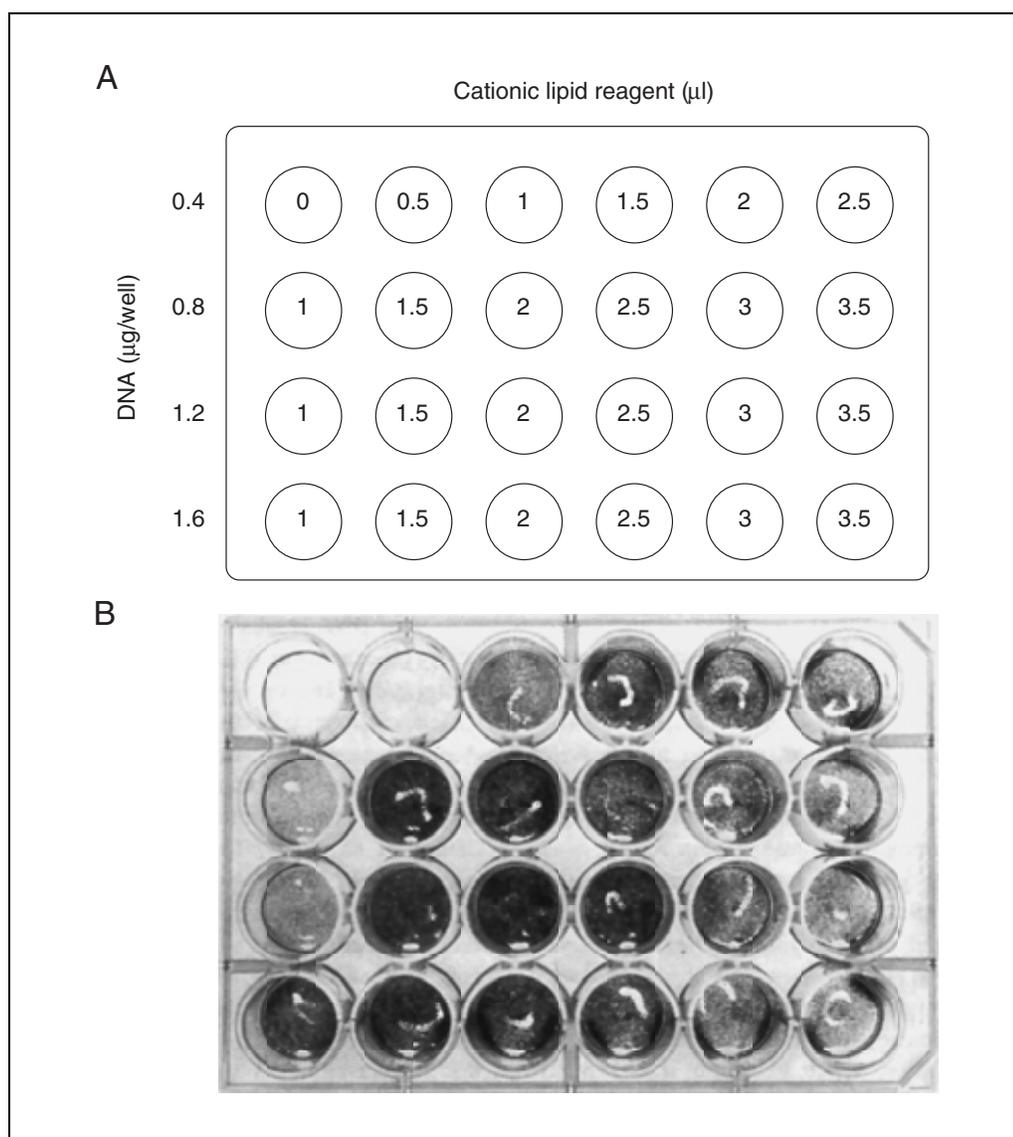


Figure 20.6.4 Results of fine-tuning or optimizing conditions for transfection. Before transfection, 293 H cells were plated at 2×10^5 /well in a 24-well plate precoated with poly-D-lysine. The following day, cells were transfected with pCMV-SPORTβgal DNA using LipofectAmine 2000 as described (see Support Protocol). One day posttransfection, cells were fixed and stained with Xgal. **(A)** Amounts of DNA and lipid reagent used. **(B)** Results of Xgal staining.

Table 20.6.5 Activity for a Scaled-up Transfection Using LipofectAmine Plus in BHK-21 Cells

Plate	Surface area ratio to 24-well plate	Cells/well ($\times 10^4$)	DNA/well (μg)	Plus reagent (μl)	LipofectAmine reagent (μl)	$\beta\text{-gal}$ (ng/cm^2) ^a
24-well	1	4	0.4	2	2	188 \pm 5
12-well	2	8	0.8	4	4	193 \pm 12
6-well	5	20	2	10	10	179 \pm 27
60-mm	10	40	4	20	20	171 \pm 16
100-mm	28	112	11.2	56	56	157

^aResults are the mean of three transfections \pm the standard deviation.

well in transfection medium containing serum (Brunette et al., 1992; Ciccarone et al., 1993, 1999; Shih et al., 1997). One exception is LipofectAmine without the Plus enhancer.

Some cationic lipid solutions are naturally cloudy. Sometimes cloudiness is observed when complexes are made with DNA. Usually this does not interfere with transfection efficiency. Most cationic liposome solutions (especially DMRIE-C and CellFectin) should be mixed gently by inversion just before use to produce a uniform suspension. With Plus reagent, it is possible to precipitate the DNA when the Plus reagent is diluted first into the DMEM and DNA is added second. Always dilute the DNA into DMEM and mix well before adding Plus reagent.

Anticipated Results

Transfection should be observed for most adherent mammalian cells transfected with DNA using Basic Protocol 1. Efficiencies vary with cell type. For example, 293, COS-7, and CHO-K1 cells yield 95% or more blue cells following Xgal staining of cells transfected with pCMVSPORT βgal plasmid DNA using LipofectAmine 2000. The authors have noted efficiencies of other cell types as high as 49% for SK-BR3 breast cancer cell lines, 77% for BE(2)C human neuroblastoma cells, and 43% for MDCK canine kidney cells (Ciccarone et al., 1999). Efficiencies also vary for suspension cells. The authors note that while LipofectAmine Plus is relatively inefficient for transfecting Jurkat cells, DMRIE-C can yield up to 85% blue cells following pCMVSPORT βgal plasmid DNA transfection, gene activation

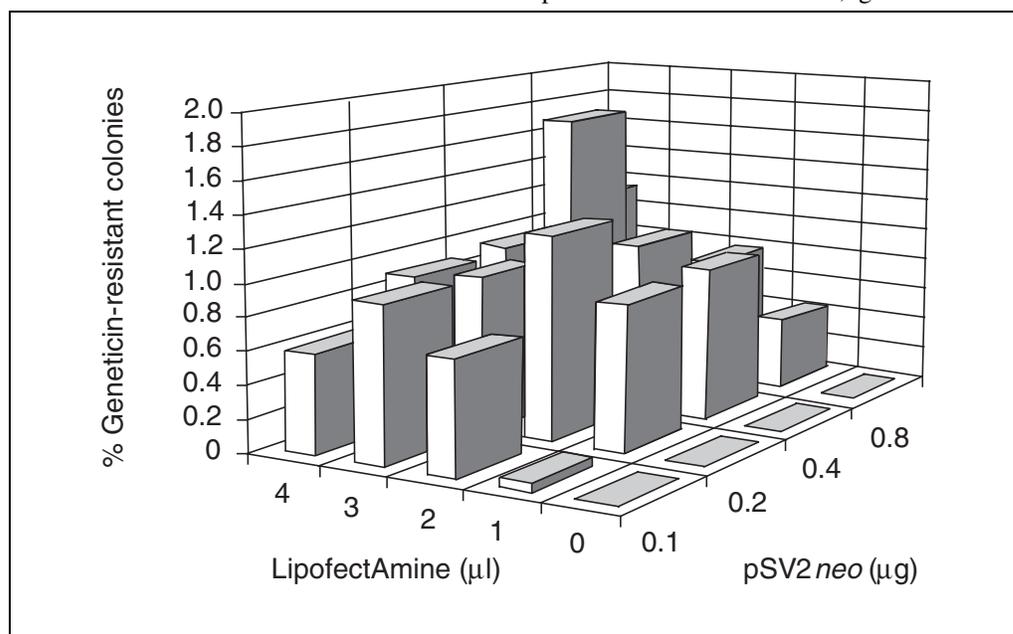


Figure 20.6.5 Stable transfection of NIH 3T3 cells. Cells were plated at 6×10^4 cells/well in 24-well plates. The day after plating, cells were transfected with LipofectAmine Plus complexed with pSV2neo DNA. The following day, cells were passaged at a total dilution of 1/150. Cells were exposed to 0.6 mg/ml Geneticin antibiotic from day 3 to day 13, and were then washed once with PBS and stained with 0.2% toluidine blue in PBS with 10% formalin.

with phytohemagglutinin and phorbol myristate acetate, and Xgal staining (Ciccarone et al., 1995, Schifferli and Ciccarone, 1996).

The result of a typical fine-tuning/optimization protocol is shown in Figure 20.6.4. The transfection reagent was LipofectAmine 2000, the DNA was pCMV-SPORT β gal, the cells were 293 H. Cells were stained the day following transfection and were allowed to stain overnight at 37°C. A selection of transfection conditions can be made.

Conditions found to be advantageous for transfection in small wells may be scaled up, the results of a typical scale-up are given in Table 20.6.5.

The results of a stable transfection of NIH 3T3 cells with pSV2neo DNA using an enhancer reagent (LipofectAmine Plus) are shown in Figure 20.6.5. The transfection was done on 24-well plates, the cells were passaged onto 6-well plates the following day, and selection with geneticin was done for 10 days. The figure shows optimization of conditions and the generally high efficiency that can be achieved using this method.

Time Considerations

Counting and plating the cells should be done the day before transfection and will usually require <1 hour. Transfection is usually done in the morning. Depending on the number of conditions being evaluated, it may require all morning plus a short period in the afternoon to increase medium volume or feed the cells. The total time required is <1 day.

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When embarking upon any transfection procedure, a critical first step is to optimize conditions. Every mammalian cell type has a characteristic set of requirements for optimal introduction of foreign DNA; there is a tremendous degree of variability in the transfection conditions that work, even among cell types that are very similar to one another. Often, an experimenter must screen a wide variety of cell types for a desired regulatory trait, such as an appropriate response to a particular effector molecule. It is thus helpful to have a straightforward, systematic approach to optimizing transfection efficiency. Transient assay systems are particularly useful for this purpose. A fusion gene that is known to function in mammalian cells can be transfected into cells under a variety of conditions, and transfection efficiency can be easily monitored by assaying for the fusion gene product. The human growth hormone (hGH) assay system is particularly useful for this purpose because both harvest and assay take very little time. However, any reporter system can be used to optimize transfection efficiency.

The single most important factor in optimizing transfection efficiency is selecting the proper transfection protocol. This usually comes down to a choice among calcium phosphate-mediated gene transfer (UNIT 20.3), DEAE-dextran-mediated gene transfer (UNIT 20.4), electroporation (UNIT 20.5), and liposome-mediated transfection (UNIT 20.6). Fusion techniques such as protoplast fusion and microinjection may also be considered. Cells are variable with respect to which transfection protocol is most efficient. It is recommended that any adherent cell line under investigation be tested for transfection ability with DEAE-dextran, calcium phosphate, and liposome-mediated transfection. Nonadherent cell lines can be transfected by electroporation and liposome-mediated transfection. Generally, if a cell can be grown in culture, it can be transfected.

CALCIUM PHOSPHATE TRANSFECTION

The primary factors that influence efficiency of calcium phosphate transfection (UNIT 20.3) are the amount of DNA in the precipitate, the length of time the precipitate is left on the cell, and the use and duration of glycerol or DMSO shock. A calcium phosphate optimization is shown in Table 20.7.1. Generally, higher concentrations of DNA (10 to 50 μg) are used in calcium phosphate transfection. Total DNA concentration in the precipitate can have a dramatic effect on efficiency of uptake of DNA with calcium phosphate-mediated transfection. With some cell lines, more than 10 to 15 μg of DNA

Table 20.7.1 Optimization of Calcium Phosphate Transfection

Dish (10-cm)	pXGH5 (μg)	pUC13 (μg)	Exposure to precipitate (hr)	Glycerol shock (min)
1	5	5	6	—
2	5	15	6	—
3	5	35	6	—
4	5	5	16	—
5	5	15	16	—
6	5	35	16	—
7	5	5	6	3
8	5	15	6	3
9	5	35	6	3
10	5	5	16	3
11	5	15	16	3
12	5	35	16	3

Contributed by John K. Rose

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added to a 10-cm dish results in excessive cell death and very little uptake of DNA. With other cell types, such as primary cells, a high concentration of DNA in the precipitate is necessary to get any DNA at all into the cell on a routine basis. For example, with human foreskin fibroblasts, transfection of 5 μg of a reporter plasmid with 5 μg of carrier DNA (e.g., pUC13) gives significantly less expression than does transfection of 5 μg of reporter plasmid with 35 μg of carrier DNA. Presumably, this is because the amount of DNA affects the nature of the precipitate and thus alters the fraction of the applied DNA that is taken up into cells.

The optimal length of time that the precipitate is left on cells varies with cell type. Some cell types, such as HeLa or BALB/c 3T3, are efficiently transfected by leaving the precipitate on for 16 hr. Other cell types cannot survive this length of exposure to the precipitate. Transfection efficiency of some cell types, such as CHO DUKX BII, is dramatically increased by glycerol or DMSO shock (UNIT 20.3). The pilot experiment listed will indicate whether the cell type is tolerant to long exposure to a calcium phosphate precipitate and whether glycerol shock should be used. Once the results of this experiment are in hand, finer experiments can be done to further optimize conditions. For example, if shocking with 10% glycerol for 3 min enhances transfection efficiency, an experiment varying the time of glycerol shock or also trying 10% and 20% DMSO shock might be done.

Once optimal conditions for transfection are found, extensive DNA curves varying the amount of reporter plasmid should be prepared. The total amount of DNA should be kept constant at the optimal level determined in the first experiment. The amount of reporter plasmid DNA (e.g., pXGH5) should be varied, and carrier DNA (e.g., pUC13) should be used to make up the difference. This is to ensure that transfections are performed under conditions where the amount of reporter plasmid in the cell is not saturating the cellular transcription and translation machinery.

DEAE-DEXTRAN TRANSFECTION

There are several factors that can be varied in DEAE-dextran transfection (UNIT 20.4). The number of cells, concentration of DNA, and concentration of DEAE-dextran added to the dish are the most important to optimize. To a first approximation, most cell types that can be transfected using DEAE-dextran will have a preference for 1 to 10 μg DNA/10-cm dish and for 100 to 400 μg DEAE-dextran/ml of medium. Table 20.7.2 shows how the dishes in an optimization might be chosen. The 20-dish experiment consists of two sets of 10 dishes; one set is plated at 5×10^5 cells/dish, the other is plated at 2×10^6 cells/dish. Each set contains dishes that will be transfected with 1 to 10 μg of a reporter plasmid and

Table 20.7.2 Optimization of DEAE-Dextran Transfection

5×10^5 cells/10-cm dish:			2×10^6 cells/10-cm dish:		
Dish	pXGH5 (μg)	DEAE-dextran ($\mu\text{g}/\text{ml}$)	Dish	pXGH5 (μg)	DEAE-dextran ($\mu\text{g}/\text{ml}$)
1	1	400	11	1	400
2	1	200	12	1	200
3	1	100	13	1	100
4	4	400	14	4	400
5	4	200	15	4	200
6	4	100	16	4	100
7	10	400	17	10	400
8	10	200	18	10	200
9	10	100	19	10	100
10	0	200	20	0	200

100 to 400 $\mu\text{g/ml}$ DEAE-dextran. If an hGH expression vector such as pXGH5 is used, a time course of expression under each condition can be determined by removing 100- μl aliquots of the medium 2, 4, and 7 days posttransfection (with a medium change after the day 4 aliquot is removed).

With the results of this pilot experiment in hand, a second experiment using a narrower range of DEAE-dextran concentrations and a wider range of DNA doses should be undertaken. For example, if the cells appear to express more hGH at 100 $\mu\text{g/ml}$ DEAE-dextran than at higher concentrations in the pilot experiment, the second experiment should cover from 25 to 150 $\mu\text{g/ml}$ DEAE-dextran. Because DEAE-dextran is toxic to some cells, a brief exposure to small concentrations may be optimal. The wide range of added DNA in this experiment is crucial in two respects. First, it is valuable to know the smallest amount of the transfected reporter gene that can give a readily detectable signal. Second, the linearity of the dose of DNA with the amount of reporter gene expression generally decays for large amounts of input DNA. When excessive (i.e., nonlinear) amounts of DNA are used in transfection experiments, it is possible that the effects observed are dose-response effects rather than the phenomenon intended for study. This serious and common problem can be eliminated by doing a careful DNA dose-response curve as above.

ELECTROPORATION

Perhaps because it is not a chemically based protocol, electroporation (*UNIT 20.5*) tends to be less affected by DNA concentration than either DEAE-dextran- or calcium phosphate-mediated gene transfer. Generally, DNA amounts in the range of 10 to 40 $\mu\text{g}/10^7$ cells work well, and there is a good linear correlation between the amount of DNA present and the amount taken up. The parameter that can be varied to optimize electroporation is the amplitude and length of the electric pulse, the latter being determined by the capacitance of the power source. The extent to which this can be varied is determined by the electronics of the power supply used to supply the pulse. The objective is to find a pulse that kills between 20% and 60% of the cells. This generally is in the range of 1.5 kV at 25 μF . If excessive cell death occurs, the length of the pulse can be lowered by lowering the capacitance. Settings between 3 and 25 μF can be tried.

LIPOSOME-MEDIATED TRANSFECTION

Three primary parameters—the concentrations of lipid and DNA and incubation time of the liposome-DNA complex—affect the success of DNA transfection by cationic liposomes (*UNIT 20.6*). These should be systematically examined to obtain optimal transfection frequencies.

Concentration of Lipid

In general, increasing the concentrations of lipid improves transfection of four cell lines examined (CV-1 and COS-7 with Lipofectin, and HeLa and BHK-21 with TransfectACE; see *UNIT 20.6*). However, at high levels ($>100 \mu\text{g}$), the lipid can be toxic. For each particular liposome mixture tested, it is important to vary the amount as indicated in Table 20.7.3.

Concentration of DNA

In many of the cell types tested, relatively small amounts of DNA are effectively taken up and expressed. In fact, higher levels of DNA can be inhibitory in some cell types with certain liposome preparations. In the optimization protocol outlined in Table 20.7.3, the standard reporter vector pSV2CAT is used; however, any plasmid DNA whose expression can be easily monitored would be suitable.

Table 20.7.3 Optimization of Liposome-Mediated Transfection

Dish (35-mm)	pSV2CAT (μ g)	Liposomes (μ l)	Dish (35-mm)	pSV2CAT (μ g)	Liposomes (μ l)
1	0.1	1	11	5	5
2	0.1	2	12	5	10
3	0.1	4	13	5	15
4	0.1	8	14	5	20
5	0.1	12	15	5	30
6	0.5	1	16	10	5
7	0.5	2	17	10	10
8	0.5	4	18	10	15
9	0.5	8	19	10	20
10	0.5	12	20	10	30

Time of Incubation

When the optimal amounts of lipid and DNA have been established, it is desirable to determine the length of time required for exposure of the liposome-DNA complex to the cells. In general, transfection efficiency increases with time of exposure to the liposome-DNA complex, although after 8 hr, toxic conditions can develop. HeLa or BHK-21 cells typically require ~3 hr incubation with the liposome-DNA complex for optimal transfection, while CV-1 and COS-7 cells require 5 hr of exposure.

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CHAPTER 21

Fluorescent Protein Technology

INTRODUCTION

The development of fluorescent proteins as molecular tags over the past decade has provided an essential new tool for understanding biological systems by allowing proteins to be visualized in living cells. The fluorescent proteins can be fused to a protein of interest and then expressed within cells to visualize, track, and quantify the behavior of a protein, providing fundamentally new insights into protein function and cellular processes in the complex environment of the cell. The fluorescent protein that has been most widely used in this regard is green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. GFP is a β -barrel-shaped protein of ~ 27 kDa that contains an amino acid triplet (Ser-Tyr-Gly) located in its center that functions as a fluorophore. Molecular engineering of the GFP coding sequence has resulted in optimized expression of GFP in different cell types, as well as the generation of GFP variants with optimal spectral properties, including increased brightness, relative resistance to the effects of pH, and photostability. When expressed within cells, GFP fusion proteins are bright and they are resistant at low illumination to photobleaching, which is the photo-induced alteration of a fluorophore that abolishes its fluorescence. These characteristics allow GFP chimeras expressed within cells to be imaged with low illumination levels over many hours, providing a simple method for visualizing the dynamics and behavior of the tagged protein.

Time-lapse observations of fluorescently tagged proteins can provide information about the steady-state distribution of the protein over time, but they usually do not reveal a protein's kinetic properties. These properties include whether a protein undergoes free or restricted diffusional motion, how rapidly it binds to and releases from other molecules, and whether it undergoes directed movement between compartments. One way to obtain this type of information with fluorescently tagged proteins is to utilize the technique of photobleaching. In this approach, a selected pool of fluorescent proteins is photobleached with a high-intensity laser pulse and the movement of unbleached molecules from neighboring areas into the bleached area is recorded by time-lapse microscopy. Analysis of the observed recovery kinetics can then be used to determine the diffusion coefficient of the fluorescent protein, its binding properties, or its transport routes. *UNIT 21.1* describes the use of fluorescent proteins in photobleaching techniques. Several specific applications of photobleaching are detailed, including fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP), and selective photobleaching. The unit also describes how to extract kinetic parameters from the photobleaching data, including a protein's diffusion coefficient and its $t_{1/2}$ and mobile fraction. Use of these techniques provides a powerful method for characterizing the movement of proteins and their interactions with cellular components in the environment of the living cell.

UNIT 21.2 describes the technique known as fluorescence localization after photobleaching (FLAP), which allows localized photolabeling of proteins and subsequent tracking of these molecules in living cells. This technique, like FRAP and FLIP described in *UNIT 21.1*, overcomes the limitations of conventional fluorescence localization by revealing the relocation of molecules during dynamic, steady-state processes such as diffusion and polymerization/depolymerization. The principle underlying FLAP is simple and relies on photobleaching to label the targeted molecules. This is accomplished by labeling

the molecular species of interest with two different fluorophores that can be imaged independently but simultaneously by fluorescence microscopy. Then one of the fluorophores is rapidly photobleached at a chosen location. Because the unbleached (reference) fluorophore remains co-localized with the target fluorophore, the subsequent fate of the photobleached molecules can be revealed by processing simultaneously acquired digital images of the two fluorophores. The FLAP method thus overcomes one drawback of FRAP and FLIP by enabling the bleached as well as unbleached molecules to be tracked in an experiment.

Jennifer Lippincott-Schwartz

Measuring Protein Mobility by Photobleaching GFP Chimeras in Living Cells

This unit describes photobleaching methods used in combination with green fluorescent protein (GFP) chimeras to analyze protein and organelle dynamics in living cells. Photobleaching is the photoinduced alteration of a fluorophore that abolishes the fluorophore's fluorescence signal. The diffusive characteristics of fluorescently tagged proteins or organelles in the cell can be studied by photobleaching a selected region of a fluorescently labeled cell with intense light and then quantitating the movement of nonbleached fluorescent molecules into the photobleached area using an attenuated light source (Lippincott-Schwartz et al., 1999, 2001; Meyvis et al., 1999). Other processes that lead to fluorescence recovery into the bleached area (including vesicle transport or flow-based movement) also may be quantitated and studied by this method. Insights into a variety of aspects of protein and organelle dynamics thus can be addressed using photobleaching. These include: (1) estimation of the diffusion coefficient, D , of a fluorescent protein, (2) determination of the fraction of fluorescent molecules able to move under different cellular conditions, (3) assessment of continuity or discontinuity of an organelle, and (4) characterization of protein rates to or from an organelle (Ellenberg et al., 1997; Hirschberg et al., 1998; Partikian et al., 1998; Dayel et al., 1999; Marguet et al., 1999; Nehls et al., 2000). Photobleaching can also be used to reduce fluorescence from background noise to reveal faint populations of fluorescent proteins.

The usefulness of photobleaching methodologies depends on the availability of fluorescently labeled molecules. Until recently, these were limited to fluorescent dyes and to fluorescently labeled antibodies, which only permitted labeling of the plasma membrane, unless microinjected into cells. With the advent of GFP, a naturally fluorescent protein from the jellyfish *A. victoria* (Tsien, 1998), this has changed. Virtually any protein of interest can be tagged with GFP. When expressed within cells, these proteins usually retain their parent protein-targeting behavior. An important property of GFP is that it can be bleached without detectable damage to the surrounding environment. This is presumably because the GFP's cage-like structure, which surrounds a small cyclic peptide fluorophore (Prendergast, 1999), shields the external environment from any damaging effects caused by reactive photobleaching intermediates. These characteristics of GFP chimeras make them ideal reagents in photobleaching experiments.

In this unit, two photobleaching techniques for confocal laser scanning microscopes (CLSM) are described: FRAP (fluorescence recovery after photobleaching; see Basic Protocol 1 and Alternate Protocol 1) and FLIP (fluorescence loss in photobleaching; see Basic Protocol 2). In FRAP, a distinct region of interest in a cell expressing a GFP chimera is briefly photobleached with a high-intensity laser, and the movement of unbleached fluorescent molecules into the bleached region is followed with low-intensity laser light. FRAP is useful for determining a protein's diffusion coefficient (D), which measures the random movement or Brownian motion of a molecule. Knowing a protein's D can be used, in turn, to obtain information about the viscosity of a protein's environment and whether the protein is part of a much larger complex. FRAP also can be used to determine the fraction of molecules capable of recovering into a photobleached area, referred to as the mobile fraction (M_f). FLIP differs from FRAP in that photobleaching is repeated several times, alternating each photobleach with a low-laser-intensity image of the whole cell. FLIP reveals the connectedness of cellular compartments and whether immobile pools of proteins are spatially segregated.

The protocols are described for mammalian cells, and the concepts can apply to other systems, including plant cells, yeast cells, and bacteria. Basic Protocols 1 and 2 (FRAP and FLIP, respectively) and Alternate Protocol 1 (FRAP with older confocal laser scanning microscopes) describe photobleaching techniques that can be used to visualize and quantitate protein mobility and organelle dynamics. Alternate Protocol 2 is a selective photobleaching protocol, which can be applied to the study of protein trafficking and protein flux through organelles. Support Protocol 1 discusses some practical issues of expressing and imaging GFP chimeras in living cells. In that protocol, preparation of transfected mammalian cells for imaging on a CLSM and setup of the CLSM is described. Data processing for FRAP is described in Support Protocol 2. Support Protocol 3 describes a simulation of inhomogeneous diffusion that can be used to obtain the diffusion coefficient D .

**BASIC
PROTOCOL 1**

**QUANTITATIVE FRAP BY STRIP PHOTBLEACHING USING A LASER
SCANNING CONFOCAL MICROSCOPE CAPABLE OF SELECTIVE
PHOTBLEACHING**

Fluorescence recovery after photobleaching (FRAP) can be performed by irreversibly photobleaching a fluorescent marker in a region of a cell with a high-intensity laser beam and then following diffusion of unbleached fluorescent proteins into the bleached region by imaging with nonbleaching attenuated laser light (Fig. 21.1.1A). Several forms of FRAP or FPR (fluorescence photobleaching recovery) have been described. Popular methods include spot bleaching, pattern photobleaching, polarized photobleaching, and strip bleaching. The protocols in this unit will focus on the strip photobleach method for confocal laser scanning microscopes. Strip photobleaching permits imaging of the whole cell while monitoring the fluorescence recovery into the photobleached region of interest (ROI). It allows the investigator to readily determine information concerning the spatial distribution of fluorescence. It also can reveal whether the cell, stage, or focus have moved during imaging, events that make FRAP analysis difficult.

There are two types of strip FRAP protocols: quantitative and qualitative. Using the quantitative FRAP protocol, it is possible to obtain an effective diffusion coefficient, D , and to calculate the mobile fraction, M_f , of a fluorescent protein. Images are acquired rapidly and the data can be used to plot a recovery curve from which D can be determined (Fig. 21.1.1B). The qualitative protocol is used to obtain high-quality images for visualizing the diffusion process in a single cell. Because the images are obtained by line averaging and slow scan speeds, they take longer to acquire and therefore are usually unsuitable for calculation of D , which requires time points immediately after bleaching.

This FRAP protocol has been designed for the Zeiss LSM 510 microscope (using the physiology software package) and for other laser scanning confocal microscopes capable of selective photobleaching (e.g., from Leica and Bio-Rad). FRAP can also be performed using older laser scanning confocal microscopes without the selective photobleaching capacity (see Alternate Protocol 1). Selective photobleaching means that the investigator can bleach any shape or size of ROI. Specific settings will vary for each microscope. The user must determine conditions for photobleaching and postbleach recovery imaging that do not result in significant photobleaching of the cell (steps 1 to 6). The actual FRAP experiment is described in steps 7 to 9, and data analysis is described in the FRAP data-processing section (see Support Protocol 2).

Materials

- Cell samples expressing GFP chimeric protein of interest (Support Protocol 1)
- Confocal laser scanning microscope capable of selective photobleaching (e.g., Zeiss LSM 510)

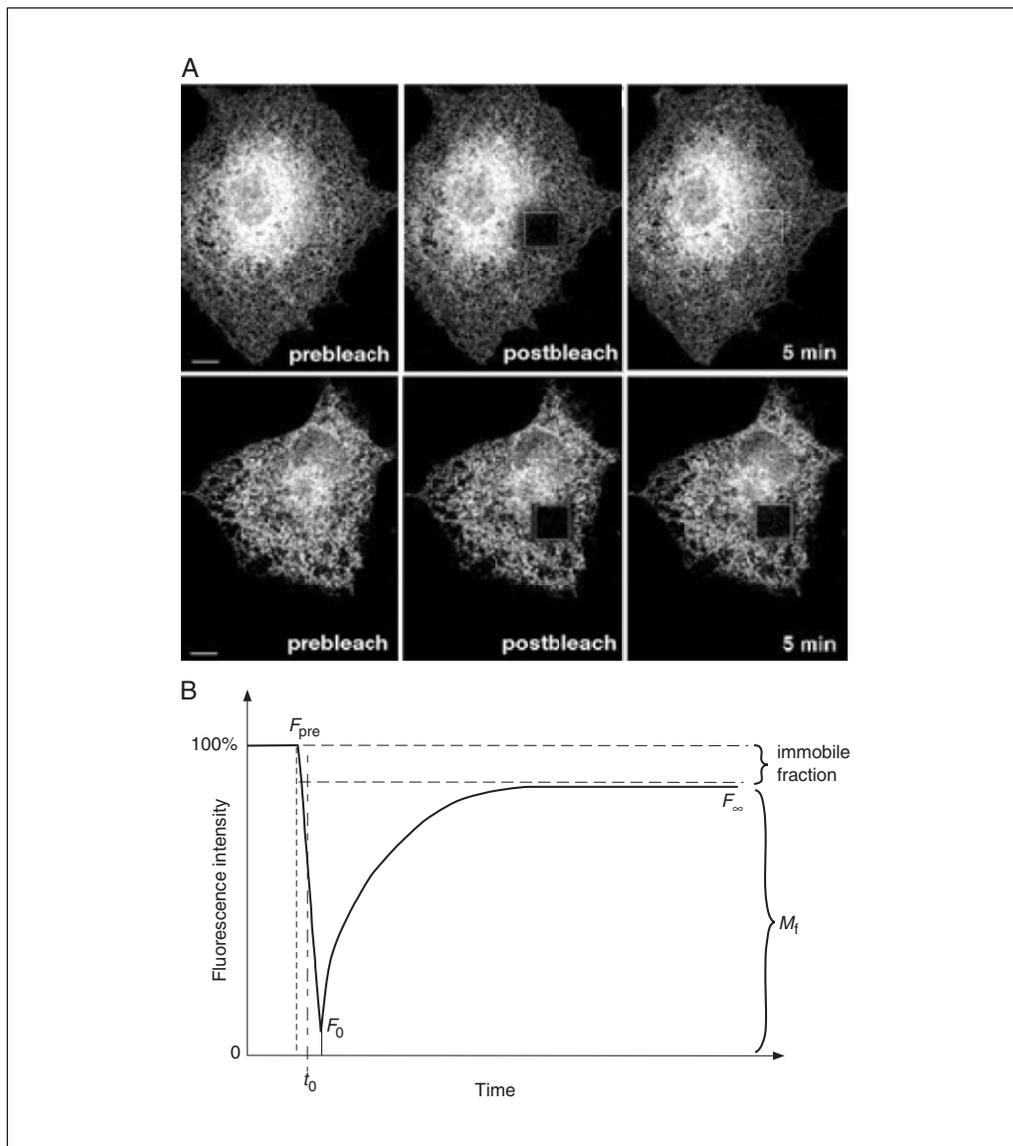


Figure 21.1.1 Photobleaching techniques. **(A)** Example of FRAP. Cells expressing VSVG-GFP were incubated at 40°C to retain VSVG-GFP in the ER under control conditions (untreated, upper row) or in the presence of tunicamycin (lower row). FRAP revealed VSVG-GFP was highly mobile in ER membranes at 40°C but was immobilized in the presence of tunicamycin. **(B)** Plot of fluorescence intensity in a ROI versus time after photobleaching a fluorescent protein. The prebleach (F_{pre}) is compared with the asymptote of the recovery (F_{∞}) to calculate the mobile and immobile fractions. Information from the recovery curve (from F_0 to F_{∞}) can be used to determine D of the fluorescent protein.

Additional reagents and equipment for preparing imaging chambers and microscope for photobleaching experiments (Support Protocol 1)

Establish FRAP conditions

1. Set up the microscope and prepare cells in imaging chamber with imaging medium as described in Support Protocol 1. Prewarm the imaging stage to 37°C or other desired temperature and warm up the microscope lasers for at least 5 min to avoid power fluctuations during imaging.
2. Identify the cell of interest on the confocal microscope. Bring it to the desired focus. Scan an image of the whole cell at the desired excitation light intensity, line averaging,

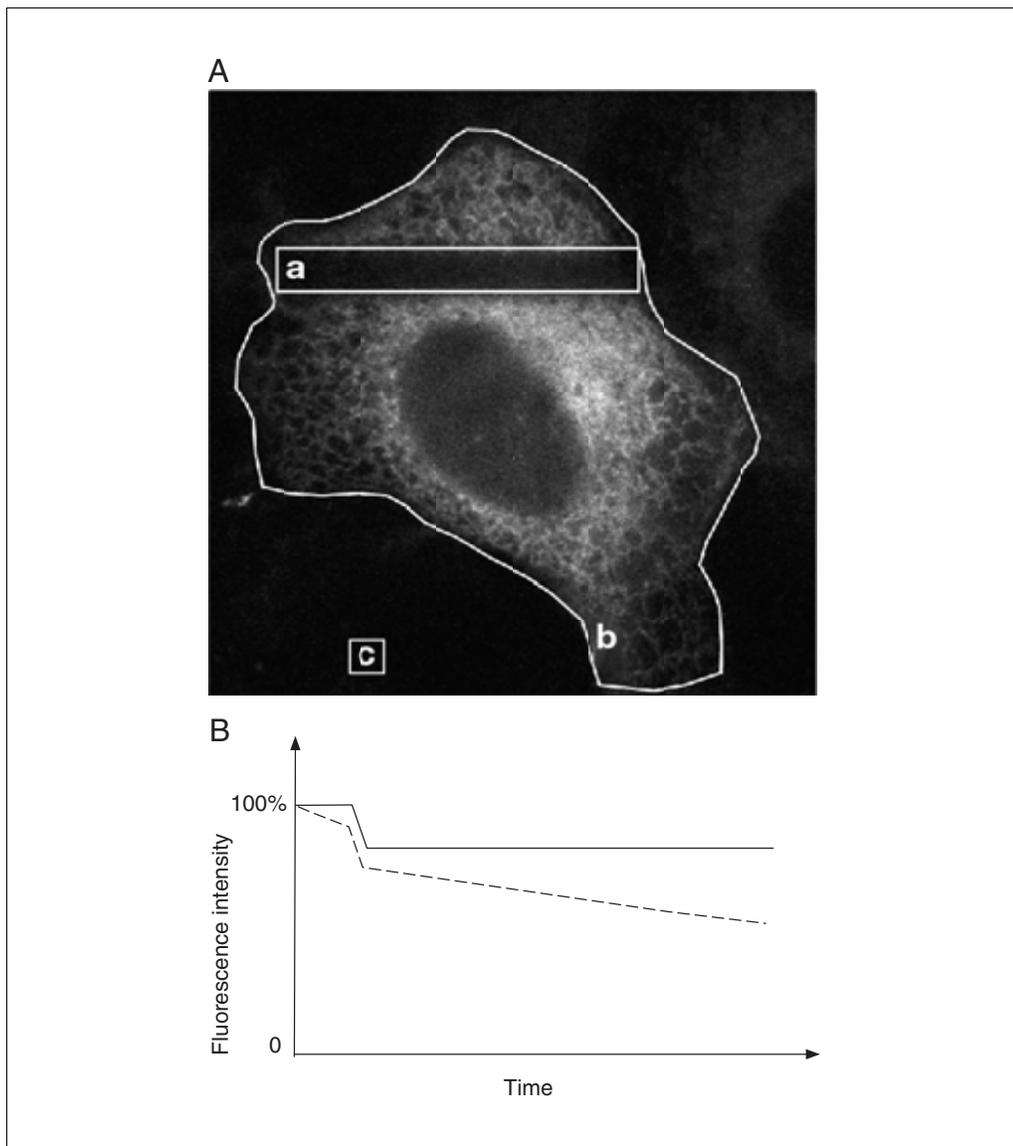


Figure 21.1.2 Defining ROIs and illustrating bleaching during imaging. **(A)** Example of ROIs, where *a* is the bleach ROI, *b* is the whole cell ROI, and *c* is the background ROI. **(B)** Plot of whole cell fluorescence (ROI *b*) versus time. The solid line represents an ideal plot in which the prebleach intensity of the cell does not change with time and where after the photobleach there is a drop in mean cellular fluorescence. There is no further loss of fluorescence. The dashed line represents a problematic plot of ROI *b*. The prebleach intensity steadily decreases with time, suggesting that the cell is being imaged at too high a laser power, resulting in photobleaching of the whole cell. The fluorescence loss continues after the photobleach of ROI *a*. The investigator must establish new imaging conditions.

zoom, and other parameters. Modify pinhole and detector gain for maximal fluorescence signal with no pixel saturation (pixel intensities that exceed the detector scale, i.e., >255 for an 8-bit image).

Saturated pixels only register as the maximum detector value, 255, so the true intensity of the pixel cannot be calculated under these conditions. Detector gain and offset will vary depending on the concentration of the fluorophore, the laser power, and the width and thickness of the fluorescently labeled organelle or region. The imaging parameters used for this image should be used for the fluorescence recovery time series. It may be useful to

record the detector gain settings to compare recoveries in cells expressing high versus low amounts of fluorescent protein.

3. Define a region-of-interest (ROI) for the photobleach (see Fig 21.1.2A), usually a 2- to 4- μm strip across the width of the cell.

For soluble proteins that diffuse rapidly, the photobleach ROI may need to be relatively large (i.e., one-third to one-half of the cell). When performing FRAP on a discrete organelle, such as the nucleus or the Golgi complex, the photobleach ROI does not need to extend across the entire cell. It is sufficient for the edges of the strip to extend beyond the edges of the structure being analyzed. Note that larger photobleach ROIs will require longer recovery times.

4. Empirically determine photobleaching conditions (i.e., scan speed, zoom, laser power, microscope objective, and the minimal number of laser iterations required for photobleaching) so that after photobleaching, the fluorescent signal of the photobleach ROI decreases to within background intensity levels. Use imaging software to quantitate fluorescence intensity in the photobleach ROI and the whole cell prior to bleaching (Fig. 21.1.2A)

It is necessary to know the fluorescence intensities in the whole cell and ROI prior to bleaching in order to determine the extent to which the photobleach ROI and the whole cell undergo bleaching during the course of imaging (Fig. 21.1.2B).

It is useful to establish photobleaching conditions on fixed samples, as rapidly diffusing species may be impossible to bleach to background levels. Fix the plated GFP-expressing cells (either on a coverslip or in a Lab-Tek chamber) for 15 min in PBS (APPENDIX 2A) containing 4% formaldehyde at room temperature. Wash twice with PBS and then either place the coverslip on an imaging chamber with imaging medium or fill the Lab-Tek chamber with imaging medium. Under no circumstances must an antifade reagent (such as Fluoromount-G or phenylenediamine) be used, because this will significantly alter the photobleaching properties of the fluorophore. Image the fixed cells in imaging medium.

Typical bleaching conditions require a 100 to 1000-fold increase in laser power (decrease in attenuation) for 1 to 5 bleach iterations (roughly 0.01 to 0.5 sec) for many organelles. If an ROI requires >20 sec to photobleach, a more powerful laser is necessary (see below).

To ensure that bleaching laser power does not damage the cell, it is useful to repeat FRAP on the same ROI for the same cell. The diffusion coefficient (see discussion of data processing under Support Protocol 2) now should not change, but the mobile fraction (see Support Protocol 2) should be close to 100%. This is because any immobile fluorophores in the first FRAP will have been bleached and therefore will not contribute to the percent recovery observed in the second photobleach.

5. For acquisition of fluorescence recovery time points, empirically determine imaging conditions that do not significantly photobleach the cell outside of the bleach ROI (Fig. 21.1.2B)

Recommended conditions for photobleaching with a 40-mW 488/514 nm argon or 25-mW argon laser are 45% to 60% power with 100% transmission. For acquisition of recovery time points, use the same power with 0.1% to 1.0% transmission. For quantitative FRAP, the whole cell is usually scanned at scan speed 8 to 10 (0.798 to 3 sec per 512×512 frame) with either two-line averaging or no-line averaging. In qualitative FRAP experiments, where the goal is to obtain high-quality images, the intervals at which images are collected during recovery need not be rapid (3- to 10-sec intervals, for membrane proteins). Soluble and luminal proteins tend to diffuse rapidly and both quantitative and qualitative FRAP may require the bleaching of a large ROI and very rapid imaging conditions.

Collect FRAP data

6. To perform data analysis of a FRAP experiment, collect a prebleach image of the cell, a series of postbleach images of the whole cell that extend from an immediate

postbleach image to several images after the bleach ROI fluorescence intensity reaches a plateau, and a table of fluorescence intensity values including the photobleach ROI, the whole-cell ROI, and a background ROI. Note the corresponding time for each image and the pixel size in microns.

For the Zeiss 510 microscope, the physiology software contains several windows which permit the user to determine the number of images to collect, laser intensities for bleaching and imaging, number of bleach iterations, how many images to collect before bleaching, and drawing of the photobleach ROI. Other microscopes may possess their own software packages or may require writing macros for the photobleach time series.

7. Collect multiple prebleach images to establish the prebleach fluorescence intensity and to confirm that the prebleach fluorescence intensities of the cell and the bleach ROI do not fluctuate significantly.
8. Using the conditions determined in step 4, photobleach the ROI with intense laser illumination. Continue to image the whole cell at low laser illumination (the same conditions as the prebleach images) until the recovery process has reached a steady state.

This must be determined quantitatively, as the human eye is incapable of distinguishing small differences in intensity. Typically, a 4- μm wide bleach ROI strip will recover within 90 to 120 sec for most freely mobile soluble luminal proteins and within 350 sec for membrane proteins in the plasma membrane, endoplasmic reticulum, or Golgi. Cytoplasmic proteins may recover as rapidly as 5 to 20 sec. In this way, the investigator can establish conditions to obtain data sufficient for estimation of $t_{1/2}$ and M_f (see Support Protocol 2). The investigator should not attempt to refocus a cell during a FRAP experiment, because the fluorescence intensities will shift and the recovery curves will no longer be smooth. Time series that contain a shift in focal plane should be discarded.

If a protein has a high protein diffusion coefficient (e.g., 5 $\mu\text{m}^2/\text{sec}$), the investigator may not observe a defined bleach region following the photobleach. Rapidly diffusing fluorescent proteins may appear homogeneously distributed immediately following a photobleach. The investigator must either: (1) increase the image collection rate, by changing the scan speed or reducing the size of the total frame to be collected, (2) increase the bleach ROI size, (3) increase the laser power, or employ a combination of these suggestions.

To ensure that FRAP did not damage the area that was being scanned, repeat FRAP on the same area and compare the diffusion coefficients obtained from both experiments. D should remain the same but the mobile fraction should now be close to 100%. This is because the immobile fraction was bleached in the previous experiment.

9. Collect at least 10 to 20 data sets for each fluorescently labeled protein and treatment for statistical analysis.

A fraction of the data sets are usually discarded because of problems that potentially bias imaging results (e.g., recovery was not complete, the focal plane shifted, or the recovery curve-fitting method failed).

In Lippincott-Schwartz et al. (1999), a method for imaging the bleach ROI alone for rapid collection of a large number of data points is described. The caveat of the method is that the cell may shift in focus or position during the course of imaging. Imaging the whole cell offers the advantage of visualizing any movement of the cell or focal plane, thus allowing the researcher to reject unusable data upon visual inspection.

FRAP WITH CONFOCAL LASER SCANNING MICROSCOPES WITHOUT THE CAPACITY FOR SELECTIVE PHOTBLEACHING

ALTERNATE
PROTOCOL 1

FRAP (see Basic Protocol 1) can also be performed with older confocal laser scanning microscopes. Photobleaching is accomplished by zooming in on a small region of the cell and scanning with full laser power. The procedure for photobleaching is described below. The actual FRAP protocol is otherwise identical to Basic Protocol 1. For quantitative FRAP experiments for determining D , images should be acquired rapidly after the bleach, and this is best done if fluorescence is collected from the ROI only. If the investigator acquires a prebleach and a postrecovery image of the whole cell, it will be possible to calculate the M_f and D .

Additional Materials (also see Basic Protocol 1)

Confocal laser scanning microscope without capacity for selective photobleaching (e.g., Zeiss LSM 410)

Photobleach by zooming

1. Define a region of interest (ROI) for the photobleach. Define the ROI to be bleached at the highest zoom possible (usually zoom 8).

At high zooms the laser will dwell longer on an ROI per line scan and thus will deliver more bleaching radiation.

2. To photobleach the ROI, set laser power at maximum and remove all neutral density filters from the path of the laser beam. Scan (photobleach) the ROI.
3. Bleach a desired ROI with high-intensity light and then rapidly and reliably switch to scanning the ROI with low levels of light and collect emission.

A photobleaching macro can be written for the confocal system to perform bleaching and recovery imaging automatically.

CELL TRANSFECTION AND IMAGING SETUP

SUPPORT
PROTOCOL 1

Cells first must be transfected with a construct in which DNA for GFP is linked to the protein of interest. Then the cells must be prepared for imaging. Transiently transfected adherent cells can be grown and imaged in a LabTek chambered slide or on a coverslip. Transfected suspension cells must be attached to a coated coverslip for imaging.

Materials

Eukaryotic cells of interest

Vector for expression of GFP chimeric protein: most laboratories today use the enhanced GFP (EGFP) variant (Clontech); although spectral variants of GFP are available, EGFP is best suited for photobleaching experiments; see Commentary for details

Cell culture medium (e.g., DMEM with serum) appropriate for cells of interest

Imaging medium (see recipe)

Silicon grease (optional)

5 to 10 mg/ml poly-L-lysine (Sigma) in PBS (see APPENDIX 2A for PBS)

Phosphate-buffered saline (PBS; APPENDIX 2A)

Confocal laser scanning microscope equipped with a filter set for GFP and a 488-nm excitation laser that is at least 10 mW and preferably ≥ 25 mW

Imaging chamber: chambered coverglass system (LabTek from Nalgene) *or* glass coverslips and silicon rubber gasket for constructing silicon rubber chamber (see recipe in UNIT 4.4; also see Fig. 4.4.1)

Immersion oil (for oil-immersion microscope objectives)

Fluorescent
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21.1.7

Stage heater (e.g., Model ASI 400 Air Stream Stage Incubator, Nevtek; for cells that grow at 37°C)

Temperature probe: e.g., Thermolyne pyrometer (Cole-Parmer)

Computer system capable of processing large image files: 350 MHz or faster processor, multiple gigabyte hard drive, and at least 128 MB RAM

Image processing software (i.e., NIH Image or Metamorph)

Additional reagents and equipment for transfection of eukaryotic cells by electroporation (*UNIT 20.5*) or use of lipid transfection reagents (*UNIT 20.6*)

Transfect cells

1. To enhance the probability of having multiple cells to choose from, transfect cells with GFP chimeric protein vector using a high-efficiency transfection method, such as electroporation (*UNIT 20.5*) or a lipid transfection reagent—e.g., FuGENE6 (Roche) or LipofectAMINE 2000 (Invitrogen)—as described in *UNIT 20.6*.

The cell density for imaging depends on cell type, cell size, and experimental design.

Before performing a photobleaching protocol, the investigator must ensure that there is sufficient GFP fluorescence in the expressing cell to maintain a significant fluorescent signal relative to background noise after photobleaching. Most standard transfection protocols are sufficient to provide bright specimens. Stable transfectants express lower levels of protein. Transient transfectants usually express higher levels of proteins; this sometimes results in overexpression artifacts, such as protein aggregation or saturation of protein targeting machinery, which lead to inappropriate localization. Whichever transfection method is selected, the investigator needs several usable cells for each experiment.

Adherent cells should be transiently transfected 16 to 36 hr prior to the experiment. The commercially available GFP expression vectors are under the control of a very strong promoter, the CMV promoter, so cell toxicity or mislocalization of GFP chimeras to other regions of the cell should always be a concern.

2. Determine the optimal level of expression and timing of imaging empirically for each sample and condition.

Set up imaging system

3. Set up the confocal laser scanning microscope and its associated hardware.

It is assumed that the investigator is familiar with the basic operation of a confocal microscope. The investigator should understand both the concept and the operation of a pinhole, as well as the considerations related to scan speed, zoom, detector gain, laser power, photobleach, and collection of a time series.

Set up the imaging chamber

To image adherent cells on chambered slides

- 4a. Plate adherent cells on LabTek chambered coverglass.

The cell density for imaging depends on cell type, cell size, and experimental design.

These chambers consist of wells with a cover glass bottom, which permits the use of high-numerical-aperture oil objectives for viewing.

- 5a. Fill the wells to the rim with imaging medium and place on the stage of an inverted microscope.

This ensures that cells have sufficient nutrients during the imaging session. For experiments that will last longer than 1 hr, the top cover of the chamber should be sealed onto the chamber using petroleum jelly or silicon grease. These steps prevent rapid evaporation of the medium above the cells and decrease the alkalization of the medium by preventing room air from entering the chamber.

To image adherent cells in silicon rubber chambers

4b. Plate cells on glass coverslips.

The cell density for imaging depends on cell type, cell size, and experimental design.

5b. Invert the cell-containing coverslips on rubber gaskets that have a hole punched in them to form silicon rubber chambers (see UNIT 4.4 and Fig. 4.4.1).

These gaskets are mounted on glass slides with petroleum jelly or silicon grease. The hole is filled with imaging medium and the coverslips are inverted and pressed onto the hole allowing the cells to face the medium. The coverslip adheres to the gasket by capillary action. Excess liquid from the top of the coverslip is wicked away with absorbant tissue. See UNIT 4.4 for full details.

To image suspension cells

4c. Precoat LabTek chambers or clean coverslips 5 to 10 mg/ml poly-L-lysine in PBS. Incubate for 15 min. Wash twice with PBS or distilled water.

The coating is usually good for up to 1 week.

5c. Wash suspension cells three times in PBS, then place them on the poly-L-lysine-coated surface and incubate 2 to 5 min. Remove nonadhering cells by washing twice with PBS. Finally, immerse the cells in imaging medium.

The cell density for imaging depends on cell type, cell size, and experimental design.

6. Keep cells warm on the microscope stage using a stage heater, and use a temperature probe to confirm that the proper temperature is maintained at the coverslip/chamber.

In addition to the chambers described above, Bioprotech offers a completely closed chamber with built-in perfusion. This type of setup permits the maintenance of cells on the microscope stage for extended periods of time (>24 hours) with minimal loss in viability. In addition, the investigator can perfuse drugs, dyes, and other reagents with controlled precision throughout the experiment. Thermal collars for objectives are another option for heating/maintaining the temperature of the chamber/coverslip. Thermal collars are available from Bioprotech.

Collect data

7. Collect FRAP data as described in Basic Protocols 1 or 2 or Alternate Protocols 1 or 2.

Many laser scanning confocal microscopes provide the investigator with a number of options for data collection, including but not limited to image size (e.g., 512 × 512 or 1024 × 1024, pixels), range of data collection (8-bit or 12-bit), and file formats. It is worth determining the requirements for image-analysis software in advance. For example, some image-analysis programs (see, e.g., Support Protocol 3) cannot process 12-bit images or only process PGM (portable graymap) image files, not TIFF (tagged-image file format) files.

See Support Protocol 2 for processing of FRAP data.

FRAP DATA PROCESSING

After collecting FRAP data using either Basic Protocol 1 or Alternate Protocol 1, the data need to be processed for quantitation. The three parameters that can be determined are the mobile fraction (M_f) of the fluorescent fusion protein, its diffusion coefficient (D), and the $t_{1/2}$ of the fluorescence recovery. When first performing FRAP experiments, it is an excellent idea to perform calibration experiments with a fluorescent protein whose D and M_f have been previously characterized (see Table 21.1.1).

**SUPPORT
PROTOCOL 2**

**Fluorescent
Protein
Technology**

21.1.9

Table 21.1.1 Diffusion Rates of GFP and GFP Chimeras Using FRAP^a

Molecule	D_{eff} ($\mu\text{m}^2/\text{sec}$)	References
GFP in water	87	Swaminathan et al. (1997)
GFP in cytoplasm	25	Swaminathan et al. (1997)
GFP in the ER lumen	5-10	Dayel et al. (1999)
Elastase-GFP in the ER lumen	0.5	Subramanian and Meyer (1997)
GFP in the mitochondrial matrix	20-30	Partikian et al. (1998)
Nucleoplasm		
GFP-HMG-17	0.45	Phair and Misteli (2000)
GFP-SF2/ASF	0.24	Phair and Misteli (2000)
GFP-fibrillarin	0.53	Phair and Misteli (2000)
Endoplasmic reticulum membrane		
Lamin B receptor-GFP (in ER)	0.35	Ellenberg et al. (1997)
GFP-MHC class I (murine)	0.46	Marguet et al. (1999)
TAP1-GFP (murine)	0.12	Marguet et al. (1999)
Galactosyltransferase-GFP (in ER)	0.48	Nehls et al. (2000)
Signal recognition particle β subunit-GFP	0.26	Nehls et al. (2000)
KDEL receptor-GFP (+BFA)	0.43	Cole et al. (1996)
VSVG-GFP ts045 (in ER+BFA, 32°C)	0.49	Nehls et al. (2000)
VSVG-GFP ts045 (in ER, 40°C)	0.45	Nehls et al. (2000)
Cytochrome P450-GFP	0.03-0.06	Szczesna-Skorupa et al. (1998)
Golgi apparatus membrane		
Galactosyltransferase-GFP (in Golgi)	0.54	Cole et al. (1996)
Mannosidase II-GFP	0.32	Cole et al. (1996)
KDEL receptor-GFP	0.46	Cole et al. (1996)
Plasma membrane		
E-cadherin-GFP	0.03-0.04	Adams et al. (1998)
GFP-Ki-Ras (12V)	0.19	Niv et al. (1999)
GFP-aquaporin (1 and 2)	0.009	Umenishi et al. (2000)
Lutenizing hormone receptor-GFP	0.16	Horvat et al. (1999)
Endosomal membrane		
GFP-rab5	0.1	Roberts et al. (1999)
<i>E. coli</i> cytoplasm		
GFP	7.7	Elowitz et al. (1999)
Maltose D-GFP	2.5	Elowitz et al. (1999)

^aAbbreviations: BFA, brefeldin A; ER, endoplasmic reticulum; GFP, green fluorescent protein; MHC, major histocompatibility complex; TAP, transporter associated with antigen processing; VSVG, vesicular stomatitis virus G protein.

Mobile fraction (M_f)

M_f refers to the percentage of fluorescent proteins capable of diffusing into a bleached ROI during the time course of the experiment (see Fig. 21.1.1B). M_f and D are distinct parameters and must be understood as such. D is a characteristic of the mobile pool of fluorescent proteins. The proteins in the immobile fraction do not diffuse.

An approximation of M_f can be calculated using the following equation, based on Feder et al. (1996), with the inclusion of a photobleaching correction (Lippincott-Schwartz et al., 1999):

$$M_f = 100 \times \frac{F_{\text{precell}} - F_{\text{background}}}{F_{\infty\text{cell}} - F_{\text{background}}} \times \frac{(F_{\infty\text{cell}} - F_{\text{background}}) - (F_0 - F_{\text{background}})}{(F_{\text{pre}} - F_{\text{background}}) - (F_0 - F_{\text{background}})}$$

Equation 21.1.1

where F_{precell} is the whole cell prebleach intensity, F_{pre} is the bleach ROI prebleach intensity, $F_{\infty\text{cell}}$ is the asymptote of fluorescence recovery of the whole cell, $F_{\text{background}}$ is the mean background intensity, F_{∞} is the bleach ROI asymptote, and F_0 is the bleach ROI immediate postbleach intensity.

In the above equation, the bleach ROI and whole cell ROIs are background-subtracted. Next, the bleach ROI data are transformed such that the prebleach fluorescence intensity is defined as 100% fluorescence intensity. The equation includes a correction for the loss of total cellular fluorescence (due to the photobleach of the ROI and bleaching of the whole cell during imaging). The correction is calculated by determining the prebleach fluorescence intensity of the whole cell ROI (F_{precell}) and dividing it by the whole cell ROI intensity at time t . Without this bleach correction, the bleach ROI intensity can never recover to 100% of the prebleach fluorescence because the photobleach depletes 5% to 20% of the total cellular fluorescence. Finally, to convert the fraction to a percentage, it is multiplied by 100.

The F_0 value is an approximation for the fluorescence intensity immediately following the photobleach. For conditions in which the ROI is bleached to near background levels, F_0 will be relatively accurate. However, for rapidly diffusing proteins or for narrow bleach ROIs, the immediate postbleach ROI intensity may be closer to 30% to 40% of the prebleach fluorescence intensity. This can lead to the appearance of an artificial immobile fraction. The true F_0 may be much lower and can potentially be derived by rapidly collecting images of the bleach ROI alone, or by attempting to determine F_0 by nonlinear regression analysis.

Diffusion Analysis

The diffusion coefficient, D , reflects the mean-square displacement (usually $\mu\text{m}^2/\text{sec}$ or cm^2/sec) that a protein explores through a random walk (i.e., Brownian motion) over time. It is important to recognize that D is not a simple linear rate (see Fig. 21.1.3). That is, the time required to cover increasing distances will not increase in a linear fashion. Time increases as the square of the distance covered divided by the D . The primary constraints on protein diffusion are the viscosity of the environment, whether the protein is soluble or integrated into a membrane, and, to a lesser extent, the molecular radius of the protein. Protein-protein interactions and collisions with other molecules also hinder free diffusion. By measuring D , the investigator can obtain information related to a protein's environment and in some cases, whether the protein is interacting with large complexes.

Several groups have derived a number of equations and computer simulations to determine D . The majority of available equations have been written for spot photobleaching. Related equations and simulations are available for strip photobleaching, including those described in Ellenberg et al. (1997) and Wey and Cone (1981). These equations, however, make several assumptions about the experimental system that may or may not be met. For example, the equation from Ellenberg et al. (1997) assumes that the bleach is complete, that there is no immobile fraction, that the cell is a uniform rectangle, and that the width of the bleach ROI is much less than the distance to either end of the cell. D values calculated with this equation can differ by 50% or more from an assumed D . Given these concerns, investigators are cautioned in the use this equation.

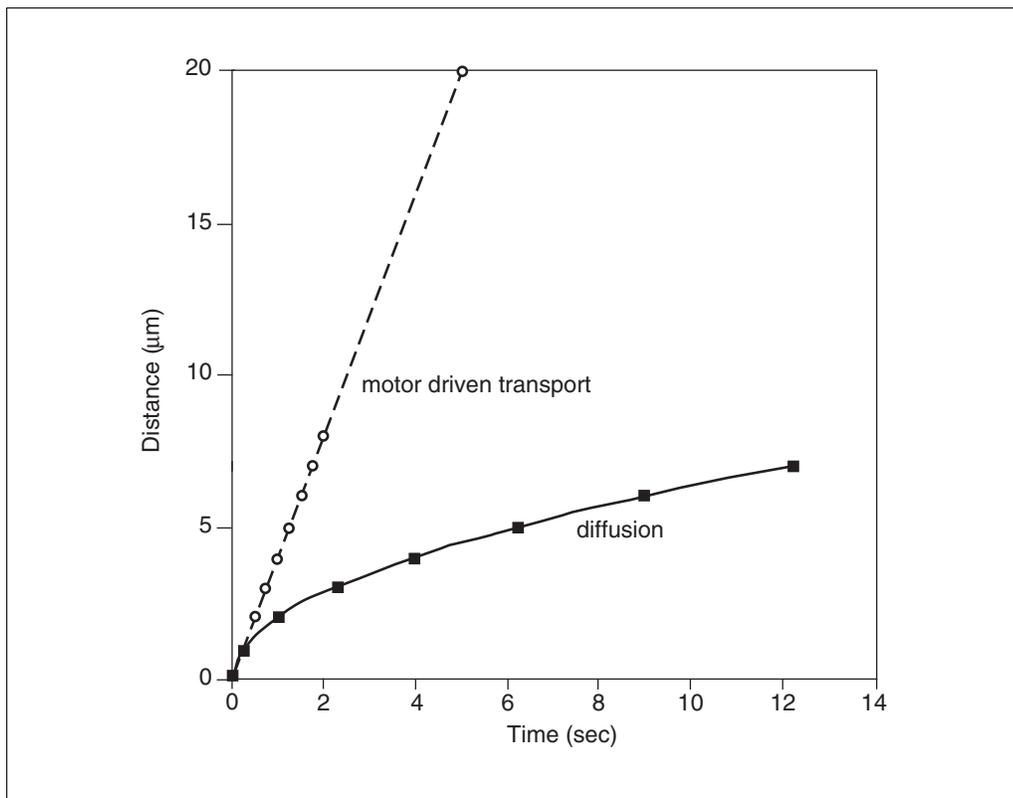


Figure 21.1.3 Diffusion versus linear movement. Plot of distance versus time for a protein, such as a motor protein, that moves directionally at a linear rate of $4 \mu\text{m}/\text{sec}$ (open circles, dashed line) and a protein that diffuses with a diffusion coefficient of $4 \mu\text{m}^2/\text{sec}$ (filled squares, solid line). Note that a protein travels $4 \mu\text{m}$ in 1 sec for the linear rate, but statistically takes 4 secs to travel the same distance by diffusion.

The best method the authors have found for obtaining D is through simulation of diffusive recovery into a strip bleach (Siggia et al., 2000). The Siggia simulation models inhomogeneous diffusion of nonbleached proteins in the cell into the photobleach ROI. The simulation then compares the simulated recovery to the actual data to determine D (Siggia et al., 2000). The program has been used for ER membrane proteins (Ellenberg et al., 1997; Zaal et al., 1999) and has been used to calculate D values comparable to values observed by other laboratories using different methods. Like other methods for determining D , simulation encounters difficulties when a significant fraction of the fluorescent protein is immobilized (Siggia et al., 2000). In such cases, the simulation either fails to fit the simulation data to the experimental recovery data or the simulation cannot calculate D . The Siggia simulation can be obtained by contacting Dr. Eric Siggia at siggia@eds1.rockefeller.edu. The simulation has been written for Unix systems and has been compiled for Macintosh computers. After signing the licensing agreement with Dr. Siggia, the Macintosh-compiled simulation can be obtained from the authors of this unit. Support Protocol 3 is a simple user's guide to the simulation.

After running any simulation or calculating a least-squares fit to determine D , it is essential to compare the simulation or fit data to the experimental data by plotting the data sets in a spreadsheet program. Even if a program or equation produces D , a poor fit means that D is questionable at best. "Goodness of fit" describes how well the simulation or equation data overlap the experimental data. The majority of the experimental data must overlap the simulation or equation plot. If the simulation misses the experimental data, the calculated D is questionable, at best. If there are any doubts about "goodness of fit," the user should consider another method of calculating the D .

A number of potential errors may occur in the course of data fitting. These errors are not unique to D calculations. They are part of the general problem of nonlinear regression analysis. Individuals that anticipate performing diffusion analysis are encouraged to become familiar with nonlinear regression analysis. An excellent introduction to the theory and problems of nonlinear regression analysis has been written by Dr. Harvey Motulsky and is available as a free download at <http://www.graphpad.com/www/nonlin.pdf>.

$t_{1/2}$

The mobilities of some proteins depend on complex behaviors, such as binding and release or populations with multiple D values. Such conditions complicate the analysis of D and may prevent fitting of data by traditional diffusion analysis. When a diffusion equation or simulation is unable to fit fluorescence recoveries, the $t_{1/2}$ measure can be used to compare relative recovery rates between samples. The $t_{1/2}$ is the time required for the fluorescence intensity in the bleach ROI to recover to 50% of the asymptote or plateau fluorescence intensity. This measure is independent of the prebleach ROI fluorescence intensity. In addition, it should be emphasized that while the $t_{1/2}$ can be a useful tool, it is only relevant for the user's system and conditions. It cannot be used to relate to $t_{1/2}$ values obtained from other experiments, since the $t_{1/2}$ value is relative to a particular experimental setup. The $t_{1/2}$ value requires two data manipulations: (1) conversion of time with the half-time of the bleach as time zero (t_0), and (2) conversion of bleach ROI recovery data into fractional fluorescence. Note that fluorescence recovery into a photobleach ROI must plateau or $t_{1/2}$ analysis cannot be performed. The $t_{1/2}$ can be determined visually or by solving the following equation (Feder et al., 1996):

$$F(t) = 100 \times [F_0 + F_\infty(t/t_{1/2})] / [1 + (t/t_{1/2})]$$

Equation 21.1.2

where t is the time for each ROI intensity value, usually in seconds or milliseconds, $t_{1/2}$ is the time required for the bleach ROI to recover to 50% of the asymptote (see discussion of $t_{1/2}$, below), and the remaining variables are defined as for Equation 21.1.1.

Fractional Fluorescence

To directly visualize and determine the $t_{1/2}$, transform the fluorescence intensity [$F(t)$] data to a 0% to 100% scale (see Fig. 21.1.4). The measurement is independent of the prebleach intensity and is not bleach corrected, as the relevant data occur after the photobleach. It is critical that the bleach ROI fluorescence recovery reaches a true plateau for accurate measurements. It is also important to have a large signal difference between the first postbleach intensity and the plateau. If the signal difference is too small, the plotted recovery will tend to be very broad and difficult to interpret. The following equation will convert the bleach ROI fluorescence recovery into fractional fluorescence data:

$$F_2(t) = 100 \times [F(t) - F_0] / [F_\infty - F_0]$$

Equation 21.1.3

where $F(t)$ is the bleach ROI fluorescence intensity at time t and the remaining variables are defined as for Equation 21.1.1. Time t must be rescaled relative to t_0 , as described above. The $F_2(t)$ data are then plotted versus time (in seconds) to determine $t_{1/2}$.

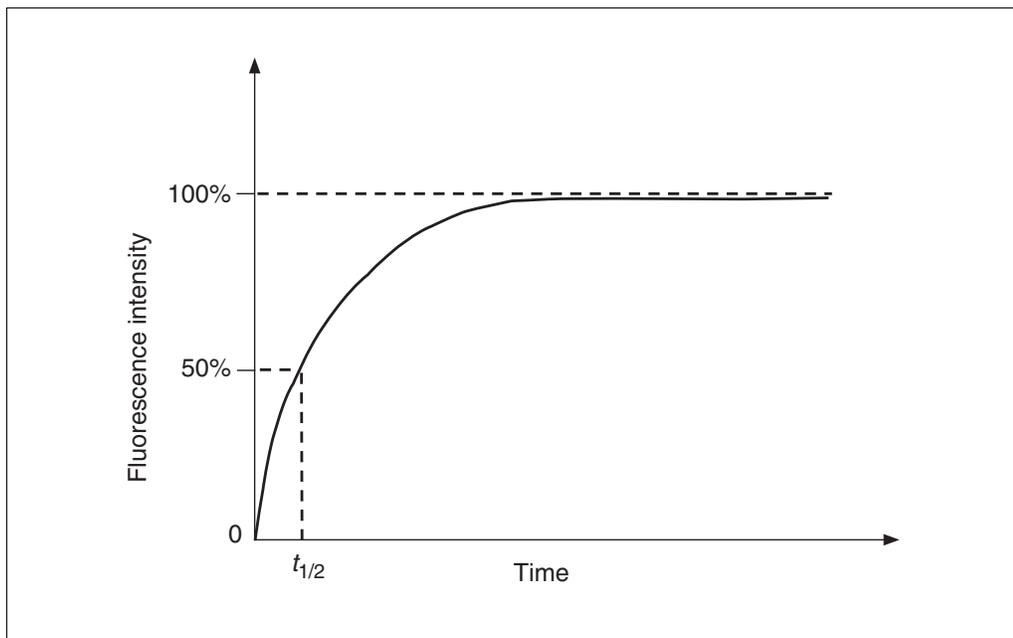


Figure 21.1.4 Fractional fluorescence. A plot of fractional fluorescence can be used to determine $t_{1/2}$, the time at which the fluorescence has recovered to 50% of the asymptote. The recovery data points (from F_0 to F_∞ in Fig. 21.1.1B) have been transformed to a scale of 0% to 100%.

The measurement is sensitive to the initial time (or t_0) of the bleach. When varying the size of the bleach ROI, the time to bleach the ROI will change. Practically, this means that the time for collection of the first postbleach image will vary between samples with different-sized bleach ROIs, which will alter the start time for data collection for each sample. Different groups define t_0 slightly differently. For this unit, t_0 is defined as the half-time of the bleach. To modify the time, subtract t_0 from each time value, where t_0 is defined by the following equation:

$$t_0 = \{[(t_{\text{post}} - t_{\text{pre}}) - (\text{time per image frame})] / 2\} + t_{\text{pre}}$$

Equation 21.1.4

where t_{post} is the time of first image after photobleach and t_{pre} is the time of image immediately prior to photobleach.

**ALTERNATE
PROTOCOL 2**

**SELECTIVE PHOTBLEACHING TO ENHANCE DIM STRUCTURES
WHILE IMAGING OR TO VISUALIZE AND MEASURE NONDIFFUSIVE
TRANSPORT INTO AN ORGANELLE**

Photobleaching has additional uses besides those defined in Basic Protocols 1 and 2 and Alternate Protocol 1. For example, a GFP chimera may localize to two organelles in close proximity. One organelle may accumulate a substantial amount of the protein, while the adjacent organelle is dim. The bright organelle can be photobleached to permit imaging of the dim structure. Another application is to use photobleaching to visualize trafficking or flux through an organelle. For example, the Golgi complex (Nichols et al., 2001) or lysosomes can be photobleached, and then fluorescence recovery—either of fluorescence into the photobleached organelle or fluorescence trafficking out of the unbleached organelle into the photobleached area surrounding the organelle—can be imaged for both qualitative and quantitative analysis. Selective photobleaching essentially follows the FRAP protocol (see Basic Protocol 1). Instead of a strip, the investigator defines an ROI

appropriate to the organelle or structure of interest. Imaging conditions must be determined to photobleach either the structure of interest or around the structure of interest and to avoid bleaching of the overall cell during the course of imaging. When bleaching a bright structure to visualize dimmer structures, the conditions need to be set such that the dim structure or structures are sufficiently bright, but not saturating. The bright structure can be saturated, as it will be photobleached.

SIMULATION OF INHOMOGENOUS DIFFUSION

This simulation (Siggia et al., 2000) is a method for obtaining the diffusion coefficient D through diffusive recovery into a strip bleach (also see Support Protocol 2, discussion of Diffusion Analysis). The Siggia simulation can be obtained by contacting Dr. Eric Siggia at siggia@eds1.rockefeller.edu. The simulation has been written for Unix systems and has been compiled for Macintosh computers. After signing the licensing agreement with Dr. Siggia, the Macintosh compiled simulation can be obtained from the authors of this unit. Before running this simulation on a Macintosh computer (note the authors collect all of their data on a PC system with the confocal microscope and process diffusion data using a Macintosh), a substantial amount of RAM (50 to 100 Mb) should be allotted to the program. When running the simulation, other programs will run very slowly; therefore it is suggested that other programs be turned off. Before running the simulation, determine the time interval between frames and the size of each pixel.

1. To run the simulation, convert the TIFF (tagged-image file format) files to PGM (portable graymap), a format recognized by the simulation program.

TIFF files can be converted to PGM files with the Macintosh program Graphic Converter (http://www.lemkesoft.de/us_gcabout.html). The authors recently discovered a bug in version 4.04 of the program, available online. The program will create PGM files; however, the simulation program does not recognize these files. The authors have used version 4.01 without incident. To run the simulation, the simulation program must be in the same folder as the PGM files. All files generated by the simulation will appear in this folder.

2. Open the first postbleach image and move the cursor to determine the coordinates of the smallest region that contains the whole cell. Write down the coordinate numbers (x , y), which should be in the range between 0 and 511, for the upper left corner of the box and the lower right corner of the box. Repeat for the photobleach ROI.
3. Start the simulation program.

The program is designed to be user-friendly. The first few steps are self explanatory.

4. Enter the name of prebleach file (include full name, e.g., b009.pgm). If any colored saturation pixels are present in the images, the program will request that the user choose a channel (r, g, or b). Assuming the user has used GFP, enter g.
5. When asked Is this is a FRAP? and Are you supplying a series of postbleach images?, answer y for each question.
6. When asked Do you want to supply a background value?, answer n .

Usually the simulation determines an accurate number, though the user is free to change the value.

7. When asked to enter the x,y coordinates of the ROI (note that the program is referring to the whole cell ROI, not the photobleach ROI), enter each coordinate for the upper left-hand corner of the box defined in step 2 (first x and then y) separated by a space (no parentheses or commas). Press the Return key and then enter the lower right-hand coordinates, each separated by a space.

8. When asked if one wants to monitor diffusion in individual rectangles, if one desires to use this option, open the postbleach_grid.pnm file and select grid rectangles of interest.

The coordinates for each rectangle are determined by numbering the rectangles (0 to 9) across and down from the upper left corner of the grid. Each rectangle will correspond to x y , entered with a single space and no punctuation. The user may opt for this method to measure diffusion differences in different regions of the cell. However, the rectangle grid generated by the program will not necessarily align with the photobleach ROI. In addition, D values will be different for different regions of the cell. This information can be useful, but the time required for equilibration of a region of the cell some distance from the photobleach ROI is often much longer than the equilibration time in the photobleach ROI.

9. To obtain D in the bleach box ROI, type n and hit the Return key. Next, enter the coordinates (x y) of the bleach box ROI that were determined using graphic converter or NIH Image. Enter a single space between coordinates with no punctuation.
10. For time, unless one has used different times between frames, type y (without punctuation) if the time intervals between postbleach frames are the same. Next, enter the time between frames in seconds.

Decimals (e.g., 0.798) are permitted.

11. Hit the Return key to run the simulation.

The program takes 3 to 10 min to run depending on computer speed, the size of the cell ROI, and the number of images to be processed. The program becomes especially slow after the words "Starting inhomogeneous diffusion" appear. Finally, the program will state the effective diffusion constant for the photobleach ROI or the rectangles selected.

IMPORTANT NOTE: At the end of the program, the user is asked, "Do you wish to plot the simulation data with a different D_{eff} (yes or no)? The user must answer y or "n, and hit the Return key or the program will not create the experimental data file. Without this file, the user cannot compare the experimental recovery relative to the simulated recovery.

12. Compare the sim001.dat versus the exp001.dat data files by simultaneously plotting both in a spreadsheet program.

It is essential to plot the simulation data to determine whether the diffusion recovery has been accurately modeled. If the simulation misses the experimental data, the calculated diffusion rate is questionable, at best. The majority of the experimental data must overlap the simulation plot. A simulation that only overlaps with the initial steep rise or only with the later more gradual rise is not correct, and these D values should not be used. It is important to use all of the recovery data because removing parts of the recovery can significantly alter the calculated D . However, if the photobleach ROI fluorescence intensity has reached a genuine plateau and then rises or drops due to a focal shift or photobleaching, then the images after the plateau may be excluded from the analysis by simply removing them from the folder.

13. If the simulation quits before stating that D cannot be determined, recheck to see if incorrect coordinates have been entered (e.g., by placing the photobleach ROI outside of the whole cell ROI). Repeat the simulation, with the correct coordinates (be sure to remove any files generated by the previous run of the simulation).

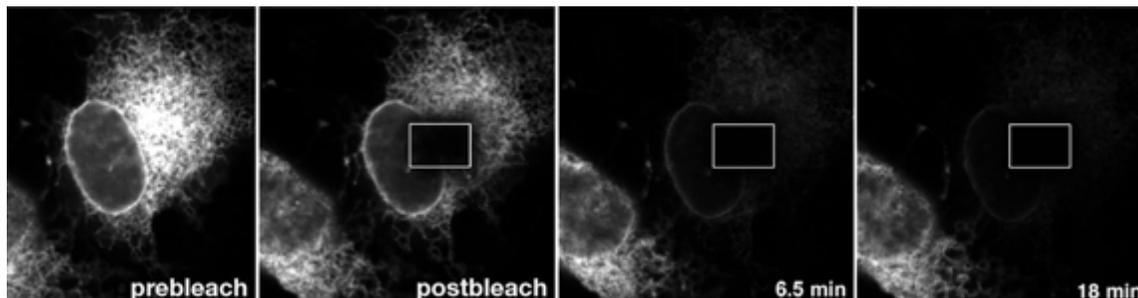


Figure 21.1.5 Example of FLIP. Protein fluorescence in a small area of the cell (box) is bleached repetitively. Loss of fluorescence in areas outside the box indicates that the fluorescent protein diffuses between the bleached and unbleached areas. Repetitive photobleaching of an ER GFP-tagged membrane protein reveals the continuity of the ER in a COS-7 cell. Image times are indicated in the lower right corners. The postbleach image was obtained immediately after the first photobleach. The cell was repeatedly photobleached in the same box every 40 sec. After 18 min, the entire ER fluorescence was depleted, indicating that all of the GFP-tagged protein was highly mobile and that the entire ER was continuous with the region in the bleach ROI.

FLIP USING A LASER SCANNING CONFOCAL MICROSCOPE CAPABLE OF SELECTIVE PHOTBLEACHING

FLIP (fluorescence loss in photobleaching) is similar to FRAP (see Basic Protocol 1) in that an ROI is photobleached with a high-power laser. However, in FLIP, the ROI is repeatedly bleached over time to examine the behavior of the entire fluorescent pool. If the fluorescent molecules are completely mobile and have access to the photobleaching ROI, the entire fluorescent pool will be depleted (see Fig. 21.1.5). Results from FLIP experiments therefore provide information about the connectedness of structures containing GFP chimeras.

For FLIP analysis, the user must obtain a prebleach image, an immediate postbleach image following the first photobleach, and images following successive photobleaches (often 2 to 3 images following each photobleach) until the entire cell is depleted of fluorescence, or until several successive photobleaches do not further deplete cell fluorescence. The time for each image, pixel size, and fluorescence intensity values for the whole cell and the background intensity must also be determined.

Materials

Cell samples expressing GFP chimeric protein of interest (Support Protocol 1)
 Confocal laser scanning microscope capable of selective photobleaching (e.g., Zeiss LSM 510)

Additional reagents and equipment for preparing imaging chambers and microscope for photobleaching experiments (Support Protocol 1)

1. Set up the microscope and prepare cells in imaging chamber with imaging medium as described in Support Protocol 1. Prewarm the imaging stage to 37°C or other desired temperature and warm up the microscope lasers for at least 5 min to avoid power fluctuations during imaging.
2. Identify the cell of interest on the confocal microscope.

The ideal situation is to have two adjacent cells of similar fluorescence intensity in the imaging field. The cell that is not bleached provides a control to ensure that the imaging conditions do not cause nonspecific photobleaching of all fluorescence in the field of view. It is essential that imaging conditions do not significantly photobleach any adjacent cells during the experiment.

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3. Bring the desired cell into focus. Scan an image of the whole cell at the desired excitation light intensity, averaging, and other parameters. Modify pinhole and detector gain for maximal fluorescence signal and minimal pixel saturation.

Detector gain and offset will vary depending on the concentration of the fluorophore, the laser power, and the width and thickness of the fluorescently labeled organelle or region. The imaging parameters used for this image should be used for the fluorescence recovery time series.

4. Define a region-of-interest (ROI) for the photobleach, which should be between 5% and 20% of the structure of interest.

The bleach ROI does not need to be a strip. A square is equally appropriate.

5. Determine imaging and photobleaching conditions (i.e., scan speed, zoom, laser power, number of laser iterations required for photobleaching, and microscope objective) that photobleach 90% or more of the fluorescent signal in the ROI, without causing significant photobleaching of the cell outside the photobleach ROI.

A 40-mW 488/514 nm argon laser can be used at 45% to 60% power with 100% transmission for photobleaching and 1% transmission for imaging. Usually, cells are scanned at 0.8 to 3 sec per image with two- to eight-line averaging.

6. Collect three to five prebleach images to establish the prebleach fluorescence intensity and to confirm that the prebleach fluorescence intensity does not significantly fluctuate.

7. Collect two to five prebleach images and then photobleach the bleach ROI. Collect two to four images of the whole cell and photobleach the ROI again. Repeat the process until the fluorescent structure intensity is similar to background.

The pauses allow unbleached molecules to diffuse into the photobleached ROI. In addition, collecting images between bleaches permits monitoring of progress and confirms that the cell remains in focus and has not moved. As this protocol requires longer time periods, the focal plane may shift. It is acceptable to refocus the cell during the experiment. However, if the cell or focus moves such that the region being photobleached also moves laterally, then the experiment should be aborted. Successful FLIP requires that the bleach ROI position within the cell is stable. If the cell migrates during the course of the experiment, then the data will be unusable.

8. Execute a quantitative FLIP.

This procedure can be automated with a macro to program the bleaching and imaging timing. A FLIP macro for the Zeiss 510 laser scanning microscope (the advanced time-series macro in combination with the physiology software package) is available from Zeiss. It is also possible to perform FLIP manually. To do this, photobleach the bleach ROI in cells for several (i.e., 20 to 50) bleach iterations. Next, collect three images of the cell. Repeat the bleach and image collection many times, up to 15 to 20 min (or longer). If the proteins are mobile and the fluorescent structure is continuous, the total structure fluorescence will be depleted. If the protein diffuses relatively slowly, the investigator can modify the routine to include a time delay between images, to reduce overall photobleaching due to laser scanning of the cell.

9. Collect at least three to five data sets for each fluorescently labeled protein and treatment.

A fraction of data sets may be unusable because the focal plane shifted, the cell moved, or other reasons.

For CLSMs that are unable to perform selective photobleaching of an ROI, it is possible to perform FLIP using essentially the same protocol as that described above. The primary differences are the need to photobleach the cell by zooming (see Alternate Protocol 1) and

the requirement for a macro to automate the FLIP experiment. Essentially, the protocol must alternate between photobleaching the ROI and scanning images of the whole cell between photobleaching. The photobleaching and imaging scans must be performed at regular time intervals such that it will be possible to directly compare the rates of fluorescence depletion between two cells. For a more extensive discussion on writing this macro, see the review by Lippincott-Schwartz et al. (1999). Some FLIP experiments may not need to be quantitative and it is possible to manually perform FLIP.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Imaging medium

Phenol-red free medium (e.g., RPMI or DMEM)

10% (v/v) fetal bovine serum (FBS)

2 mM glutamine

25 mM HEPES, pH 7.4

Prepare fresh

COMMENTARY

Background Information

Diffusion is defined as the rate of movement or flux of particles due to random or Brownian motion. An example of diffusion is the mixing of a drop of dye in a liquid solution. Random movements of proteins in a lipid bilayer (lateral diffusion), in the cytosol, or within an organelle are all examples of diffusion in a cell. Diffusion primarily depends on the viscosity of the environment and the size of the molecule. For soluble spherical molecules, the diffusion coefficient, D , is inversely proportional to the cube root of the radius of the molecule (Stokes-Einstein equation). This means that a molecule must increase 8- to 10-fold in size to decrease D by one-half. The size dependence of D for membrane proteins relates primarily to the radius of the transmembrane segments, rather than aqueous domains. This is because the lipid bilayer is more viscous than cytoplasm or organelle lumina. Other factors, such as protein-protein interactions or binding to a matrix can also profoundly affect the D of a protein.

Since Frye and Edidin (1970) demonstrated that proteins on the plasma membrane of cells move by diffusion, investigators have sought to directly measure diffusion coefficients of cellular proteins in their cellular context. Poo and Cone (1974) and Liebman and Entine (1974) performed some of the first FRAP experiments by photobleaching retinal rod outer segments; these experiments successfully measured the D value of the transmembrane protein rhodopsin. The analytical equations and methodology that form

the basis of FRAP were described originally by Axelrod et al. (1976). Labeling of proteins that are exposed to the extracellular face of the plasma membrane of living cells, using fluorescent antibodies, has been the primary labeling method for FRAP studies for 20 years. However, with that method, studies were restricted primarily to proteins on the cell surface, leaving all of the vesicles, organelles, and cytoskeletal components of cells unexplored. Some researchers have exploited microinjection techniques to place fluorescent antibodies or fluorescently labeled proteins in the cytoplasm. However, standard microinjection does not deliver proteins to the lumina of organelles, leaving these important cell environments unprobed. An additional barrier to investigating protein mobility was that traditional FRAP methodology required a dedicated custom microscope (Wolf, 1989).

Today, the combination of advances in fluorescence imaging methods, user-friendly commercially available laser scanning microscopes, and powerful cost-effective computing resources has made it possible for nonspecialists to exploit GFP chimeras and to probe their mobilities and interactions. For examples and more information, the reader is referred to several reviews of FRAP theory and techniques (Edidin, 1992; Ellenberg et al., 1997; Lippincott-Schwartz et al., 1999, 2001; Meyvis et al., 1999; White and Stelzer, 1999; Nichols et al., 2001).

Critical Parameters and Troubleshooting

The critical parameters for data collection are described in the protocols. There are several considerations for data analysis and interpretation that require additional emphasis. The major concerns are discussed below.

EGFP is better suited than other spectral variants for photobleaching experiments

Photoconversion and reversible photobleaching are phenomena that can seriously complicate photobleaching analysis by generating artificially high D values. Photoconversion is the process by which a fluorophore is excited and becomes transiently or permanently altered in its fluorescence excitation and emission spectra. Reversible photobleaching occurs when a fluorophore's excitation state is changed by intense illumination, which appears to an observer as destruction of the fluorophore. However, the fluorophore reverts to its native excitation and emission spectra and becomes fluorescent again. While many fluorophores exhibit varying degrees of both phenomena, photoconversion and reversible photobleaching are minimal for enhanced green fluorescent protein (EGFP). In contrast, wild-type GFP readily undergoes photoconversion. Thus, FRAP experiments using wild-type GFP chimeras should be avoided.

Correct for photobleaching due to bleaching of the ROI and repeated scanning of the cell

One method to measure the amount of photobleaching is to directly measure the change in the fluorescence of the whole cell used in an experiment. A decrease in fluorescence can be used for data correction and can also help the investigator modify imaging conditions to achieve minimal sample bleaching. In addition, this method offers an additional advantage in that the investigator can monitor fluorescence changes in every frame and can be alerted to cell movement and focal drift, which can change the apparent fluorescence recovery. Alternatively, the investigator can fix the sample (using 4% formaldehyde in PBS for 15 min) and acquire the time-lapse images of the fixed sample using exactly the same imaging parameters (e.g., excitation light intensity, duration, zoom, and number of images) as in living cells. Loss of fluorescence under these conditions will be due only to photobleaching and not to biological degradation processes. Once the photobleaching rate has been determined,

the experimental values obtained from living cells can be corrected.

Photobleaching experiments must produce recoveries with a true plateau or asymptote

M_f and $t_{1/2}$ calculations cannot be performed reliably when the asymptote has not been reached. Often, in the course of data processing, what appears to be a flat plateau with raw data may become an incomplete recovery following data processing. This result emphasizes the importance of determining the optimal imaging time to obtain a true plateau.

D and M_f must be interpreted in the context of the cell

Data analysis requires a commitment on the part of the investigator not to blindly accept numbers generated by the equations without attempting to determine whether the results are biologically and physically reasonable. For example, a D of $2 \mu\text{m}^2/\text{sec}$ for an integral membrane protein exceeds the highest recorded diffusion coefficient (0.4 to $0.5 \mu\text{m}^2/\text{sec}$) for a membrane protein in a cell by four-fold. Such a number could indicate that a protein is no longer membrane-associated or that the GFP chimera is incorrectly processed or targeted. Another less likely possibility is that processes besides diffusion are occurring (e.g., flow; Sciaky et al., 1997) that do not exhibit diffusive behavior.

Interpretation of D and M_f can be confounded by the connectedness of fluorescent structures

The problem concerning compartment connectedness is not always readily apparent. That is, an organelle or compartment may appear connected at the light level, but not actually be connected. This situation will result in a low apparent mobile fraction since fluorescent molecules in nearby but unconnected areas will be unable to diffuse into the recovery box. One way to clarify whether a structure is continuous or disconnected is to perform FLIP and check to see whether fluorescence of an otherwise mobile protein is depleted from all areas of the compartment (indicating connectivity) or only specific areas (indicating a lack of connectedness). If the molecule being studied resides within a discontinuous compartment, it will be difficult to estimate its D , since the equations and simulation programs used for calculating D assume free diffusion throughout a compartment.

Table 21.1.2 Troubleshooting Guide for Photobleaching

Problem	Possible cause and solution
Autofluorescence noise	Lysosomes are notorious for autofluorescence when excited with light between 400 and 488 nm. To decrease autofluorescence, lower the intensity of the excitation beam and use narrower-bandpass emission filters. Also, avoid phenol red and high serum concentrations (>20%) in the medium.
Fluorophore bleaches too rapidly during acquisition	Decrease the excitation light intensity using either neutral density filters or by lowering the voltage to the Acousto-Optical Modulator and increase the gain on the detector side to collect light more efficiently. If this does not work try adding Oxyrase [®] , an oxygen scavenger, to the medium.
Cells round up during imaging	This could be due to gross photodamage to the cells from long periods of exposure to an intense excitation beam. To correct, decrease the intensity of excitation light. Alternatively, the cells may have depleted some nutrient in the medium due to remaining in coverslip chambers for too long. Replenishing the imaging medium should help.
Cells are excited with a constant light intensity, but the fluorescence intensity varies over time	If this is not due to a biologically relevant process such as recruitment/degradation of fluorophore, then either the focus is shifting during acquisition or the laser power output is unstable. Maintain the focus either manually or by using autofocus software, which is available for some CLSMs. If laser output is a concern, then check it by exciting fluorescent beads and quantifying the emission over time. Fluctuating laser output may be due to the laser being operated at low power output. Increasing the power output to 50% may help. If this fails to correct the problem, then contact your confocal service representative.
Focus drifts	Try using relatively flat cells (i.e., COS-7) which may alleviate the problem. Try imaging with the pinhole partly or entirely open and use lower-NA objectives. Be sure to pre-equilibrate the sample to the temperature of the objective, as this will prevent expansion/contraction of the coverslip/chamber during imaging. Make sure that the stage insert is mounted securely and that the sample is seated properly in the holder.
D values of two subsequent experiments of the same ROI in the same cell do not match	This suggests the area that has been photobleached has been damaged. Try using a different GFP variant that requires a lower (and less damaging) wavelength of laser light, such as YFP. Note that YFP may reversibly photobleach.
Slow recovery or lack of recovery after bleaching	Either the fluorophore is relatively immobile or there are discontinuities within the structure where the fluorophore is located. This is relevant for isolated membrane structures with little connection between membranes, such as structures of the endocytic system (e.g lysosomes and endosomes).
Recovery is significantly faster than expected	A faster than expected <i>D</i> for a membrane protein may indicate that the protein is no longer correctly assembled or inserted in the membrane. This can be tested by performing immunofluorescence colocalization with a marker for the organelle of interest or performing immunoblotting to determine whether the GFP chimera has become partially degraded. Another condition that may affect fluorescence recovery is loss of cell integrity or viability.

continued

Table 21.1.2 Troubleshooting Guide for Photobleaching, *continued*

Problem	Possible cause and solution
Nondiffusive behavior	A failure to fit recovery-curve data by a simulation or to a least squares fit equation may suggest that the protein is not moving by simple diffusion. There are conditions that cannot be described by equations for simple diffusion, e.g., in the case of anomalous diffusion ^b and when there are multiple populations diffusing at different rates. ^c

^aProduct of Oxyrase Inc.; see *SUPPLIERS APPENDIX*.

^bPotential mechanisms of anomalous diffusion include binding and collisional interactions with mobile and immobile obstacles (Periasamy and Verkman, 1998) and recovery due to vesicular transport.

^cThe labeled protein in the FRAP experiments may alternate between multiple states, i.e., different-sized oligomers or bound and unbound to a membrane or complex. These conditions pose significant problems to diffusion analysis. Gordon et al. (1995) describe the mathematical difficulties of separating multiple *D* coefficients. The main problem is that the investigator does not usually know the fraction of each population or the actual number of populations. It is worth noting that at least one group has successfully teased out multiple diffusing populations (Marguet et al., 1999). In this example, the group independently determined the *D* coefficients for the two populations and biochemically determined the relative percentages of each population.

Troubleshooting

A number of common problems that may arise in photobleaching experiments and potential solutions for them are presented in Table 21.1.2.

Anticipated Results

The methods described in this unit should permit the investigator to obtain and interpret fluorescence intensity recovery data following photobleaching of GFP chimeras with a CLSM. The investigator should be able to calculate the M_f and either the *D* or $t_{1/2}$ of fluorescence recovery of a protein in a cell. The investigator should also be able to perform a FLIP experiment to determine the connectivity of GFP-labeled cellular compartments and organelles. Finally, the investigator may take advantage of selective photobleaching either to reveal dimer fluorescent structures obscured by bright structures or to visualize dynamic processes under steady-state conditions, such as vesicular trafficking.

Time Considerations

Creation of GFP chimeras involves standard cloning procedures and biochemical or genetic assays to confirm that the properties of the chimera are similar to those of the parent protein. Preparation for photobleaching experiments consists of transferring cells to coverslips or imaging chambers and transfecting the cells with a GFP construct 16 to 48 hr prior to imaging. The initial setup for photobleaching experiments requires a time investment of a few hours to determine conditions for

each protein to be bleached. The actual FRAP experiments can be very rapid, ranging from 90 sec for the recovery of a soluble luminal ER protein into a 4- μ m bleach ROI, to 6 min for a membrane protein. FLIP experiments often take 10 to 20 min. FRAP experiments require at least 5 (and preferably 10) data sets for useful statistics to compare *D* values. Data processing depends on the method used and the power of the computer. The Siggia simulation will process a 250-image data series in 2 to 3 min on a Macintosh G4 450 MHz computer. Due to the large numbers of data sets to be processed, data analysis can be time consuming.

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Fluorescence Localization After Photobleaching (FLAP)

Fluorescence localization after photobleaching (FLAP) is a new method for localized photolabeling and subsequent tracking of specific molecules within living cells. The molecular species to be located carries two different fluorophores that can be imaged independently but simultaneously by fluorescence microscopy. For the method to work, these two fluorophores should be accurately colocalized throughout the cell so that their images are closely matched. One of the fluorophores (the target fluorophore) is then rapidly photobleached at a chosen location. The unbleached (reference) fluorophore remains colocalized with the target fluorophore; thus the subsequent fate of the photobleached molecules can be revealed by processing simultaneously acquired digital images of the two fluorophores. For example, an absolute FLAP image, which shows the location of the photobleached molecules, is calculated simply by subtracting the target intensity from the reference intensity at each pixel. This image is effectively the same as images obtained directly by photoactivation methods, so absolute FLAP can be considered to be a method of virtual photoactivation. In addition, however, a relative FLAP image can be calculated to show the photobleached fraction of molecules within each pixel. This useful information is not available with other methods.

The Basic Protocol in this unit demonstrates the simplicity and effectiveness of the FLAP method in revealing both fast and slow molecular dynamics in living cells. As an example, cDNA fusion constructs of β -actin with yellow and cyan fluorescent proteins (YFP and CFP) are microinjected into the nuclei of transformed rat fibroblasts. Using a Zeiss LSM 510 laser-scanning microscope, the authors show that it is possible to follow simultaneously the fast relocation dynamics of monomeric (globular) G-actin and the much slower dynamics of polymeric (filamentous) F-actin in expressing cells.

In order to give a step-by-step protocol for the FLAP method, it is necessary to focus on a particular configuration of fluorophores and microscopy methods. CFP-actin and YFP-actin fusion proteins have been chosen as the fluorescent molecules, since YFP can be efficiently and rapidly photobleached. Moreover, the predominance of native actin in the cell means that noise-free images can be obtained at low relative expression levels of the tagged molecules. Using wide-field fluorescence microscopy, it is possible to separate the emission spectra of these two fluorophores and thus obtain simultaneous images. For this, both fluorophores are excited simultaneously, and a commercially available arrangement of fixed dichroic mirrors and filters (Cairn Research Ltd.) enables the two images to be projected side-by-side onto a single CCD chip. However, in view of the large overlap of emission spectra of CFP and YFP, laser scanning microscopy, which permits alternately exciting the two fluorophores line-by-line and yields images that are effectively simultaneous, has been chosen for the Basic Protocol. Some laser scanning microscopes, such as the Zeiss LSM 510 used here, have the further advantages of versatile choice of size, shape and location of the bleach region, and rapid resumption of image scanning after bleaching. Time-lapse recording using no intervals between scanned frames will reveal rapid diffusion dynamics, whereas intervals of several seconds or longer may be required to reveal the movements of polymerized or bound molecules without excessive fading of the fluors.

FLAP OF ACTIN IN LIVING CELLS

This protocol describes the FLAP method using transformed rat fibroblasts of the lines K2 and T15 (Pavel Vesely, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Flemingovo nam. 2, 166 37 Prague 6 Dejvice, Czech Republic; e-mail, *pvy@zeus.img.cas.cz*). Many other cell types that the authors have tried work equally well. A notable exception is the Swiss 3T3 line, which shows significant autofluorescence on the YFP channel. Extensive details on preparation of constructs, cell culture, and microinjection methods will not be given, as these are standard procedures. Also a basic familiarity with the software and operation of the Zeiss LSM 510 upright microscope will be assumed. Once the microscope and software have been prepared (Support Protocol 1), there are two main sequential stages to the Basic Protocol: (1) preparation of cells and setting up the microscope (steps 1 to 4) and (2) microscopy (steps 5 to 17). Post-processing and analysis of images (Support Protocol 2) can be performed later.

Materials

Rat fibroblast cell line K2 or T15
Hanks' Minimal Essential Medium (MEM; Cancer Research UK; *daniel.zicha@cancer.org.uk*) containing 10% bovine serum and no antibiotics
cDNA constructs of eCFP- β -actin and eYFP- β -actin (see recipe)
Experimental reagents of interest (e.g., myosin light chain kinase inhibitor, ML-7)
Hot wax mixture (see recipe)
Non-toxic immersion oil optimized for 37°C, refractive index 1.515 (Cargille Labs)
18 × 18-mm glass coverslips
35-mm plastic petri dishes (Costar)
Microinjection system (also see *UNITS 4.10 & 17.1*) including:
 5171 micromanipulator (Eppendorf)
 5246 transjector (Eppendorf)
Zeiss Axiovert 35 microscope
Microneedles (GC120TF-10, Harvard Apparatus)
P97 Flaming/Brown micropipette puller (Sutter)
Optical chambers (see recipe)
Zeiss upright LSM 510 microscope (see Support Protocol 1 for full configuration) contained within a 37°C environmental control incubator (e.g., Microscope Temperature Control System, Life Imaging Services) *or* a similar apparatus assembled in house (Fig. 21.2.1)
Software:
 Zeiss LSM 510 operating software for image acquisition
 Zeiss LSM Reader for image review (free download; see Internet Resources)
Additional reagents and equipment for cell culture (*UNIT 1.1*), microinjection (see *UNITS 4.10 & 17.1*), and use of LSM 510 operating software (see Support Protocol 1)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified. Some media (e.g., DMEM) require altered levels of CO₂ to maintain pH 7.4.

Prepare cells and set up microscope

1. Seed cells at the required density onto the 18 × 18-mm coverslips in 35-mm plastic petri dishes and flood with Hanks' Minimal Essential Medium/10% bovine serum (see *UNIT 1.1* for basic cell culture techniques). Incubate 72 hr.



Figure 21.2.1 Zeiss upright LSM 510 microscope contained within a 37°C environmental control incubator. In the authors' laboratory, the environmental control chamber is a specially built Plexiglass box with access hatches (Cancer Research UK workshop). The essential components for the heater and control system are a centrifugal fan (part no. 40BTFL from Air Flow Developments), and a temperature controller (208-2739), thermocouple type T (219-4680), box (584-615), mini-3-pole plug (449-269), mini-3-pole socket (449-275), fuse holder (418-603), optical relay (394-535), type T panel socket (219-4860), type T line plug (219-4876), enclosed heater (224-565), and thermocouple connector (219-4876), all from RS Components. A similar commercially available system (Microscope Temperature Control System) may be obtained from Life Imaging Services (see Internet Resources).

MEM has been optimized by the authors at Cancer Research UK for the cell lines used here. However, it might be possible to obtain satisfactory results with the MEM formulation supplied by Sigma.

In the authors' laboratory, experiments usually require that the cell density still be well below confluence (~50%) after this incubation.

2. Microinject the two cDNA constructs at concentrations of 50 ng/μl (YFP-actin) and 75 ng/μl (CFP-actin) into the nuclei of 20 to 30 cells located near the center of each coverslip. Return to incubator for 2 to 3 hr.

Expression and folding times may vary widely with other fluorescent protein constructs.

3. During the incubation, switch environmental heating and argon laser on the LSM 510 to on and open a database with previous software settings (see Support Protocol 1).
4. Assemble a coverslip culture (from step 2) onto an optical chamber filled with medium and any experimental reagents. Seal chamber with hot wax mixture, wash outside of chamber with clean distilled water, and blow dry before placing on the stage of the LSM 510.

Perform microscopy

5. Restore settings for tile scanning by selecting the previous tile scan from the saved database and reusing the settings (Support Protocol 1). Check that scan rate is now at fastest setting and laser power settings are correct.

Tile scanning provides a montage of adjacent fields acquired sequentially in order to overview a large area for identification of expressing cells.

6. Use the stage control joystick to locate the center of the chamber on the microscope axis, switch to direct viewing using a low-power phase objective ($5\times$ to $25\times$), change phase setting of condenser as necessary, and focus on cells manually.

7. Start Tile scan in Stage controls.

The authors generally use a 4×4 tile array with 256×256 pixel images.

8. Mark the expressing cells. Create an image database and use Save As to store the tile scan.
9. Restore software settings for FLAP imaging by selecting a FLAP sequence from the saved database and reusing the settings (Support Protocol 1). Use the stage-control joystick to move the chamber away from objective, apply immersion oil, and recentralize chamber by moving to the first marked cell. Switch to high-aperture oil-immersion objective ($63\times$ Plan apochromat Ph3) and change phase setting of condenser as necessary.

CAUTION: Do not attempt to switch objectives automatically unless it has been ascertained that they are accurately parfocal (Support Protocol 1) and that the lower surface of the upper coverslip is in focus. The high-power objective may have a very short working distance and any error could cause a disastrous crash between the objective and the specimen.

10. Start a fast scan while displaying split channels and manually focus on a cell.

The interference reflection microscopy (IRM) image will brighten suddenly when one is focused on the lower surface of the upper coverslip. A small distance below this surface (turn top of fine focus knob away from you) will usually be the optimal setting with maximal brightness of the fluorescence images.

11. If necessary, centralize the cell in image field using the X/Y buttons and the nudge wheel on the joystick control. Cease fast scanning as soon as possible.

Note that the IRM image will often be out of focus at the optimal setting for fluorescence imaging; a compromise is necessary if good IRM images are required.

12. Select orientation and zoom factor (the authors usually keep a standard zoom of 2) and do a single scan.

When it is critical to record fast molecular dynamics immediately after bleaching, note that the top of the reoriented image will be scanned first.

13. Click the use ROI button to set up region of interest (ROI) if required in order to reduce scan times.

It is sometimes advantageous to image the whole cell for accurate estimates of fluorophore fade during imaging (Support Protocols 2). Note that the top one or two scan lines will sometimes show a defective match between channels and should be avoided (see Troubleshooting).

14. Do a single scan while displaying split channels. Switch to the custom Hall palette (Support Protocol 1). Using a minimal number of further scans, adjust laser gains and offsets so that the two fluorescence channels are accurately matched with no saturation and with a low but finite background intensity. Adjust settings for the other two channels if necessary. Return to the no palette display.

If matched, the fifth combined image should show the fluorescent regions as pure white (Fig. 21.2.2). The phase-contrast and IRM channels should show good contrast.

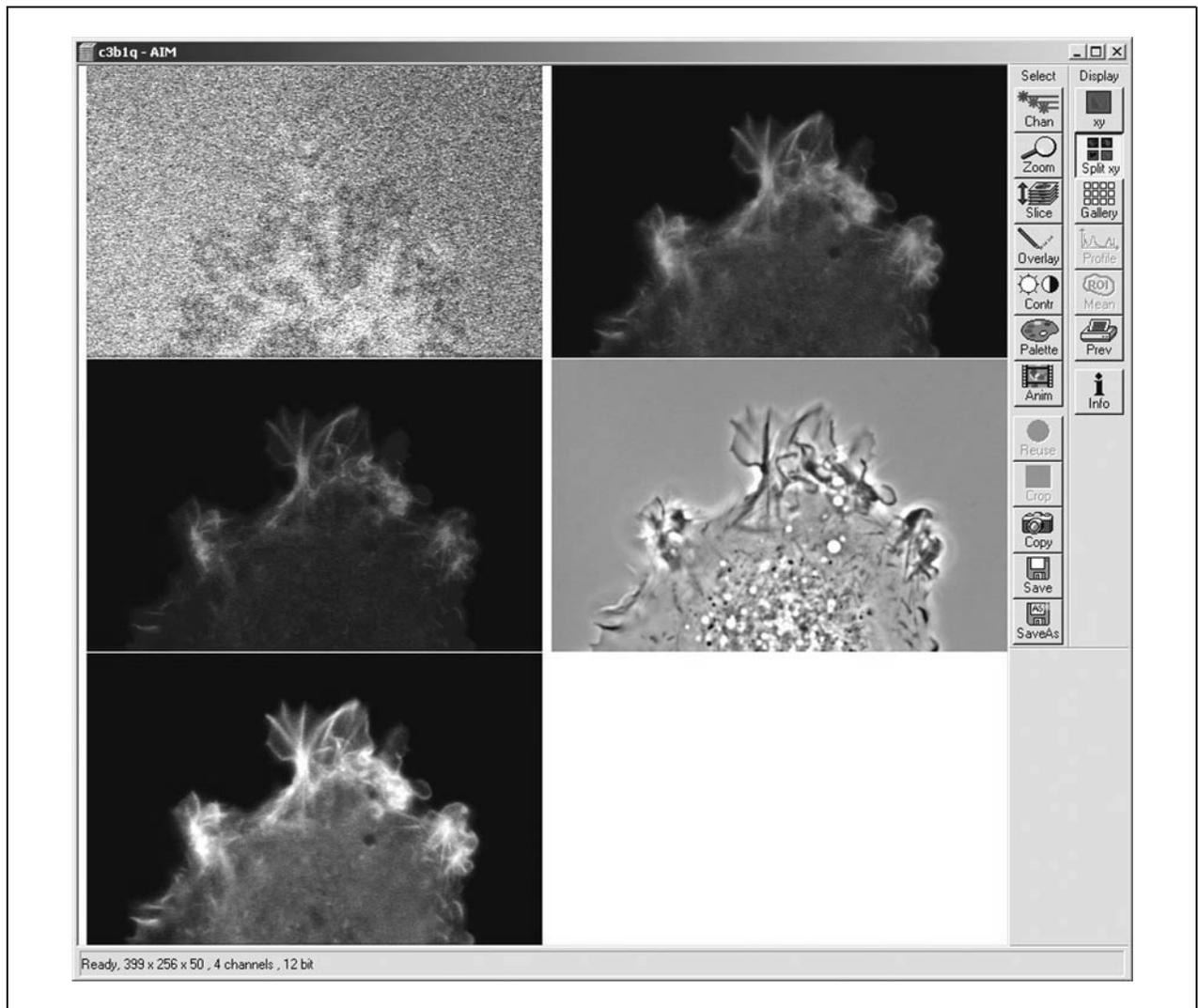


Figure 21.2.2 Window from Zeiss LSM software showing four channels and fifth combined channel after setting gains and offsets and laser powers. Courtesy of Carl Zeiss, Germany; reprinted with permission of Zeiss UK. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to <http://www.currentprotocols.com/colorfigures>.*

15. Select the bleach panel, choose a bleach region, and change the bleach parameters if necessary.

The authors use 50 bleach scans at the full-power laser setting. Note that bleach times depend on the height of the bleach region (regardless of its shape) but not on its width, so a horizontal narrow strip will bleach a large area rapidly.

16. Select the time series panel and change the time-lapse parameters if necessary. Do one single scan to check that everything is still OK (i.e., that the current setup still provides a satisfactory image of the selected cell). Choose the number of scans to average and start the recording.

The authors normally average four scans per frame and record two frames before bleaching and 50 afterwards with no time-lapse interval. Recording of the FLAP signal is best monitored in a large window showing the combined image. After bleaching, the bleach-labeled molecules will then show as a bluish color against a white background (Fig. 21.2.3). Avoid transmitting any vibrations to the microscope during recording.

17. Save the recorded sequence if satisfactory, export the bleach region image for later reference (showing the bleach region and with the zoom aspect set to 1:1) as a

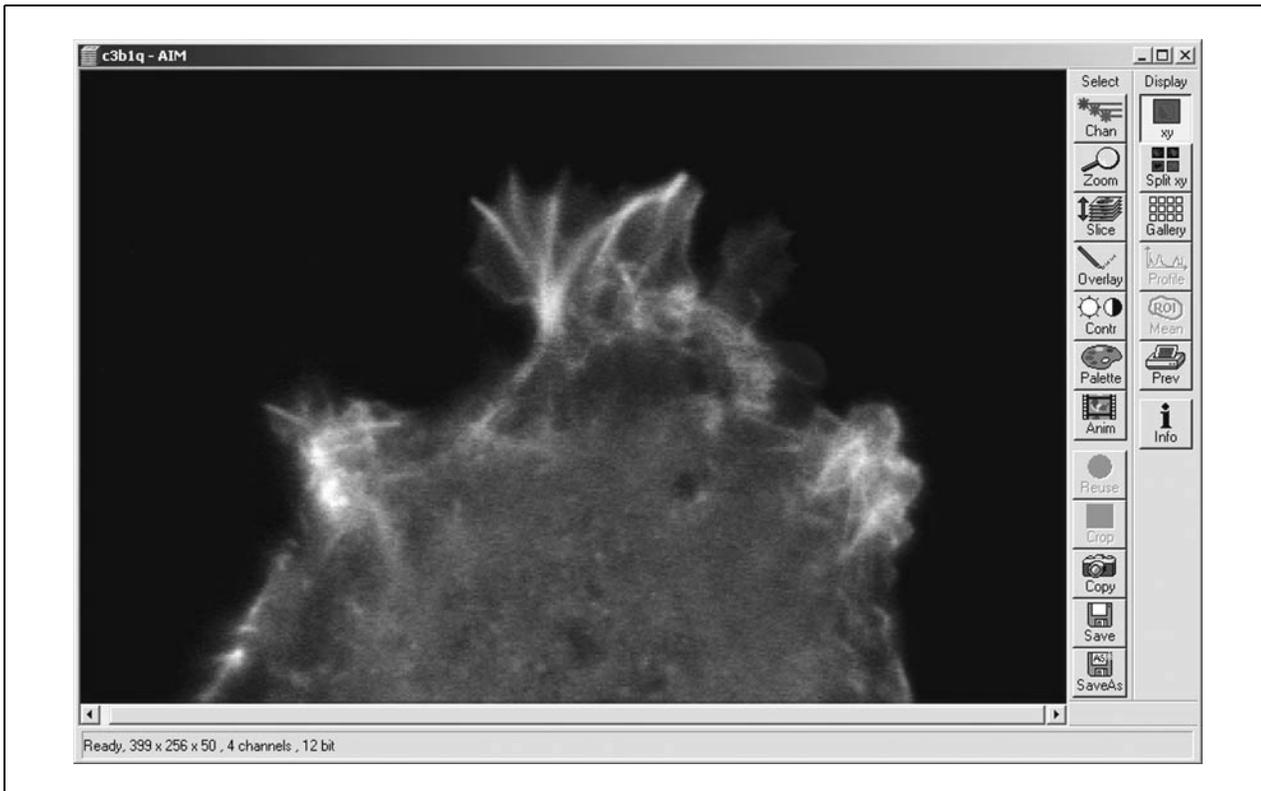


Figure 21.2.3 Window from Zeiss LSM software showing only the combined channel after bleaching during data recording. Courtesy of Carl Zeiss, Germany; reprinted with permission of Zeiss U.K. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to <http://www.currentprotocols.com/colorfigures>.*

single-image TIFF file, reset the scan averaging to 1, move to the next marked cell using the stage-control panel, and repeat steps 10 through 16 for all marked cells. Return to step 5 to start a new culture chamber.

**SUPPORT
PROTOCOL 1**

SETTING UP THE LSM 510 AND ITS SOFTWARE

It is important that the microscope be configured optimally—e.g., pinhole alignment should be set correctly. Optimization of settings for tile scans and FLAP imaging will require a trial run through the Basic Protocol.

Materials

Zeiss upright LSM 510 microscope and software (see Basic Protocol 1)
Small beads (e.g., TetraSpeck microspheres, 0.2 μm ; Molecular Probes)

1. Make sure that the objectives to be used are accurately parfocal using small beads and Parfocal Settings from the CLM32 . EXE program.
2. Define the pseudocolor lookup table (LUT) for the custom Hall palette (Fig. 21.2.4) to be used for gain and offset adjustments and for viewing fully processed FLAP images.

Other preferred LUTs may be used, but it is critical that minimum and maximum gray levels be easily distinguishable from the rest.

3. For tile scans, use three channels (CFP fluorescence, YFP fluorescence, and phase contrast), a low number of pixels (256×256), and fast scanning.

**Fluorescence
Localization
After
Photobleaching
(FLAP)**

21.2.6

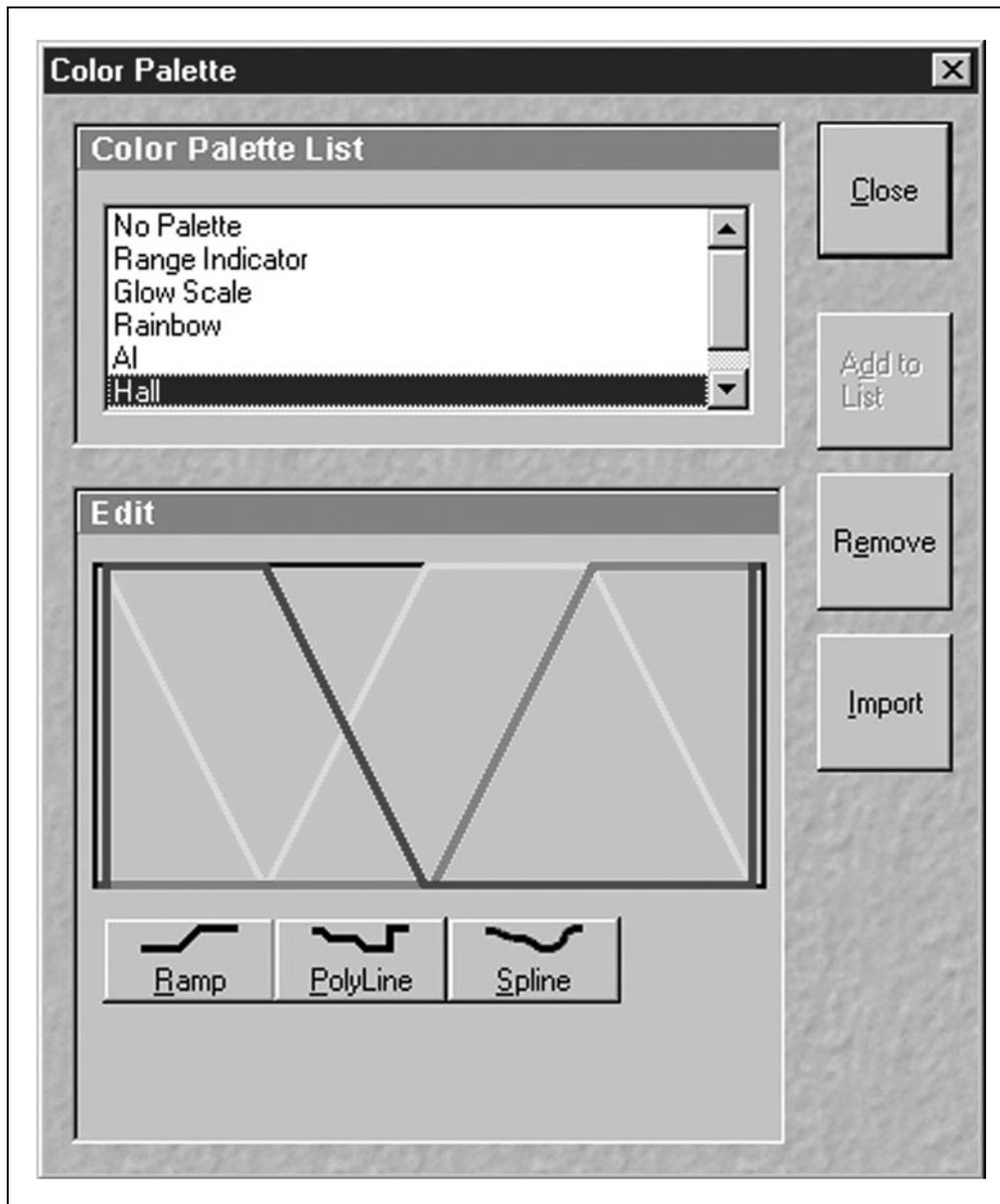


Figure 21.2.4 Window from Zeiss LSM software showing custom Hall pseudocolor palette. Note that the lowest intensity level is coded as black and the highest as white. Courtesy of Carl Zeiss, Germany; reprinted with permission of Zeiss UK.

4. For FLAP imaging, set the microscope to use 4 channels: CFP fluorescence, YFP fluorescence, phase contrast, and IRM (Fig. 21.2.5) with 512×512 pixels.

Tile scan is used to identify expressing cells for FLAP imaging.

5. Set (typically) laser line 514 nm power to 1% and 548 nm power to 21%; pinhole diameters Ch1 to 408 μm ; Ch2 to 409 μm ; and Ch3 to 414 μm .

These pinhole sizes give optical sections of 3 μm in all channels.

6. Set dichroic mirror HT to HTF 458/514; set dichroic mirror NT1 to NFT 635 VIS; set dichroic mirror NT2 to NFT 545; set dichroic mirror NT3 to Plate; set filter Ch1 to None for IRM; set filter Ch2 to BP 475-525 for CFP fluorescence; set filter Ch3 to LP 530 for YFP fluorescence; and set filter ChD for phase-contrast microscopy (Fig. 21.2.5).

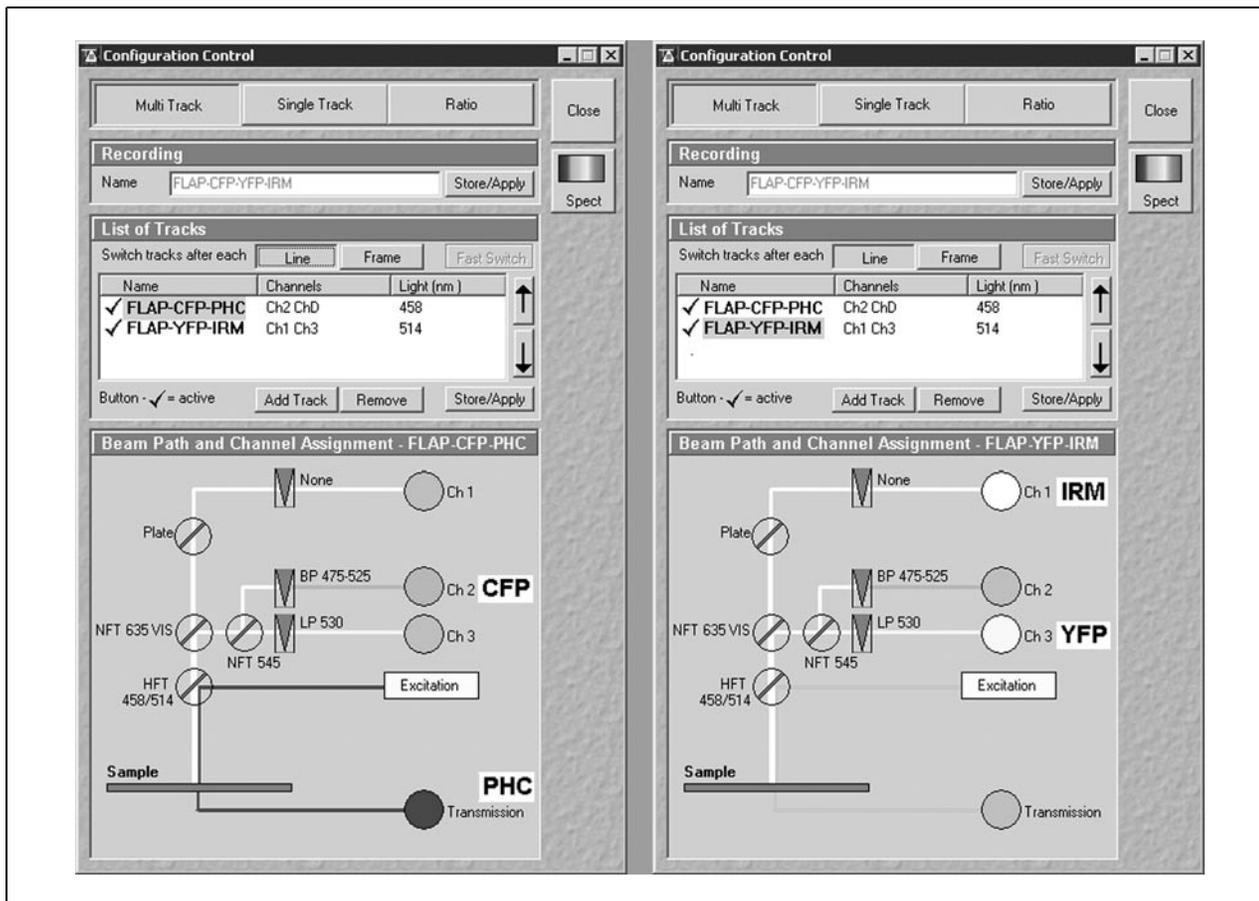


Figure 21.2.5 Window from Zeiss LSM software showing both tracks on the configuration panel. Courtesy of Carl Zeiss, Germany; reprinted with permission of Zeiss UK.

7. Set pixel size to $0.29 \mu\text{m} \times 0.29 \mu\text{m}$ and pixel dwell time to $1.6 \mu\text{sec}$.
8. Use laser line 514 nm under the bleach setup at maximum power measured as 1.32 mW.
9. Set palette of the CFP fluorescence channel to $\text{RGB} = (0, 128, 255)$ and YFP fluorescence channel to $\text{RGB} = (255, 128, 0)$ so that the cyan and yellow channels are easily identified and equal intensities combine to a white image when the phase contrast and IRM are switched off from the overlay panel.
10. Save settings by saving the trial run as a database for future Reuse. Do not check Re-use Objective in the Save Settings menu, to avoid unexpected movements of the objective revolver.

Further runs will be required to refine gain and laser power settings for fade matching of channels (Support Protocol 2).

SUPPORT PROTOCOL 2

Fluorescence Localization After Photobleaching (FLAP)

21.2.8

IMAGE PROCESSING AND ANALYSIS

The authors perform the image processing in Mathematica (Wolfram Research). This has the advantage of immense flexibility over dedicated image-processing software and can be programmed to read the information in the *.lsm Zeiss file headers. A commercial image acquisition and processing program from Kinetic Imaging (<http://www.kineticimaging.com>) can also read these file headers as, of course, can the LSM Reader software distributed as a free download by Zeiss. The LSM Reader will also export the individual images of the four channels as 12-bit TIFF format files. The required

image processing is fairly basic, and it will be assumed that a package is available that can read and operate on the raw 12-bit TIFF files exported from LSM Reader.

Materials

Mathematica v 4.2 or 5 (Wolfram Research) or a dedicated image-processing package capable of processing 12-bit TIFF images

1. Subtract a constant from the intensity values of all the CFP and YFP images (it may be different for each image) so that the background regions outside the cell(s) have a mean intensity of zero. Set all negative intensity values to zero.

The intensity values should now be proportional to the number of fluorescent molecules at each location if care was taken to avoid saturation in step 14 of the Basic Protocol.

2. Multiply the intensity values of the last pre-bleach YFP image by a factor so that the total image intensity matches that of the last pre-bleach CFP image.

The two images should now look identical.

3. Multiply all the remaining YFP images by the factor obtained in step 2.

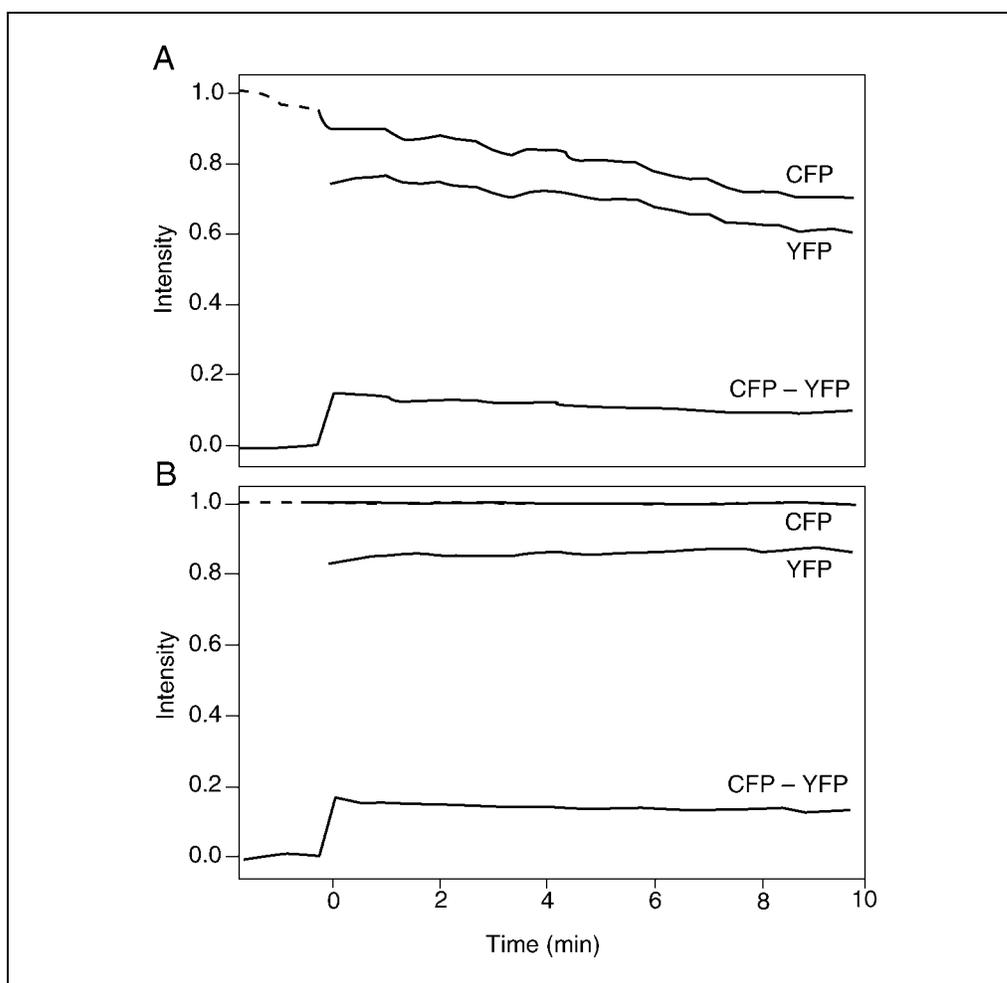


Figure 21.2.6 (A) The summed intensity values for a whole cell during a 10-min time series after step 3 in Support Protocol 2. These values have been normalized so that the total intensity of the first CFP image is 1. (B) The same intensity values after fade compensation as in step 4 of Support Protocol 2. This consists of dividing each CFP image by a factor so that the total intensity is 1 and then dividing the corresponding YFP image by the same factor. Note that fluctuations due to cyclical focus drift have also been compensated.

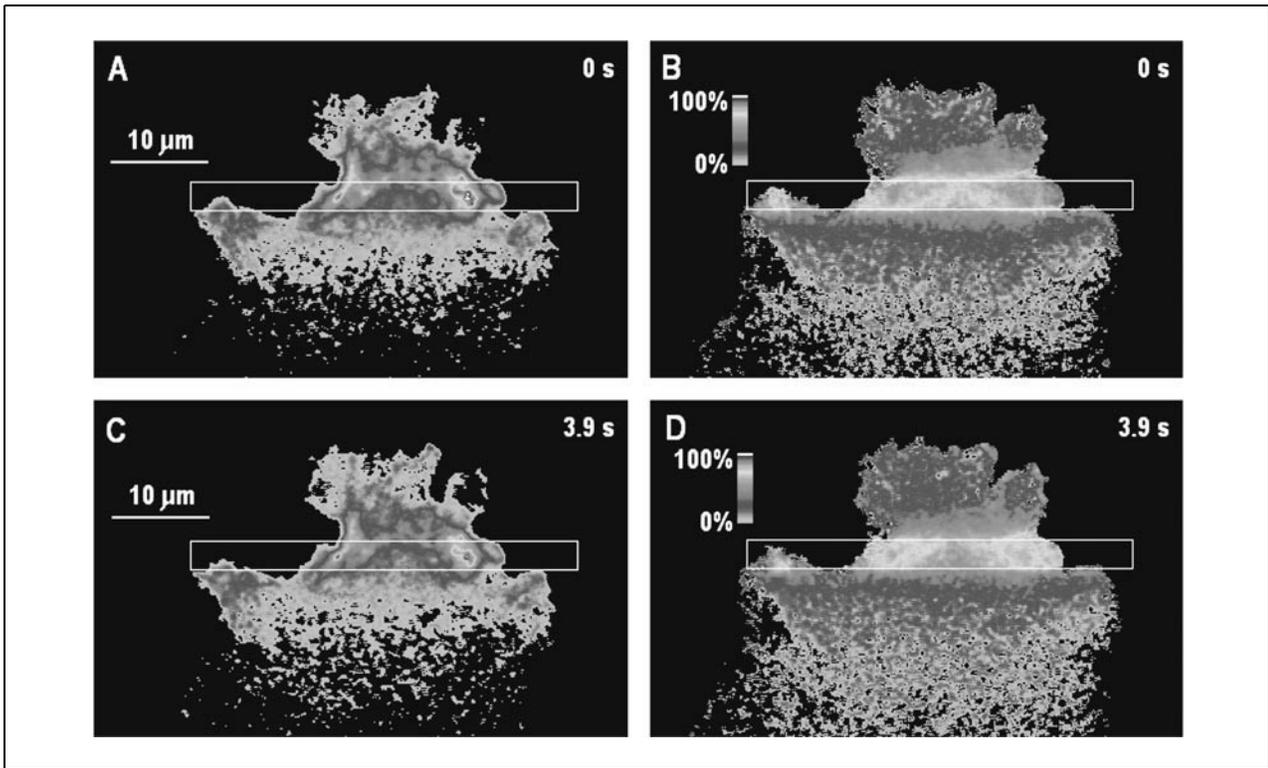


Figure 21.2.7 Absolute FLAP (A, C) and relative FLAP (B, D) images of the cell featured in Figures 21.2.2 and 21.2.3 recorded immediately after bleaching (A, B) and 3.9 sec later (C, D). Bleach box is shown as white rectangle. Hall palette. This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to <http://www.currentprotocols.com/colorfigures>.

If the YFP and CFP channels do not fade at the same rate (see Support Protocol 1), it will be necessary to change this factor for each YFP image. This is best calculated if a whole cell is contained within the image. Plots of the total image intensity of each channel against time will then reveal the fade rates and any compensation that is necessary (Fig. 21.2.6A).

4. If planning to calculate absolute FLAP images, multiply all images after the first pair by a factor to compensate for fade.

The fade rate is best calculated on whole cells as in step 3. After compensation, plots of the total image intensity of each channel against time should be horizontal lines with a step fall at bleaching in the YFP channel (Fig. 21.2.6B).

5. For each pair of simultaneous images, subtract the YFP image from the CFP image. Set all negative intensity values to zero.

If fade compensation has been performed as in step 4, the resulting images are now absolute FLAP images.

6. Divide each image obtained in step 5 by the corresponding CFP image to obtain the relative FLAP images.

Note that this operation has an infinite or undefined result for pixels in which the CFP intensity is zero and an unreliable result where intensity values are low. A threshold level of CFP intensity should be chosen (by trial and error) below which the result of this operation is set to zero.

7. View the final FLAP images, if desired, using a pseudocolor lookup table or palette.

The authors often use the Hall palette (Support Protocols 1). The relative FLAP images (Fig. 21.2.7B) have intensity values in the range 0 to 1, and a scale bar showing the corresponding pseudocolors can be calibrated from 0% to 100%, showing the fraction

of bleached YFP molecules in each pixel. Absolute FLAP images (Fig. 21.2.7A) could in theory be calibrated for numbers of molecules, but this would require knowing numbers of expressed and native molecules for the whole cell. If the images are noisy and the noise levels in the raw images cannot be improved further (see Troubleshooting), there are two helpful image-processing procedures. A 3×3 or higher-order smoothing filter can be applied to the raw images before or after step 1, and/or a threshold can be used below which values are displayed as zero when viewing the FLAP images. Note that smoothing filters other than a block mean may change the intensity value total.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

cDNA constructs of eCFP- β -actin and eYFP- β -actin

Use a pEGFP- β -actin construct (Dunn et al., 2002; daniel.zicha@cancer.org.uk) as the basic starting vector. Use enhanced yellow and cyan fluorescent protein coding regions from pEYFP-C1 and pECFP-C1 (Clontech), respectively, to substitute the eGFP coding region using conserved restriction enzyme sites (James Monypenny and Daniel Zicha, Cancer Research UK; daniel.zicha@cancer.org.uk). Maintain the 45-bp linker (TCC GGA CTC AGA TCT CGA GCT CAA GCT TGC GGC CGC GCC GCG GCC) between eGFP and β -actin in the pEGFP- β -actin vector in the new fusions. Microinject the DNA in distilled water. The amino acid translation (SGLRSRAQACGRAAA) has net positive charge and 40% hydrophobic residues, ensuring linker flexibility in a cytosolic environment.

Hot wax mixture

Prepare a 1:1:1 (w/w/w) mixture of beeswax, soft yellow paraffin, and paraffin (melting point 46°C) maintained at 54°C in a wax bath.

All of the above materials are available from Fisher.

Optical chambers

Fabricate optical chambers from 76 \times 26-mm (as cut) glass microscope slides (Chance Propper), No. 3, 76 \times 26-mm glass coverslips, and No. 1 $\frac{1}{2}$, 18 \times 18-mm glass coverslips (Chance Propper). Drill a \sim 10 mm hole centrally through the glass slide using a diamond-tipped drill (Proxon) under water. Fix a 76 \times 26-mm coverslip to one face using Sylgard 184 silicone elastomer (Dow Corning) and allow it to set overnight at room temperature.

While in use, the other face of the medium-filled chamber is sealed with an 18 \times 18-mm coverslip carrying the cell culture (see Basic Protocol).

COMMENTARY

Background Information

Fluorescence microscopy has long been the most important tool for revealing the changing distribution of specific proteins within living cells, but it is only recently that fluorescence methods have enabled the study of other aspects of molecular dynamics (see reviews by Lippincott-Schwartz et al., 2001; Holt et al., 2004). One serious limitation of conventional fluorescence localization is that it reveals little of the relocation of molecules during cycli-

cal, steady-state dynamics. Thus critical activities such as the constant depolymerization, diffusive transport, repolymerization, and treadmill of structural molecules of the cytoskeleton cannot be visualized by this means.

Two methods developed more recently for studying the relocation of molecules, fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP), can have a sufficiently high time resolution to enable diffusion rates of proteins

within the cytoplasm to be estimated (see review by Lippincott-Schwartz et al., 2001). Their chief disadvantage is that the bleached molecules themselves cannot be tracked. On the other hand, fluorescent speckle microscopy (FSM) permits the study of slow molecular movements such as treadmilling and translocation within polymerized structures (*UNIT 4.10*; Waterman-Storer and Salmon, 1997). It relies on a low percentage of tagged molecules (<1%) to produce a discontinuous pattern that can then be used to determine whether the labeled polymer is stationary or translocating. It cannot detect the much more rapid movements of freely diffusing molecules, but this is considered an advantage because it identifies which molecules are polymerized. A refinement of this technique detects the signals from single fluorophores and permits the sites of polymerization and depolymerization of the tagged molecule to be located and their rates to be estimated (Watanabe and Mitchison, 2002). Although complementary to some extent, FRAP, FLIP, and FSM cannot easily provide a full picture of the dynamics within a cyclical polymerization/depolymerization system because the depolymerized molecules cannot be tracked directly.

In contrast, photoactivation of fluorescence (PAF) allows specific molecules to be “flash” labeled at a chosen site and then tracked directly, regardless of whether or not they are polymerized. Earlier PAF methods relied on caged fluorophores and, although they yielded much significant information in the hands of skilled investigators (see review by Mitchison et al., 1998), results were generally disappointing. Uncaging the fluorophore requires exposure to intense UV light, and radiation damage cannot be ruled out. Moreover, molecules tagged with caged fluorophores have to be microinjected or otherwise introduced directly into the cell, and often clump or fail to distribute to their natural locations within the cell. This latter shortcoming led to the development of fluorescent proteins that could be expressed by the cells from cDNA constructs and photoactivated directly. Again, early results were disappointing, and it is only very recently that efficiently photoactivatable fluorescent proteins have been developed (Patterson and Lippincott-Schwartz, 2002; Chudakov et al., 2003). These promise to contribute a great deal to the knowledge of cellular dynamics in the future.

The FLAP method is an alternative approach to the problem of direct and localized photolabeling of a molecular species (Dunn

et al., 2002). There is no need to use specialized fluorophores. The principle is simple and relies on photobleaching instead of photoactivation to label the targeted molecules. Suppose that, instead of a single fluorophore, each molecule carries two fluorophores that have differing spectral properties so that they can be imaged separately. If only one of the fluorophores is photobleached, then the resulting molecules are unambiguously labeled: they are the only molecules carrying just one active fluorophore. Although the bleached molecules cannot be imaged directly, one fluorescence image shows where all the molecules are, while the other shows only where the unbleached molecules are. If image intensities have been matched, simply subtracting the second image from the first generates an absolute FLAP image that shows where the bleach-labeled molecules are. Thus FLAP combines the best features of FRAP and PAF. In FRAP, the molecules can no longer be imaged after they have been targeted, whereas in PAF they cannot usually be imaged before they have been targeted. With FLAP, full information is retained on the distributions of both targeted and nontargeted molecules. It is therefore possible to construct other useful virtual images such as relative FLAP, which shows the percentage of bleach-labeled molecules at any given site (Zicha et al., 2003).

It is not an absolute requirement of the FLAP method that a single molecule carry both fluorophores. Indeed, there are some advantages when they are carried by different individual molecules of the same species. In this case, if each colocalizes with the corresponding native molecules as expected, they will still colocalize with each other. However, colocalization now depends on the statistical properties of molecular distribution and, in order to obtain noise-free FLAP images, it is important that both tagged molecules be present in large numbers. This is usually not a problem if the corresponding native molecules are abundant in the cell. It is hard to specify how many molecules need to be carrying fluorophores compared to the number of native molecules, since this depends on many other factors such as detection efficiency, pixel size, and post-processing. A general guideline is that results comparable to PAF or better will be obtained if the two fluorophores give well matched, noise-free images prior to photobleaching. The effect of having the fluorophores on different molecules is, roughly, that the error noise in the final FLAP image is doubled (see Critical Parameters).

The chief competitors to FLAP for flash labeling molecules at specific sites are PAF techniques. Each method has advantages and shortcomings. Although first in the field, the earliest PAF methods using caged fluorophores are probably now the ones with the most disadvantages. First, there are difficulties in conjugating and purifying the protein in sufficient quantity without impairing its activity, as well as in introducing it into cells so that it colocalizes with native protein. A serious limitation is that caged compounds cannot yet be incorporated into cDNA constructs and expressed by the cells. The photoactivatable fluorescent proteins are much more promising in this respect, and recent developments suggest that some will soon be able to be activated and deactivated repeatedly and efficiently (Chudakov et al., 2003), which will be a decided advantage over current FLAP methods.

On the other hand, an interesting feature of the FLAP method as described here is that there is much less need to worry about photodamage occurring to the photolabeled molecules. The method calculates where the virtual bleached molecules are located, i.e., where the bleached molecules would be assuming that they were not damaged, and it is not of much concern what happens to the real bleached molecules. While it is still possible that reactive intermediates generated by photobleaching might cause problems, the compact barrel-like structure of GFP and its variants is thought to shield the external environment from these (Lippincott-Schwartz et al., 2001). Coupled with this, FLAP has the advantages of not requiring specialized fluorophores, and the bleaching process is rapid and efficient using the 514-nm line of a standard argon laser on the LSM 510, whereas photoactivation may require an additional blue diode laser (the alternative two-photon photoactivation is very slow; *UNIT 4.11*). The main advantage of FLAP, however, is that all the tagged molecules are available for imaging throughout the experiment, whether or not they have been photobleached. In the authors' experiments (see Anticipated Results) it has been possible to see throughout where all the actin is, as well as where it has been FLAP-labeled. It may turn out to be possible to do this in conjunction with PAF, but it will require either a second reference fluorophore, as with FLAP, or a second way of exciting the molecules to fluoresce before photoactivating them.

A potential shortcoming of the FLAP method is that colocalization of the two fluorophores is not perfect, but relies on the sta-

tistical properties of large numbers of individual molecules. In practice, the authors have not found this to be a problem and have obtained results that compare very favorably with photoactivation methods (but see Critical Parameters). For the future, however, one way around this problem would be to achieve almost perfect colocalization by fusing both fluorophores to the same molecule. This has not been done in this unit because, besides the potential problems of creating a bulkier molecule that may no longer colocalize with corresponding native molecules, it would no longer be possible to ignore any photodamage that might occur to the bleached molecules. Moreover, there is an additional complication that the two fluorophores used in this unit are capable of fluorescence resonance energy transfer (FRET; *UNIT 17.1*) when very close together on the same molecule. Even so, there are early indications that the FRET complication may not be a serious disadvantage and may actually enhance the FLAP signal; however, there are still worries that the FRET efficiency may alter during configuration changes such as polymerization.

Critical Parameters

Expression levels are critical. At high expression levels, there is a danger that FRET may occur in regions of high fluorophore density even when the two fluorophores are carried on separate molecules as described in this unit (see Troubleshooting). On the other hand, the FLAP images would be unusably noisy if the density of fluorescent molecules were very low, as is required by the FSM method. This is especially true when the fluorophores are carried on separate molecules. The binomial theorem can be used to predict these statistical errors in the FLAP signals. Consider first the case in which there happen to be 1000 molecules of CFP-YFP-actin in a pixel and half of them are expected to have a photobleached YFP. Theory predicts that, in repeated experiments, the number of molecules found to be bleached by absolute FLAP would be 500 ± 16 (mean \pm SEM). In the case of relative FLAP, the bleached fraction would be 0.5 ± 0.016 . Now, consider the case in which the two fluorophores are not on the same molecule but there still happen to be 2000 fluorophores in the pixel: half are expected to be CFP-actin and half YFP-actin. Half of the latter are expected to have been photobleached. Theory now predicts that the number of molecules found to be bleached by absolute FLAP would be 500 ± 37 , and the fraction of YFP-actin molecules

found to be bleached by relative FLAP would be 0.5 ± 0.027 . Thus, one can conclude that having the fluorophores on different molecules approximately doubles the error of the FLAP estimates. This also holds true if there are only 200 fluorophores in the pixel, in which case the figures are: 50 ± 5 ; 0.5 ± 0.05 ; 50 ± 12 ; and 0.5 ± 0.09 , respectively. The relative errors are proportional to the square of the number of molecules, so it is necessary to express four times as many fluorescent molecules in order to halve the relative error.

A further requirement, as with most fluorescence microscopy, is that the tagged molecules accurately colocalize with native molecules of the same species. There is evidence that GFP variants, fused to actin in the configuration used by the authors, do this well (Choidas et al., 1998). Ensuring that the two different variants are incorporated into otherwise identical cDNA constructs will give the best colocaliza-

tion for FLAP even when colocalization with the native species is slightly impaired.

Even the best apochromatic objectives show a significant lateral and axial chromatic aberration. This means that the CFP and YFP images may not exactly coincide both laterally and vertically, and it is necessary for good results that the cell be close to the optical axis of the objective (see Troubleshooting) and that there be no drift in focus. Temperature stabilization is essential for maintaining a fixed focal plane. In the past, the authors have obtained satisfactory results using a fan and temperature-controlled heater within a spacious microscope dust cover placed over the entire microscope. However, a properly constructed air-flow chamber (Fig. 21.2.1) is essential for consistently good results. The thermocouple is placed very close to, but not touching, the optical chamber (see Troubleshooting). The temperature controller is

Table 21.2.1 Troubleshooting Guide to FLAP

Problem	Possible cause	Solution
Regions of cell edge show in FLAP images even before bleaching	Chromatic aberration	Minimize by using fluorescent beads to find best region of objective.
Rhythmic noise or parallel lines	Vibration from heater, thermocouple, table, or computer	Eliminate fan/computer vibration; make sure that table is properly isolated.
Focus drift	Temperature fluctuation	Check heater stability and presence of drafts.
Artifacts on top 1-2 lines of first image scanned after a frame interval	Software bug in acousto-optic driver	Avoid frame intervals if possible. Avoid placing critical part of cell near top of image.
Difference signal is not zero everywhere before bleach	Images not matched	See Basic Protocol, step 14 or match images during post-processing.
	Fluorophores not colocalized	Choose new fluorophore(s).
Striped or fluctuating phase image	Polarizing filter in the light path	Remove polarizing filter from the light path.
Lower (negative) FLAP signal in areas of dense expression before bleach	FRET	If unacceptable, use shorter expression times, compensate during post-processing, or choose new fluorophore(s).
Noisy fluorescence images on one or both channels.	Low expression	Wait longer for expression. Adjust cDNA injection ratio.
	Too much gain in photodetector amplifier	Increase laser power and reduce gain (but keep fade rates matched if possible).
	Too much magnification (pixels too small)	Use lower zoom factor or average more frames.

trained to maintain a temperature of 37°C, and should do so, ideally, within a range of $\pm 0.1^\circ\text{C}$.

Troubleshooting

See Table 21.2.1 for a troubleshooting guide.

Anticipated Results

FLAP has been used to estimate the diffusion rate of G-actin in cytoplasm (Zicha et al., 2003), and the result agrees well with previous estimates using FRAP (McGrath et al., 1998). Relative FLAP has proven particularly useful for detecting the rapid transport of actin to the cell's leading edge from regions up to 12 μm behind the edge (Zicha et al., 2003). Four FLAP images showing some of the results obtained from the cell featured in Figures 21.2.2 and 21.2.3 are shown in Figure 21.2.7. Note that the absolute FLAP images (Fig. 21.2.7A and C) show the highest intensity in parts of the bleach region where the density of actin is greatest (see Fig. 21.2.3), whereas relative FLAP (Figure 21.2.7B and D) gives a more uniform signal throughout the bleach region. In the later relative FLAP image which started 3.9 sec after bleaching (Figure 21.2.7D) highly labeled actin (~60%) can be seen at small regions of the cell's leading edge. This phenomenon is not noticeable in the absolute FLAP image (Figure 21.2.7C) because these thin marginal regions have a relatively low density of actin. When interpreting the FLAP images, bear in mind that the top of the image has been scanned first and that there can be a time difference of several seconds between the top and bottom of the image. It is especially important to compensate for the scanning time when fitting mathematical models of fast molecular processes such as diffusion of monomer (see Internet Resources).

Time Considerations

Cell culture

Plan ahead by at least 72 hr to allow for sufficient cell density, depending on growth rate of cell type.

Microinjection

This should take no more than 15 min if injecting cells at room temperature without CO_2 .

Expression of constructs

Allow at least $2\frac{1}{2}$ hr for expression and maturation of GFP and variants.

Filming chamber preparation

Chambers should be prepared well in advance (usually left overnight) and kept sterile in a sealed container until needed.

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Key References

Dunn et al., 2002. See above.

First description of the technique.

Zicha et al., 2003. See above.

Describes an application of the technique.

Internet Resources

<http://www.lis.ch>

Web site of Life Imaging Services, which supplies microscope temperature control systems.

<http://www.zeiss.com/us/micro/home.nsf/Contents-FrameDHTML/286BA4D22B14DEE985256B4A007C3686>

Zeiss Web site from which LSM Reader can be downloaded.

<http://www.sciencemag.org/cgi/content/full/300/5616/142/DC1>

Supplementary online material for Zicha et al. (2003). Describes diffusion modeling.

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CHAPTER 22

Cell Biology of Chromosomes and Nuclei

INTRODUCTION

Organizing and expressing the information within the genome is an enormous challenge faced by all living organisms. This chapter is concerned with methods that address the structure and maintenance of chromosomes. Notably, the isolation of chromosomal DNA within the nucleus is one of the defining characteristics of eukaryotic cells. This isolation has important consequences for the organization of the genome, as well as for expression, trafficking, and interpretation of genetic information. Thus, additional aspects of nuclear organization may be included in this chapter in future updates.

UNITS 22.1 to 22.5 concern the cytogenetic analysis of vertebrate chromosomes. *UNIT 22.1* provides an overview of cytogenetic analysis techniques. These techniques are particularly important in analyzing subtle changes in chromosome structure that are associated with genomic instability in many contexts. *UNIT 22.2* describes culture methods and the preparation of mitotic and interphase samples for this analysis. *UNIT 22.3* describes traditional banding methods that permit the identification of chromosomes by number and allow the identifications of abnormalities. *UNIT 22.4* describes the use of fluorescence in situ hybridization (FISH) for the analysis of chromosomal structure. FISH analysis of mitotic chromosomes has provided a wealth of information about genetic changes in cancers and in genetic syndromes, such as chromosomal translocations and inversions. These methods have been extended with multicolor FISH applications to identify aberrations in genomic structure (*UNIT 22.5*). These techniques include M-FISH, wherein individual chromosomal paints are distinguished from one another during acquisition using distinct excitation filters. Alternatively, individual chromosomal paints within a mixture can be distinguished through spectral karyotyping (SKY) methods, which rely on Fourier transform methods for their analysis and separation. Moreover, fluorescence in situ hybridization analysis through comparative genomic hybridization (CGH) provides sensitive determination of the net genomic gains and losses in test samples in comparison to normal reference genomic DNAs, giving a detailed picture of how genomes change during the progression of human disease.

Readers should note that additional methods for studying the nuclear structure or nuclear processes can be found in earlier chapters of *Current Protocols in Cell Biology*. An extensive collection of relevant in vitro protocols is provided in Chapter 11. *UNIT 11.5* describes systems for studying mammalian cell replication using the large T antigen–dependent replication of plasmids within mammalian cell extracts. *UNIT 11.6* contains protocols for the preparation and use of mammalian and *Drosophila* extracts that accurately initiate basal and activated polymerase II transcription. *UNIT 11.17* describes protocols for in vitro analysis of post-transcriptional mRNA splicing, and *UNIT 11.14* describes protocols for RNA export assays, using microinjected *Xenopus* oocytes. Protocols for the analysis of nuclear trafficking in digitonin-treated cells are described in *UNIT 11.7*. Chapter 11 also contains protocols for the production and use of extracts from *Xenopus laevis* eggs. Extracts prepared from these eggs are able to reconstitute sperm pronuclear assembly, resulting in nuclei that are functional for nuclear transport and DNA replication (*UNIT 11.10*). By varying the conditions of preparation slightly, mitotic egg extracts can be produced that have the capacity to assemble intact and functional spindles from added sperm

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22.0.1

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chromatin (*UNIT 11.13*). Other aspects of nuclear and chromosomal remodeling are reconstituted within extracts that spontaneously alternate between interphase and mitosis (*UNIT 11.11*). *UNIT 11.12* also discusses variant *Xenopus laevis* egg extracts that undergo programmed cell death (apoptosis) in vitro.

Other experimental approaches to nuclear processes are provided in Chapters 13, 17, and 18. *UNIT 13.5* describes the application of photobleaching techniques to proteins within the nucleus, providing temporally and spatially resolved data regarding the dynamics of nuclear structures. The measurement of protein-DNA interactions in the context of chromatin, through chromatin immunoprecipitation (ChIP) assays, is described in *UNIT 17.7*. Analysis of nuclear functions during apoptosis is provided in Chapter 18, particularly in *UNIT 18.4*, which discusses monitoring of telomeres through quantitative in situ hybridization, and *UNIT 18.6* which discussed other assays for telomere status and telomerase activity.

Together, these methods provide experimental avenues to address questions about the organization, maintenance, and expression of the genome—some of the most complex and fascinating problems in modern biology.

Mary Dasso

Overview of Cytogenetic Chromosome Analysis

This set of units describes the common techniques used in the preparation and analysis of mammalian chromosomes. Although the protocols mainly describe analysis of human chromosomes, the techniques are often applicable, with minor modifications, to the study of cytogenetics in other species. The amount of cytogenetic information that can be generated from a given piece of tissue or from a cell line is vast. Thus, the specific application of the techniques described depends on the type of question being asked by the researcher.

The first visualization of chromosomes in the late 1950s opened up the field of cytogenetics. Successful culturing of primary tissues or cell lines (UNIT 22.2) is paramount for obtaining a sufficient number of high-quality metaphase preparations for chromosome analysis. Once a culture has been established from a tissue or cell line, chromosome spreads need to be prepared (UNIT 22.2).

Banding techniques such as Giemsa banding (G-banding) have permitted the identification of chromosomes by number as well as the detection of chromosomal abnormalities (UNIT 22.3). For the past 40 years, these banding techniques have enabled the identification of chromosomal abnormalities in hematological malignancies, sarcomas, carcinomas, and genetic syndromes.

The 1990s brought about a second golden age for the field of cytogenetics with the advent of fluorescence in situ hybridization (FISH), where labeled DNA probes (UNIT 22.4), and, more recently, protein nucleic acid (PNA) probes, could now be hybridized to the chromosome target, detected, and analyzed using fluorescence microscopy (UNIT 22.4). This provided an important tool for gene mapping and for the identification of specific chromosomal aberrations associated with disease. Commercial diagnostic FISH tests could now routinely be included as an adjunct to classical banding analysis in the clinical hospital cytogenetics laboratory. In addition, with FISH, the drawback of having few metaphase spreads in specimens could be overcome by using gene/locus-specific probes and examining interphase nuclei. Tissues embedded in paraffin can also be examined by interphase FISH.

Multicolor FISH analysis of all human and mouse chromosomes can be carried out in one experiment. This recent development is a technological milestone and brings the field of cytogenetics full circle. Now, very subtle chromosomal aberrations such as small translocations or inversions can be readily identified without having to rely solely on the subjective interpretation of classical banding patterns. Chromosomes that were once dubbed as marker chromosomes, indicating that they provide insufficient banding information for chromosomal identification, can now easily be identified using advanced cytogenetics techniques. In conjunction with standard FISH experiments, small structural changes such as inversions, microdeletions, and amplifications can easily be assessed using locus-specific probes. Improvements in microscopy and fluorochromes make it possible to carry out many tests on one specimen.

Another FISH-based technology now routinely used is comparative genomic hybridization (CGH). This technique permits the determination of net genomic gains and losses in a given DNA sample. The test/tumor DNA is compared to a normal reference DNA sample and hybridized to normal metaphase slides. A two-color FISH detection experiment is performed, and image-analysis software calculates the change in the green/red ratio along the length of the chromosomes, corresponding to the tumor (green) DNA and normal reference (red) DNA that has hybridized, competitively, to the normal metaphase target. Increase in green/red ratios indicates a gain of genomic material at that chromosomal locus in the test/tumor, while a decrease in green/red ratio indicates a loss of genomic material at that chromosomal locus. CGH can provide important information from specimens where short-term culture has proven unsuccessful or impossible. DNA for CGH can readily be extracted from fresh or frozen tissue, paraffin-embedded tissue, or microdissected cells. More recently, CGH methods are being used to identify chromosomal imbalances using microarray target slides.

Sister chromatid exchange (SCE) refers to the interchange of DNA between replication

products. Each exchange represents a point of DNA template exchange during strand synthesis. SCEs are visualized as asymmetric chromatid staining or as harlequin chromosomes, because BrdU incorporation during strand synthesis causes differential staining with Hoechst dye and Giemsa. SCEs can be used to identify

chromosomal anomalies and errors in the mitotic machinery.

Finally, improvements in antibody systems and identification of new proteins permit the study of the events that occur during DNA replication and the components involved in the mitotic machinery.

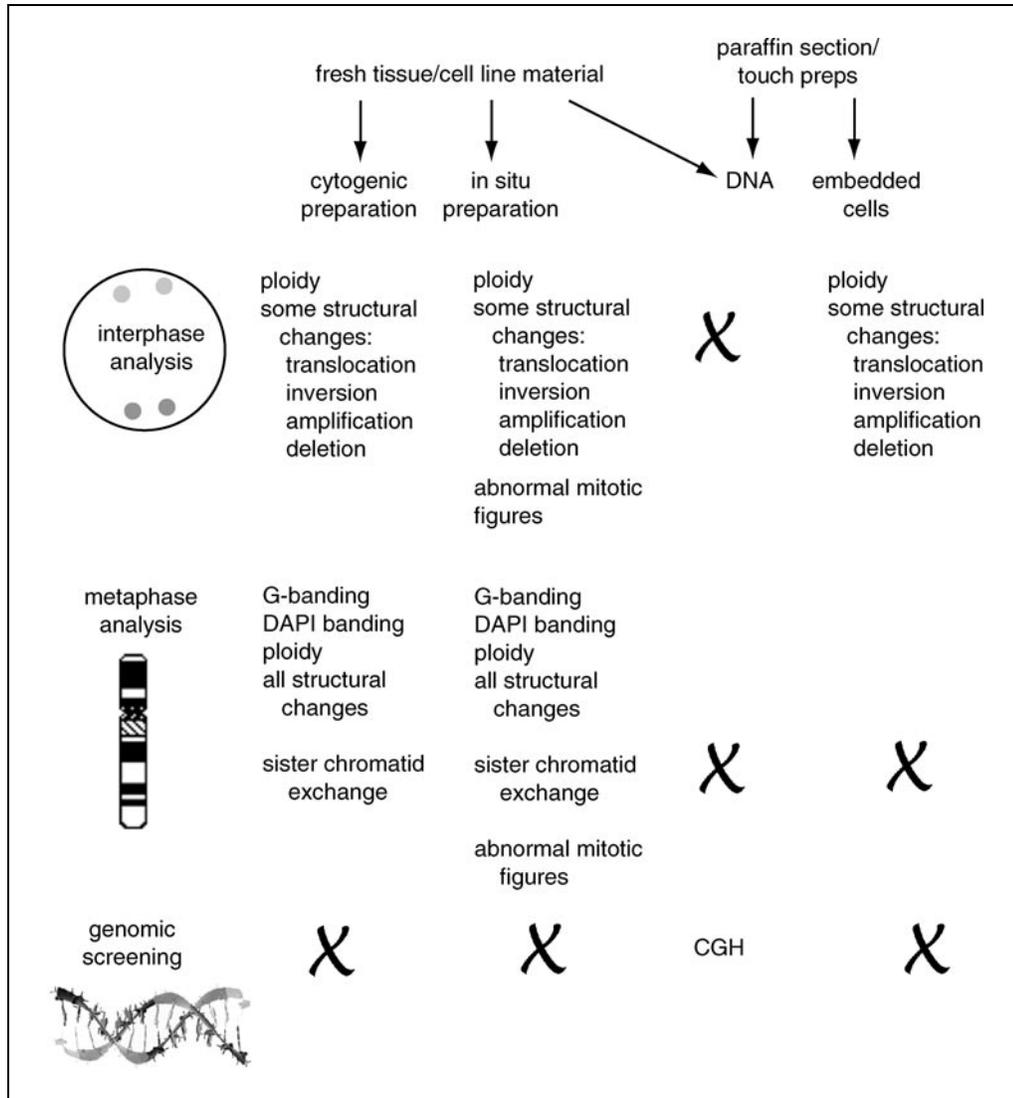


Figure 22.1.1 Overview of techniques for chromosomal analysis. Information regarding the status of whole chromosomes or specific chromosomal loci can be obtained from fresh material, such as fresh tissues and cultured cell lines, as well as from archival material including paraffin-embedded tissues, using FISH analysis. However, only cultured cells make it possible to perform specific chromosomal analysis using metaphase spreads as well as interphase cells. Tissues embedded in paraffin material can only provide information derived from interphase nuclei. Metaphase analysis provides information regarding ploidy, as well as the presence of structural changes such as balanced and unbalanced translocations, inversions, deletions, and additions. Complex extra-chromosomal structures such as double-minute chromosomes (dmns), homogeneously staining regions (HSRs), ring chromosomes (r), and multicentric or acentric chromosomes can also be revealed by metaphase analysis; these features can only be indirectly inferred by interphase analysis. Both fresh and archival material will yield DNA that can be used for comparative genomic hybridization (CGH), a genome-screening technique. Other techniques that reveal chromosomal abnormalities and errors in the mitotic machinery include analysis of sister chromatid exchange and in situ hybridization using antibodies against proteins involved in the mitotic machinery, such as centrosomes.

Figure 22.1.1 describes the types of chromosomal analysis that can be performed on a given specimen and the information that can be generated from such analysis. Since each assay provides different information, it is important to determine which assay is most applicable for addressing a specific research question. Furthermore, the type, amount, and status of the tissue will greatly influence the type of analysis that can be carried out.

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Preparation of Cytogenetic Specimens from Tissue Samples

UNIT 22.2

This unit describes basic tissue culture techniques that enable the investigator to prepare metaphase or interphase cells suitable for cytogenetic analysis from short-term cultured cells derived from tissue or body fluids. Established cell lines from sources such as the American Type Cell Collection (ATCC) are well adapted to *in vitro* growth and can also be used. A comprehensive discussion of tissue culture techniques for long-term cell lines can be found in *UNIT 1.1* (also see Barch, 1991; Freshney, 1993). This unit will describe the general parameters for establishing a short-term culture from primary tissue for cytogenetic studies (see Basic Protocol 1 and Alternate Protocols 1 to 3).

Success in obtaining chromosomes for analysis depends on achieving a mitotically active cell population. Different tissues and cell types, depending on the nature of the experiment, may require specific growth factors and medium supplements (*UNIT 1.2*). In addition, some cell types may sustain mitotic activity for a few days in culture because of residual *in vivo* growth-promoting effects. It is suggested that the investigator refer to current literature sources for the most effective growth conditions and media required for specific tissue types to ensure successful *in vitro* mitotic activity. These factors may be essential when maintaining primary or cell line cultures for several days. Moreover, abnormal cells (e.g., tumors, xenografts, or transgenic tissues) will have different growth kinetics than normal cells, and optimal conditions to favor growth of the cell type of interest should be selected. The culture techniques described here are applicable to tissues acquired from human as well as murine and other rodent sources.

After the cultures are established, the viable cells must be harvested and fixed to make a cytogenetic suspension (see Basic Protocol 2 and Alternate Protocol 4). Growing cultures are treated with a microtubule stabilizer called Colcemid that arrests cells in metaphase. Following exposure to Colcemid, the cells are hypotonically treated to enhance chromosome morphology and to increase chromatin accessibility for appropriate fixation using methanol and acetic acid. Once fixed, the cytogenetic preparation can be stored as cell pellets, under fixative, at -20°C for several months or even longer. Metaphase slides are prepared by resuspending the cells and applying them to glass slides. Obtaining adequate-quality chromosome spreads is multifactorial, and is discussed in detail by Henegariu et al. (2001) and Barch (1991). Oftentimes there is insufficient material to establish a culture, and it may be necessary to harvest any remaining viable cells before the culture dies. Sometimes the cell type of interest will only grow adherently, so short-term cultures may have to be grown on a glass slide or coverslip and treated *in situ*. The techniques for obtaining metaphase spreads for these types of cultures are also described here and in the cited literature.

CULTURING CELLS DERIVED FROM SOLID TISSUES FOR CHROMOSOMAL ANALYSIS

**BASIC
PROTOCOL 1**

This protocol describes the preparation and culture of cells from solid tissues; these cells may be fully or partially adherent. The tissue is subjected to enzymatic disaggregation (if required) of the solid tissue, and the cells are plated and maintained *in vitro*.

Materials

- Solid tissue specimen maintained in transfer medium
- Appropriate culture medium (*UNIT 1.2*)
- 10 \times collagenase IV stock (see recipe)

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22.2.1

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Sterile medium-sized petri dishes (e.g., 60-mm)
Tabletop centrifuge
Scalpel and scalpel blades
Vented 25-cm² tissue culture flasks or 10-cm diameter petri dishes
15-ml conical centrifuge tube

Additional reagents and equipment for cell culture (Chapter 1)

NOTE: All tissue culture incubations are performed in a humidified 37°C, 5% CO₂ incubator. Some media, e.g., DMEM, require increased levels of CO₂ to maintain the medium at pH 7.4.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique must be used.

Dissociate solid specimen

1. Using aseptic techniques (see Chapter 1), remove solid tissue specimen from transfer medium/container and place in a sterile petri dish.

If required, transfer transport medium and remaining cells to a sterile centrifuge tube, centrifuge 5 min at 200 to 300 × g in a tabletop centrifuge, room temperature, and transfer the cell pellet to a sterile tissue culture flask as in step 5. Transfer medium typically contains the same basal medium, growth factors, serum, and other ingredients as the medium for culture; it may contain higher concentrations of antibiotics or fungicide.

2. Mince tissue, using scalpels into a fine pulp.
3. Add a few milliliters of tissue culture medium to the minced tissue and gently draw into a 5-ml pipet. Transfer to a vented 25-cm² tissue culture flask (or 10-cm diameter petri dish) containing sufficient medium (~10 to 15 ml) to cover the surface of the flask.

The culture should be densely seeded to promote growth.

4. Continue mincing the large tissue pieces (remaining in the original petri dish) until they are a fine pulp, and transfer to the flask.
5. Depending on the nature of the tissue, add sufficient 10× collagenase IV stock to the tissue culture flask containing the medium and minced tissue such that the final collagenase concentration is 1×.

Tissues that are fibrous or contain bone may benefit from collagenase treatment. Other, softer tissues, such as brain, may not require collagenase treatment. Additionally, some tissues may be sensitive to collagenase treatment and die. Thus, if there is sufficient tissue, one flask should be prepared with collagenase IV and one without collagenase IV. For the culture without collagenase IV, proceed to step 12.

6. Cap the flask securely and place it on its side in the 37°C CO₂ incubator. Incubate overnight.
7. The following day, use a sterile pipet to gently pipet the treated tissue up and down to break up remaining solid masses and obtain a single-cell suspension.
8. Examine the culture using phase-contrast microscopy to assess the extent of digestion. Also assess the extent of cell adhesion and colony formation. Transfer the single-cell suspension to a 15-ml conical centrifuge tube. Add fresh medium to flask with the adherent cells

Any adherent cells in the original flask should remain untouched.

9. Centrifuge the single-cell suspension 5 min at 200 to 300 × g, room temperature, in a tabletop centrifuge. Pour off the supernatant and resuspend the pellet in 5 ml of fresh medium.

There may still be large fragments of tissue remaining. These tissues can be treated again with collagenase IV in a separate culture.

10. Add fresh medium and transfer the resuspended pellet back to the flask.

If there is a very high density of cells, one or more other cultures can be established.

Establish culture

11. Place the cultures back into the incubator and leave undisturbed overnight.
12. Check cultures daily to obtain a general estimate of doubling time, and feed cultures as required (UNIT 1.1; also see Critical Parameters).
13. Once culture is ~80% confluent, proceed to Basic Protocol 2.

If required, subculture cells as described in UNIT 1.1.

CULTURING TISSUES IN SITU

This protocol is especially useful when a very small amount of tissue is available for analysis. The procedure is essentially the same as that in Basic Protocol 1.

Additional Materials (also see Basic Protocol 1)

Poly-L-lysine-coated glass coverslips *or* slides (see Support Protocol) *or* chambered glass tissue culture slides (Nunc)

Phase-contrast microscope

NOTE: All tissue culture incubations are performed in a humidified 37°C, 5% CO₂ incubator. Some media, e.g., DMEM, require increased levels of CO₂ to maintain the medium at pH 7.4.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique must be used.

1. Proceed with transfer and mincing of the solid specimen (see Basic Protocol 1, steps 1 to 2), except transfer the minced tissue to a 15-ml conical centrifuge tube.
2. Mince remaining large tissue pieces again and transfer to the 15-ml conical centrifuge tube.
3. Depending on the nature of the tissue, add sufficient 10× collagenase IV to the 15-ml conical centrifuge tube for a final collagenase concentration of 1×.

Tissues that are fibrous or contain bone may benefit from collagenase treatment. Other softer tissues, such as brain may not require collagenase treatment. Furthermore, some tissues may be sensitive to collagenase treatment and die. Thus, if there is sufficient tissue, prepare one culture with collagenase IV and one without collagenase IV. For the culture without collagenase IV, proceed to step 6.

4. Cap the tube loosely and place in the 37°C CO₂ incubator. Incubate overnight.
5. The following day, gently pipet the treated tissues up and down to further disaggregate to a single-cell suspension. Centrifuge 5 min at 200 to 300 × g, room temperature, in a tabletop centrifuge. Pour off the supernatant and resuspend the pellet in 3 to 4 ml fresh medium.
6. If using coverslips or nonchambered glass slides, carefully place each in a sterile petri dish and add a few drops of cell suspension, followed by a few drops of fresh

ALTERNATE PROTOCOL 1

medium. If using chambered slides, add sufficient medium to cover the chambered area. Using a sterile plastic transfer pipet, add a few drops (~500 μ l total) of cell suspension. View using a phase-contrast microscope and add more cell suspension if the cell density appears low.

Depending on preference, use glass coverslips or glass slides directly for in situ culturing or coat with an appropriate matrix (see Support Protocol). Commercially available chambered, coated tissue culture slides can also be used. The investigator should refer to the current literature to determine the ideal matrix for promoting attachment of the specific cell type.

Do not use plastic coverslips if subsequent fluorescent-based work will be carried out because plastics produce autofluorescence.

7. Place the slides or coverslips with the cells in the 37°C CO₂ incubator. Incubate overnight, undisturbed.
8. The following day, carefully examine the slides/coverslips with a phase-contrast microscope to determine the extent of adherence. Add fresh medium as required by carefully pipetting fluid from the slide or coverslip and replacing with new medium. Monitor growth daily until the cultures are 80% confluent, then proceed to Basic Protocol 2.

SUPPORT PROTOCOL

PREPARE GLASS COVERSLEIPS OR GLASS SLIDES FOR IN SITU CULTURING

In many cases, cells will adhere to uncoated glass or plastic surfaces; however, the adherence process can be facilitated by the pretreatment of regular glass coverslips and slides. This protocol describes the steps to coat and store glass slides and coverslips for in situ culturing as in Alternate Protocol 1. Poly-L-lysine-coated slides can also be purchased commercially from many companies.

Materials

Glass coverslips *or* slides, autoclaved
1 mg/ml poly-L-lysine stock solution: mix 100 mg poly-L-lysine (Sigma) with
100 ml sterile H₂O
Sterile petri dishes or Coplin jar

1. Place autoclaved glass coverslips or slides in a petri dish or Coplin jar containing the 1 mg/ml poly-L-lysine stock solution. Incubate at least 1 hr at room temperature.
2. Carefully remove the coverslips and/or slides and allow to dry.
3. Store in a dry cool place up to 1 month at room temperature, in a sterile petri dish sealed with Parafilm.

ALTERNATE PROTOCOL 2

CULTURING NONADHERENT OR FLUID TISSUES FOR CHROMOSOMAL ANALYSIS

This protocol describes the culturing of fluid tissue sources and nonadherent cells for cytogenetic preparations.

Additional Materials (also see Basic Protocol 1)

Fluid specimen maintained in transfer medium

NOTE: All tissue culture incubations are performed in a humidified 37°C, 5% CO₂ incubator. Some media, e.g., DMEM, require increased levels of CO₂ to maintain the medium at pH 7.4.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique must be used.

1. Using aseptic techniques, transfer fluid tissue-containing specimen to a sterile 15-ml conical centrifuge tube and centrifuge 10 min at 200 to 300 × g, room temperature, in a tabletop centrifuge.
2. Pour off supernatant and resuspend pellet in 5 ml fresh culture medium.
3. Transfer cells to a sterile 25-cm² vented tissue culture flask containing 10 ml of medium.

Alternatively, if there is abundant material, the investigator may wish to establish a larger culture in a 75-cm² vented tissue culture flask. Keep in mind that high cell density tends to promote growth.

4. Place in a 37°C CO₂ incubator and incubate overnight.
5. Monitor growth daily by phase-contrast microscopy and pH changes as revealed by changes in medium color.
6. If required, subculture the cells (*UNIT 1.2*).

CULTURE OF PERIPHERAL BLOOD CELLS

This is a standard protocol for obtaining metaphase spreads from blood. Depending on the nature of the specimen (e.g., specimens from hematological malignancies), most blood specimens will be processed in the same fashion. The blood is collected in heparin and processed for culturing as soon as possible. Although whole blood can simply be cultured with the medium, this protocol describes the separation of the white blood cells using a Ficoll-Hypaque density gradient and centrifugation. The isolated white blood cells are then cultured for 72 hr in medium containing phytohemagglutinin, a cell synchronizer. At a time point ~24 hr prior to harvesting, the cultures may be half-fed (see Critical Parameters).

Materials

Blood collected in heparin
Phosphate-buffered saline (PBS; *APPENDIX 2A*), sterile
Ficoll-Hypaque (density 1.077; Amersham Biosciences; store at 4°C protected from direct light)
Supplemented RPMI 1640 medium (see recipe)

50-ml conical tubes (e.g., Falcon)
15-ml conical centrifuge tubes (e.g., Falcon)
Refrigerated centrifuge
Sterile disposable plastic transfer pipets (Fisher)
25-cm² tissue culture flasks

NOTE: All tissue culture incubations are performed in a humidified 37°C, 5% CO₂ incubator. Some media, e.g., DMEM, require increased levels of CO₂ to maintain the medium at pH 7.4.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique must be used.

Isolate white blood cells by Ficoll-Hypaque gradient

1. Transfer 12 ml of blood collected in heparin to a sterile 50-ml tube and add 20 ml sterile PBS. Cap tube and mix by inversion.

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2. To each of four 15-ml conical centrifuge tubes, add 3 ml Ficoll-Hypaque.
3. Layer 8 ml of the blood/PBS mixture onto the top surface of the Ficoll-Hypaque.
Add the blood/PBS mixture slowly, in such a way that it does not mix with the Ficoll.
4. Cap the tubes and carefully place them into a 4°C centrifuge. Centrifuge 20 min at $300 \times g$, 4°C, without the brake.
If a refrigerated centrifuge is not available, a room temperature centrifuge can be used, but the investigator is cautioned to work quickly, as Ficoll can be toxic to cells.
5. At end of centrifugation, carefully remove the tubes from the centrifuge without disturbing the separated phases.
6. Using a sterile disposable plastic transfer pipet carefully remove the band of white blood cells located at the interface above the packed red cell pellet, and transfer into a new 15-ml conical tube.
Cells from all four tubes may be combined into the same conical tube.
7. Fill the conical tube almost to the top with supplemented RPMI 1640 medium. Cap and mix by inversion. Centrifuge 5 min at 200 to $300 \times g$, room temperature.
8. Remove the supernatant and resuspend the pellet by gently tapping the tube. Add ~4 ml of fresh medium.

Establish cultures

9. Add 9 ml of supplemented RPMI 1640 medium to each of four 25-cm² tissue culture flasks. Add 1 ml of the resuspended cells to each flask for a total volume of 10 ml.
10. Place flasks on their sides in a 37°C CO₂ incubator and incubate 48 hr. At end of incubation, add 5 ml of medium to the flask and gently pipet the cells up and down to break up any clumps of cells. Return flasks to incubator.
11. After another 24 hr of incubation (total culture time of 72 hr), proceed to Basic Protocol 2.

A normal peripheral blood culture usually requires a 1-hr Colcemid treatment at the standard concentration (see Basic Protocol 2).

BASIC PROTOCOL 2

PREPARATION OF METAPHASE SPREADS

The following protocol describes the steps required to obtain metaphase spreads from both adherent and nonadherent cultures as well as those from cells grown in situ. Briefly, the cultures are treated with Colcemid, pelleted by centrifugation, and hypotonically swollen. Finally the cells are fixed to maintain cellular architecture and to ensure that cellular proteins do not interfere with subsequent detection steps. The hypotonically swollen and fixed cytogenetic suspension is then applied to glass slides and air dried under controlled humidity. The slides are then ready for various analyses. The exposure time and concentration of Colcemid varies and depends on factors including tissue type and overall growth characteristics. The incubation times and concentrations listed below are typical starting points that will require modification. Barch (1991) provides a detailed summary of harvesting conditions for many of the hematopoietic malignancies and clinical samples such as amniocytes and chorionic villus cells. Harvesting solid tumors is far less predictable and requires experience borne from trial and error. Human, murine, and rodent cells are treated in the same fashion; however just as different cultures may respond differently to harvesting techniques within the same tissue type, there may also be some variability between species. An empirical approach to optimizing harvesting procedures is critical.

Materials

Adherent or nonadherent cell culture (Basic Protocol 1 or Alternate Protocol 2 or 3), 80% confluent
10 $\mu\text{g/ml}$ Colcemid (Invitrogen) in H_2O (store up to 2 months at 4°C)
Appropriate tissue culture medium with serum
1 \times trypsin/citrate saline (see recipe)
0.075 M KCl (see recipe), prewarmed to 37°C in oven or water bath
3:1 (v/v) methanol/acetic acid fixative (see recipe)

15-ml conical centrifuge tubes
Tabletop centrifuge
Sterile disposable plastic transfer pipets (Fisher)
Coplin jars
Glass slides
Slide box

Additional reagents and equipment for cell culture (Chapter 1), including trypsinization (*UNIT 1.1*)

Treat cells with Colcemid

1. To 80% confluent adherent or nonadherent culture, add sufficient 10 $\mu\text{g/ml}$ Colcemid stock to give a final concentration of 0.1 $\mu\text{g/ml}$.
2. Return the cultures to the 37°C CO_2 incubator and continue incubating for 1 hr.

See Troubleshooting for discussion of Colcemid sensitivity and incubation time.

Prepare single-cell suspension

- 3a. *For adherent and partially adherent cells:* At end of incubation, transfer the medium to a 15-ml conical centrifuge tube and centrifuge 5 min at 200 to $300 \times g$ in a tabletop centrifuge, room temperature. Discard the supernatant. Trypsinize attached cells in the flask using 1 \times trypsin/citrate saline (also see *UNIT 1.1*) and transfer to the 15-ml tube containing the pellet of cells from the medium. When the cells have detached, add fresh medium, collect the cells, add to the tube, and centrifuge 5 min at 200 to $300 \times g$, room temperature. Discard supernatant.
- 3b. *For nonadherent cells:* Transfer cell suspension to a 15-ml conical centrifuge tube and centrifuge 5 min at 200 to $300 \times g$, room temperature. Discard supernatant.
4. Resuspend the cell pellet in the remaining solution by gently tapping the bottom of the conical tube. Resuspend as much of the pellet as possible into a uniform cell suspension.

Swell and fix cells

5. To the resuspended cells, add the first milliliter of prewarmed (37°C) 0.075 M KCl (hypotonic solution) in a dropwise fashion using a plastic disposable transfer pipet, gently tapping the bottom of the tube between drops. Once the first milliliter has been added, slowly fill the tube with the hypotonic solution. Gently mix by inversion and incubate 10 min at 37°C .
6. Again using a plastic disposable transfer pipet, add 5 to 10 drops of 3:1 methanol/acetic acid fixative. Cap tube and gently mix by inversion.
7. Centrifuge 5 min at 200 to $300 \times g$, room temperature, in a tabletop centrifuge.

The pellet should look white and should have doubled in size.

8. Discard supernatant and resuspend the pellet thoroughly by flicking the bottom of the tube.
9. Using a plastic disposable transfer pipet, add 3:1 methanol/acetic acid fixative in a dropwise fashion for the first milliliter with gentle flicking between drops. Slowly fill the tube with 3:1 fixative after the addition of the first milliliter.
10. Centrifuge again as in step 7. Discard supernatant and resuspend pellet by gentle flicking of the bottom of the tube.
11. Repeat fixation by simply filling the conical tube with fixative, capping, and mixing by inversion.
12. Repeat steps 10 and 11 once more. If necessary, store the cytogenetic suspension as a pellet under fixative at -20°C .

Prepare cytogenetic slides

13. Discard fixative and resuspend pellet by gentle flicking of the bottom of the tube. Add sufficient fixative such that the suspension appears opaque.

After preparing the first slide, the cell density may be adjusted as needed with fixative.

14. To a clean Coplin jar containing distilled water, add a clean glass slide.

Different techniques for slide making exist. A comprehensive discussion can be found in Henegariu et al. (2001) and Barch, (1991).

15. Remove one slide and hold roughly at a 45° angle (Fig. 22.2.1).

A thin layer of water will still remain.

16. Using a 1-ml plastic disposable transfer pipet, add 1 to 2 drops of the cytogenetic suspension. Allow the slide to dry.

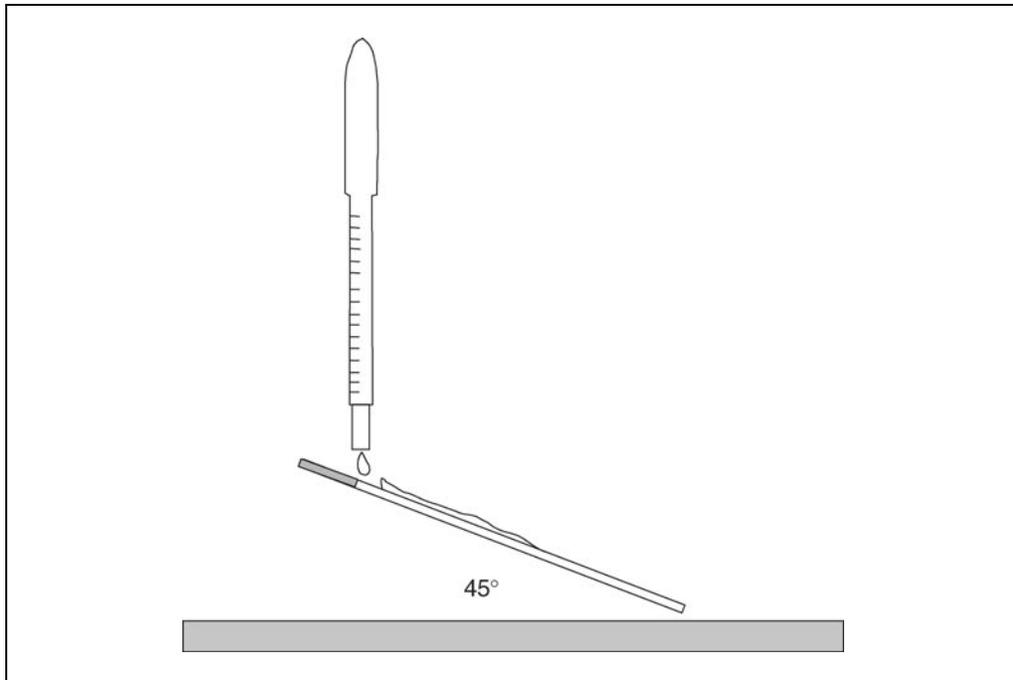


Figure 22.2.1 Schematic representation of “drop-method” of slide making. Shown in the figure is the general method for preparing slides for molecular cytogenetic analysis from a fixed suspension. Using a plastic disposable transfer pipet, add 1 or 2 drops (or $200\ \mu\text{l}$) to a clean slide held at $\sim 45^{\circ}$ angle. The slide may be pretreated with water or fixative. Allow the suspension to run down the slide. Expose the slide to humidity or heat as required to achieve the proper drying conditions.

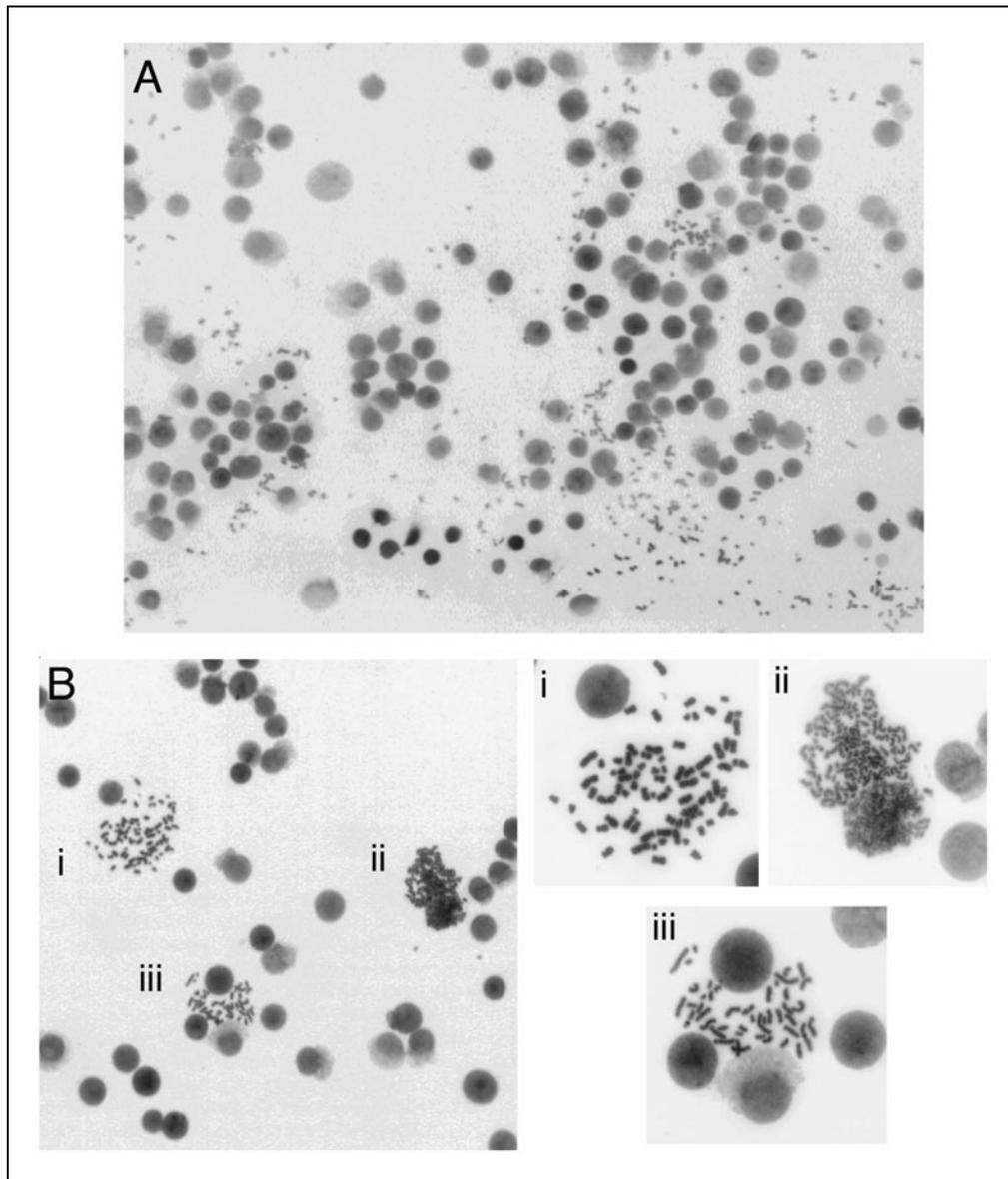


Figure 22.2.2 Phase-contrast images of slides that are appropriate and not appropriate for molecular cytogenetic analysis. **(A)** This panel is a 40 \times view of a prepared cytogenetic slide where the cell density is too high; thus, less suspension should be applied to the slide, or the main suspension should be diluted with fixative. This slide would be appropriate for interphase analysis, since the nuclei are intact and flattened, and chromosome analysis would not be accurate on this slide, since the metaphase spread is not intact. There is the appearance of “chromosome soup,” making it difficult to discern the chromosomal content of one cell. The bursting of the cells may have occurred from rapid fixation following hypotonic treatment, or from excessive hypotonic treatment. If the entire slide has this appearance, a change in dropping technique could be used to prevent overspreading of chromosomes; however it is suggested that another suspension be made. **(B)** This panel shows a cell density that is manageable, with many intact metaphase spreads acceptable for analysis. Each metaphase spread in this field has been magnified. Metaphase “i” shows a well spread metaphase with few overlapping chromosomes that are at a reasonable length for banding analysis. Metaphase “ii” shows a tight spread with longer chromosomes. This metaphase is not appropriate for banding analysis. Metaphase “iii” shows a spread with good-length chromosomes and few overlaps. Its location between nuclei may make access to the spread difficult if there is residual cytoplasmic debris; however this is an analyzable metaphase.

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PROTOCOL 4**

17. Examine by phase-contrast microscopy. Change slide-making strategy as required based on visual assessment of slides (see Troubleshooting and Fig. 22.2.2).
18. Store slides in a slide box in a dry area until ready for use (see Troubleshooting).

CYTOGENETIC PREPARATIONS FROM IN SITU CULTURES

This protocol describes the technique used for cells grown on slides or coverslips (Alternate Protocol 1). The principles of hypotonic treatment and fixation are similar to those of Basic Protocol 2.

Additional Materials (also see Basic Protocol 2)

In situ cultures (Alternate Protocol 1), 80% confluent
Cytoseal 60 mounting medium (Richard-Allan Scientific)

1. To 80% confluent cultures, add sufficient 10 µg/ml Colcemid stock to give a final concentration of 0.1 µg/ml.

The amount of Colcemid used will depend on the amount of medium present (i.e., after adding fresh medium to the initial drops of cell suspension).

2. Return the cultures to the 37°C CO₂ incubator and continue incubating for 1 hr.
See Troubleshooting for discussion of Colcemid sensitivity and incubation time.
3. Carefully remove medium and add fresh medium to wash the Colcemid from the cells.
4. Remove medium and carefully add prewarmed (37°C) 0.075 M KCl. Place in a 37°C incubator or dry oven for 10 min.
5. Add 5 to 10 drops of 3:1 methanol/acetic acid fixative and gently mix by rocking the petri dish or chambered slide.
6. Using a plastic disposable transfer pipet, remove solution. Slowly add fresh 3:1 methanol/acetic acid fixative and let stand at room temperature for 5 min.
7. Remove fixative and repeat fixation twice. Air dry slides
8. Mount coverslip on slide using mounting medium. Examine slides using phase-contrast microscopy. Change slide-making strategy as required based on visual assessment of slides (see Troubleshooting and Fig. 22.2.2).
9. Store slides in a slide box in a dry area until ready for use (see Troubleshooting).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Citrate-saline, 10×

0.44 g sodium citrate (0.44% w/v)
1.0 g potassium chloride (1% w/v)
100 ml H₂O
Dilute to 1× using H₂O

Collagenase IV, 10×stock

153 mg of 260 U/mg Collagenase IV (Sigma)
20.0 ml sterile culture medium or H₂O
Filter sterilize
Store in 500- μ l single-use aliquots indefinitely at -20°C

KCl, 0.075M

2.8 g KCl (Sigma; 0.075 M final)
500.0 ml H₂O
Store at room temperature or 37°C up to 1 month

Methanol/acetic acid fixative, 3:1

90.0 ml methanol (molecular biology grade)
30.0 ml glacial acetic acid (molecular biology grade)
Fixative should be made fresh, however it may be stored at -20°C for several days.

Supplemented RPMI 1640

415.0 ml RPMI 1640 medium with antibiotics (e.g., Life Technologies)
75.0 ml fetal bovine serum (15% v/v final)
5 ml 100 \times L-glutamine (Invitrogen; 1 \times final)
5 ml phytohemagglutinin (M Form, Invitrogen; reconstitute lyophilized powder per manufacturer's instructions)
Store up to 1 month at 4°C

Trypsin/citrate saline, 1 \times

10 ml 10 \times trypsin/EDTA (Invitrogen) (1 \times final)
90 ml 1 \times citrate-saline (see recipe)
Store indefinitely at 4°C
Place at room temperature or 37°C before use

COMMENTARY

Background Information

Cell cultures

Growing tissues in culture has given investigators the ability to analyze and control cellular events. However, it has become increasingly important to characterize the gross chromosomal changes and correlate them to abnormal cellular processes and phenotypes identified. A comprehensive discussion on tissue culture techniques can be found in Chapter 1 of this manual, as well as in Freshney (1993) and Barch (1991). The brief discussion in this unit covers basic tissue culture issues relevant for preparing cytogenetic preparations.

Slide preparation

The first karyotypes were made from peripheral lymphocytes by Hungerford et al. (1959), using a squash technique that is now considered primitive. Later, Moorehead et al. (1960) published the method for air drying peripheral blood chromosomes, a modification of the method of Rothfels and Siminovitch (1958)

using methanol/acetic acid. Prior to this, plant studies had revealed that mitotic-spindle disruptors such as colchicine made it possible to discern the beginning and end of distinct chromosomes, since the chromatin material was condensed and arrested at metaphase; this technique was later applied to mammalian cells. In the procedure used here, the hypotonic swelling of the cells causes a concentration gradient and stretches the cell membrane. Fixation helps to maintain this state until the cell is forced to flatten out when applied to the slide. It is evident from the many cytogenetic publications since those early reports that identifying the chromosomal content of a cell has significance in diagnosing, monitoring, and treating disease and genetic abnormalities. The success of fluorescence in situ hybridization (FISH)-based technologies lies largely in the meticulous principles of harvesting and slide making established by early cytogeneticists.

Finally, obtaining optimal chromosome spreads is an art in and of itself, and techniques

vary from laboratory to laboratory as well as between individuals in the same laboratory. It can be compared to a group of people who are given the same recipe and same ingredients and asked to prepare the same meal. Invariably some will prepare a better-tasting meal than others, based on their careful observations and years of culinary experience. This analogy is worth considering when preparing slides. The sections below (see Critical Parameters and see Troubleshooting) will discuss some of the more critical aspects of slide making, but the onus is on the individual to determine the best method of slide making from trial and error.

Critical Parameters

Cell cultures

Aseptic techniques and proper biohazard safety practices should always be implemented when handling tissue, especially human tissue. These precautions serve not only to protect the investigator and the laboratory but also to ensure that unwanted microbes or fungi in the cultures are minimized.

As mentioned, a search of the current literature will provide the requirements necessary for optimal short-term culture of the tissue of interest. Since not all tissues will have the same growth rate, the investigator should monitor the progress of the cultures daily to ensure that they are not becoming contaminated with normal cells and to estimate the likelihood that sufficient cells will grow. Investigators are urged to consult the current literature concerning the cells being used to determine the best way of limiting the growth of unlimited cell populations. Feeding should be carried out just as the medium starts to become more acidic, based on the change in color of the indicator present in most media. Cultures may be heterogeneous with respect to the types of cells present in the culture, ranging from fully adherent or nonadherent to partially adherent with floating viable cells. When feeding cultures, it is suggested that the cultures be half-fed. To do this, remove half of the tissue culture medium and centrifuge to bring down any floating cells. Discard the supernatant. Add fresh medium to the pellet, resuspend the pellet, and return the cells back to the culture. This ensures that the culture obtains fresh nutrients and at the same time retains critical growth factors or cytokines present in the original medium, which may have been produced by the cells themselves. Finally, some tissues may fail to thrive in culture after enzymatic treatment.

In this unit, collagenase IV is featured as the main enzyme for tissue disaggregation; however, there are several types of collagenase as well as other enzymes that may be used for tissue disaggregation. Caution should be exercised when treating with an enzyme, because some cells do not recover well from enzymatic treatment.

Preparing slides

Cell division may be stimulated by subculturing the cells 1 or 2 days before harvesting for cytogenetic suspension (Basic Protocol 2 or Alternate Protocol 4). Cultures do not necessarily need to be split into new flasks or dishes, but they can be passaged into a new flask simply to induce cell division by disaggregation while keeping cell density at a level just below confluence to avoid contact inhibition of proliferation.

The cultures to be harvested should be actively dividing. When using established cell lines, it is usually easy to identify and count rounded-up mitotic pairs of daughter cells at anaphase/telophase. However, cultures from primary tissues often do not have a uniform morphology, and it can be difficult to distinguish between proliferating and apoptotic or necrotic cells. Oftentimes there is not sufficient material to stain cells with trypan blue; thus the investigator should monitor primary cultures daily. Some tissues fail to thrive in vitro and may need to be harvested within 72 hr. Cellular heterogeneity with respect to cell cycle will also influence the mitotic index of the final cytogenetic suspension. Treatment with cell synchronizers is discussed in Barch (1991) and may help to increase the mitotic index.

Treatment with the drug Colcemid will also influence the mitotic index of a cytogenetic suspension. Since Colcemid arrests cells by disrupting the spindle, increasing treatment times will improve the mitotic index by accumulating cells arrested at metaphase. However, prolonged exposure or use of higher concentrations of the drug will cause increasing chromosome contraction, resulting in shorter chromosomes. As the chromatin within chromosomes becomes condensed, the ability to identify banding patterns continues to decrease.

Hypotonic swelling is generally carried out using KCl. However some have used a mixture of KCl with culture medium or water (Squire, 1983; Barch, 1991; Henegariu et al., 2001). These variations help to prevent the sudden rush of water into the cells. Should the hypotonic treatment occur too quickly, the cell

membranes may burst, yielding “chromosome soup” (Fig. 22.2.2A). Too little swelling will prevent the membranes from adequately releasing the chromosomes from the confines of the cell membrane, preventing them from spreading. Hypotonic treatment is typically carried out slowly, with gentle mixing. Vortexing is usually too harsh, although some individuals have used this method with success. A successful hypotonic treatment will yield a pellet that has doubled in size and that often looks translucent due to increased water content within cells.

The first fixation, termed the pre-fix, occurs after the hypotonic treatment. It involves the addition of a few drops of fixative to the swollen cells. This pre-fix serves to harden cell membranes to maintain cellular architecture and the swollen cell state. The first full fixation is carried out gradually. Once the first full fixation is complete, the subsequent fixations can be carried out in normal fashion. Repeated fixations help to remove cytoplasmic and other cellular debris as well as to preserve the cells.

Fixed cells can be stored for several years, provided that they are stored as pellets under fixative in tightly capped tubes at -20°C . The reasons why cytogenetic suspensions degrade over time are that the cells were not pelleted, they were stored at room temperature or 4°C , or that the methanol has evaporated, leaving an increased concentration of acetic acid, which will damage the cells.

The consensus of many experienced cytogeneticists is that the relative humidity in the room where slides are made is the most critical parameter for slide making. It is not uncommon to find laboratories hoping for a rainy or humid day so that their slide preparation will be optimal. In the authors' experience, a relative humidity of at least 30% to 35% is optimal for preparing well spread chromosome preparations. This can be achieved by having a boiling kettle, beaker of water, or water bath nearby, over which to pass the freshly prepared slide. Humidity in excess of 50% will require aided drying, usually with a warm-to-the-touch hot plate. Determining the best conditions for slide making requires frequent changes in test conditions and trips to a phase-contrast microscope to check the results. Fast drying can be controlled by applying the suspension to slides that are wet (with water). In more humid conditions, suspensions may be applied to dry slides. Others choose to wet their slides with fixative, but this may speed up the drying process. In many state-of-the-

art cytogenetics laboratories, a humidity- and temperature-controlled unit (about the size of a fume hood) called a Thermotron (<http://www.thermotron.com/cryogen.html>) has been used to establish the correct humidity and temperature for slide making.

Storing of cytogenetic slides is also subjective. Some laboratories simply store slides at room temperature, while others store slides at -20°C (in or free of ethanol), and still others store in dessicators. Each laboratory will need to determine the best storage conditions. However, if there is left-over cytogenetic suspension, fresh slides can always be made as needed.

Troubleshooting

Cell cultures

Depending on the likelihood that tissues were handled aseptically before reaching the cell culture facility (i.e., from an operating room or pathology laboratory), it may be necessary to include antifungal agents in addition to the routine antibiotics supplementing both the culture and transport media. Once again, caution should be exercised because antifungal agents are often toxic to cells.

If bacterial or fungal contamination does occur, particularly in adherent cultures, and there are no backup cultures, the contaminants may be removed from the culture, the cultures washed with sterile PBS, and fresh medium added. The cultures must be harvested (Basic Protocol 2) within 24 hr so that the remaining contaminants do not have sufficient time to overgrow. The investigator should be warned that the presence of contaminating bacteria or fungi will cause subsequent background problems for banding analysis and FISH analysis because they contain DNA and antigens that bind the antibodies conjugated to a fluorochrome.

Slide preparation

See Table 22.2.1 for a troubleshooting guide to slide preparation for cytogenetic analysis.

Anticipated Results

Establishing short-term cultures from primary tissues requires careful and systematic monitoring to determine the optimal time to initiate harvesting. Longer-term cell lines will generally yield better quality cytogenetic preparations, as they have undergone adaptation to *in vitro* growth and usually exhibit high mitotic activity. The investigator should make

Table 22.2.1 Troubleshooting Guide for Cell Harvesting and Slide Preparation for Cytogenetics Analysis^a

Problem	Possible cause	Solution
No metaphase spreads	Slowly dividing cell culture	No immediate solution to increasing cell growth; however growth can be stimulated by providing a half-feed of the culture the day before harvesting
	Insufficient Colcemid incubation Insufficient Colcemid concentration	Treatment time with the standard Colcemid concentration of 0.1 µg/ml may need to be increased in increments from 60 to 90 min If the culture appears to be dividing (empirically determined), then increase the Colcemid concentration, but either maintain the same incubation time, or decrease it
Chromosomes too long	Short Colcemid incubation	Every cell type reacts differently to Colcemid treatment; if chromosomes are too long, this suggests that they can tolerate longer incubations or higher concentrations of Colcemid
	Insufficient Colcemid concentration	As described above, Colcemid incubation time and/or concentration can be increased to achieve shorter chromosomes
Chromosomes too short	Cells sensitive to Colcemid treatment	This is more of a problem than the situation above. Decrease amount of Colcemid but maintain the same incubation time, or maintain the same concentration of Colcemid, but decrease the incubation time.
	Colcemid incubation too long	Decrease the amount of Colcemid but maintain the same incubation time, or maintain the same concentration of Colcemid, but decrease the incubation time.
Metaphase spreads too tight	High cell density	If the concentration of cells in the working suspension is too high, it will not permit the chromosomes to spread. Either add less cell suspension to the slide, or dilute the suspension further while maintaining the amount applied to the slide.
	Insufficient hypotonic swelling	The chromosomes must burst out of the plasma membrane and spill onto the slide. If the hypotonic treatment is insufficient to build the necessary tension, then the chromosomes will fail to spread or burst from the plasma membrane. Increase the hypotonic treatment to by 5 to 10 min. However, too much swelling will cause the cells to be too fragile.
Presence of cytoplasm	High cell density	If the concentration of cells in the working suspension is too high, it will not permit the chromosomes to spread. Either add less cell suspension to the slide, or dilute the suspension further while maintaining the amount applied to the slide.
	Insufficient hypotonic swelling	The chromosomes must burst out of the plasma membrane and spill onto the slide. If the hypotonic treatment is insufficient to build the necessary tension, then the chromosomes will fail to spread or burst from the plasma membrane. Increase the hypotonic treatment to by 5 to 10 min. However, too much swelling will cause the cells to be too fragile
Refractile-looking chromosomes	Slides drying too quickly or too slowly	This is the “art” of slide making. Ambient temperatures in the lab and the time of year will greatly affect slide making and their appearance. During the dry months of the year, keep a beaker or kettle of water boiling, or have a water bath on in the lab to increase the relative humidity. For the humid days of the year, keep a “warm-to-the-touch” hotplate on to facilitate drying.

^aA comprehensive discussion can be found in Barch (1991).

note of increasing debris from dead cells and the number of cells that appear to be inactive or senescent, and thus decide the best time to harvest the cells for optimal cytogenetic preparations.

Every harvest will be different, even when harvesting multiple cultures of the same cells. However, with experience, the investigator will learn when to change Colcemid concentration, alter hypotonic conditions, and vary slide-making techniques.

Under the perfect conditions, a slide will have a relatively high mitotic index, such that at least three metaphases can be seen under low power (Figure 22.2.2). Chromosomes should not be too long or too short. Cytoplasmic debris and other cellular debris should be minimal with nuclei and chromosomes appearing gray to dark gray.

Time Considerations

It takes several days to establish a primary culture from tissue and for the cells to reach 80% confluence, depending on the growth characteristics of the cells in question.

It takes 2 to 3 hr to prepare slides for cytogenetic analysis depending on the skill of the investigator and the number of slides to be prepared.

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Internet Resources

<http://www.thermotron.com/cryogen.html>

Thermotron Web site for equipment specifications and description.

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Traditional Banding of Chromosomes for Cytogenetic Analysis

UNIT 22.3

This unit describes the two most commonly used methods for banding chromosomes, Giemsa banding (G-banding; see Basic Protocol) and DAPI banding (see Alternate Protocol). G-banding is the conventional means of staining chromosomes to reveal their banding patterns. It is a nonfluorescent technique requiring only a standard light microscope for visualization. DAPI banding utilizes a DNA stain that fluoresces. With appropriate pretreatment (if required), DAPI banding identifies the same major banding patterns revealed by G-banding. Imaging tools can convert the DAPI image into a black-and-white image, which can then be converted into a G-banding-like image, more familiar to cytogeneticists. Both techniques permit the identification of chromosomes and detection of the presence of grossly abnormal chromosomes and abnormal structures. G-banding requires sufficient chromosome aging, by enzymatic digestion, to remove cytoplasmic debris and allow access of the Giemsa stain to the DNA. The incubation times for each step may be variable depending on the type and quality of specimen. DAPI staining, on the other hand, is not as finicky; slides can be used as is and can normally be stained directly with minimal or no pretreatment.

GIEMSA BANDING (G-BANDING) OF METAPHASE CHROMOSOMES

This protocol describes the basic steps for G-banding metaphase spreads. The enzymatic treatment of the slides requires optimization, either with respect to duration or trypsin concentration. Similarly, the time for staining requires adjustments to suit the investigator's preference and the slide quality. The investigator must be able to recognize the morphology of overtrypsinized or undertrypsinized chromosomal banding patterns. It is suggested that a few practice slides be used first to determine the optimal conditions for digestion and staining. Practice slides should ideally be slides made from the same suspension at the same time and under the same aging conditions as the test slides.

**BASIC
PROTOCOL**

Materials

Metaphase slide preparation made 1 to 2 days before banding (UNIT 22.2)
Trypsin working solution (see recipe)
1% (v/v) fetal bovine serum (FBS): mix 1 ml FBS with 99 ml H₂O
Gurr's buffer, pH 6.8: dissolve 1 Gurr's buffer table (Bio/medical Specialties) in 1 liter sterile H₂O
Giemsa stain working solution (see recipe)

55° to 60°C drying oven
Forceps
Coplin jars
Phase-contrast microscope

1. Age the air-dried slide overnight in a drying oven at 55° to 60°C. Remove and bring to room temperature just prior to banding.
2. Grasp the slide with forceps and immerse it in a Coplin jar containing the 0.025% trypsin working solution for 8 to 10 sec, moving the slide back and forth.

The exposure to trypsin will need to be adjusted depending on the quality of the cytogenetic specimen and the resulting banding (see Troubleshooting and Fig. 22.3.1).

**Cell Biology of
Chromosomes
and Nuclei**

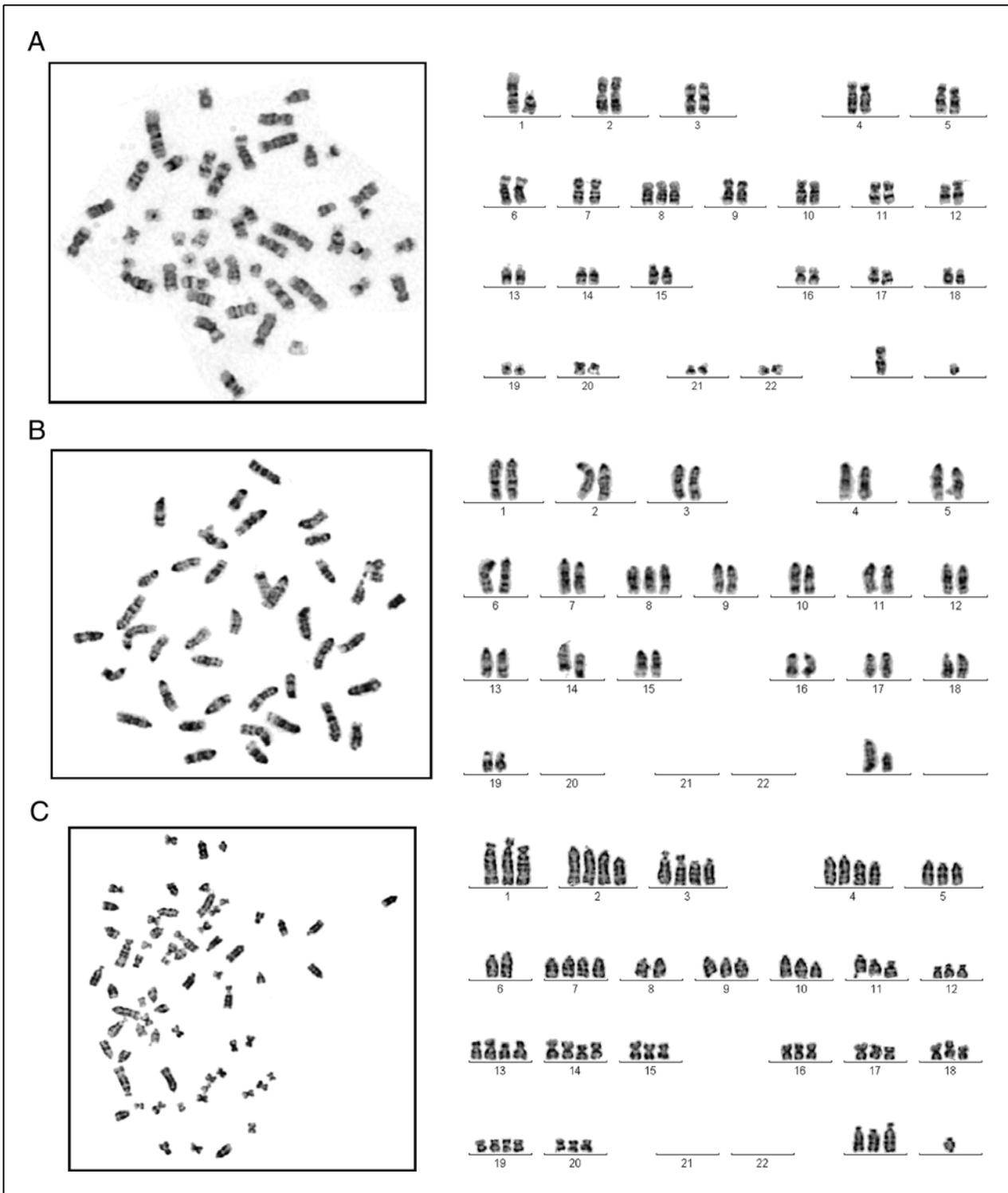


Figure 22.3.1 Images of G-banded metaphase spreads and karyotypes from human, mouse and rat tumors. For each panel, a G-banded metaphase spread and its karyotype has been shown for tumors derived from (A) human, (B) mouse, and (C) rat. All spreads show banding patterns that are well defined with few or no overlapping chromosomes.

**Traditional
Banding of
Chromosomes
for Cytogenetic
Analysis**

22.3.2

3. Briefly rinse the slide in a Coplin jar containing 1% FBS to inactivate the trypsin.
4. Pre-rinse the slide by dipping in a Coplin jar containing Gurr's buffer, using the same agitation technique as in step 2.
5. Place slide in a Coplin jar containing Giemsa staining solution for 8 to 10 min.

The degree of staining will also require adjustment following visualization by phase-contrast microscopy.

6. Rinse slide in autoclaved distilled water until the stain no longer discolors the water, using the same agitation technique as in step 2.

CAUTION: *Long exposure to water will result in destaining of the slide.*

7. Allow slide to air dry. Examine by light microscopy using a phase-contrast microscope to determine the quality of banding. Adjust trypsin exposure or duration of staining as required.
8. Once the optimal banding quality has been achieved, analyze the slides. Store the slides in a slide box free of dust and dirt at room temperature.

4'-6-DIAMIDINO-2-PHENYLINDOLE (DAPI) STAINING OF METAPHASE CHROMOSOMES

ALTERNATE PROTOCOL

In this protocol, cytogenetic specimens are stained with DAPI, which is typically mixed with an antifade mounting medium. Antifade mounting medium can be made in-house or bought commercially (e.g., Vectashield from Vector Laboratories). Unlike G-banding, DAPI staining requires no slide pretreatment and is fluorescently based.

Materials

Metaphase slide preparation (*UNIT 22.2*)
DAPI/antifade medium (see recipe)

Glass coverslips
Fluorescent microscope with DAPI filters

1. Add 50 μ l of the DAPI/antifade medium to the metaphase slide preparation. Add a coverslip.
2. Visualize the chromosome banding pattern using fluorescence microscopy (Fig. 22.3.2). Store the slide at -20°C .

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

DAPI/antifade medium

DAPI stock solution: Dilute an entire container of DAPI (4',6-diamidino-2-phenylindole, Sigma) powder with sufficient water to prepare a 100 $\mu\text{g}/\text{ml}$ stock solution. Store in small aliquots up to several months at -20°C .

DAPI/antifade medium:

Combine in the following order:

5 ml PBS (*APPENDIX 2A*)

500.0 μ l of 100 $\mu\text{g}/\text{ml}$ DAPI stock (see above; 1 $\mu\text{g}/\text{ml}$ final)

0.5 g *p*-phenylenediamine (Sigma; 10 mg/ml final)

Dissolve well, then add:

45 ml glycerol (90% final)

Total volume, 50.0 ml

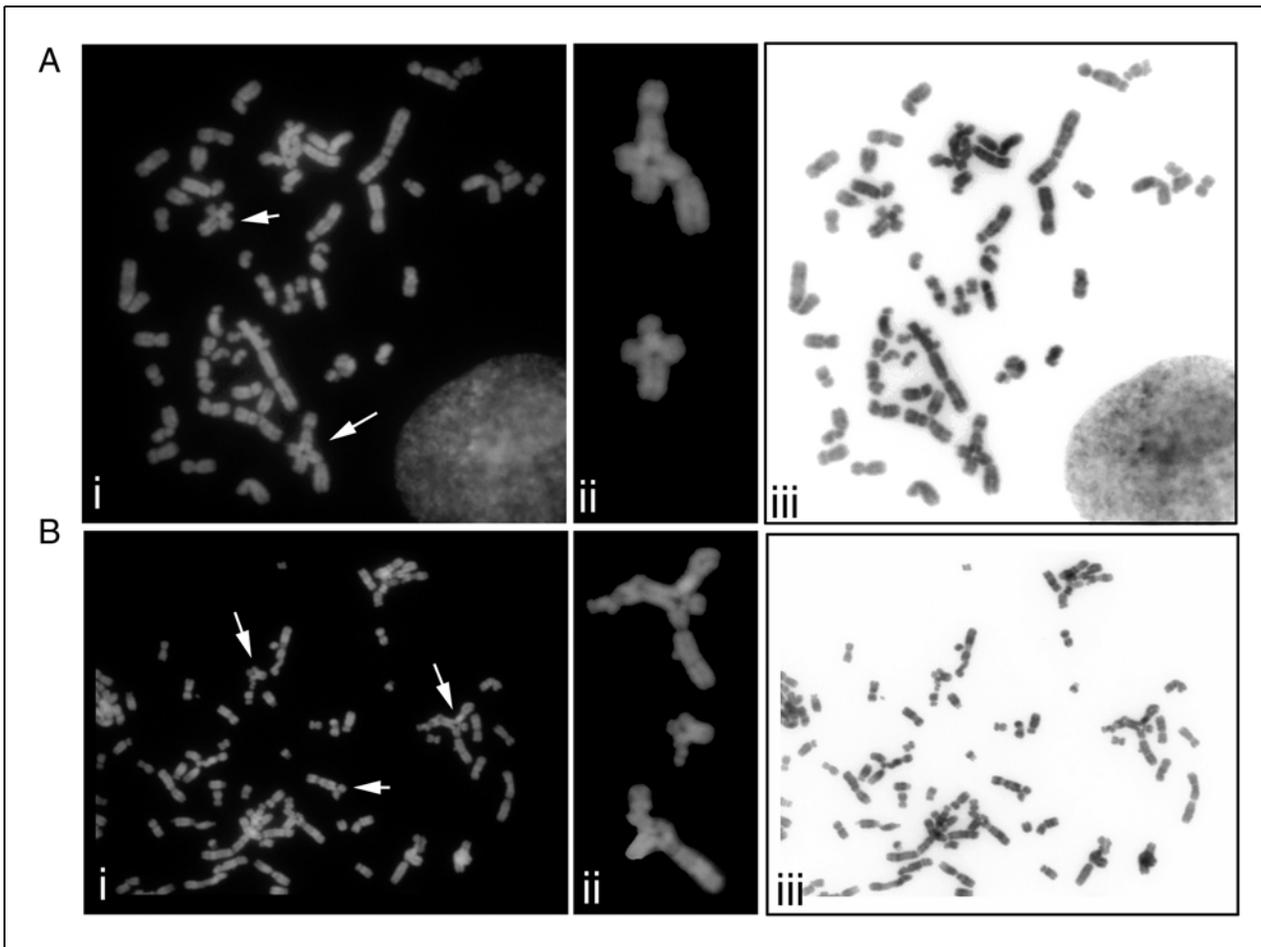


Figure 22.3.2 DAPI staining of a prostate cell line subjected to a chemotherapeutic agent and 4 Gy radiation. Shown in panel **A**, labeled “i,” is a DAPI image of a prostate cell line subjected to mitomycin C treatment. Structural aberrations, e.g., radial structures, are labeled “ii.” Also in panel **A** is the inverted DAPI image (labeled “iii”) demonstrating that DAPI banding provides similar banding patterns as G-banding, but at a lower resolution. Panel **B** illustrates the same prostate cell line subjected to 4 Gy of ionizing radiation, showing complex structural aberrations. Other aberrations, including DNA fragmentation, chromatid breaks, and dicentrics, can be identified. Arrows indicate multiradial chromosomal structures as shown enlarged in (ii).

The resulting medium is very viscous. Transfer to a 50-ml conical polypropylene centrifuge tube (e.g., Falcon), wrap tube in foil (product is light-sensitive), and place on an end-over-end rotator to ensure proper mixing (30 min). Store, protected from light, in 1-ml aliquots for several months at -20°C .

Giemsa stain working solution

1 ml Giemsa stain (Azure Blend, Harleco 620G/75, from EM Science; 2% v/v final)
 50 ml Gurr's buffer solution, pH 6.8: dissolve 1 Gurr's buffer table (Bio/medical Specialties) in 1 liter sterile H_2O
 Store up to 2 months at room temperature

Trypsin working solution 0.025%

0.5 g trypsin (Difco, 0.025% w/v final)
 200.0 ml Earle's balanced salt solution (e.g., Life Technologies)
 Store up to 1 day at room temperature

COMMENTARY

Background Information

Since chromosomes were first visualized in 1959, there has been an obvious need to determine ways of identifying individual chromosomes. Although size, shape, and the position of the centromere is clearly a means of identifying chromosomes, it became readily apparent that there were many chromosomes that were very similar in size and shape. Solid staining and chromosome measurement were used in the early 1960s; however, this still did not provide an adequate method for unequivocal chromosome identification. The Paris Conference of 1971 first systematically described chromosome bands that could be distinguished from adjacent segments on the basis of darker or lighter appearance by one or more techniques. Banded chromosomes were seen as appearing with a consistent series of dark and light bands. Human banding patterns and their nomenclature can be found in the International System of Cytogenetic Nomenclature (1995). Briefly, convention states that the short arm of a normal chromosome is called the p arm and the long arm is referred to as the q arm. A centromere is identified as a constriction along the length of the chromosome that defines the transition from the p to the q arm. Individual bands along each arm are designated numerically, with subdivisions of bands denoted as fractions of the main band. The resolution of bands is influenced by the length of the chromosome; hence, longer chromosomes produce more bands than shorter chromosomes.

There are several banding techniques that are described in Barch (1991). Today, G-banding is considered the gold standard for cytogenetics. Introduced in 1971 by Sumner et al. (1971), G-banding permits stable and effective banding of chromosomes without the need for fluorescence, which was used in an earlier technique called Q-banding (quinicrine banding). Through investigations by Sumner and colleagues, and others (reviewed in Barch, 1991), it was determined that the most effective means of revealing the banding pattern of human chromosomes was through digestion with a protease. Trypsin is used as the protease because it was found to effectively hydrolyze the proteins surrounding the chromatin. The differential protein composition along the length of the chromosome then reveals the variation of chromatin compaction associated with euchromatin and heterochromatin. These differences in chromatin condensation are easily revealed through the staining procedure.

Giemsa is a complex mixture of dyes including azure A, azure B, azure C, thionin, methylene blue, and eosin. Each dye component binds different protein moieties along the length of the chromosome, resulting in the typical banding patterns seen in Figure 22.3.1.

4'-6-diamidino-2-phenylindole (DAPI) is a fluorescent dye that has an emission of 450 nm. It has AT specificity, which enables the elucidation of constitutive heterochromatin-containing regions. DAPI staining is typically used to detect centromeric regions, and it is useful in identifying pericentromeric breakpoints in chromosomal rearrangements. DAPI is also a good method for determining the quality of DNA on the slide and for identifying chromosomes when performing FISH. If the DNA fails to stain, it is often indicative of damaged DNA or slides that are too old to use.

Typical applications

Banding and staining of chromosomes permit the visualization of gross chromosomal abnormalities, including gains and losses of whole or partial chromosomes, the presence of extrachromosomal material, double-minute chromosomes, ring chromosomes, homogeneously staining regions (HSRs), chromosome fragments, and chromatid breaks (see Fig. 22.3.2). Banding and staining can also identify large structural abnormalities such as additions, deletions, inversions, and translocations. The technique has usefulness in xenograft studies, where the investigator can identify contaminating cells from the host (i.e., murine) and estimate the purity of the tissue. It is also useful for determining the extent of *in vitro* chromosomal (genetic) change that a given culture may have developed. Recently, investigations of genomic instability have used these simple methods for determining the rate of change of normal, tumor, or transfected cells using drug or radiation treatment (Gebhart et al., 1981; Ellis et al., 1999).

Critical Parameters

The most critical parameter in successful banding is a well spread metaphase with few overlapping chromosomes that are of good length. Generally there are spreads of varying chromosome lengths; thus the investigator is able to carry out effective analysis. Sometimes, particularly in abnormal cells, metaphase spreads will typically be too long or too short (see UNIT 22.2), a result of physiological responses to Colcemid treatment.

Table 22.3.1 Troubleshooting Guide to Banding Chromosomes

Problem	Cause	Solution
<i>G-banding</i>		
Solid staining	Insufficient trypsin treatment	Increase the trypsin incubation time in increments of 2-3 sec
	Excessive Giemsa staining time	Decrease the time in Giemsa. This will help to determine whether the trypsin time is sufficient.
No stain uptake	Excessive trypsin treatment	Decrease trypsin incubation time. Puffy chromosomes are also an indication of overtrypsinization.
	Insufficient Giemsa staining time	If the chromosomes do not appear overtrypsinized, increase the staining time in Giemsa. If the stain has been used for many specimens, the dye may be more dilute than before. If this is the case, prepare a fresh dye mixture.
Puffy chromosomes	Excessive trypsin treatment	Decrease trypsin incubation time. If the slides were not sufficiently aged prior to treatment, try more aggressive aging.
<i>DAPI banding</i>		
Solid staining	Insufficiently aged slides	Heat-treat the slides prior to DAPI staining or pass through an ethanol dehydration series.
	DAPI concentration too high	If the concentration of DAPI is too high (typically the case of in-house formulations), the bright signal intensity may obscure banding patterns. Remove coverslip and wash in $2 \times$ SSC for 10 min at room temperature. Mount in a diluted DAPI/antifade medium (i.e., add more glycerol and PBS) and visualize again.
Puffy chromosomes	Insufficiently aged slides	Although no enzymatic treatment is required, very fresh chromosome preparations, and preparations made during high humidity (i.e., summer time), will produce puffy chromosomes with poor banding. Age slides artificially.

By phase-contrast microscopy, chromosomes should appear flat and range from pale gray to dark gray. The artificial aging of slides helps to sharpen the chromosome contours and increase band resolution. For G-banding, slides not aged sufficiently appear fuzzy after banding; this is due to the protease treatment. Very fat or puffy chromosomes, with little banding or stain uptake, are an indication of excessive trypsin treatment. Trypsin treatment (duration and concentration) must be optimized with each slide and for each batch of trypsin solution made. Duration adjustments will often be on the order of seconds. The brand of trypsin and the supplier also influences

trypsin digests markedly. Fetal bovine serum is used to inactivate the action of trypsin; however, other rinses can be used such as 0.85% saline. Time spent in the Giemsa stain will also vary depending on the extent of trypsinization and the investigator's preference.

The criteria for DAPI banding are similar to those described above. Aged slides are preferred for DAPI banding, as discussed for Giemsa staining.

Careful consideration should be given to solution preparation and quality control. The trypsin solution may be used for the course of the day, although it loses its enzymatic activity as a function of use during this time period.

Be sure to take this into consideration, since a freshly prepared solution will digest more quickly than a solution that has been sitting for a few hours at room temperature or that has already been used to prepare several slides. Similarly, the concentration of Giemsa stain will become progressively more dilute, and the stain will start to precipitate as specimens are passed through it. As the day progresses, the stain requires an increase in incubation time to produce the same results. The stain is known for producing a slight film along the stain surface in the container. This can be removed by skimming the surface of the stain with some filter paper or a paper towel to prevent deposition of purple scum or precipitate on the slide as it is pulled out of the Coplin jar.

Troubleshooting

See Table 22.3.1 for troubleshooting chromosome banding techniques. The techniques are applicable to human and animal chromosomes.

Anticipated Results

Under the proper conditions, the resulting banding patterns should permit identification of the chromosomes and detection of gross anomalies or abnormal chromosomal DNA structures if present.

Time Considerations

Although the bench time is minimal, the most time-consuming portion of chromosomal

banding is determining the correct trypsinization and staining times. Each specimen may require some minor adjustments.

Acknowledgements

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Fluorescence In Situ Hybridization (FISH)

As early as 1988 (Lichter et al., 1988), FISH was used to visualize labeled DNA probes hybridized to chromosome and interphase nuclei preparations. The improvement of cloned DNA sources, antibodies, fluorochromes, microscopy and imaging equipment, and software has permitted a variety of scientific investigations.

This unit is divided into five parts—probe preparation, slide preparation, hybridization, post-hybridization washes, and interpretation. Probes are prepared by nick translation (see Basic Protocol 1) or degenerative oligonucleotide primer PCR (DOP-PCR; see Alternate Protocol 1) using hapten- or fluorochrome-labeled nucleotide. The amount of hapten label incorporated is quantified by dot blotting (see Support Protocol 1). Cytogenetic slide preparations are suitable for FISH (see Basic Protocol 2) if they are appropriately aged (see Support Protocol 2); these slides may be used even if they have previously been G-banded (see Alternate Protocol 2). In addition, slides made from paraffin-embedded tissues are suitable specimens for hybridization with DNA (see Alternate Protocol 3) or protein nucleic acid (PNA) probes (see Alternate Protocol 4). Hybridization conditions must be appropriate for both the sample and probe materials: cytogenetic slides can be hybridized with DNA (see Basic Protocol 3) and PNA (see Alternate Protocol 5) probes, as can paraffin sections (see Alternate Protocols 6 and 7). Post hybridization wash and detection conditions vary depending on the probe—indirectly labeled DNA probes (see Basic Protocol 4), directly labeled DNA probes (see Alternate Protocol 8 or 9), and peptide nucleic acid (PNA) probes (see Alternate Protocol 10).

NOTE: Coplin jars containing solutions at temperatures other than room temperature should be prewarmed and maintained in a water bath at the specified temperature.

LABELING DNA PROBES FOR FISH

This section describes methods for labeling probes for fluorescence in situ hybridization (FISH) analysis. The first protocol (see Basic Protocol 1) describes DNA labeling by nick translation either indirectly with a hapten (biotin or digoxigenin) or directly with a fluorochrome (*APPENDIX 1E*) conjugated to dUTP. This method is appropriate for most DNAs listed in Table 22.4.1; however, smaller DNAs, such as cDNAs, may require labeling by PCR methods, which are also described (see Alternate Protocol 1). The choice of label, whether direct or indirect, is based on the type of DNA probe, its size, and the application. Small probe inserts (such as cDNAs) should be labeled with a hapten, enabling the option of signal amplification. Larger inserts can be both directly or indirectly labeled (Table 22.4.1). The probe's signal strength can vary depending on the application and sequence specificity (Table 22.4.2). Furthermore, the choice of label or fluorochrome will also depend on the availability of conjugated antibodies, the nature of the experiment, and the availability of filters for the microscope utilized for visualization. A list of fluorochrome properties can be found in Table 22.4.3. For both labeling methods discussed here, assessment of probe labeling is determined by size, gel electrophoresis, and for indirectly labeled probes, dot blot analysis of incorporation (see Support Protocol 1). The labeled DNA is ethanol precipitated in the presence of excess unlabeled DNAs, which serve as carriers (sonicated salmon sperm DNA) and as suppressors of repetitive sequences (Cot-1 DNA). The final product is resuspended in a hybridization buffer and is then ready for use in FISH experiments (see Hybridization). The labeling procedures outlined can be applied to all species of DNA.

Table 22.4.1 Sources of DNA Probes for FISH Analysis^a

Type	Application	Amplification of signal required	Labeling methods applicable	Indirect or direct labeling
Plasmids (10–20 kb insert)	Locus specific	Yes	Nick translation and PCR	Indirect
Cosmids (15–30 kb insert)	Locus specific	Yes	Nick translation and PCR	Indirect
PACs (~100 kb insert)	Locus specific	No	Nick translation and PCR	Both
BACs (~200 kb insert)	Locus specific	No	Nick translation and PCR	Both
YACs (300–1.5 Mb insert)	Locus specific	No	Nick translation and PCR	Direct
Genomic DNA from flow sorted chromosomes	Chromosome paints	No	PCR labeling	Both
Genomic DNA from microdissected DNA	Locus specific	Yes	PCR labeling	Both

^aShown are the most common types of DNAs used as probes for FISH analysis. Many of these DNAs can be labeled by nick translation (see Basic Protocol 1) or PCR (see Alternate Protocol 1). For probe inserts <2.0 kb, labeling by PCR is recommended.

Table 22.4.2 Different Classes of Commonly Used Probes for FISH Analysis^a

Type	Fluorescence intensity	Typical applications
Centromere probes	Strong	Ideal probe for the beginner to learn basic FISH techniques. Commonly used to enumerate chromosomal monosomies and trisomies, and for sex determination in transplantation studies.
Subtelomere specific probes	Moderate/weak	Used for determining whether small terminal rearrangements near telomeres have taken place.
Chromosome paints	Strong/moderate	Useful for identifying small marker chromosomal aberrations where a specific chromosome is suspected. Also useful in confirming SKY or MFISH findings.
Translocation junction unique-sequence probes	Moderate	Used for detecting the presence of specific translocations in interphase cells or in metaphase spreads.
Microdeletion unique-sequence probes	Moderate	Identification of small submicroscopic deletions using metaphase preparations.
Probes detecting gene amplification	Strong	For detecting oncogene copy number increases (gene amplification) in interphase and metaphase cells

^aShown are the most common types of commercially available probes used in FISH experiments and their typical applications. Signal strength will vary depending on the size and application of the probe as well as the target DNA specimen.

Another choice for FISH analysis is a peptide nucleic acid (PNA) probe. These probes also bind DNA in a sequence-specific manner but are created by oligonucleotide synthesis. The fabrication of PNA probes is not easily accomplished in a typical molecular cytogenetic laboratory, so they are currently only available commercially (see Internet Resources).

BASIC PROTOCOL 1

Labeling FISH Probes by Nick Translation

This protocol, adapted from Beatty et al. (2002), outlines the basic steps for either directly or indirectly labeling DNA probes for FISH analysis. Once labeled probes are ethanol precipitated, they are ready for final preparation prior to hybridization to the target specimen.

Materials

DNA probe (i.e., cosmid, plasmid, PAC, BAC, or YAC; Table 22.4.1)
10× nick translation buffer (see recipe)

Fluorescence In Situ Hybridization (FISH)

22.4.2

Table 22.4.3 Fluorescent Properties of Labels and DNA Stains Used for FISH^a

Label	Excitation (nm)	Emission (nm)
<i>Haptens^b</i>		
Digoxigenin	NA	NA
Biotin	NA	NA
<i>Fluorochromes</i>		
Alexa 488 ^c	490	520
Alexa 532 ^c	525	550
Alexa 546 ^c	555	570
Alexa 594 ^c	590	615
Amino-methyl coumarin	399	445
Cascade Blue	400	420
Cyanine 2 ^d	489	506
Cyanine 3 ^d	550	570
Cyanine 5 ^d	649	670
Cyanine 7 ^d	743	767
Fluorescein isothiocyanate (FITC)	495	523
Rhodamine B	560	580
Spectrum Aqua ^e	433	480
Spectrum FRed ^e	655	675
Spectrum Green ^e	497	524
Spectrum Gold ^e	530	555
Spectrum Orange ^e	559	588
Spectrum Red ^e	587	612
Tetramethylrhodamine isothiocyanate (TRITC)	550	570
Texas Red	595	610
<i>DNA stains</i>		
Chromomycin A3	430	570
4',6-Diamindine-2-phenylindole (DAPI)	538	461
Ethidium bromide	518	615
Hoechst 33258 (bis-benzimide)	352	461
Propidium bromide	535	617

^aShown are the commonly used fluorochromes and DNA stains used in FISH analysis. Of these, DAPI is the most widely used.

^bHaptens, such as digoxigenin and biotin are detected with antibodies conjugated to one of the fluorochromes listed.

^cThe Alexa family of fluorochromes can be purchased from Molecular Probes.

^dThe Cyanine family of fluorochromes can be purchased from Amersham Biosciences.

^eThe Spectrum family of fluorochromes can be purchased from Vysis.

DNase I dilution buffer (see recipe)

1 mM dNTP mixture (see recipe)

Fluorochrome/dTTP or hapten/dTTP mixture (see recipes)

3 mg/ml DNase I (see recipe)

10 U/μl *E. coli* DNA polymerase I (Roche)

5 × loading dye (see recipe)

2% (w/v) agarose gel (see recipe)
100-bp DNA ladder
Sonicated salmon sperm DNA standards (i.e., 12.5, 25.0, and 500 ng/μl; see recipe)
1× TBE buffer (see APPENDIX 2A)
300 mM EDTA (APPENDIX 2A)
10 mg/ml sonicated salmon sperm DNA (Invitrogen)
1 μg/μl human or mouse Cot-1 DNA (Invitrogen)
3 M sodium acetate (APPENDIX 2A)
100% ethanol
70% ethanol, cold
Hybridization buffer (see recipe)

Water bath or PCR machine
0.5-ml PCR tube (optional)
Additional reagents and equipment for agarose gel electrophoresis (APPENDIX 3A)
and determination of hapten incorporation by dot blot analysis (see Support
Protocol 1)

Prepare equipment and reagents

1. Set water bath or PCR machine to 15°C.
2. Put DNA probe and all labeling reagents (i.e., 10× nick translation buffer, DNase I dilution buffer, 1 mM dNTP mixture, and fluorochrome/dTTP or hapten/dTTP mixture) on ice.
3. Prepare 10× DNase I solution (1.0 ml total) by combining 1.0 μl of 3 mg/ml DNase I and 999.0 μl DNase I dilution buffer. Place on ice.

Perform labeling reaction

4. For each DNA to be labeled, prepare the following mixture in a 1.5-ml microcentrifuge or 0.5-ml PCR tube, adding the 10× DNase I solution and *E. coli* polymerase I last:
 - 2 μg DNA probe
 - 10.0 μl 10× nick translation buffer
 - 5.0 μl 1 mM dNTP mixture (i.e., dATP, dCTP, dGTP)
 - 4.0 μl fluorochrome/dTTP or hapten/dTTP mixture
 - 10.0 μl 10× DNase I solution
 - 2.5 μl *E. coli* DNA polymerase.Adjust the volume to 100 μl with water.

The amount of DNase I solution added will vary (see Commentary).

A probe prepared using hapten/dTTP is indirectly labeled, while one prepared with fluorochrome/dTTP is directly labeled.

5. Incubate 90 to 120 min at 15°C (see Table 22.4.22 and Critical Parameters).
6. After incubation, place tubes on ice.

Determine size of products from labeling reaction

7. Transfer 10 μl labeling reaction to a fresh tube and mix with 4 μl of 5× loading dye. Load onto a 2% agarose gel (APPENDIX 3A) along with a 100-bp DNA marker and 10 μl of each salmon sperm concentration standard.

The salmon sperm standards are used to help determine the amount of labeled DNA probe, as well as to act as a molecular-size marker.

8. Electrophorese at 100 V for ~20 min or until the dye front is two thirds of the way through the gel.

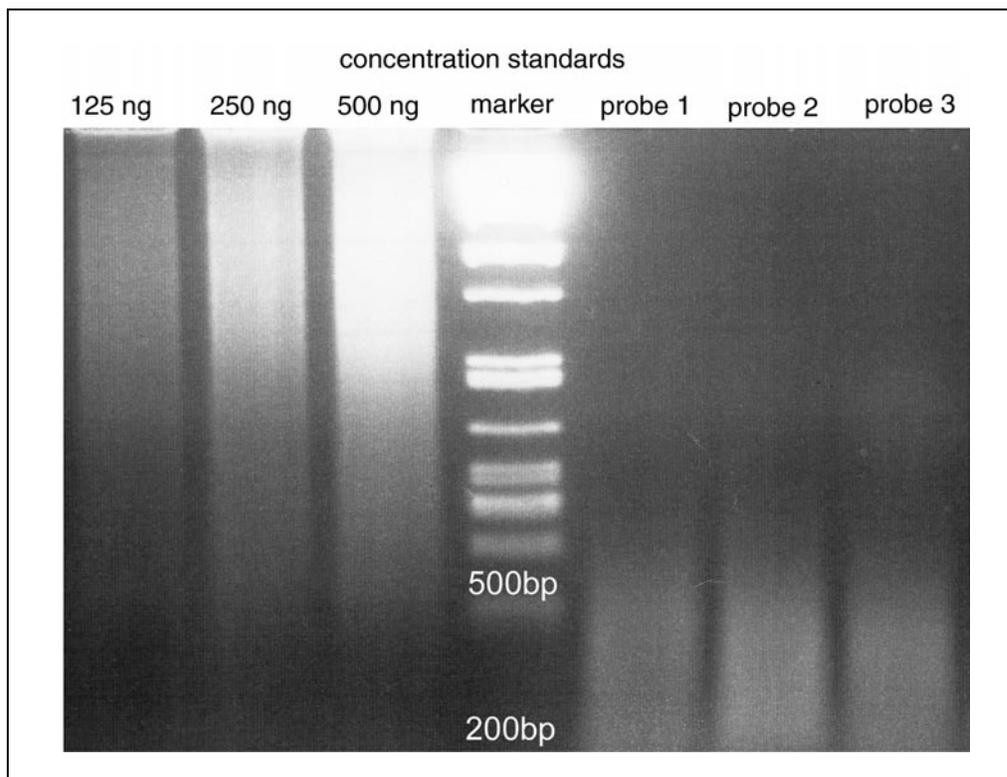


Figure 22.4.1 Nick translated DNA fragments electrophoresed on a 2% agarose gel. A volume of 10.0 μl labeled product was loaded. The fragment sizes range from 200 to 500 bp as determined from the molecular marker. The estimated concentration of the probe is 200 ng in 10 μl , yielding ~ 20 ng/ μl .

The final fragment sizes should be between 200 and 500 bp, with ~ 200 ng labeled DNA present in the gel, assuming the original amount of labeled probe was 2 μg . This can be ascertained using the sonicated salmon sperm concentration standards (Figure 22.4.1).

- If the fragment sizes are too large, return the labeling reaction to 15°C for 20 to 30 min (or time as required) and reassess the size of the fragments (i.e., repeat steps 5 to 8).

*It may be necessary to spike the labeling reaction with additional DNase I solution and *E. coli* DNA polymerase I. If the fragments are too small, labeling (step 4) will need to be repeated with an adjustment in the amount of DNase I added to the labeling reaction. If there is no significant change in fragment size following further digestion, the investigator should start again and review Table 22.4.22 for possible reasons of insufficient digestion.*

Determine amount of labeled probe

- Once the proper labeling size has been achieved, add 10 μl of 300 mM EDTA to the labeling reaction to stop the action of the enzymes.
- Estimate the amount of labeled probe on the gel using the salmon sperm standards as a guide. Calculate the amount of labeled DNA remaining in the tube.

Be sure to consider the volumes removed from the starting volume for loading onto the gel(s) when calculating the amount of labeled DNA left in the tube.

Based on the concentration standards, the labeled probe in Figure 22.4.1 appears to be somewhere between 125 ng/10 μl (1.25 ng/ μl) and 250 ng/10 μl (25.0 ng/ μl). A conservative estimate is 200 ng/10 μl (20 ng/ μl). Thus, from an original 100 μl starting volume, 10 μl was removed for a gel, leaving 90 μl . If the estimated concentration is 20 ng/ μl , the remaining labeled probe is 1800 ng (20 ng/ μl \times 90 μl = 1800 ng).

Add carrier and suppressor of repetitive sequences

12. For each 1 μg labeled DNA in the tube, add 5 μl of 10 mg/ml sonicated salmon sperm DNA (50 μg).

For example, for 1.8 μg DNA, add ~ 100 μg sonicated salmon sperm DNA.

13. For each microgram labeled DNA in the tube, add 5 to 10 μg human or mouse Cot-1 DNA.

For the example given above (1.8 μg labeled DNA), ~ 20 μg Cot-1 DNA is required

The amount of Cot-1 used is dependent on the number and extent of repeat elements in the DNA insert used as the probe. Generally, cDNA probes will have fewer repeats than genomic probes, so they will require relatively less Cot-1 suppression. BAC and YAC probes may need more suppression.

Determine incorporation

14. If the DNA probe has been labeled with hapten, check incorporation by dot blot analysis (see Support Protocol 1).

Ethanol precipitate DNA

15. Add 0.1 vol. of 3 M sodium acetate followed by 2.5 vol of 100% ethanol.

16. Incubate overnight at -20°C or 2 to 3 hr at -80°C .

The recovery of DNA during the precipitation process can be increased by lengthening the time at -20°C or -80°C .

17. Microcentrifuge 20 min at 13,000 rpm, 4°C .

Resuspend DNA

18. Carefully remove the supernatant and wash the pellet with cold 70% ethanol.

19. Microcentrifuge 20 min at 13,000 rpm, 4°C .

20. Remove the ethanol and allow the pellet to air dry.

21. Resuspend the pellet in hybridization buffer to a final concentration of 10 ng/ μl . Store labeled probe at -20°C up to several months (directly labeled probe) or several years (indirectly labeled probe).

The final concentration should be based on the labeled DNA content, not the total DNA content, which includes the unlabeled Cot-1 and salmon sperm DNA. Thus, for a labeled DNA amount of 1.8 μg , 180 μl hybridization buffer should be added to reach a final concentration of 10 ng/ μl .

The labeled probe can now be used for FISH experiments.

The amount of labeled probe used will depend on the area of the slide to be hybridized. For example, 10 μl is sufficient to cover a 22×22 -mm coverslip, 20 μl is sufficient to cover a 22×30 -mm coverslip, and 30 μl is sufficient to cover a 22×50 -mm coverslip.

ALTERNATE PROTOCOL 1

Labeling Probes for FISH by Degenerative Oligonucleotide Primer Polymerase Chain Reaction (DOP-PCR)

This protocol describes direct and indirect labeling procedures using PCR labeling. The user has various choices in the primer sets. These may be specific primers for a region, specific primers for vector sequences, universal primers, or DOP primers.

Additional Materials (also see Basic Protocol 1)

DNA template (Table 22.4.1)

$10\times$ PCR buffer: 100 mM Tris-Cl, pH 8.3 (APPENDIX 2A)/500 mM KCl (store up to several months at -20°C)

2 mM dNTP mixture (i.e., dATP, dCTP, dGTP; see recipe)
 5 μ M each primers 1 and 2 (*APPENDIX 3F*)
 50 mM MgCl₂
 5 to 10 U/ μ l *Taq* DNA polymerase
 Water, sterile
 Mineral oil, sterile

1. For each labeling reaction, mix the following:

1 to ng DNA template
 5.0 μ l 10 \times PCR Buffer
 4.0 μ l 2 mM dNTP
 2.0 μ l 1 mM fluorochrome/dTTP or hapten/dTTP
 1.5 μ l 5 μ M primer 1
 1.5 μ l 5 μ M primer 2
 1.5 μ l 50 mM MgCl₂
 0.5 μ l 5–10 U/ μ l *Taq* DNA polymerase.

Adjust final volume to 50.0 μ l with sterile water. Overlay the mixture with sterile mineral oil and put on ice until ready to place in the thermocycler.

The 10 \times PCR buffer is often supplied with the enzyme.

2. Place in a thermocycler and program the following steps:

Initial step:	5 min	93°C	(denaturation)
35 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	90 sec	72°C	(extension)
Final step:	5 min	72°C	(extension)
Hold:	indefinitely	4°C.	

3. Mix 5 μ l labeled reaction with 1 μ l of 5 \times loading dye and run on a 2% (w/v) agarose gel with molecular weight markers and sonicated salmon sperm DNA concentration standards as described (see Basic Protocol 1, steps 7 and 8).

The final PCR product should fall between 200 and 500 bp.

4. Calculate the amount of labeled DNA remaining in the tube, add carrier and suppressor DNA, precipitate, and resuspend in hybridization buffer as described (see Basic Protocol 1, steps 11 to 20).
5. If the DNA probe has been labeled with hapten, check incorporation by dot blot analysis (see Support Protocol 1).

Determination of Hapten Incorporation by Dot Blot Analysis

Gel electrophoresis of the labeled DNA FISH probe allows the investigator to determine whether the DNA has been nicked (see Basic Protocol 1) or amplified (see Alternate Protocol 1) to the appropriate sizes. It does not, however, allow determination of whether the hapten has been incorporated into the DNA. Using a dot blot assay, the degree of hapten incorporation can be assessed to determine the efficiency of the labeling protocol and the activity of the DNA polymerase I or *Taq* DNA polymerase. This method is useful in determining whether the lack of FISH signal is due to a poorly labeled probe or to hybridization or slide preparation factors. This method involves spotting labeled product onto a nylon filter along with a control. The labeled DNA is detected by immunolabeling and colorimetric visualization.

SUPPORT PROTOCOL 1

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Materials

100 mM Tris·Cl (pH 7.5)/15 mM NaCl (*APPENDIX 2A*)
Indirectly labeled probe (i.e., with biotin or digoxigenin; Table 22.4.3)
Control DNA labeled with biotin or digoxigenin (i.e., labeled DNA probe known to produce good signal strength)
0.5% (w/v) BSA in 100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)/15 mM NaCl (store up to 6 months at 4°C)
AP-labeled antibody mixture (see recipe)
100 mM Tris·Cl (pH 9.5)/100 mM NaCl/50 mM MgCl₂ (*APPENDIX 2A*)
NBT/BCIP (see recipe)

Charged nylon membrane (~5 × 5 cm per sample)
Filter paper
37°C dry oven
Rotating platform

Prepare and load nylon membrane

1. Cut a charged nylon membrane to a size appropriate for the number of samples.
2. Soak the membrane 5 min in 100 mM Tris·Cl (pH 7.5)/15 mM NaCl, room temperature. Blot the membrane with filter paper.
3. Pipet aliquots of indirectly labeled probe and control DNA labeled with biotin or digoxigenin in a dilution series (e.g., 200, 100, 50 ng) onto the membrane, leaving ample space between spots. Incubate 5 to 10 min, room temperature.

Block and label membrane

4. Incubate the membrane 1 min in 100 mM Tris·Cl (pH 7.5)/15 mM NaCl, making sure it is saturated.
5. Incubate the membrane 30 min in 0.5% (w/v) BSA in 100 mM Tris·Cl (pH 7.5)/15 mM NaCl at room temperature.
6. Transfer the membrane to an alkaline phosphatase–labeled antibody mixture in a plastic container and incubate 30 min in a 37°C dry oven on a rotating platform, making sure the membrane is completely saturated.

Develop dot blot

7. Remove the membrane and wash 15 min with 100 mM Tris·Cl (pH 7.5)/15 mM NaCl.
8. Remove the membrane and wash 2 min with 100 mM Tris·Cl (pH 9.5)/100 mM NaCl/50 mM MgCl₂, room temperature.
9. Remove the membrane and incubate 5 to 10 min in NBT/BCIP solution under dimmed lighting until the blot develops.
10. Wash the membrane with water and air dry.

The membrane can be stored at room temperature. Over time the intensity of the chemical stain will fade. Typical results are shown in Figure 22.4.2.

TARGET SLIDE PREPARATION

Slide quality can greatly influence the success of FISH assays. When slide preparations are less than optimal, treatment with a protease can decrease the risk of high background and increase access of the probe to the DNA target by removing protein barriers to allow efficient hybridization. FISH carried out on paraffin sections requires more aggressive

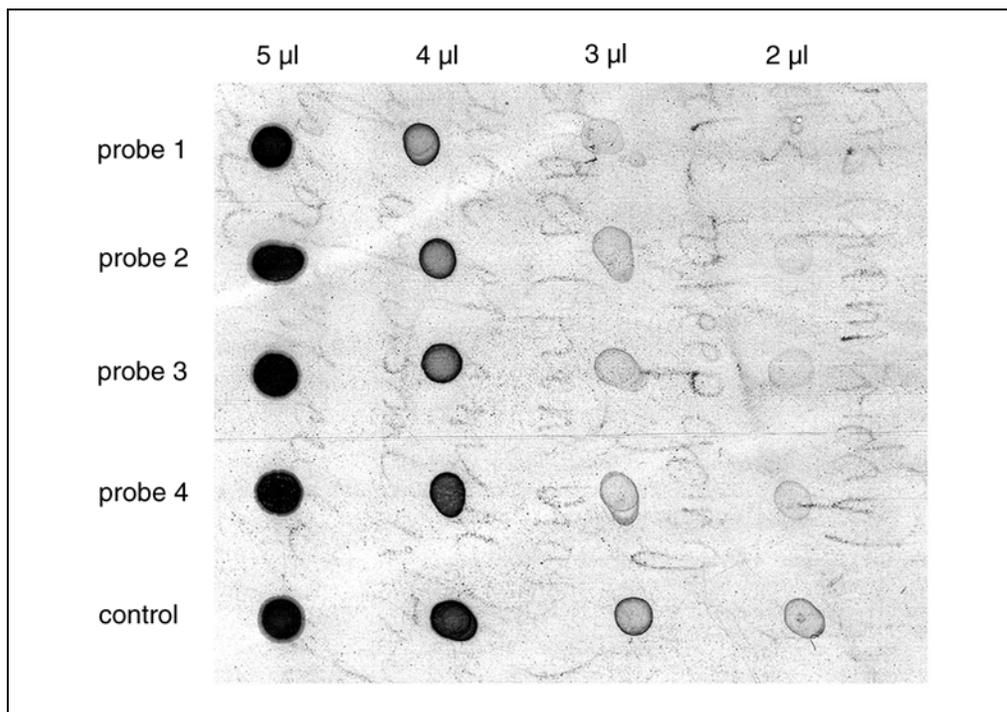


Figure 22.4.2 Dot-blot analysis of biotin incorporation in probes shown in Figure 21.4.1. Compared to control labeled DNA, the labeled probes appear to have incorporated biotin well, with probe 4 showing the greatest incorporation compared to probe 1. The concentration of each probe was estimated at 20 ng/ μ l; however, it is evident that the labeling efficiency was greater in some probes over others.

protease treatment and assessment. Following optimal slide pretreatment, the specimen is subjected to denaturation, causing double-stranded DNA to become single stranded. This will permit the efficient hybridization of the denatured probe to the DNA target on the slide.

Preparation of Cytogenetic Specimens for FISH

The following protocol describes the pretreatment of cytogenetic slides (*UNIT 22.2*) for FISH analysis with DNA probes (in-house or commercial) or commercially obtained PNA probes. In this protocol, pepsin is recommended for use as the protease; however, proteinase K is also often used. The concentration and conditions for proteinase K treatment are also noted in the protocol. The treatment with pepsin will vary depending on the extent of digestion required on the slide. In some cases, pepsin treatment is not needed.

Materials

Cytogenetic slide preparation (*UNIT 22.2*): age naturally at least 2 days at room temperature or artificially (see Support Protocol 2)

10% (w/v) pepsin—100 mg pepsin powder (Sigma) in 1.0 ml H₂O; store in 20- μ l aliquots up to several months at -20°C —and 0.01 M HCl, 37°C or

14 mg/ml proteinase K (Roche Diagnostics) and proteinase K buffer—i.e., 20 mM Tris·Cl, pH 7 (*APPENDIX 2A*)/0.2 mM CaCl₂; store up to several months at room temperature

1 \times PBS (*APPENDIX 2A*)

70% formamide/2 \times SSC (pH 7.0), 72°C (see *UNIT 18.6* for SSC): prepare fresh

70% ethanol, ice-cold (for DNA probes)

BASIC PROTOCOL 2

Table 22.4.4 Coplin Jars Needed for Preparation of Cytogenic Specimens for FISH^a

No. Coplin jars	Contents	Temperature
1	70% ethanol	Room temperature
1	70% ethanol ^b	4°C
1 or 2 ^b	80% ethanol	Room temperature
1 or 2 ^b	100% ethanol	Room temperature
1	70% formamide in 2× SSC ^b	72°C
1	1× PBS	Room temperature
1	Pepsin/0.01 HCl <i>or</i> proteinase K/buffer	37°C or room temperature (respectively)

^aSee Basic Protocol 2.

^bFor DNA probes only.

Phase contrast microscope (*UNIT 4.1*)
Coplin jars

Additional reagents and equipment for hybridization (see Basic Protocol 3 and Alternate Protocol 5)

CAUTION: Formamide is a carcinogen and should be handled with care. Discard according to biohazard rules of the institution.

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.4.

Detect and remove cytoplasmic contamination by protease treatment

- Using a phase-contrast microscope, determine the extent of cytoplasmic residue on the cytogenetic slide preparation. If there is no cytoplasm, proceed to step 6.
- For pepsin digestion:* Add 10 to 15 μ l of 10% (w/v) pepsin to 50 ml warm 0.01 M HCl in a Coplin jar. Incubate the slides in the protease 5 min at 37°C.
- For proteinase K digestion:* Dilute 14 mg/ml proteinase K to a final concentration of 0.1 μ g/ml in proteinase K buffer in a Coplin jar. Incubate the slides in the protease 6.5 min at room temperature.

Remove protease and determine efficacy

- Wash the slide 5 min at room temperature in a Coplin jar containing 1× PBS.
- Pass the slide through a dehydrating series of 70%, 80%, and 100% ethanol in Coplin jars for 5 min each. Allow to air dry after the final ethanol treatment (~5 min).
- View the slide using phase-contrast microscopy to determine the extent of protein digestion. If necessary, repeat the protease step.

Prepare slides for hybridization

- For PNA probes:* Proceed to hybridization (see Alternate Protocol 5).
- For DNA probes:* Denature the slide for the recommended time (see Table 22.4.5) in 70% formamide/2× SSC (pH 7.0), 72°C.

The time will vary according to the age and quality of the slide.

- Promptly place the slide into a Coplin jar containing ice-cold 70% ethanol for 5 min. Pass the slides through a dehydration series of 80% and 100% ethanol for 5 min each.

Table 22.4.5 Formamide Denaturation Guide for Cytogenetic Specimens^a

Slide criteria	Suggested denaturation time ^b
Fresh slide stored 1–2 days at room temperature	60–90 sec
Fresh slide artificially aged 1–2 days	60–90 sec
Aged slide stored 1–2 weeks at room temperature	1.5–2 min
Aged slide stored 2–4 weeks at room temperature	2 min
Aged slides stored 1–2 months	2–3 min
Previously G-banded slides >1-week old	30–40 sec
Previously G-banded slides <1-week old	20–30 sec

^aShown are general guidelines for denaturation of slides of varying ages and conditions. The investigator should pay attention to the slide quality and monitor the changes in denaturation conditions as the slides age or are processed. Each laboratory will possess different environmental conditions as well as slide making procedures, which will affect the denaturation procedures significantly.

^bDenaturation conditions are 72°C in 70% formamide/2× SSC.

8b. Air-dry the slide after the final ethanol treatment and proceed to hybridization (see Basic Protocol 3).

Artificial Aging of Cytogenetic Slide Preparations for FISH

The chromosomes on freshly prepared slides are often too fragile for immediate use—i.e., the high temperatures used during the slide denaturation procedure can damage the DNA, making it less optimal for hybridization. Such slides require aging, which can be achieved by allowing them to naturally age a few days at ambient temperature; however, an experiment must occasionally be performed immediately. The protocol below outlines a method for artificially aging freshly prepared slides so that FISH results are available within 12 to 24 hr after preparation from the cytogenetic suspension. This method may also be used when the relative humidity in the laboratory is high due to local weather conditions.

To age, incubate a freshly prepared cytogenetic slide (*UNIT 22.2*) at least 1 hr (up to 3 hr) in a Coplin jar containing 2× SSC (*UNIT 18.6*), 37°C. Remove the slide and pass through a dehydration series of 70%, 80%, and 100% ethanol for 5 min each, and air dry (5 to 10 min). Proceed with enzyme digestion if required.

Note that a list of all Coplin jars used in this protocol is given in Table 22.4.6.

Preparation of Previously G-Banded Cytogenetic Specimens for FISH

The following method outlines the steps involved in pretreating a previously Giemsa (G)-banded cytogenetic slide (*UNIT 22.3*) for subsequent use in FISH analysis. The slides must be completely free of any oils and must also be destained. Since the slide has already been treated with a protease, it will require no additional protease digestion; however, stripping the proteins, which are protective to the DNA, will cause it to become much more sensitive to degradation during the denaturation process. The ability to re-use a previously banded slide for FISH will be dependent on the age of the slide, the degree of trypsin digestion during the banding, and whether residual immersion oils or mounting buffers have degraded the DNA.

Materials

Banded cytogenetic slide specimen (*UNIT 22.3*)
Xylene

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ALTERNATE PROTOCOL 2

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Table 22.4.6 Coplin Jars Needed for Artificial Aging of Cytogenetic Slide Preparations for FISH^a

No. Coplin jars	Contents	Temperature
1	70% ethanol	Room temperature
1	80% ethanol	Room temperature
1	100% ethanol	Room temperature
1	2× SSC	37°C

^aSee Support Protocol 2.

Table 22.4.7 Coplin Jars Needed for Preparation of Previously G-Banded Cytogenetic Specimens for FISH^a

No. Coplin jars	Contents	Temperature
1	70% ethanol	4°C
1	70% ethanol	Room temperature
2	80% ethanol	Room temperature
2	100% ethanol	Room temperature
1	70% formamide/2× SSC, pH 7.0	72°C
1	Methanol	Room temperature
1	Xylene ^b	Room temperature

^aSee Alternate Protocol 2.

^bCAUTION: Maintain in a fume hood.

Methanol

70%, 80%, and 100% ethanol

70% formamide in 2× SSC (pH 7.0; UNIT 18.6), 72°C: prepare fresh

70% ethanol, ice-cold

Coplin jars

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.7.

1. Remove residual oils from previously banded cytogenetic slide specimen by incubating 5 min in a Coplin jar containing xylene under a vented chemical hood.
2. Destain by incubating the slide ~5 to 10 min in a Coplin jar containing room-temperature methanol.
3. Pass the slide through a dehydrating series of 70%, 80%, and 100% ethanol for 5 min each. Allow the slide to air dry (5 to 10 min) after the final ethanol treatment.
4. Denature slide 20 to 30 sec in 70% formamide/2× SSC (pH 7.0), 72°C.

The time will vary according to the age and quality of the slide (Table 22.4.5).

5. Promptly place the slide into a Coplin jar containing ice-cold 70% ethanol for 5 min following denaturation.
6. Proceed through a dehydration series of 80% and 100% ethanol for 5 min each. Air-dry the slide after the final ethanol treatment and proceed with hybridization (see Hybridization).

Preparation of Paraffin-Embedded Specimens for Hybridization with DNA Probes

FISH analysis on paraffin sections is becoming increasingly important in both diagnostic and research laboratories. This protocol outlines the steps involved in pretreating paraffin sections for FISH. While the preparation of paraffin-embedded specimens can be modified, the most critical component is the quality of the starting section (see Commentary). Different histopathology laboratories will have various methods of tissue fixation which should be taken into consideration when assessing the success of an experiment.

Materials

- 5- to 10- μ M paraffin sections on silanized slides
- Xylene
- 100% ethanol
- 0.5% (w/v) pepsin in 0.85% (w/v) NaCl (pH 1.5), 45°C (see recipe)
- 2 \times SSC, pH 7.0 (UNIT 18.6)
- Propidium iodide (PI) or DAPI in antifade (see recipes)
- 70%, 80% and 100% ethanol

- Coplin jars
- 45°C water bath
- Fluorescence microscope (UNIT 4.2) with a FITC, and PI or DAPI filter set

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.8.

Remove wax

1. Add 5- to 10- μ M paraffin sections on silanized slide to a Coplin jar containing xylene. Gently agitate 5 min at room temperature. Transfer slides to a second Coplin jar containing fresh xylene for an additional 5 min with gentle agitation.
2. Transfer the slide to a Coplin jar containing 100% ethanol. Soak 5 min, then transfer slides to fresh 100% ethanol and soak another 5 min. Agitate 2 to 3 times during each 5-min period.
3. Remove the slide from ethanol and allow to air dry (5 to 10 min).

Perform protease digestion and counterstain

4. Place slide in a Coplin jar containing 0.5% (w/v) pepsin in 0.85% NaCl, 45°C. Incubate 15 to 20 min in a 45°C water bath (see Table 22.4.5).
5. Rinse the slide 20 to 30 sec in a Coplin jar containing 2 \times SSC, pH 7.0.

Table 22.4.8 Coplin Jars Needed for Preparation of Paraffin-Embedded Specimens for Hybridization with DNA Probes^a

No. Coplin jars	Contents	Temperature
1	70% ethanol	Room temperature
1	80% ethanol	Room temperature
3	100% ethanol	Room temperature
1	0.5% pepsin in 0.85% NaCl	45°C
4	2 \times SSC, pH 7.0	Room temperature
2	Xylene ^b	Room temperature

^aSee Alternate Protocol 3.

^b**CAUTION:** Maintain in a fume hood.

Table 22.4.9 Troubleshooting Guide for Pretreatment of Paraffin-Embedded Sections

Problem	Cause	Solution
Green autofluorescence upon inspection/or poor uptake of counterstain	Under-digestion with pepsin solution	Remove coverslip and wash with 2× SSC as described (see Alternate Protocol 3, step 5). Return the slide to pepsin digestion buffer (see Alternate Protocol 3, step 4). The time required for subsequent protease treatment will be dependent on the existing degree of digestion. Should repeated attempts at digestion fail, consider another enzyme such as proteinase K (use at 10 mg/ml in 2× SSC for 20 min at 37°C) in place of the pepsin buffer (see Alternate Protocol 3, step 4) or in combination with the pepsin buffer.
Lifting of tissue from slide	Loosening by protease treatment	Occasionally the protease treatment will loosen the tissue from the slide. Try to work carefully such that the tissue is relatively undisturbed.
	Glass slides not coated with silane	Associated with this is the use of charged or silanated slides. Many pathology labs will use coated slides. Check if the sections were mounted onto such slides. If not, try to obtain sections on these slides.
Holes in tissue or “ghostly” appearance	Over-digestion	Over-digestion will be evident from the degradation of the tissue. Start again and decrease the tissue treatment.

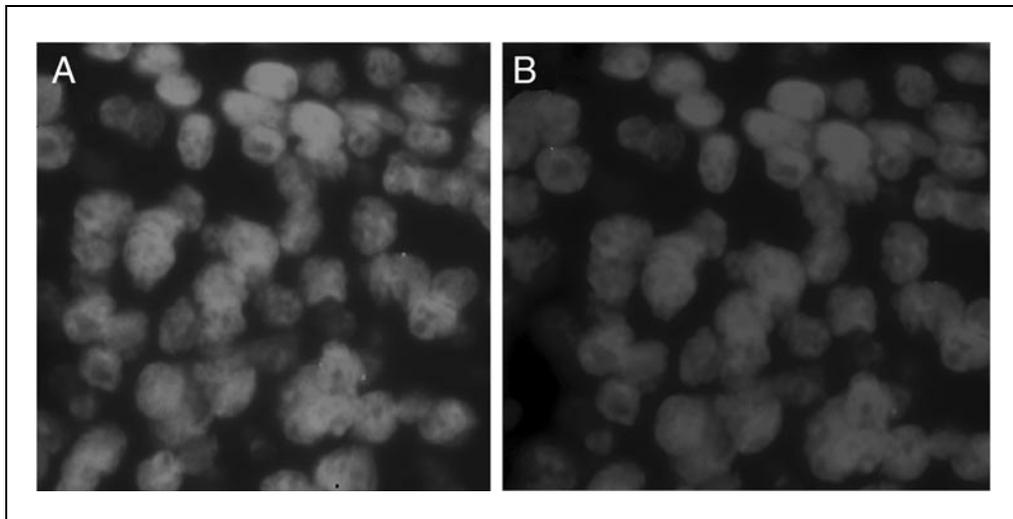


Figure 22.4.3 Digestion of paraffin-embedded specimen for FISH. **(A)** Under-digestion is indicated by weak uptake of the DAPI stain. **(B)** Increased digestion permits better access to the DNA and increase uptake of DAPI. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure, see color plates.*

6. Apply 10 μ l PI or DAPI in antifade to counter stain and coverslip.

The choice between PI or DAPI depends on the label color of the probe being analyzed.

7. View the slide using a fluorescence microscope with FITC, and PI or DAPI filter set. Evaluate the tissue sections for under-digestion, appropriate digestion, or over-digestion using the guidelines in Table 22.4.9 and Figure 22.4.3.

For certain types of tissue additional troubleshooting steps may also be necessary (see *Troubleshooting*).

8. Carefully remove the coverslip and rinse slide three times in separate Coplin jars containing 2× SSC, pH 7.0, for 5 min each, agitating 5 to 10 sec in each rinse.
9. Dehydrate slide in a series of 70%, 80%, and 100% ethanol washes for 1 min each.
10. Allow the slide to air dry. Proceed to hybridization with labeled DNA probes.

Preparation of Paraffin-Embedded Specimens for Hybridization with PNA Probes

PNA probes are also applicable to paraffin sections. Like pretreatment for hybridization with DNA probes (see Alternate Protocol 3), pretreatment for hybridization with PNA probes requires some minor modifications as described below.

Materials

5- to 10- μ M paraffin sections on silanized slides
Xylene
100% ethanol
100 μ g/ml RNase I (see recipe)
2× SSC (UNIT 18.6)
1 M sodium thiocyanate, 80°C
5 mg/ml pepsin in 0.85% (w/v) NaCl, 45°C (see recipe)
0.1 M triethanolamine, pH 8.0
Acetic anhydride (Sigma)
1× PBS (APPENDIX 2A)
70%, 80%, and 100% ethanol
45°C hot plate or slide warmer
Coplin jars
37°C oven

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.10.

Remove wax

1. Preheat 5- to 10- μ m paraffin sections on silanized slide on a 45°C slide warmer or hot plate for ~30 min (i.e., until the wax melts).

Table 22.4.10 Coplin Jars Needed for Preparation of Paraffin-Embedded Specimens for Hybridization with PNA Probes^a

No. Coplin jars	Contents	Temperature
1	70% ethanol	Room temperature
1	80% ethanol	Room temperature
4	100% ethanol	Room temperature
2	1× PBS	Room temperature
1	5 mg/ml pepsin in 0.85% NaCl	45°C
1	1 M sodium thiocyanate	80°C
5	2× SSC	Room temperature
1	Triethanolamine, pH 8.0	Room temperature
2	Water	Room temperature
3	Xylene ^b	Room temperature

^aSee Alternate Protocol 4.

^b**CAUTION:** Maintain in a fume hood.

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2. Dewax by transferring the slide to a Coplin jar containing 50 ml xylene and incubating 5 to 10 min at room temperature. Repeat twice with fresh xylene.

Time for dewaxing is dependent on many factors (e.g., tissue section thickness, surface area, type of tissue).

3. Remove xylene by soaking slide 5 to 10 min in 100% ethanol. Repeat twice with fresh ethanol.

4. Allow slides to air dry (5 to 10 min).

The tissue should turn white at this point.

Treat with RNase, SSC, and sodium thiocyanate

5. Add 40 μ l of 100 μ g/ml RNase I to the slide. Cover with a coverslip and incubate 1 hr at 37°C.
6. Wash twice in Coplin jars containing fresh 2 \times SSC for 5 min each time.
7. Incubate slide 8 min in a Coplin jar containing 1 M sodium thiocyanate, 80°C.
8. Rinse twice in Coplin jars containing fresh distilled water for 1 min each.

Digest with protease

9. Incubate 7 to 9 min at 45°C in a Coplin jar containing 5 mg/ml pepsin in 0.85% NaCl, pH 1.5.
10. Wash twice for 1 min each in Coplin jars containing fresh 2 \times SSC.

Acetylate

11. Start acetylation by placing slide in a Coplin jar containing 0.1 M triethanolamine, pH 8.0.
12. While stirring gently, slowly add 125 μ l acetic anhydride to give a final concentration of 0.25% (v/v). Incubate 10 min at room temperature.

The acetylation procedure is carried out to reduce the electrostatic binding of probe to positive charges on the tissue, thereby reducing the background.

Wash and dehydrate

13. Rinse 5 min in a Coplin jar containing 1 \times PBS. Repeat the rinse using a fresh Coplin jar of PBS.

Washes from this point forward can be reduced in duration if adherence of tissue to the slide is poor.

14. Rinse 5 min in a fresh Coplin jar containing 2 \times SSC.
15. Dehydrate through a series of 70%, 80%, and 100% ethanol and allow to air dry.
16. Proceed to hybridization with PNA probes (see Alternate Protocol 7).

HYBRIDIZATION

In the following section, hybridization of the probe to the slide is described. The prepared DNA probe is denatured to a single-stranded state in the presence of denaturing buffer. Depending on the probe type, there may be a preannealing step. The denatured probe is applied to the denatured cytogenetic specimen and allowed to hybridize overnight. Additionally, this section also describes the hybridization conditions for paraffin sections, where the slide and probe are simultaneously denatured. If commercial DNA probes are being used, the manufacturer's instructions for probe denaturation are followed and the hybridization conditions are adjusted accordingly. If a PNA probe is utilized, the probe

is applied to the slide, and together the probe and target are denatured and permitted to hybridize for at least 1 hr.

Hybridization of Labeled DNA Probes to Cytogenetic Specimens

Hybridization of a cytogenetic specimen with the probe requires heat-denaturation of the DNA probe in the hybridization buffer, and a pretreated and denatured slide specimen; together they are permitted to hybridize at 37°C. This protocol can be applied to cytogenetic specimens that are unstained or were previously G-banded (see Alternate Protocol 2).

Materials

- DNA probe (see Basic Protocol 1 and Alternate Protocol 1)
- Pretreated and denatured cytogenetic slide specimen (see Basic Protocol 2 and Support Protocol 2)
- Rubber cement
- 75°C water bath or PCR machine
- 37°C dry oven
- Glass coverslips
- Hybridization box, slightly dampened (e.g., black plastic video tape box lined with slightly moistened gauze or paper towel)
- Hybridization container (i.e., any plastic container with lid or a black video cassette box)

1. Heat-denature the required amount of labeled DNA probe 5 min in a 75°C water bath or PCR machine. Preanneal 1 hr in a 37°C dry oven (see Critical Parameters).
2. Add the appropriate amount of DNA probe to the pretreated and denatured cytogenetic slide specimen using guide given in Table 22.4.11. Coverslip and ring the perimeter with rubber cement to seal in place.

Table 22.4.11 Guide to Appropriate Probe Volume for a Given Coverslip Size

Probe volume	Coverslip size (mm)
10 μ l	22 \times 22
20 μ l	22 \times 30
30 μ l	22 \times 50

Table 22.4.12 Examples of Minimal Hybridization Times Required for Representative DNA Probes

Probe type (300–500 bp)	Minimal hybridization time (37°C)
Centromere Probes ^a	4 hr
Subtelomere chromosome-specific probes ^a	24 hr
Chromosome paints	4 hr
Translocation junction unique-sequence probes	24 hr
Microdeletion unique-sequence probes	24 hr
Probes detecting gene amplification	24 hr

^aProtein nucleic acid (PNA) probes, which are commercially available, require a minimum of 60 min for hybridization.

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3. Place slide in a slightly dampened hybridization box.
4. Transfer slide to a hybridization container and incubate overnight in a 37°C dry oven (see Table 22.4.12).
5. Proceed to post-hybridization washes and immunofluorescent detection of indirectly (see Basic Protocol 4) or directly labeled DNA probes (see Alternate Protocol 8)

Hybridization of PNA Probes to Cytogenetic Specimens

PNA probes, as discussed below (see Critical Parameters), can be commercially obtained. Use of PNA probes requires minor modifications in the hybridization to both cytogenetic specimens and paraffin-embedded sections, as described below.

Additional Materials (also see Basic Protocol 3)

Labeled PNA probe (Applied Biosystems)
Pretreated cytogenetic specimen, not denatured (Basic Protocol 2)
80°C oven, hot plate, or HYBrite (Vysis)

1. Add the required amount of labeled PNA probe to the pretreated, undenatured, cytogenetic specimen.
2. Coverslip and seal with rubber cement. Allow the rubber cement to set and dry.
3. Place the slide in an 80°C oven or HYBrite, or on an 80°C hot plate for 90 sec.
4. Remove the slide and place it into a hybridization box. Hybridize at least 1 hr at room temperature.
5. Proceed to post-hybridization washes for specimens hybridized with PNA probes (see Alternate Protocol 10).

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Hybridization of Labeled DNA Probes to Sections from Paraffin-Embedded Material

Unlike cytogenetic specimens, tissue sections from paraffin-embedded material require higher denaturation temperatures and longer denaturation times. Hybridization to these sections requires co-denaturation of the probe and tissue section simultaneously. All experiments using previously paraffin-embedded material and a DNA probe should include a minimal 24-hr hybridization.

Additional Materials (also see Basic Protocol 3)

Deparaffinized and enzyme-digested specimen (see Alternate Protocol 3)
90°C oven, hot plate, or HYBrite (Vysis)

1. Apply DNA probe to the deparaffinized and enzyme-digested specimen using the guide given in Table 22.4.11. Add a glass coverslip and apply rubber cement along the perimeter of the coverslip. Allow the rubber cement to dry.
2. Denature the probe and target DNA simultaneously by placing the slide in a 90°C oven or HYBrite, or on a 90°C hot plate for 12 min.
3. *For slide denatured in an oven or on a hot plate:* Transfer the slide to a hybridization container lined with a wet paper towel or damp gauze, and incubate overnight in a 37°C dry oven.
4. *For slide denatured in a HYBrite:* Program the unit to hold at 37°C. Incubate overnight.

5. Proceed to post-hybridization washes and immunofluorescent detection of indirectly or directly labeled DNA probes (see Basic Protocol 4 or Alternate Protocols 8 or 9).

Hybridization of PNA Probes to Sections from Paraffin-Embedded Material

The following protocol describes hybridization of commercially available PNA probes to pretreated sections from paraffin-embedded samples. Unlike DNA probes, PNA probes require a minimum of 60 min for hybridization.

Additional Materials (also see Basic Protocol 3)

PNA probes (Applied Biosystems)

Deparaffinized and enzyme-digested specimen (see Alternate Protocol 4)

80°C oven, hot plate or HYBrite (Vysis)

1. Apply the appropriate amount of PNA probe (as suggested by the manufacturer) to the deparaffinized and enzyme-digested paraffin section using the guidelines provided in Table 22.4.11. Coverslip and seal with rubber cement. Allow the rubber cement to dry.
2. Place in an 80°C oven or HYBrite, or on an 80°C hot plate for 3 min.
3. Place in a hybridization container and allow to hybridize at least 1 hr at room temperature.
4. Proceed to post-hybridization wash for slides hybridized with PNA probes (see Alternate Protocol 10).

POST-HYBRIDIZATION WASHES AND DETECTION

Following hybridization, unbound probes, whether DNA or PNA, must be removed from the specimen. This is accomplished through washes of appropriate stringency, using formamide and SSC in varying amounts. After immunodetection, final washes contain detergents to remove unbound antibodies. The slides are counterstained and mounted in an antifade medium for visualization. Variations on washing procedures reflect the nature of the probe, whether directly or indirectly labeled.

Post-Hybridization Washes and Immunofluorescent Detection of Indirectly Labeled DNA Probes

This protocol describes post-hybridization washes and detection of indirectly labeled DNA probes. Following overnight hybridization, the coverslip is removed and the slide is immersed in a solution of formamide to remove any unbound probe. The slide is then washed in stringent SSC washes and blocked with a solution of BSA. Depending on the type of DNA probe used, amplification of the signal may be required. This is achieved by the addition of primary and secondary antibodies that may or may not be conjugated with a fluorochrome. Detergent washes are carried out after each antibody incubation; it is critical that the slide not be permitted to dry out at any point of the assay. The slide is then counterstained and ready for visualization. In multicolor FISH experiments, the user may have both indirectly and directly labeled probes on the same specimen. If this is the case, it is important to keep the ambient light dim to prevent quenching of the signal; Coplin jars with lids are useful for this purpose. If using multiple indirectly labeled probes, be sure that the primary and secondary antibodies are raised in different animals such that there is no cross-reaction. This protocol is applicable to cytogenetic slides or sections from paraffin-embedded samples; however, more stringent or additional washes may be required for paraffin experiments.

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PROTOCOL 7**

**BASIC
PROTOCOL 4**

**Cell Biology of
Chromosomes
and Nuclei**

22.4.19

Table 22.4.13 Coplin Jars Needed for Post-Hybridization Washes and Immunofluorescent Detection of Indirectly Labeled DNA Probes^a

No. Coplin jars	Contents	Temperature
3	50% formamide in 2× SSC	45°C
3	1× SSC	45°C
9	0.1% Tween 20 in 4× SSC	45°C

^aSee Basic Protocol 4.

Materials

Hybridized slides in a hybridization box (see Basic Protocol 3 and Alternate Protocol 6)

50% formamide in 2× SSC, 45°C (prepare fresh)

1× SSC, 45°C (UNIT 18.6)

Blocking solution: 1% BSA (w/v)/0.1% (v/v) Tween 20 in 4× SSC (store indefinitely at –20°C or up to several months at 4°C)

Primary, secondary, and tertiary antibodies (see recipe for antibodies)

0.1% (v/v) Tween 20 in 4× SSC, 45°C

DAPI in antifade (see recipe)

Clear nail polish

Coplin jars

22 × 50–mm glass coverslips

37°C oven

Fluorescent microscope and appropriate filter sets (UNIT 4.2)

CAUTION: Formamide is a carcinogen and should be handled with care. Discard according to biohazard rules of the institution.

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.13.

Remove coverslip

1. After 24 hr, remove hybridized slide from the hybridization box.
2. Carefully peel the rubber cement from the hybridized slide and immerse in a Coplin jar containing 50% formamide in 2× SSC, 45°C.
3. Allow the coverslip to fall off and let stand 5 min in solution with gentle agitation.

Wash and block

4. Remove slide and transfer to second Coplin jar containing formamide/SSC, 45°C, for 5 min. Agitate gently. Repeat with a third Coplin jar containing formamide/SSC, 45°C.
5. Wash the slide for 5 min each in three consecutive Coplin jars containing 1× SSC, 45°C, agitating gently between washes.
6. Drain excess solution but do not allow the slides to dry. Add 80 μl blocking solution, coverslip with 22 × 50–mm glass, and place back in hybridization box. Incubate 40 min at 37°C.

Label with primary antibody

7. Remove coverslip and add 80 μl primary antibody. Coverslip and place back into the hybridization box. Incubate 40 min at 37°C.

Table 22.4.14 Common Primary and Secondary Antibody Systems and Final Concentrations^a

Hapten	Primary antibody	Secondary antibody	Tertiary antibody	Color	Probe Type
Biotin (no signal amplification)	5 µg/ml FITC-avidin	—	—	green	Centromere chromosome paints
Biotin	5 µg/ml FITC-avidin	5 µg/ml biotinylated goat anti-avidin	5 µg/ml FITC-avidin	Green	Locus-specific cDNAs
Biotin	5 µg/ml rhodamine-avidin	5 µg/ml biotinylated goat anti-avidin	5 µg/ml rhodamine-avidin	Red	Locus-specific cDNAs
Digoxigenin (no signal amplification)	2 µg/ml rhodamine anti-Dig	—	—	Red	Centromere chromosome paints
Digoxigenin (no signal amplification)	2 µg/ml FITC anti-Dig	—	—	Green	Centromere chromosome paints
Digoxigenin	0.5 µg/ml mouse anti-digoxigenin	2 µg/ml digoxigenin anti-mouse	2 µg/ml rhodamine anti-Dig	Red	Locus-specific cDNAs
Digoxigenin	0.5 µg/ml mouse anti-digoxigenin	2 µg/ml digoxigenin anti-mouse	2 µg/ml FITC anti-Dig	Green	Locus-specific cDNAs

^aShown in this table are the most common antibody systems for the detection of biotinylated and digoxigenin (Dig)-labeled DNA. Final concentrations for antibodies are also stated but may require adjustment. A variety of antibody systems are available from Molecular Probes and Roche Diagnostics. If a two-color FISH approach is being used, be sure that respective primary, secondary, and tertiary antibodies do not cross-react. This may require sequential hapten detection rather than concomitant hapten detection. The scheme above uses antibodies raised against mouse and goat, thus no cross-reaction will occur. Antibodies raised in rabbits will also offer more variety in hapten detection.

This antibody may or may not have a fluorescent moiety conjugated to it, depending on the nature of the probe (see Critical Parameters). Common primary antibody and secondary antibody systems are outlined in Table 22.4.14.

8. Remove coverslip and wash slide for 5 min each in three consecutive Coplin jars containing 0.1% Tween 20/4× SSC, 45°C, with gentle agitation. If proceeding with signal amplification (see Table 22.4.15) continue to step 9. Otherwise, proceed to step 14.
9. Drain excess wash solution, but do not allow slide to dry. Add 80 µl blocking solution, coverslip, and place back into the hybridization box. Incubate 10 min at 37°C.

Label with secondary antibody

10. Remove coverslip and add 80 µl secondary antibody (Table 22.4.14). Coverslip and place back into the hybridization box. Incubate 30 min at 37°C.
11. Remove coverslip and wash slide 5 min each in three consecutive Coplin jars containing 0.1% Tween 20 in 4× SSC, 45°C, with gentle agitation.

Label with tertiary antibody

12. Remove coverslip and add 80 µl tertiary antibody (conjugated to fluorochrome). Coverslip and place back into the hybridization box. Incubate 30 min at 37°C.
13. Remove coverslip and wash slide 5 min each in three consecutive Coplin jars containing 0.1% Tween 20 in 4× SSC, 45°C, with gentle agitation.

Counterstain and seal

14. Drain excess wash solution, but do not allow the slides to dry. Add 40 µl DAPI in antifade counterstain. Coverslip and seal with clear nail polish.

The slides are now ready for visualization. When not in use, store slides up to several weeks or months at –20°C, depending on the frequency of visualization.

15. Examine using a fluorescent microscope and appropriate filter sets.

Table 22.4.15 Interpretation of Parallel Positive Control Experiments

Problem	Slide hybridized with new probe	Slide hybridized with good known probe	Analysis
Background	Present	Not present	New probe may still contain unincorporated dNTPs or many small labeled fragments are present and did not hybridize. Slide quality is not the problem. Washing conditions also do not appear to be the cause.
Background	Present	Present	Problems likely related to washing conditions or slide quality rather than issues with the probe.
Weak Signal (indirect-labeled probe)	Yes	No	If using antibody detection system for indirectly labeled probes, this scenario indicates that the antibodies are in good working order. Look to problems with incorporation of the hapten into the DNA.
Weak signal (direct-labeled probe)	Yes	No	This indicates a problem with the labeling of the probe. Check that all enzymes and fluorochromes are within their shelf life. This also indicates that washing conditions are correct.
Weak signal (indirect labeled probe)	Yes	Yes	This indicates a problem with the antibody detection system. Ensure that the concentrations are correct and that the antibodies are fresh. It may also indicate a problem with hybridization, insufficient denaturation of the target DNA on the slide, or overall quality of the DNA specimen.
Weak signal (direct-labeled probe)	Yes	Yes	The problem may be in the post-hybridization washes: too stringent or temperatures too high.
Poor chromosome morphology with weak signal	Yes	Yes	Over denaturation if the chromosomes appear puffy.
Good chromosome morphology but no signal	Yes	Yes	Slides are likely too old and resistant to denaturation. Change to a more fresh preparation. Otherwise the experiment can be repeated but the denaturation of the slide should be increased.

**ALTERNATE
PROTOCOL 8****Post-Hybridization Washes and Detection of Directly Labeled DNA Probes**

The protocol below outlines steps for washing slides hybridized with directly labeled probes. If using commercially produced probes, follow the manufacturer's instructions. This protocol is applicable to hybridized cytogenetic slides or paraffin sections. See Basic Protocol 4 for materials.

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.16.

1. After 24 hr hybridization, carefully peel off rubber cement from slide and immerse in a Coplin jar containing 50% formamide in 2× SSC, 45°C. Allow the coverslip to fall off and let stand 5 min.
2. Remove slide and transfer to a fresh Coplin jar containing formamide/SSC, 45°C. Incubate 5 min. Repeat with a third Coplin jar containing formamide/SSC, 45°C.

**Fluorescence
In Situ
Hybridization
(FISH)****22.4.22**

Table 22.4.16 Coplin Jars Needed for Post-Hybridization Washes and Detection of Directly Labeled DNA Probes^a

No. Coplin jars	Contents	Temperature
3	50% formamide in 2× SSC	45°C
3	1× SSC	45°C
3	0.1% Tween in 4× SSC	45°C

^aSee Alternate Protocol 8.

Table 22.4.17 Coplin Jars Needed for Rapid Wash of Directly Labeled Probes^a

No. Coplin jars	Contents	Temperature
1	0.3% (v/v) NP-40 in 2× SSC	72°C
1	0.3% (v/v) NP-40 in 2× SSC	Room temperature
1	2× SSC	Room temperature

^aSee Alternate Protocol 9.

3. Wash slide 5 min each in three consecutive Coplin jars containing 1× SSC, 45°C.
4. Wash slide 5 min each in three consecutive Coplin jars containing 0.1% Tween 20 in 4× SSC, 45°C, with gentle agitation.
5. Drain excess wash solution, but do not allow the slide to dry. Add 40 μl DAPI in antifade counterstain. Coverslip with 22 × 50–mm glass and seal with clear nail polish. Store slide up to several months at –20°C.

Fluorescence fading will depend upon the frequency of viewing.

6. Examine using a fluorescent microscope equipped with the appropriate filters.

Rapid Wash of Directly Labeled Probes

This protocol describes the use of high temperatures and stringent SSC washes for the removal of unbound probe from target DNA. This protocol is generally effective for directly labeled DNA, particularly from commercial sources. It is important to consider the differing stringency requirements of each probe being used when planning this protocol since excessive temperature can strip bound probe from the target. This protocol is applicable to cytogenetic slides or paraffin sections.

Additional Materials (also see Basic Protocol 4)

2× SSC (UNIT 18.6)

0.3% (v/v) NP-40 in 2× SSC, room temperature and 72°C

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.17.

1. Carefully remove rubber cement from the hybridized slide. Place the slide and coverslip in a Coplin jar containing 2× SSC to gently remove coverslip.
2. Once the coverslip has fallen off, incubate the slide 1 to 2 min in a Coplin jar containing 0.3% NP-40 in 2× SSC, 72°C.
3. Remove slide and wash 1 min in a Coplin jar containing 0.3% NP-40/2× SSC, room temperature.
4. Drain solution from slide and add DAPI in antifade counterstain. Add a 22 × 50–mm glass coverslip and seal with clear nail polish.
5. Examine the slide with a fluorescent microscope equipped with the appropriate filters.

ALTERNATE PROTOCOL 9

Post-Hybridization Washes for Specimens Hybridized with PNA Probes

This protocol describes a procedure for removing unbound PNA probe from cytogenetic specimens or from paraffin sections.

Materials

- Slides hybridized with PNA probes (see Alternate Protocol 5 or 7)
- 70% (v/v) formamide/10 mM Tris·Cl (pH 7.0 to 7.5)/0.1% (w/v) BSA (see recipe)
- 0.1 M Tris·Cl (pH 7.0 to 7.5)/0.15 M NaCl/0.08% (v/v) Tween 20 (store up to several weeks at room temperature)
- 70%, 90%, and 100% ethanol
- DAPI in antifade solution (see recipe)
- Fluorescent microscope and appropriate filters

NOTE: A list of all Coplin jars used in this protocol is given in Table 22.4.18.

1. Remove coverslip from slide hybridized with PNA probes and wash 15 min in a Coplin jar containing 70% formamide/10 mM Tris·Cl (pH 7.0 to 7.5)/0.1% BSA. Repeat once.
2. Wash 5 min each in three consecutive Coplin jars containing 0.1 M Tris·Cl (pH 7.0 to 7.5)/0.15 M NaCl/0.08% Tween 20.
3. Dehydrate slide by incubating 5 min each in Coplin jars containing 70%, 90%, and 100% ethanol. Air dry (5 to 10 min).
4. Apply DAPI in antifade and coverslip.
5. Examine the slide using a fluorescent microscope and appropriate filters.
6. Store slides up to several months at -20°C

INTERPRETATION OF FISH FINDINGS

This section discusses interpretation of FISH experiments and relevant troubleshooting measures. Each laboratory may adopt a different means of assessing positive and negative results as is applicable to the experiment and the question being asked. The guidelines below can generally be applied to both interphase and metaphase analysis.

Evaluating FISH

FISH encompasses four main components: the slide and its preparation (see Target Slide Preparation), the DNA probe and its preparation (see Labeling DNA Probes for FISH),

Table 22.4.18 Coplin Jars Needed for Post-Hybridization Washes for Specimens Hybridized with PNA Probes^a

No. Coplin jars	Contents	Temperature
2	70% formamide/10 mM Tris·Cl (pH 7.0–7.5)/0.1% BSA	Room temperature
3	0.1 M Tris·Cl (pH 7.0–7.5)/0.15 M NaCl/0.08% Tween 20	Room temperature
1	70% ethanol	Room temperature
1	90% ethanol	Room temperature
1	100% ethanol	Room temperature

^aSee Alternate Protocol 10.

denaturation and hybridization of the probe to the slide (see Hybridization), and finally, post-hybridization washes and detection (see Post-Hybridization Washes and Detection). Each of these components is, in itself, multisteped. The discussion below outlines many of the problems encountered in FISH experiments, including factors influencing signal strength, background, and preserving optimal chromosome morphology. Other sources for FISH optimization and parameters can be found in Beatty et al. (2002), Henegariu et al. (2001), Schwarzacher and Heslop-Harrison (2000), and van de Rijke et al. (1996).

Signal strength

Slide age. In the author's experience, optimal results have been obtained from slides not older than 2 months. As the slides age further (3 to 6 months), they become harder to denature. Conversely, very old preparations (> 1 year) often have partially degraded DNA that may reduce or preclude effective FISH experiments. In such situations, preparations tend to become very sensitive to denaturation. Previously G-banded slides (UNIT 22.3) have an even shorter life span and should be processed within 2 weeks (refer to Table 22.4.5).

Cytoplasmic debris. The presence of cytoplasm on the slide may inhibit binding and contribute to overall background. A more aggressive protease pretreatment may be required to reduce cytoplasmic proteoinacious noise. This can be accomplished by increasing the time of digestion or the amount of protease added (maintain the same digestion time). One must also consider whether the slide is made from a dropped suspension or FISHED to a slide made from an in situ culture, since the latter slides tend to possess more cytoplasmic and cellular debris. A high background obscures true signals. If there is minimal background but the signal is weak in different parts of the slide, then there may be a gradient or patchiness of cellular protein in areas of the slide with a higher density of fixed cells.

Excessive slide pretreatment. Excessive enzymatic treatment may damage the target DNA, making it less efficient for hybridization with the probe. This is usually indicated by weak uptake of the counterstain and the presence of bright centromeres.

Denaturation time. As the slide ages (see above), the chromosomes become harder to separate into single strands. Slides used within 1 to 2 weeks of preparation should be denatured for 1.5 to 2 min. Slides that are older may require times that range from 2 to 3 min. Under-denaturation results in insufficient strand separation of the target DNA, decreasing the effective hybridization efficiency. Over-denaturation of the target DNA causes DNA damage and reduces the amount of target DNA that is able to hybridize with the probe; it also results in reduced hybridization efficiency. Over-denaturation usually also results in poor uptake and banding using the DNA counterstain and very bright centromeres.

Sealing of coverslip. It is critical that the coverslip be adequately sealed with rubber cement during incubations to prevent any moisture from entering the hybridized area, thus diluting the probe. Do not use contact cement or other ultra-adhesive glues.

Proper temperature for washes and incubations. Check that the temperature in the oven, hot plate unit, or water bath is correct for hybridization and other incubations. High temperatures during post-hybridization washes may remove bound probe with weak sequence homology (see Critical Parameters), thus decreasing the amount of DNA available for signal detection or antibody binding. Avoid taking shortcuts during incubation times with blocking reagents or antibody detection reagents. Also be sure that the reagents do not dry up on the slide; this makes washing more difficult.

Detergent type and concentration. Increasing the detergent content or choice of detergent may influence signal strength. Tween 20 is generally less harsh than NP-40. Increasing

the detergent concentration in combination with increasing temperature will remove more probe and/or bound antibodies. Be sure that the concentration and temperatures are correct.

SSC concentration. Decreasing SSC concentration increases stringency. Thus $0.1 \times$ SSC destabilizes double-stranded DNA more readily than $2 \times$ SSC. Double-stranded DNA stability is also affected by the extent of sequence homology between the probe and target DNAs. Ensure that the proper SSC concentrations are used during washes.

Proper filter sets. For fluorescence, make sure that the proper filter sets are used for image acquisition. The use of filters with incorrect spectral characteristics for the fluorochrome being used can severely impair the ability to detect the correct signals or they may increase the amount of autofluorescence. Also refer to *UNIT 4.2* and *APPENDIX 1E*.

Amplification of signal. When using an indirectly labeled DNA probe, signal amplification may be required. If the signal is amplified but still weak, check the concentrations and shelf lives of antibodies used. If the antibodies are in good order, relabel the probe and check the labeling efficiency (see Critical Parameters). For directly labeled probes, consider the labeling efficiency of the probe.

Probe characteristics

In-house probes. If probes are labeled in-house (see Labeling DNA Probes for FISH), strict controls must be undertaken to ensure that proper hapten or fluorochrome incorporation has been obtained to produce a high-quality DNA probe. Haptens may be assessed using the dot blot method of incorporation. Directly labeled probes can be assessed by removing a small aliquot of probe, placing it on a slide, and viewing it by fluorescence microscopy.

Cot-1 suppression. Excessive Cot-1 suppression may be the cause of reduced signal. Smaller DNA probes or cDNAs contain fewer repeat elements compared to larger inserts. Normally, 5 to 10 μg Cot-1 is sufficient per 100 ng labeled DNA; adjust as required.

Commercial probes. Usually commercial suppliers properly process the product with the necessary quality controls. Check that the probes were properly stored and used before the expiration date. For some experiments it is possible to optimize procedures using concentrations of commercial probes at levels slightly below those recommended by the supplier. This can prove very cost effective if a specific commercial probe is going to be used routinely.

Probe concentration. The probe is usually used in excess of the target DNA; however, make sure that sufficient probe volume has been added to adequately cover the area of interest on the slide (see Table 22.4.11).

Preannealing. Preannealing at 37°C is usually carried out for 1 hr following denaturation. This step is performed to permit the annealing of repetitive sequences in the DNA probe with unlabeled Cot-1 DNA to prevent cross-hybridization. If there are few repetitive elements in the probe, or the probe is a cDNA or small insert, this step may be omitted. The probe is simply denatured and applied to the denatured slide.

Background

Cytoplasmic debris. This is the most common cause of background. Increase the incubation time during protein digestion or maintain the same time but change the concentration of the enzyme.

Bacterial/yeast contamination. Microorganisms that contaminate cultures used to make the slide, or reagents used to prepare the slide or for hybridization are deposited onto the slide. If the contamination is minor, then it is only necessary to analyze the slide in areas that contain fewer deposits of fungi or bacteria. If the contamination is heavy, the slide or reagent must be prepared again.

Coverslips. Coverslips should be clean and dust free. If cells are to be grown in situ, use only glass coverslips as plastic coverslips autofluoresce.

Residual oils. Slides that have been previously visualized using immersion oil (e.g., G-banded slides) should be cleaned with xylene. Residual oils will prevent hybridization and cause background problems.

Dust and other particles. Particles may be deposited on the slide during various transfers into solutions. Be sure that solutions are well mixed and filtered as needed. Some solutions may form precipitates that may bind to the slide.

In-house probes. Ensure that nonincorporated conjugated nucleotides are removed from the final probe preparation.

Commercial probes. These probes are usually not the cause of background, especially when the same probe on another slide preparation has not produced background; however, note the lot number for future reference. It is also very helpful to introduce a new batch of a commercial probe into routine use before the previous lot is exhausted in case the supplier has had production difficulties

Unlabeled DNA: carrier and Cot-1. An excess of unlabeled DNA is added to the probe mixture. Although it is unlabeled, it can contribute to background by trapping antibodies. To reduce this problem, ensure that the resuspended probe pellet is fully dissolved in sufficient hybridization buffer. If the pellet does not completely dissolve, add more buffer. This will not significantly alter the reaction.

Denaturation time. The probe is usually heat denatured a minimum of 5 min. This should be sufficient time to denature the probe and dissolve any remaining DNA. The denaturation time may be increased to 10 min without any damage to the DNA probe.

Post-hybridization washes. Make sure that the correct temperature has been maintained for washes and incubations. Agitation during the washes can help to remove unbound probe and antibodies. Increasing the stringency of the washes by either increasing the temperature or altering the amount of SSC in the washes can also help considerably if background is encountered. Avoid drying the slide with any residual blocking or detection reagents.

Detergent concentration. Increasing the detergent concentration in post-hybridization washes in combination with increasing temperature removes more probe and/or bound antibodies. Be sure that the concentration and temperatures are correct.

Fading signals

In-house probes. Ensure that stocks of directly conjugated dNTPs are properly stored in the dark and used well before the expiration date. Unlike indirectly labeled probes, directly labeled DNAs have a much shorter shelf life.

Commercial probes. Although these probes are supposed to be quality controlled, these too are subject to the same concerns as in-house probes (see above). During denaturation

and hybridization, keep lights dimmed and hybridize in a light-proof container for directly labeled probes.

Directly-labeled DNA probes. When directly labeled probes are used, slides should be washed with the lights dimmed to prevent unnecessary exposure to extraneous light.

Mounting medium. Check that the DAPI/antifade mounting solution is used before its expiration date. Normally, the medium is clear with a slight pink tinge; medium that is degrading will turn increasingly amber. Expired antifade medium causes rapid signal degradation and displays a red glow when viewed under the microscope.

Image acquisition. Extended exposure of the hybridized slide to UV sources leads to signal quenching. When not in use, store slides at -20°C . During visualization, keep the light source off when not in use.

Poor chromosome morphology

Slide age. See Signal Strength (above) for information concerning this parameter.

Cytoplasmic debris. See Signal Strength (above) for information concerning this parameter.

Excessive slide pretreatment. Excessive enzymatic treatment may damage the target DNA, making it less efficient for hybridization with the probe

Previously G-banded slides. G-banding (UNIT 22.3) causes DNA to become sensitive to subsequent denaturation. For this reason, denaturation times are greatly reduced. This also has an effect on the quality of hybridization and signal strength.

Over-denaturation. The cause of poor chromosome morphology is usually over-denaturation, which causes the DNA to be destroyed. This can be sample specific and/or slide-age related.

Denaturation temperature. Check that the temperature for denaturation is accurate. The final internal temperature of the Coplin jar should be 72°C . Add 1°C for each slide that is denatured in the jar. Avoid denaturing more than five slides in one jar at one time. Note that plastic Coplin jars have a greater differential temperature between bath and internal temperatures as compared to glass.

Probe cross-hybridization

Clone identity. When using in-house FISH probes for research applications, ensure that the clone identity and the insert are correct for the experiment. Some genes have highly homologous sequences elsewhere in the genome and may cross-hybridize to other family members.

DNA contamination. Consider whether stock DNA was contaminated with DNA from another clone. Increase the hybridization temperature if contamination is suspected. Typically, the hybridization temperature is 37°C . If cross-hybridization occurs, try increasing the hybridization temperature to 42°C .

Stringency of washes. Some probes may cross-hybridize, particularly centromere probes. Usually cross-hybridization signals are significantly weaker than true signals. More stringent washes can be undertaken by decreasing the SSC concentration, increasing wash temperature, or adding additional washes.

Evaluation of a New DNA Probe

The most critical factor in optimizing a new FISH probe which was labeled in-house is first determining whether the probe of interest maps to the correct location. Many vectors (e.g., plasmids, cosmids, BACs) have traveled from laboratory to laboratory and have changed hands many times. These DNAs, as well as cloned DNA obtained from reputable repositories, can be mislabeled or misidentified. After a cloned DNA has been extracted and labeled, the first FISH experiment should be carried out on normal lymphocyte spreads and should address the following questions.

Is a signal visible on a pair of chromosomes?

If there is a weak signal, refer to the section above.

Does the DNA probe map to the right chromosomal location? Does it map to only one location?

It helps to have someone who can identify the chromosomes by DAPI banding. Most people are not as proficient as trained cytogeneticists, thus it may be useful to carry out a two-color experiment using a commercially available centromere probe for the chromosome of interest. Refer to *UNIT 22.3* for general conventions for identification of chromosomes according to ISCN nomenclature. Some DNA sequences/genes may belong to families with similar sequences. It is possible that hybridization to these similar regions may occur. Usually the true signals are stronger than the cross-hybridized ones. If this is the case, wash conditions can be adjusted or another clone should be obtained.

Does a positive control probe work properly?

It is useful to perform parallel experiments with a probe known to give good signals in the same experiment. This will help to identify any problems that are not related to the newly labeled probe (see Table 22.4.15 for interpretation of such control experiments).

For example, in panel iii of Figure 22.4.4A, two mouse BAC probes were labeled (one green and the other red) and assessed. A separate slide with a previously labeled BAC known to give good signal and no background was prepared as a control at the same time (not shown). The newly labeled BAC probes shown in panel iii of Figure 22.4.4A mapped to the proper location with weak signal intensity and high background. Analysis of this experiment is as follows.

The BAC probes map to the correct location. Because the signal intensity was weak consider (1) starting DNA quality and quantity, (2) labeling efficiency, (3) fidelity of DNA polymerase I and DNase I, (4) labeling time and temperature, (5) effective removal of nonincorporated nucleotides, and (6) insufficient Cot-1 suppression. Because the control probe displayed no background problems, one can assume that the hybridization and wash conditions were sufficient.

Evaluation of Hybridization Efficiency

The minimum number of cells or metaphase spreads required to obtain a given result reflects the clinical context of the finding, the limitations of the patient material available for study, and the question being asked. With tissues or cells that are hard to obtain, a single abnormal metaphase may be significant. For example, in some situations, limited FISH data may be supported by results obtained using PCR and/or Southern blot analysis.

Prior to enumerating or analyzing FISH results on a test sample, it is important to carefully assess the overall quality, uniformity, and effectiveness of hybridization as discussed

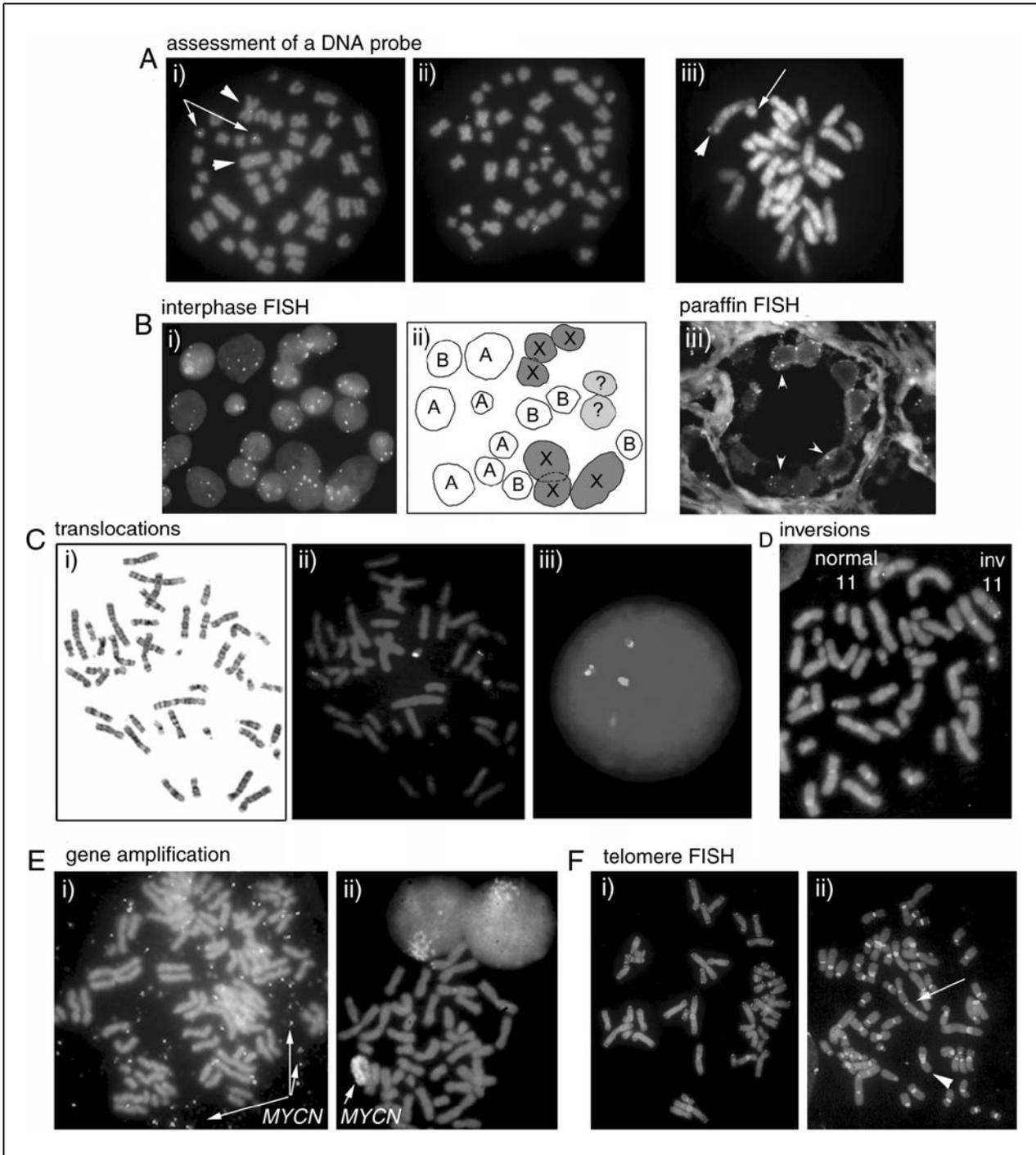


Figure 22.4.4 Legend at right.

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22.4.30

above. Each hybridized slide should be evaluated for the specificity of the hybridization, the probe signal intensity, and the signal-to-background noise, to determine if the hybridization was optimum for the given analyses. There should be minimal background or nuclear fluorescent noise. At least 85% of all nuclei in the target area should be easily innumerable. For some applications (such as the detection of mosaicism or minimal residual disease), more rigorous analytical sensitivities and hybridization efficiencies are required.

1. Perform the FISH protocol appropriate for the type of patient and control slides in the experiment.
2. Prior to determining the hybridization efficiency, quickly scan the whole slide, noting the general signal-to-noise levels in different regions of the slide and any areas with high background or unusually weak signals. It may be useful to mark the underside surface of the slide in these areas with a diamond pen. If the signal intensity far exceeds background levels, proceed with estimating the hybridization efficiency.
3. Pick several representative areas of the slide and score at least 200 nuclei from the areas selected, following the selection criteria described below in Selecting Cells for FISH Microscopy. Keep a running log of the number of cells scored and the observed signal counts for the patient and control slides.
4. For all slides, add up the number of cells with no signal. In general, hybridization is considered to be adequate if >85% of the cells scored have one or more signals. Lower hybridization efficiencies may be encountered with smaller probes (generated

Figure 22.4.4 (at Left) Composite figure illustrating FISH hybridization. **(A)** Assessment of a DNA probe. (i) Cross-hybridization of probe (arrowhead) to chromosome 1. True signals are located on chromosome 22. (ii) Changes in stringency washes and post-hybridization removes cross-hybridization. (iii) High background associated with a insufficient repetitive sequence (Cot-1) suppression in this mouse metaphase spread. Weak signals (arrows) indicate poor labeling efficiency. **(B)** Interphase FISH analysis. (i) A cytogenetic specimen from a short-term ovarian primary culture hybridized with PNA probes for centromeres 7 (green) and 8 (red). When scoring interphase nuclei, it is especially important to focus through the cells since signals may be present at different z planes. (ii) Schematic indicating the nuclei that are acceptable for scoring: A, cell acceptable for scoring; B, cell is likely acceptable for scoring, but requires careful attention; ?, cell has qualities that make it questionable for scoring; X, cell is not acceptable for scoring. (iii) Interphase analysis using PNA probes specific for centromeres 7 (green) and 8 (red) on a paraffin section from a prostate carcinoma. Arrowheads indicate cells containing changes in ploidy. **(C)** Analysis of translocation probes. (i) and (ii) Results of consecutive hybridization of a G-banded metaphase spread with the Vysis *BCR/ABL* translocation probe. Two fusion signals (yellow) are produced from the hybridization of *BCR/ABL* fusion on the Philadelphia chromosomes (Ph) on chromosome 22 and the reciprocal *ABL/BCR* on the derivative chromosome 9. Separate green and red signals from the normal chromosomes 9 and 22 are also seen. (iii) Interphase pattern from this specimen. **(D)** Example of a chromosomal inversion on chromosome 11. An inversion was identified involving the terminal portion of chromosome 11 by gross cytogenetic analysis. Clones 200 kb apart and in the 11p15.5 region, containing the *IGF2* gene (green) and *H19* (red) were hybridized to the patient specimen. The normal chromosome 11 shows the red and green signal hybridizing on top of each other at 11p15.5. The inverted 11 shows the clear split of signal along the abnormal chromosome 11 indicating the breakpoint lies within the 200 kb between *IGF2* and *H19*. **(E)** Gene amplification of *MYCN* in neuroblastoma specimens. (i) Double minute chromosomes (dmns) containing hundreds of copies of the *MYCN* gene. This is in contrast to amplification of *MYCN* in (ii) as a large block of signal called a homogeneously staining region (HSR). Interphase nuclei nearby show the typical hybridization pattern for an HSR. **(F)** Use of subtelomeric and pan-centromeric PNA probes. (i) Hybridization of a prostate cell line with PNA subtelomeric probes. (ii) Hybridization of another prostate cell line with subtelomeric and pan-centromeric PNA probes. Loss of telomeric sequences are indicated by the arrow while the presence of multicentric chromosomes are indicated by arrow heads. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure, see color plates.*

for example in a research laboratory). Extreme caution must be exercised when using probes with lower hybridization efficiencies or elevated background signal to provide clinical information.

Selecting Cells for FISH Microscopy

Generally, look at all areas of the slide and analyze regions with uniformity in signal strength. Compare the intensity of the background signals to the intensity of the signals in the nuclei or metaphases of interest. The FISH signal intensity should consistently be greater than the background intensity in the regions of the slide chosen for analysis. If the background signals are equivalent to signals in the nuclei, then the counts will be skewed and the results biased. The following provides a guide to selecting targets for analysis.

1. Ideally analyze cells from all areas of the slide. Systematically select representative areas from different regions of the slide. Any regions that have unacceptable background or weak signals identified in prescreening evaluations should be excluded.
2. Select nuclei or metaphase cells that do not touch or overlap (Fig. 22.4.4B).
3. Select intact nuclei that have smooth well-rounded borders. Partially ruptured nuclear membranes may have lost informative chromatin. Similarly select metaphase spreads that have no evidence of preparation artifacts or breakage.
4. Select cells that are not surrounded by cytoplasmic material and that are without evidence of potential drying artifacts such as rings or clumped cells.
5. Do not evaluate interphase nuclei with signals located on the extreme periphery of the nucleus.
6. Do not score regions of the slide containing nuclei that have no signals. Absence of signals may represent uneven or patchy hybridization, resulting in some areas of the slide having very weak or absent signals.

Analysis of Interphase Nuclei

Interpretation of interphase FISH is very much dependent on statistical analyses and has inherent technical challenges. For instance, the presence of signal is dependent upon the probe and its fluorescent label successfully entering the cell and hybridizing to the target DNA. Detection of the correct number of signals can be complicated by signals overlapping or splitting. Any background hybridization whatsoever leads to major complications in interpretation. It is not uncommon to find monosomy or trisomy in nuclei that reflect technical artifact or false positive background signals. Therefore, the accuracy of interphase FISH analysis is dependent upon recognizing these technical issues, correcting for them, and standardizing the scoring criteria accordingly. Interphase analysis is typically used for enumeration of chromosomes using centromere probes, detection of gene amplification or deletion (see below), and detection of the presence of translocations, so that many cells should be scored. Metaphase FISH analysis, although more informative, is more difficult, especially when there are few metaphase spreads present.

1. Select nuclei in which signals generally have the same intensity.
2. Focus up and down in the z axis to accommodate spatial configurations of probe signals within the nucleus (Fig. 22.4.4B).
3. Signals that are more intense in some nuclei than the specific signal indicate the presence of regional background. Care must be taken when analyzing any sample with this type of background noise.

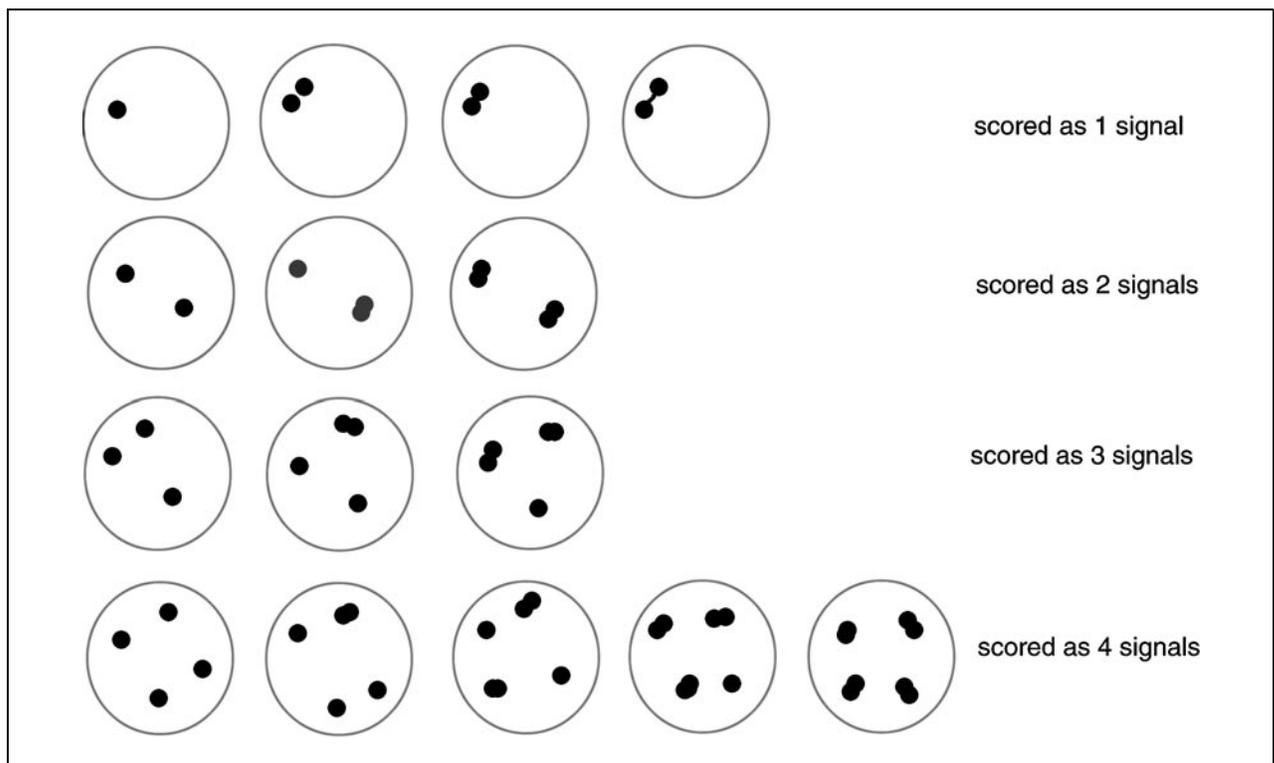


Figure 22.4.5 Schematic representation of scoring criteria. Shown in the illustration are typical hybridization signal configurations. These images pertain to a single probe, such as a centromere- or locus-specific probe. Signals must be more than one signal width apart to be considered one signal. Signals joined by a string of hybridization are also considered as one signal.

4. Do not count lower level nonspecific hybridization signals. These signals can usually be recognized by their lower intensity and different shape.
5. Some nuclei will have passed through the S phase of the cell cycle and may be present as G2-paired signals (i.e., two smaller signals in very close proximity). These paired signals represent a single chromosome already divided into chromatids and should be counted as one signal (Fig. 22.4.5).
6. Count two signals connected by a strand of fluorescence as one signal. Sometimes centromere or long genomic probes will generate signals that are not spherical. Typically, FISH signals appear as separate fluorescent dots on each chromatid of a metaphase chromosome when the target size is 100 to 250 kb. Similarly, in interphase nuclei, such probes will also generate discrete easy-to-interpret signals. Larger probes can appear as fused signals straddling both chromatids, and in interphase nuclei, these probes can generate signals that present more diffuse or dispersed hybridization spots in the chromatin of interphase nuclei. Knowledge of the probe size and anticipated configuration in both metaphase spreads, and interphase nuclei is essential. As long as the signal is continuous it should be scored as one signal.
7. Count only nuclei in which a definite enumeration can be made. Do not analyze or enumerate inconclusive cells.
8. Use two people to score 200 consecutive nuclei from each sample such that each person scoring will analyze ~100 nuclei from a given sample. The slides should be coded and scored independently by two analysts. Any discrepancies may mean the established scoring criteria for the FISH assay are not being adhered to rigidly.

Analytical sensitivity of interphase FISH assays

Once a particular probe set is made available as a routine FISH test for a clinical service, it is important that the laboratory perform assay validation and create a database establishing reportable range and general laboratory experience with each probe. The analytical sensitivity assay measures the success of a given FISH test in a particular laboratory environment and on a given tissue type. Since there are known differences in the cell populations and tissue types, it is important to use the appropriate positive control tissue for the assessment of analytical sensitivity and for the establishment of the database. Analytical sensitivity analyses are performed by scoring 200 interphase nuclei representing at least five normal, preferably male, individuals. (Pooling samples on one slide is acceptable.) The nuclei are scored for the percentage of nuclei exhibiting the appropriate number of distinct signals.

For constitutional studies using FISH, the recommended analytical sensitivity for probes intended for nonmosaic detection is 90%, while for probes intended to detect mosaicism, it is 95%. Similarly, for detection of minimal residual disease, sensitivities $\geq 95\%$ are helpful. Databases for each probe should be established so that it will then be possible to determine the mean and standard deviation of results from a series of normal samples processed and analyzed in the same manner as clinical samples. False positive rates can then be calculated and used for final scoring reports. More discussion on this issue is available from the following sources: VYSIS guidelines for single (http://www.vysis.com/tech_sup_fishproto_quality_single.asp) and dual probes (http://www.vysis.com/tech_sup_fishproto_quality_dual.asp), and scoring criteria for preimplantation genetic diagnosis of numerical abnormalities for chromosomes X, Y, 13, 16, 18, and 21 (Munne et al., 1998).

Statistical considerations concerning interphase FISH analysis of paraffin sections

Due to truncation of the nuclei during sectioning, loss of signal from areas of the nucleus excluded from the target slide will be encountered when enumerating signals after FISH has been performed on paraffin sections. The criteria for determining the significance of loss or gain of signals in interphase nuclei will depend on a number of parameters (e.g., nuclear diameter, age of patient, type of tissue). Readers are referred to some of the scientific literature where suggested cutoff values are adopted from the available literature (Qian et al., 1996). In the authors' experience with FISH analysis of prostate cancer, chromosomal gains can be identified when more than $\sim 10\%$ of the nuclei exhibit more than two signals. Panel iii of Figure 22.4.4B, shows an example of a prostate section hybridized with centromere probes for centromeres 7 (green) and 8 (red). Chromosomal losses have been identified when more than 50% of the nuclei exhibit a reduction of signal number, and tetraploidy has been assumed when all chromosomes investigated show signal gains up to four. For some classes of tumor, extreme polyploidy together with complex patterns of chromosomal rearrangement means that it is not realistic to select a suitable control chromosomal region in which two signals are expected. In such situations, it may be helpful to perform flow cytometric analysis of DNA content in parallel with interphase FISH analysis.

Analysis of Translocation and Inversion Probes

If the probes based on green and red fluorescence used for FISH are close to specific translocation breakpoints on different chromosomes, they will appear joined as a result of the translocation, generating a yellow color fusion signal. Commercial probes are now available for many of the common translocations in cancers (Table 22.4.19). One such probe from Vysis detects the Philadelphia chromosome (Ph) resulting from the

Table 22.4.19 Commonly Used Commercial Probes for Detection of Translocations in Sarcomas and Hematological Malignancies

Neoplasm	Chromosomal location	Probe	Scoring method
CML/pediatric ALL	9q34/22q	<i>BCR/ABL</i>	Color fusion observed in metaphase and interphase
Various leukemias	11q23	<i>MLL</i>	Split signal, metaphase
Various leukemias	5q31	<i>EGFR1</i>	Loss of signal, metaphase/interphase
Various leukemias	7q31	<i>DSS486</i>	Loss of signal, metaphase/interphase
AML M4 EO	Inv(16)	<i>CBFB</i>	Split signal, metaphase
Various leukemias	20q13.2	<i>ZNF217, D20S183</i>	Loss of signal, metaphase/interphase
Various hematologic malignancies	8q24 /14q32	<i>MYCC/IgH</i>	Color fusion observed in metaphase and interphase
AML-M1	12p13/21q22; 8q22/21q22	<i>TEL/AML1</i> ; <i>AML1/ETO</i>	Color fusion observed in metaphase and interphase
AML-M3	15q22/17q21.1	<i>PML/RARA</i>	Color fusion observed in metaphase and interphase
Ewings sarcoma	t(11;22) (q24;q12)	<i>FLII/EWS</i>	Color fusion observed in metaphase and interphase
Rhabdomyosarcoma	t(2;13)(q35;q14)	<i>PAX/FKR</i>	Color fusion observed in metaphase and interphase

translocation between ABL on chromosome 9 and BCR on chromosome 22. Shown in Figure 22.4.4C is an example of combined G-banding and FISH analysis using the Vysis *BCR/ABL* probe set. Panel i of Figure 22.4.4C shows the G-banded metaphase spread to which the *BCR/ABL* probe was subsequently hybridized. In panel iii of Figure 22.4.4C, detection of a Ph chromosome in interphase nuclei of leukemia cells is achieved by the presence of two double-fusion (D-FISH) signals. All nuclei positive for the translocation contain one red signal (*BCR* gene), one green signal (*ABL* gene), and two intermediate fusion yellow signals because the 9;22 chromosome translocation generates two fusions, one on the 9q+ and a second on the 22q-. The following general guidelines may be helpful for performing this type of assay.

1. Green and red signals that are juxtaposed but not overlapping should be scored as ambiguous.
2. Do not score nuclei that are missing a green or red signal. This assay is looking for the presence or absence of a fusion signal, not the absence of a green or red signal.
3. Atypical signal patterns have been reported and are now considered to be clinically important (Kolomietz et al., 2001).

Table 22.4.19 lists some of the commonly used FISH assays in hematological cancers as well as sarcomas. In addition to the scientific literature, readers are referred to the suppliers web sites (see Internet Resources and *SUPPLIERS APPENDIX*), which will provide the most up-to-date listing of currently available probes and the preferred scoring method.

Inversions are related to translocations such that a break and rejoining occurs within the resident chromosome. Probes are available to detect common inversions present in AMLs

(see Table 22.4.19). Mapping of breakpoints is common in many research cytogenetic laboratories and involves chromosome walking. To identify the locus containing the breakpoint, rough cytogenetic analysis locates the band region. Clones are then obtained spanning the putative breakpoint. These clones are then FISHed to the specimen of interest. Such is the case in Figure 22.4.4D. An inversion was identified involving the 11p15.5 region. Clones 200 kb apart, containing the *IGF2* gene (green) and *H19* (red) were hybridized to the patient specimen. The normal chromosome 11 shows the red and green signal hybridizing on top of each other at 11p15.5. The inverted 11 shows the clear split of signal along the abnormal chromosome 11 indicating the breakpoint lies within the 200-kb distance between *IGF2* and *H19*.

Use of FISH Probes in Assessing Solid Tumors and Gene Amplification

Gene amplification is one of the mechanisms by which cancer cells achieve over expression of some classes of oncogenes, which involves an increase in the relative number of copies of a gene per cell. This can range from one or two additional copies per cell to extreme examples where over a thousand copies per cell have been reported. Gene amplification can occur in association with the over-expression of oncogenes, thus conferring a selective growth advantage or mechanism of acquired resistance to chemotherapeutic agents leading to poor prognosis. Gene amplification is highly suited to FISH analytical approaches that have the added benefit of excellent sensitivity and the ability to address cellular heterogeneity.

Neuroblastoma is characterized by the frequent occurrence of a highly amplified oncogene, *MYCN*. It has been known for many years that the presence of this aberration is strongly associated with poor outcome. More aggressive management is usually required when *MYCN* is found to be amplified. Similarly, breast cancer can be accompanied by an amplified oncogene *HER2/Neu*, and presence or absence of this aberration may determine which of different treatment regimens are followed. Examples of metaphase and interphase FISH assays for gene amplification are shown in Figure 22.4.4E. In this figure, a DNA probe containing the *MYCN* gene was hybridized to a cytogenetic specimen from a neuroblastoma patient. Panel i of Figure 22.4.4E illustrates double minute (dms) chromosomes containing hundreds of copies of the *MYCN* gene as extrachromosomal bodies. This is in contrast to another patient where amplification of the *MYCN* gene occurs as a homogeneously staining region (HSRs) inserted in a chromosome other than the resident site of *MYCN* (normally at 2p24). Interphase nuclei in this image show a large patch of hybridization signal characteristic of HSRs. Some of the commonly detected aberrations observed in solid tumors which are amenable to FISH analysis are presented in Table 22.4.20.

Analysis of Telomere Probes

Telomeres are located at the ends of chromosomes and are characterized as (T₂AG₃) repeat sequences and their associated proteins (Poon et al., 1999). Maintained by the ribonucleoprotein complex, telomerase, they function to protect chromosomes from end-to-end fusions. In most normal tissues, telomerase is expressed at very low levels. As such, each round of DNA replication results in the gradual shortening of the telomeres. The shortening of telomeres is associated with replicative cell senescence. The up-regulation of telomerase extends the proliferative lifespan of a normal cell. In abnormal cells, expression of telomerase is associated with the maintenance of telomere length or telomere lengthening. Conventional Southern blot analysis gives average telomere length but fails to yield information on individual chromosome ends. It also underestimates the size and number of short telomeres. Although DNA probes for these sequences are available commercially, the use of commercial PNA probes for such sequences, as well as for centromeric and pancentromeric sequences, has enabled researchers to determine the overall

Table 22.4.20 FISH Probes Used For Assessing Solid Tumors and Gene Amplification

Neoplasm	Chromosomal location	Probe	Scoring method
Neuroblastoma	2p24; 2p23-24	<i>MYCN</i>	Interphase, metaphase; amplification
Ewings sarcoma	t(11;22) (q24;q12)	<i>FLII/EWS</i>	Color fusion observed in metaphase and interphase
Breast cancer	17q11.2-q12	<i>HER2/neu</i>	Interphase amplification
Glioblastoma	7p10-p21	<i>EGF-R</i>	Interphase amplification
Bladder cancer	Centromere regions of chromosomes 3, 7,17 and 9p21 region of chromosome 9	Centromere 7, 13, 9	Interphase enumeration

telomere lengths of individual cells (interphase nuclei) and chromosomes (metaphases) using quantitative digital imaging, as described by Poon (1999), with greater specificity and accuracy. Both fixed cytogenetic cells (Poon et al., 1999) and paraffin-embedded samples (Vukovic et al., 2003) have been used for telomere analysis. Panel i of Figure 22.4.4F illustrates subtelomeric PNA probes hybridized to a prostate cell line metaphase spread. Digital imaging and signal intensity ratios determine the relative telomere length. In some cases, loss of telomere signals can also be identified as in panel ii of Figure 22.4.4F (arrow). In this figure, a prostate cell line was hybridized with subtelomeric PNA probe as well as a PNA pan-centromeric probe. The pancentromeric probe confirmed the presence of multicentric chromosomal structures indicative of chromosomal instability.

For those who wish to engage in telomere studies, access to digital imaging and analysis software capable of determining telomere length is suggested. Like all other FISH experiments, the background should be minimal. Telomere signals are relatively small and located at the ends of chromosomes. Background, such as antibody speckling can greatly affect the sensitivity of the analysis. The proper controls must also be established. Since telomere length is dependent on the number of cell divisions, age- and sex-matched controls should be included in all experiments.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Agarose gel, 2% (w/v)

Dissolve 2 g molecular biology–grade agarose in 1 × TBE buffer (APPENDIX 2A) by either microwaving or placing on a hot plate, and allow to cool slightly. Add 5 μl of 10 mg/ml ethidium bromide in a well vented chemical hood. Pour into casting trays (APPENDIX 3A) and allow to solidify. Store up to 1 week at 4°C covered with foil.

AP-labeled antibody mixture

Dilute anti-biotin or anti-digoxigenin conjugated to alkaline phosphatase (AP; In-vitrogen) to a final concentration of 0.75 U/ml in 100 mM Tris·Cl (pH 7.5)/15 mM NaCl (APPENDIX 2A). Prepare fresh for each experiment.

Antibodies

Prepare antibodies stocks according to the manufacturer's instructions. Tertiary antibodies are conjugated to fluorochromes. Store up to several months as frozen aliquots. Prepare working solutions of primary and secondary antibodies according to the manufacturer's instructions using the guidelines for final antibody listed in Table 22.4.14. Diluted antibodies should be kept at 4°C in light-proof containers for 2 to 3 months.

Minor adjustments in concentration will need to be made to account for background and signal intensity.

DAPI, 100 µg/ml

Dilute all of the powder from a 1-mg bottle of 4',6-diamidino-2-phenylindole (DAPI; Sigma) in 10 ml buffer to produce a 100 µg/ml stock solution. Store in small aliquots up to 1 year at -20°C.

CAUTION: DAPI is a potential carcinogen and should be handled with care.

DAPI in antifade

Combine the following in order:

5.0 ml 1× PBS (APPENDIX 2A)

500.0 µl 100 µg/ml DAPI stock (see recipe; 1 µg/ml final)

0.5 g *p*-phenylenediamine (Sigma; 10 mg/ml final; dissolve well)

45.0 ml glycerol (90% v/v final)

Transfer to a 50.0-ml conical tube, wrap with aluminum foil (product is light sensitive), and place on a rotator 30 min to ensure proper mixing. Store in 1-ml aliquots up to 1 year at -20°C.

The resulting solution is very viscous

This reagent is available commercially as Vectashield (Vector Laboratories).

DNase I, 3 mg/ml

3.0 mg DNase I powder

500.0 µl glycerol (50% v/v final)

50.0 µl 1 M Tris·Cl, pH 7.5 (50 mM final; APPENDIX 2A)

5.0 µl 1 M MgCl₂ (5 mM final)

1.0 µl 1 M 2-mercaptoethanol (1 mM final)

1.0 µl 10 mg/ml BSA (10 µg/ml final)

Adjust volume to 1.0 ml with H₂O

Store in 50-µl aliquots up to 1 year at -20°C.

DNase I dilution buffer

250.0 µl 1 M Tris·Cl, pH 7.0 (50 mM final; APPENDIX 2A)

25.0 µl 1 M MgCl₂ (5 mM final)

5.0 µl 1 M 2-mercaptoethanol (1 mM final)

20.0 µl 10 mg/ml BSA (4 µg/ml final)

4.7 ml H₂O

Store in 1-ml aliquots up to 6 months at -20°C *or*

In 1 ml aliquots up to 1 month at 4°C.

dNTP mixture, 1 and 2 mM

For a 1 mM mixture:

1.0 μ l 100 mM dATP

1.0 μ l 100 mM dCTP

1.0 μ l 100 mM dGTP

97.0 μ l H₂O, sterile

Store in 100- μ l aliquots up to several months at -20°C

For a 2 mM mixture: Double the volume of each 100 mM dNTP added (i.e., use 2 μ l of 100 mM dNTP) and reduce the amount of water to 94 μ l. Store up to several months.

The total volume of either solution is 100 μ l.

Fluorochrome/dTTP mixture

The amount of fluor-dUTP will vary. Thus, the recipes below are guidelines. Refer to the manufacturer's suggested final concentrations. All labeled nucleotides should be stored in 20 to 30- μ l aliquots up to 6 months at -20°C in light-proof containers.

FITC-dUTP/dTTP:

3.5 μ l 1 mM dTTP (0.6 mM final)

1.75 μ l 1 mM fluorescein (FITC)-dUTP (0.3 mM final; Roche)

The total volume, 5.25 μ l, is appropriate for one labeling reaction and can be scaled up as needed.

Rhodamine-dUPT/dTTP mixture:

3.5 μ l 1 mM dTTP (0.6 mM final)

1.75 μ l 1 mM rhodamine-dUTP (0.3 mM final; Roche)

The total volume, 5.25 μ l, is appropriate for one labeling reaction and can be scaled up as needed.

Texas Red-dUPT/dTTP mixture:

3.5 μ l 1 mM dTTP (0.7 mM final)

1.0 μ l 1 mM Texas Red-dUTP (0.2 mM final; Roche)

The total volume, 4.5 μ l, is appropriate for one labeling reaction and can be scaled up as needed.

Cy3-dUPT/dTTP mixture:

3.5 μ l 1 mM dTTP (0.7 mM final)

1.25 μ l 1 mM Cy3-dUTP (0.2 mM final; Amersham Biosciences)

The total volume, 4.75 μ l, is appropriate for one labeling reaction and can be scaled up as needed.

Cy5-dUPT/dTTP mixture:

3.5 μ l 1 mM dTTP (0.7 mM final)

1.25 μ l 1 mM Cy5-dUTP (0.2 mM final; Amersham Biosciences)

The total volume, 4.75 μ l, is appropriate for one labeling reaction and can be scaled up as needed.

Formamide, 70%/Tris·Cl (pH 7.0 to 7.5), 10 mM/BSA, 0.1% (w/v)

Dissolve 100 mg BSA in 29 ml water. To this solution, add 70 ml formamide (Invitrogen) and 1 ml of 1 M Tris·Cl, pH 7.0 to 7.5 (*APPENDIX 2A*). Store up to several weeks.

CAUTION: Formamide is a carcinogen and should be handled with care. Discard according to institution's biohazard rules.

Total volume is 100.0 ml; however, the solution can be made in larger quantities and stored up to several weeks at 4°C.

Hapten/dTTP mixture

The amount of hapten-dUPT will vary. Thus, the recipes below are guidelines. Refer to the manufacturer's suggested final concentrations. Store up to several months or a year at -20°C.

Biotin-dTTP/dUTP mixture:

3.5 µl 1 mM dTTP (0.6 mM final)

1.75 µl 1 mM biotin-16dUTP (0.3 mM final; Invitrogen)

The total volume, 25 µl, is appropriate for one labeling reaction and can be scaled up as needed.

Digoxigenin-dTTP/dUTP mixture:

3.5 µl 1 mM dTTP (0.6 mM final)

1.75 µl 1 mM dig-11dUTP (0.3 mM final; Roche)

The total volume, 5.25 µl, is appropriate for one labeling reaction and can be scaled up as needed.

Hybridization buffer

500.0 µl high grade formamide (50% v/v final; Invitrogen)

100.0 µl 20× SSC (2× final; *UNIT 18.6*)

100.0 µl dextran sulfate (10% final)

300.0 µl H₂O

Store in 100-µl aliquots up to several months at 4°C

Total volume is 1.0 ml.

Hybridization buffer can also be purchased from DAKO.

Loading dye, 5×

0.125 g bromphenol blue (0.25% final)

15.0 ml glycerol (30% final)

35.0 ml H₂O

Store in 1.0-ml aliquots up to several months at 4°C

Total volume is 50.0 ml.

NBT/BCIP

22.5 µl 75 mg/ml NBT (Invitrogen)

17.5 µl 50 mg/ml BCIP (Invitrogen)

4.96 ml 100 mM Tris·Cl (pH 9.5; *APPENDIX 2A*)/100 mM NaCl/50 mM MgCl₂

Prepare fresh for each experiment.

Volumes can be scaled up or down as required.

Nick translation buffer, 10×

1.0 μl 10 mg/ml BSA (0.1 $\mu\text{g}/\mu\text{l}$ final)
10.0 μl 1 M 2-mercaptoethanol (0.1 M final)
50.0 μl 1 M Tris·Cl (0.5 M final)
5.0 μl 1 M MgCl_2 (50 mM final)
34.0 μl H_2O
Store in 100- μl aliquots up to several months at 4°C
Total volume is 100.0 μl .

PBS, 1 \times /MgCl₂, 0.05 M

950.0 ml 1 \times PBS
50.0 ml 1 M MgCl_2
Store at room temperature until ready for use.
The total volume is 1 liter.

Pepsin, 0.5% (w/v) in NaCl, 0.85%

Dissolve 500 mg pepsin in 100.0 ml of 0.85% (w/v) sodium chloride. Adjust pH to 1.5 with 12 N HCl. Prepare fresh for each experiment.

PI, 100 $\mu\text{g}/\text{ml}$

Dissolve the powder from an entire 10-mg bottle of propidium iodide (PI; Sigma) in 10 ml water to a final concentration of 100 $\mu\text{g}/\text{ml}$. Store in small aliquots indefinitely at -20°C .

PI in antifade

Combine in the following order:
5.0 ml 1(PBS; *APPENDIX 2A*)
150.0 μl 100 $\mu\text{g}/\text{ml}$ PI (0.3 $\mu\text{g}/\text{ml}$ final; see recipe)
0.5 g *p*-phenylenediamine (10 mg/ml final; Sigma; dissolve well)
45.0 ml glycerol (90% final)

Transfer to a 50.0-ml conical tube, wrap in aluminum foil (the product is light sensitive), and place on a rotator 30 min to ensure proper mixing. Store in 1-ml aliquots up to several months at -20°C .

The resulting solution (50 ml total) is very viscous.

This solution is available commercially available from Vectashield (Vector Laboratories).

RNase I, 100 $\mu\text{g}/\text{ml}$

Prepare a final 100 $\mu\text{g}/\text{ml}$ solution of RNase I in 2 \times SSC (*UNIT 18.6*) fresh for each experiment using any molecular-grade RNase enzyme.

Sonicated salmon sperm DNA standards

Prepare 50.0 ng/ μl salmon sperm DNA standard by combining 5.0 μl of a 10 $\mu\text{g}/\mu\text{l}$ stock (Invitrogen), 300 μl of 5 \times loading dye (e.g., *UNIT 18.6*), and 695 μl water. Prepare a 25.0 ng/ μl standard by combining 500 μl of the 50 ng/ μl standard, 100 μl of 5 \times loading dye, and 400 μl water. Prepare a 12 ng/ μl standard by combining 500 μl of the 25.0 ng/ μl standard, 100 μl of 5 \times loading dye, and 400 μl water. Store all standards up to several months at 4°C or indefinitely at -20°C .

The average size of the commercial salmon sperm is \sim 500 to 2.0 kb

Triethanolamine (pH 8.0), 0.1 M

660.0 µl triethanolamine (final 0.1 M; Sigma)

50.0 ml H₂O

Prepare fresh for each experiment.

Total volume is 50.0 ml.

COMMENTARY

Background Information

Applications of FISH

As early as 1988 (Lichter et al., 1988), FISH was used to visualize labeled DNA probes hybridized to chromosome and interphase nuclei preparations. The improvement of cloned DNA sources, antibodies, fluorochromes, microscopy and imaging equipment, and software has permitted a variety of scientific investigations. FISH analysis is routinely used in clinical cytogenetic laboratories for detection of syndromes in prenatal assessments, including Prader-Willi/Angelman Syndrome, DiGeorge Syndrome, and Cri-du-chat. Cancer cytogenetics laboratories use FISH to confirm and monitor hematological malignancies such as CML and AML (Table 22.4.19 and Table 22.4.20). Gene amplification studies for neuroblastoma (MYCN amplification), and breast cancer (HER2/NEU amplification) are also routinely carried out. Mapping of newly identified genes have relied heavily on FISH (Squire et al., 1993; Lichter et al., 1988) both in humans and other species (Boyle et al., 1990; Giguere et al., 1995). Metaphase spreads may not always be easy to obtain, thus interphase cells offer a means of obtaining information, albeit via an indirect method. This has been useful for gauging chromosome instability (Speicher et al., 1995; Ghadimi et al., 1999; Al-Romaih et al., 2003; Vukovic et al., 2003) and determining normal versus abnormal content, as well as gene amplification or deletion. FISH applied to paraffin-embedded sections appeared in the early 1990s (Thompson et al., 1994) and was applied to both thick (>5 µm) and thin (5 µm) paraffin sections. Application of FISH to paraffin-embedded sections provides a tremendous opportunity to correlate the histopathological classification to the genomic changes detected. Furthermore, it enables the investigator to study concepts of cellular heterogeneity, tumor focality, and metastasis (Squire et al., 1996). Paraffin FISH analysis requires patient and careful technical expertise. Proper controls must be used when interpreting final results. Depending on the type of information sought, paraffin FISH

is an acceptable form of data collection when fresh tissues for cytogenetic suspensions are not available; however, paraffin FISH will not provide chromosome-based information.

Preparation of probes

The development of reliable cloning strategies in the 1980s facilitated the genomic analysis and sequencing of specific DNA fragments. Mapping of these genes to their chromosomal locations was previously laborious and infrequently reliable. The emergence of FISH in the early 1990s paved the way for an effective and direct means of mapping specific DNA fragments to their chromosomal locations.

Types of probes. The creation of comprehensive genomic libraries as a result of the human genome project provides renewable resources for FISH probes. At present, bacterial artificial chromosomes (BACs) are the most popular cloned forms of genomic DNA used for FISH probes. Although yields are generally considered low in comparison to plasmids and cosmids (predecessors of BACs), BAC inserts are larger (200 kb) and can produce a stronger FISH signal compared to cosmids and plasmids, which are considerably smaller in insert size (i.e., ranging from 2 to 30 kb). P1 artificial chromosomes (PACs) and yeast artificial chromosomes (YACs) have also been used in the past and are still used in many research laboratories. Other probes, such as RNA and oligonucleotide probes are options and are reviewed in Speel (1999), and Schwarzacher and Heslop-Harrison (2000). More recently, peptide nucleic acid (PNA) probes (Lansdorp, 1996), have been used for FISH experiments with great success (Martens et al., 1998; Vukovic et al., 2003.). Using the same nucleotide bases as DNA probes, PNA probes do not have a phosphate or deoxyribose sugar backbone. Specific fluorochromes, haptens, or enzymes can be chemically attached to the bases and used in the same fashion as DNA probes. PNA probes are sequence specific. Thus, sequence information must accompany a PNA custom probe

order to companies such as Applied Biosystems (<http://www.appliedbiosystems.com>). There are several advantages of PNA probes. For instance, PNA has a neutral backbone, which provides stronger binding and greater specificity of interaction. Also, the uncharged PNA structure creates stronger binding independent of salt concentration, allowing more robust hybridization applications. Finally, the fact that PNA oligomers have resistance to nucleases and proteases, permitting more robust *in situ* experiments, is also an advantage. The drawback of PNA probes are in the cost of their fabrication. The typical molecular (cytogenetic) laboratory cannot generate these probes *in-house*. The cost-effectiveness of PNA probes, therefore, can only be appreciated if the laboratory is carrying out a particular hybridization frequently, as in the case of a clinical cytogenetic laboratory.

Methods for preparing probes. By far the most commonly used strategy for labeling DNA probes is by nick translation. Nick translation involves the simultaneous actions of two enzymes: DNase I and DNA polymerase I. DNase I randomly nicks the DNA fragment in each strand of the double-stranded DNA molecule. DNA polymerase I (derived from *E. coli*), with its three activities—i.e., exonuclease function (removes bases in the 5' to 3' direction), polymerase function (adds nucleotides from the 3' nick site), and the 3' to 5' proofreading function—incorporates the label, whether indirectly using a hapten (i.e., biotin or digoxigenin) or directly (i.e., fluorescein or rhodamine). Nick translation can be applied to all cloned DNA sources without the need to remove vector sequences. Polymerase chain reaction (PCR) is another commonly used method of labeling DNA probes from cloned sources, as well as from microdissected genomic DNA or flow-sorted chromosomes. PCR employs multiple rounds of template denaturation, primer annealing, and template replication, facilitated by *Taq* DNA polymerase. Primers are variable and may include sequence-specific primers to amplify those targeted fragments, vector sequence primers to amplify and label cloned DNA, and/or universal primers. Compared to nick translation, which requires micrograms of starting DNA, PCR requires only nanograms of template. Furthermore, nick translation is most efficient when the starting DNA is of high molecular weight with little or no degraded material. PCR labeling is a little more forgiving of DNA quality; thus, suboptimal DNA may still be used when not acceptable for nick translation. De-

pending on the template, several rounds of amplification may be required before labeling, as in the case of *in-house* production of chromosome painting probes. Other labeling strategies, including random priming and end-labeling, are also options and are described in Schwarzbacher and Heslop-Harrison (2000).

For both nick translation and PCR labeling, commercial kits and enzyme mixes are readily available and optimized. Usually, commercial kits combine DNase I and DNA polymerase I as an enzyme mix. The drawback of combining them is the fact that one enzyme (usually the DNA polymerase I) will lose its enzymatic activity before the other. Commercial kits can also be costly. Making an *in-house* kit allows the investigator to alter enzyme concentrations as necessary to better optimize the reaction. This alleviates the combined-enzyme problem with commercial kits. *In-house* kits are also expensive and they do require rigorous internal quality control and troubleshooting from time to time.

See Critical Parameters for discussion of slide preparation, hybridization, post-hybridization washes and detection, and interpretation of FISH findings.

Critical Parameters

Preparation of probes

DNA quality. Ensure that the starting DNA to be labeled has a high molecular weight with no degraded DNA fragments or protein. Contaminating RNA will contribute slightly to the spectrophotometric quantification of the DNA and cause a miscalculation of the actual starting amount of DNA. DNA should be assessed using an accurate spectrophotometer and by gel electrophoresis. Compare the concentration of DNA as determined using a spectrophotometer and gel with a DNA of known concentration. The DNA used as a standard for concentration can be another undigested clone or genomic DNA.

The starting DNA should be dissolved in water rather than Tris/EDTA (TE) buffer. TE is normally used to inhibit any DNases that may be present in the water. By that reasoning, TE buffer has been known to inhibit the enzymatic activity of the DNase during the nick translation reaction. Furthermore, it may inhibit the polymerase activity for nick translation and PCR labeling.

Reagents. Ensure that reagents are used prior to their expiration dates. The most critical reagents in the labeling reactions are:

1. DNA polymerase *I/Taq* DNA polymerase. Failure of polymerase activity in the reaction will lead to inefficient incorporation of the labeled nucleotide into the DNA, resulting in a very weak FISH signal.

2. Conjugated nucleotides. Nucleotides conjugated to a fluorochrome are light sensitive. Repeated and prolonged exposure to direct light will quench the fluorescent signal. Store these reagents in small aliquots at -20°C in light-proof containers. Restrict exposure to light during the labeling procedure and store labeled DNA at -20°C in light-proof containers. Unlike nonfluorescently labeled probes, these directly-labeled DNAs will have a shorter shelf life. The concentration of nucleotides can vary from as much as 1 mM to as low as 0.2 mM in different nick translation protocols. The labeled nucleotide should always be in at least a 2-fold excess of the corresponding unlabeled nucleotide. Furthermore, the labeled nucleotide can be interchangeable between hapten-dUTP or hapten-dATP. If the investigator chooses to use Biotin 14-dATP rather than the dUPT form, the nucleotide mixture should be adjusted accordingly.

3. 2-Mercaptoethanol. Buffers containing 2-mercaptoethanol should be replaced every 3 to 4 months, particularly if not stored at -20°C .

4. DNase I. Although the activity of DNase I is usually potent enough that it may be used for up to a year when stored at -20°C , the specific activity may decrease over time. This is evident when the fragment sizes are larger than expected when the standard DNase I concentration and incubation times are used. Simply increase the amount of DNase I used in the reaction and keep the same period of incubation, or keep the same amount of DNase I used in the reaction and increase the time of incubation.

5. PCR factors. Treat PCR labeling like any other PCR experiment and consider the following factors: amount of template, pH, magnesium ion concentration, and primer sequence.

Equipment. Small volumes are used for both labeling strategies. Pipets should be calibrated and serviced often. Use an accurate spectrophotometer and water bath that can effectively maintain a constant temperature, if a thermocycler is not available for nick translation.

Preparation of slides

FISH involves the hybridization of the labeled and denatured DNA probe to its denatured DNA target located on the slide. Each

component requires care to obtain the most effective results. Pretreatment of the slide with a protease before denaturation permits the removal of any cellular and cytoplasmic debris. In the case of paraffin-embedded sections, more aggressive treatment is required to remove residual wax as well as to allow sufficient protease treatment to permit access of the probe to the intended target DNA.

Protease treatment. Treatment with pepsin has been featured in this protocol because its action is relatively mild as compared to other proteases such as proteinase K; however, many laboratories use proteinase K alone or in combination with pepsin, particularly for the digestion of paraffin-embedded sections, to obtain the proper digestion conditions for FISH (see Table 22.4.9). The incubation times of any protease treatment will vary according to the type of tissue (e.g., paraffin-embedded), quality of the slide (i.e., quantity of cytoplasmic debris/cellular debris), as well as the lot or batch of pepsin used. It is suggested that incubation times be increased rather than the concentration of pepsin used in the digest. The temperature at which the incubations occur is also critical. Higher temperatures facilitate faster digestions as opposed to those at room temperature or below. While variations in the pretreatment protocol can be made, the starting quality of the fixed tissue is the most critical factor. Ideally, the tissue should be fixed shortly after removal from the host.

Paraffin-embedded samples. In the case of paraffin-embedded tissues, formalin is the fixative of choice for routine use in most clinical laboratories; however, superior results for most research needs may be obtained if tissue sections are fixed using ethanol or paraformaldehyde prior to embedding. Buffered formalin solutions have also been implemented to provide better recovery of DNA, RNA, and protein for future studies. The time spent in fixation is equally as important as the type of fixative. The longer the time spent in fixative, the more difficult it may be to denature the DNA to a single-stranded state. This is due to the cross-linking action of the fixative to proteins that enables the cells to maintain their morphology. Sections for FISH should be cut fresh from the original block, preferably a few sections from the exposed face. This will help to ensure that the DNA has not been exposed to DNases, or oxidative damage from the handling of the block or from the local environment. Several sections should be taken so that different pretreatment conditions can be tested. It is suggested that the investigator take the time to contact the

histology laboratory preparing the specimens to document the fixation procedure for troubleshooting later.

Denaturation. Denaturation is also a critical parameter for successful FISH. Too much denaturation of the target DNA causes excessive DNA damage, yielding poor banding and/or hybridization. Too little denaturation results in ineffective hybridization due to limited access to the target DNA. The extent of denaturation will depend on several factors.

1. Quality of the slide. Namely, the presence of cytoplasmic and/or cellular debris.

2. Age of the slide. Whether using a fresh slide, a fresh slide with artificial aging, a slide that is 1 week or 1 month old, and so forth.

3. Previous assays. Namely if banding has previously been performed on the slide.

4. Tissue type. Whether fibrous, bony, or muscle tissue, or the like, is being used.

5. Age and method of fixation. See above.

6. Section thickness. Normally 5- μ m-thick sections are used.

Hybridization

Optimal hybridization is influenced by many factors that will affect the target DNA and the probe itself. These factors are sources for inefficiencies and should be carefully monitored and documented such that effective troubleshooting can be carried out.

Stringency. The hybridization of a DNA probe to its DNA target is dependent on the extent of sequence homology, the complexity of the DNA sequence, and the chemical factors that influence the success of maximal binding efficiency. Stringency refers to the percentage of matches and mismatches between the probe and target such that the hybridized double helix remains stable. FISH experiments are typically run at 70% to 90% stringency, with 70% representing a lower relative stringency than 90%. This is critical when determining the feasibility of carrying out cross-species FISH experiments (i.e., human probes on mouse targets or vice versa), where sequence homology may be considerably lower in parts of the probe. The reagents present in hybridization buffers, as well as the temperature significantly influence stringency.

Temperature. DNA strand melting and annealing are influenced by temperature. Since reagents such as formamide are present in the hybridization buffer, high temperatures (i.e., 80° to 90°C) are not required for DNA denaturation. This is the reason denaturation of the DNA can be accomplished at 72°C in

the presence of formamide. Increasing temperatures also increases stringency, such that a 1°C alteration increases the stringency by 1%. Thus, increasing the hybridization temperature from 37° to 42°C will increase the stringency of the hybridization step, requiring the probe to possess higher sequence homology with its target. This may be particularly useful if there is a chance of cross-hybridization, which is typically the case with locus-specific probes of genes that belong to a class of genes/sequences that are very similar. Pseudogenes may also cross-hybridize. Lowering the hybridization temperature under 37°C will result in lower stringency conditions permitting the hybridization of the probe to targets with greater mismatch. This also has advantages and disadvantages. At low stringency, mismatched spurious hybridization events will be more common and contribute to more experimental noise at the detection step.

Probe size. The rate of hybridization is also influenced by probe size. Generally, longer fragments (>500 bp) will require a longer time to hybridize than shorter ones. Once again, the hybridization is affected by the extent of sequence homology.

PNA probes. In the case of PNA probes, the specificity of the probe, as a required function of their fabrication through oligosynthesis, eliminates some of the complexities experienced by DNA probes. Since the PNA probe has an uncharged backbone, there is no charge repulsion when the probe hybridizes to the DNA, thus creating a stronger bond. Furthermore, PNA/DNA bonds will melt 15°C above DNA/DNA bonds, almost independently of salt concentration. The specificity of the PNA/DNA bond lies in the increased destabilization of the PNA/DNA bond when a mismatch occurs.

Post-hybridization washes and detection

Stringency. The factors that affect hybridization also influence the conditions involved in post-hybridization washes and antibody incubations. Incubation at 37°C under a range of 50% to 60% formamide in 2 \times SSC sets a stringency of ~70% to 80%. This permits hybridization of sequences with a 20% to 30% mismatch and maintains duplex stabilization. Increasing hybridization temperatures raises the stringency, requiring greater sequence homology for effective hybridization. Conversely, decreasing temperatures permits hybridization with greater mismatch. Decreasing salt ion strength (i.e., SSC) also increases

Table 22.4.21 Stringency of DNA/DNA Hybridization and Post-Hybridization Washes^a

SSC concentration	Temperature ^b	Stringency as a function of % formamide						
		60	55	50	45	40	35	30
5×	37°C	76	73	70	67	64	61	58
2×	37°C	83	80	77	74	71	68	65
1×	37°C	88	85	82	79	76	73	70
0.75×	37°C	90	87	84	81	78	75	72
0.5×	37°C	93	90	87	84	81	78	75
0.2×	37°C	100	96	93	90	87	84	81
0.1×	37°C	105	101	98	95	92	89	86
5×	42°C	81	78	75	72	69	66	63
2×	42°C	88	85	82	79	76	73	70
1×	42°C	93	90	87	84	81	78	75
0.75×	42°C	95	92	89	86	83	80	77
0.5×	42°C	98	95	92	89	86	83	80
0.2×	42°C	105	101	98	95	92	89	86
0.1×	42°C	110	106	103	100	97	94	91

^aThis Table describes the changes in stringency for probes with 43% GC content at 300 bp (modified from Schwarzacher and Heslop-Harrison, 2000). Increasing the formamide content or temperature, or decreasing the SSC strength will increase stringency. A combination of these three factors can be used to modulate the stringency.

^bHybridization of probe to DNA typically occurs under 50% formamide in 2× SSC at 37°C, giving a 77% stringency. Washing of unbound probe following hybridization typically occurs under 50% formamide in 2× SSC at 42°C, giving an 82% stringency.

stringency. The combination of temperature and salt concentration can affect hybridization kinetics considerably. The same holds true for removal of unbound probe.

Typically, post-hybridization washes are carried out at 42° to 45°C. This temperature, coupled with 50% formamide and 2× SSC, permits 90% stringency for probes with a 43% GC content, at a probe size of 300 bp (Schwarzacher and Heslop-Harrison, 2000). Table 22.4.21 describes the stringency conditions based on these parameters.

Detergents. Some suppliers of commercial probes may suggest higher temperature washes of 72°C in a low ion (high stringency) SSC wash containing a detergent. This too is based on high sequence homology and the relative GC sequence content of the probe. This rapid wash method eliminates the need for formamide. Caution should be exercised when using high temperatures, since there is the risk of partial denaturation of the hybridized probe and target. This will result in loss of signal. A rapid wash should only be used with probes that possess high sequence homology and high labeling efficiency. Commercial probes tend to fit these criteria.

The subsequent SSC washes and washes with detergents serve to remove any remaining unbound probe and antibodies. As before, decreasing the SSC content will create a more stringent wash. The choice of detergent is subjective, largely based on the preference and experience of the investigator. The most commonly used detergents include Tween 20, NP-40, IPEGAL, SDS, and Triton X-100 (reviewed in Speel, 1999). In general, the actions of these detergents are comparable when used at the standard 0.1% in 4× SSC; however, SDS, Triton X-100, and NP-40 are generally harsher than Tween 20. If high background persists, the number of washes with the detergent can be increased or the percentage of detergent and SSC strength can be altered.

Antibodies. The choice of antibody systems is also fairly standard. Many companies provide antibodies raised in different animals and conjugated to different fluorochromes so that a variety of probes may be used and detected at one time. Some antibodies may require a higher concentration; thus, it is important that the signal strength be monitored carefully for background and the antibody concentration adjusted as required. As discussed

above, caution should be exercised when using various antibody combinations for detecting more than one probe: ensure that there is no cross-reactivity between antibodies raised in different species.

Counterstains. Finally the use of a counterstain helps to visualize the cells or spreads. DAPI stains DNA blue upon fluorescence and is most commonly used. DAPI, however, may obscure a green signal if using a dual filter (i.e., FITC/DAPI), so a red counterstain, such as propidium iodide (PI), may be more appropriate. If the color of probe detected is red, do not use PI as a counterstain. The signals are protected from rapid quenching through the reagents used in the mounting medium. These counterstains in mounting (antifade) medium can be purchased commercially. If the counterstain is prepared in the laboratory, be sure to use high quality glycerol, since some lower quality glycerols can cause autofluorescence. The concentration of counterstain may require adjustment. Higher counterstain concentrations may obscure FISH signals. If this is the case, dilute the counterstain in antifade by adding more glycerol and PBS in amounts that will be consistent with the final concentrations.

Storage. All slides should be stored at -20°C for long-term storage (i.e., up to 1 year) and at 4°C for short-term storage (i.e., up to 6 months).

Troubleshooting

Preparation of probes

See Table 22.4.22 for a troubleshooting guide to labeling.

Preparation of slides

Cytogenetic specimens. The influence of pretreatment and denaturation cannot be readily assessed until the entire FISH protocol has been carried out and the slides are visualized by fluorescence microscopy (Henegariu et al., 2001). The slides, however, may be visualized by phase-contrast microscopy for cytogenetic preparations after the dehydration step following pepsin treatment to determine the extent of digestion. The slides may be returned for digestion if the cytoplasmic debris does not appear to have been removed. Similarly, for treatment of paraffin sections, following visualization for autofluorescence, the slides may be returned for digestion and reassessed.

If there are no cells remaining on the slide following pepsin treatment, then the treatment was too harsh. If many cells have lifted off,

the treatment was too harsh. Adjust the time or pepsin concentration as required.

Paraffin sections. The diameter of nuclei in various tissue types will vary between 4 and $8\ \mu\text{m}$. The estimated diameter for the tissue of interest should be ascertained from the literature and sections of the same thickness or 2 to $4\ \mu\text{m}$ greater should be cut, so that it is likely that the entire nucleus is included in the section.

Pepsin digestion works for most tissue types. Occasionally, the tissue will exhibit persistent green autofluorescence of nuclei and no signal (see Table 22.4.19). Variation in histology fixation procedures and quality control performed by the hospital pathology laboratory necessitates careful assessment of treatment times and/or concentration of pretreatment (also see Table 22.4.9). Certain tissue sections are more resistant to effects of protein digestion than others. This may be due to the tissue type or amount of protein cross-linking elicited by excessive formalin fixation. If surplus fresh tissue can be obtained, then ethanol or paraformaldehyde fixation will cause less cross-linking and provide better results for most research applications. It should also be noted that normal tissues are often more refractory to protein digestion than tumor material derived from the same tissue type. If there are a limited number of slides remaining from the patient sample of interest it is sometimes helpful to use a superfluous slide from another sample of the same age and cell type to derive the optimal digestion parameters with respect to aging and storage. More comprehensive guidelines for improved paraffin results are summarized by Hyytinen et al. (1994).

Anticipated Results

Preparation of probes

Successful labeling for FISH will yield fragment sizes between 200 and 500 bp. Fragments up to 1 kb are acceptable, but larger fragments will contribute to background. Investigators should expect that the actual amount of DNA aliquoted onto the gel for visualization should be fairly close to the expected amount loaded onto the gel. The labeling reaction is fairly tolerant to variations in concentration from half the actual amount to two-times greater than the actual amount.

Once the labeling conditions have been established and tested in control FISH experiments, the investigator can be confident that subsequent labeling experiments will proceed without incident. Minor adjustments must

Table 22.4.22 Troubleshooting Guide to FISH Probe Labeling^a

Problem	Cause	Solution
DNA failed to digest	DNase I not present	Ensure that DNase I was added to the labeling reaction.
	Loss of DNase I activity	If DNase I was added, check the expiration date or change to a fresh aliquot.
Fragments too small	TE buffer used to dissolve DNA (EDTA inactivates DNase I)	If TE buffer was used to resuspend the DNA, reprecipitate the DNA, resuspend in water, quantify by spectrophotometry, and relabel.
	Too much DNase I added	Pipetting errors are the leading cause of many experimental problems. Use accurate pipettors.
	DNA starting concentration inaccurate	Refer back to spectrophotometer readings and gel images of undigested DNA to determine whether there were any discrepancies. Contaminating RNA and proteins may contribute to an inaccurate reading, such that the actual amount of starting DNA was less than expected, thus changing the labeling (digestion) kinetics for a specified amount of DNA over a specific time at a specific DNase I concentration. Relabel and increase the amount of starting DNA as required
	Starting DNA was degraded	If the starting DNA already contains degraded fragments, they will simply continue to degrade into relatively smaller fragments. Refer back to the gel of the undigested DNA and determine the percentage of degraded fragments present. Compensate for the fragments that will be digested away and increase the amount of DNA required to relabel. Consider PCR labeling strategies or obtain a better DNA sample.
	Labeling time too long	Be sure that the correct incubation time was used.
	Unstable water bath temperature	The labeling reaction has been optimized for a specific temperature. Increasing the temperature will facilitate faster nicking, while decreasing the temperature will slow it.
Fragments too long	Insufficient DNase I	Use accurate pipettors (see above).
	DNA starting concentration inaccurate	As described above, check the spectrophotometer reading and gel image. If too much DNA was added to the labeling reaction, the same amount of DNase I used to nick a given amount of DNA in a specific time will increase when there is more DNA present. The labeling of this DNA can be salvaged by continuing to incubate for 20 to 30 min (or as required). Be sure to spike the reaction with fresh enzymes.

continued

Table 22.4.22 Troubleshooting Guide to FISH Probe Labeling^a, *continued*

Problem	Cause	Solution
No fragments present	Starting DNA was dissolved in TE buffer	Check whether the starting DNA was dissolved in TE as this will inhibit the nicking of DNA. The labeling of this DNA can be salvaged by continuing to incubate as required. Be sure to spike the reaction with fresh enzyme.
	Decreasing activity of DNase I	Over time, the specific activity of DNase I will decrease. If this is the case, increase the amount of DNase I used in the reaction, while keeping the same incubation period. Otherwise, keep the same DNase I concentration and increase the labeling time.
	Absence of DNA added to reaction	Make sure that DNA was added to the reaction
	Insufficient DNA added	As discussed above, check to see that the concentration of DNA is accurate and pipettors are accurate.
Expected amount of DNA too low/too high (but fragmented to the right size)	Starting DNA degraded	If a significant portion of the starting DNA was degraded to begin with, as shown by gel electrophoresis, this will cause an increase in fragmentation speed, with the smaller fragments running off the gel. A new DNA specimen should be used, or the investigator should consider PCR methods.
	Too much DNase I added	Check that the proper DNase I concentration and/or volume was used in the labeling reaction.
PCR failed	DNA starting concentration inaccurate	Assess using a concentration standard. If the actual amount of DNA is less than expected, as determined by gel electrophoresis, then the starting amount of DNA was insufficient or caused by some of the factors described above. If the investigator chooses to relabel, increase the amount of starting DNA as required. If the actual amount of DNA is greater than expected, again as determined by gel electrophoresis, then the starting amount of DNA was incorrect. The investigator may choose to proceed and use this probe for FISH, but the overall incorporation of label into the DNA will be lower since the labeling reaction contains sufficient dNTPs for a specified DNA quantity. If the investigator chooses to relabel, decrease the amount of starting DNA material as required.
	Amount of template, pH, or MgCl ₂ concentration incorrect, or quality of template poor	Refer to <i>APPENDIX 3F</i> and <i>APPENDIX 2A</i> .

^aThe guide assumes that the labeling conditions have generally been optimized but occasionally anomalies occur.

be made when using new reagents. Once a probe has been FISHed, the signal should be clearly visible with little or no background or cross-hybridization. The investigator should be aware that other factors, such as hybridization efficiency, slide quality, antibody quality, stringency of post-hybridization washes can also affect the signal strength.

Preparation of slides

Optimal pretreatment of cytogenetic or paraffin-embedded slides should produce slides that are relatively free of cytoplasm/cellular debris, free of paraffin, and have adequately denatured DNA for FISH. The samples should not be autofluorescent.

Hybridization

Due to the multistep nature of FISH, it is difficult to determine the success of hybridization until the entire assay is completed. Successful hybridization will depend on adequate denaturation of the target DNA on the slide, as well as adequate denaturation of the probe. Proper suppression of repetitive sequence elements with Cot-1 DNA can increase the specificity of hybridization. The specificity of hybridization is also regulated by stringency factors described above (see Background information; also see Critical Parameters). Successful hybridization will allow the identification of chromosomal location of the probe sequence.

Post-hybridization washes and detection

The protocols described provide a basic framework for adjusting post-hybridization washes and detections. Under ideal conditions, the stringency of the post-hybridization washes is sufficient to adequately remove any unbound/nonhybridized probe from the target DNA. The specificity of the probe and the suppression of repetitive elements during preannealing will also decrease the number of cross-hybridization signals. The concentration of detergent used in the washes is also optimized to remove any unbound antibodies, although minor modifications, as required, should be made when using a different brand, lot, or type of antibody. The result, upon visualization with a microscope, is a clean preparation showing minimal background and an easily visualized signal(s) on the DNA target.

Time Considerations

Preparation of probes

The most time consuming portion of labeling for FISH is in the optimization of the la-

beling procedure if using an in-house protocol. As discussed previously, commercial kits for labeling are available and give relatively consistent results.

Preparation of slides

The preparatory work for preparing cytogenetic slides requires minimal time and the protocol is fairly robust. Optimization for paraffin-embedded sections requires more careful time and trial-and-error. The preparation of cytogenetic slides for FISH can be accomplished in ~1 hr. Once optimized conditions have been obtained for paraffin-embedded tissues, it too can be accomplished in 1 to 2 hr. However, initial optimization may require several experimental attempts.

Hybridization

The hybridization step of FISH typically occurs overnight (i.e., DNA probes), ~18 to 24 hr at 37°C. The time may be lengthened to 48 or 72 hr (as in the case with weekends), with no adverse effects. Hybridization with PNA probes require less time, at least 1 hr at room temperature. Caution, however should be taken that the slides are adequately sealed such that the probe in hybridization solution does not evaporate. Maintaining a slightly dampened slide container will help alleviate this problem.

Post-hybridization washes and detection

The time allotted for post-hybridization washes and detection will vary depending on the type of DNA probe used in the experiment. Directly labeled probes (DNA or PNA probes) require very little bench time since no antibody detection is required; typically they take up to 30 minutes. Probes are simply washed using high stringency washes as described in the methods. Indirectly labeled probes require incubations with antibodies (primary, secondary and/or tertiary), with the final incubation with a fluorochrome-conjugated antibody, as well as detergent washes and/or blocking steps between each antibody incubation. If the signal requires amplification, the bench time increases as each antibody incubation should last at least 30 minutes. A wash protocol using an indirectly labeled probe with signal amplification will range from 3.5 to 4 hours.

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Internet Resources

<http://www.appliedbiosystems.com>

The Applied Biosystems website. This company supplies PNA probes and custom orders PNA probes.

<http://www.cytocell.co.uk>

The Cytocell website. This is a commercial supplier of DNA FISH probes

<http://www.biochem.roche.com>

The Roche Molecular Biochemicals website. This company is a supplier of antibody and labeling reagents.

<http://www.vysis.com>

The Vysis website. This company is a supplier of commercial DNA FISH probes.

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Multi-Color FISH Techniques

UNIT 22.5

Small structural chromosomal aberrations are often difficult to determine with certainty using conventional cytogenetic banding methods alone (UNIT 22.3). The problems that can typically arise in both clinical and cancer cytogenetics are the presence of structural chromosome aberrations with unidentifiable chromosomal regions, or very complex chromosomes (sometimes called marker chromosomes) in which no recognizable region appears to be present. Confirmation of the cytogenetic origins of such chromosomal aberrations can sometimes be obtained by the judicious application of locus-specific FISH analysis (UNIT 22.4) if the investigator has some general impression regarding a possible identity. However, such an approach is very subjective and risky and requires some knowledge of the specific loci and available probes likely to be involved in the aberration. A more systematic approach is to use whole-chromosomal paints in succession, until the marker chromosome and its constituents can be identified. While this strategy will eventually identify each chromosomal region involved, it is both costly and time-consuming and may lead to the depletion of valuable patient samples. Recently, some generalized screening FISH techniques utilizing sensitive and differentially labeled chromosome-specific paints (also see UNIT 22.4) have been developed, and these allow the full chromosome complement to be analyzed to identify unknown aberrations. In this unit, the commonly available methods will be described with suggested protocols and approaches to troubleshooting.

There are currently two to three slightly different imaging systems available that utilize the mechanical rotation of fluorescence excitation filters to distinguish the distinct fluorescence of a mixture of chromosomal paints during image acquisition. Such filter-based systems are generically termed multicolor FISH (M-FISH; Speicher et al., 1996; each supplier has their own modified acronym for this technique). The second more frequently used system, called spectral karyotyping (SKY), utilizes image analysis based on Fourier transformation to spectrally analyze the differential fluorescence of each chromosomal paint (Schrock et al., 1996). Both the SKY and M-FISH methods require the use of human whole-chromosomal paints that are differentially labeled, so that each chromosome emits a unique combination of colors following hybridization, this color combination is used for identification purposes. Each method can be performed as a laboratory procedure and is capable of identifying the cytogenetic origins of all chromosomes in the complement in one image acquisition step (as in SKY) or through sequential imaging with specific filters (M-FISH).

The protocols in this unit employ the basic FISH techniques already discussed in UNIT 22.4. However, since multi-chromosome full-painting probe cocktails are different for specific assays, it is prudent to discuss the labeling strategy and denaturation, hybridization, and post-hybridization steps in detail. The probe labeling protocol is similar to those discussed in UNIT 22.4, where normally, genomic DNA from cloned sources serves as the template. However, the technique described for SKY and M-FISH requires multiple rounds of amplification. The in situ hybridization steps, whether for SKY or M-FISH, are very similar, differing only in the post-hybridization washes and specific reagents. The critical difference between the two assays is the method of image acquisition and analysis. All of the critical parameters of slide making, probe labeling, and washing stringency apply to SKY and M-FISH (see UNIT 22.4).

This unit includes protocols for labeling SKY and M-FISH probes (see Basic Protocol 1) and for multi-color FISH (see Basic Protocol 2 and Alternate Protocol 1), including

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multi-color FISH of previously G-banded slides (see Alternate Protocol 2) and using locus-specific probes with previously SKY or M-FISH hybridized slides (see Alternate Protocol 3).

Multi-color FISH assays can be easily completed if the reagents are purchased commercially. If the investigator plans to produce the probes and antibody mixtures in-house, many more experiments and quality-control experiments will be required.

LABELING WHOLE-CHROMOSOME DNA PROBES FOR MULTI-COLOR FISH ASSAYS

This labeling protocol has been kindly provided by the laboratory of Dr. Thomas Ried; it outlines the general labeling strategy for in-house production of whole-chromosome paints to be used in SKY and M-FISH experiments. Some modifications may be required for optimization. The labeling protocol is divided into three parts: (1) primary amplification of flow-sorted chromosomes, (2) secondary amplification of the degenerate oligonucleotide primed (DOP) PCR-amplified primary product, and (3) labeling of the secondary amplification product with fluorochromes or haptens. Each labeling experiment involves the use of DOP primers as described by Telenius et al. (1992). The labeling protocol below includes tables of the human fluorochrome/hapten combinations currently used by Applied Spectral Imaging for use with the SKY system, the labeling combinations used by Vysis for use with the SpectraView System, and the combinations used by Applied Spectral Imaging for a Mouse SKYPaint (see Tables 22.5.3, 22.5.5, and 22.5.6). These labeling combinations can be used to custom produce probes for use with any standard FISH imaging system, provided that the proper filters are available on the microscope and there is a means of identifying and analyzing custom multi-color FISH experiments. Custom experiments may involve all the chromosomes, a subset of them, or specific bands along one chromosome (MBand). MBand, which differentially labels major chromosomal bands along the length of a chromosome is offered by Metasystems but is not covered in this unit.

Materials

- Template DNA (flow-sorted chromosomes in a volume of 32.0 μ l; Gray et al., 1986)
- 5 \times buffer D (accompanying 10 mM dNTP from Invitrogen)
- 10 mM dNTP nucleotide mix (see recipe)
- 5 μ M 5'-CCGACTCGAGNNNNNNATGTGG-3' primer
- 15 U/ μ l Super *Taq* polymerase (CPG)
- 5 \times loading buffer (see recipe)
- 2% (w/v) agarose gel with ethidium bromide (see recipe)
- 5 U/ μ l *AmpliTaq* polymerase (Perkin Elmer)
- 10 \times PCR buffer
- 25 mM MgCl₂
- 2 mM dNTP for secondary amplification (see recipe)
- 2 mM dNTP for labeling (see recipe)
- 100 μ M Rhodamine 110 (Perkin Elmer)
- 1 mM FITC
- 1 mM Texas Red (see recipe for *x*-dUTPs)
- 50 mM biotin (Boehringer Mannheim)
- 50 mM digoxigenin (Boehringer Mannheim)
- 1 mM Spectrum green (see recipe for *x*-dUTPs)
- 1 mM Spectrum orange (see recipe for *x*-dUTPs)
- 1 mM Spectrum aqua (see recipe for *x*-dUTPs)
- 1 mM Spectrum gold (see recipe for *x*-dUTPs)

1 mM Spectrum far red (see recipe for *x*-dUTPs)
 1 mg/ml human Cot-1 DNA (GIBCO)
 10.0 mg/ml sonicated salmon sperm DNA (Invitrogen)
 3 M sodium acetate (APPENDIX 2A)
 100% and 70% ethanol
 Hybridization buffer (see recipe)

PCR tubes
 PCR thermal cycler
 Gel system and power source
 0.5- and 2-ml microcentrifuge tubes
 Refrigerated centrifuge

NOTE: Use aseptic technique throughout. Be sure to change pipet tips for each reagent and before addition to each tube.

DOP-PCR amplify flow-sorted chromosomes

1. Vortex flow-sorted chromosomes in PCR tubes well in order to detach chromosomes from the inner walls. Microcentrifuge for ≥ 3 min (~ 32.0 μ l volume) at maximum speed, or 200 to 300 $\times g$, room temperature.

This will be the tube to which the PCR mix will be added. The total volume will be 50 μ l.

2. Combine the following PCR mixture for each sample (make sure to change tips for each reagent):

10.0 μ l 5 \times buffer D
 4.0 μ l 10 mM dNTP
 1.5 μ l 5 μ M 5'-CCGACTCGAGNNNNNNATGTGG-3' primer
 2.0 μ l ddH₂O
 0.25 μ l 15 U/ μ l Super *Taq* polymerase.

3. Lightly vortex each tube and microcentrifuge 30 sec at maximum speed.
4. Immediately transfer tubes to PCR thermal cycler and run PCR with the following parameters:

1 cycle:	10 min	93°C
4 cycles:	1 min	94°C
	1.5 min	30°C
	3 min	30°–70°C
	3 min	72°C
34 cycles:	1 min	94°C
	1 min	62°C
	3 min, add 1 sec/cycle	72°C
1 cycle:	10 min	72°C (final extension)
		4°C (hold).

5. When the program is complete, mix a 2- μ l aliquot with 1 μ l 5 \times loading buffer from each reaction and run on a 2% agarose gel to determine the efficiency of the initial amplification reaction.

The resulting smear migrates at ~ 500 bp. The efficiency of the amplification can be assessed by comparing the intensity of the resulting smear with concentration standards by gel electrophoresis or by standard spectrophotometry. The amplified product may be stored indefinitely at -20°C .

Table 22.5.1 Master Mix for Secondary Amplification

Reagent	Volume per reaction (μl)	Total for 13 reactions (μl)	Total for 12 reactions (μl)
Sterile ddH ₂ O	65	845	780
10 \times PCR buffer	10	130	120
25 mM MgCl ₂	8	104	96
2 mM dNTP	10	130	120
5 μM primer (for 13 reactions)	4	52	48
AmpliTaq DNA polymerase	1	13	12

Perform secondary amplification of product

- Label twenty-five 0.5-ml tubes with chromosome number and date (1 to Y and a control tube without DNA).

Secondary amplification using DOP-PCR is used to amplify the primary product above. Future labeling experiments will start from this step.

- Thaw template DNA at 37°C if previously frozen, vortex, and centrifuge briefly in a microfuge prior to dispensing the DNA into aliquots.
- Pipet 2 μl of the amplified flow-sorted chromosomal DNA into each PCR tube and place closed PCR tubes temporarily at 4°C.
- Since there are a total of 25 reactions (22 autosomes, 2 sex chromosomes, and a negative control), prepare the master mix in two 2-ml microcentrifuge tubes according to Table 22.5.1. Vortex the two tubes, centrifuge, and place on ice.

One tube will contain enough master mix for 13 reactions and the other will contain enough for 12 reactions.

- Take out the AmpliTaq polymerase, mix carefully (tap with finger), centrifuge, and then add an appropriate amount to each master mix. Vortex the master mix tubes, centrifuge, and put back on ice.
- Place PCR tubes containing DNA in order and open each tube by handling only the outside of the tube. Pipet 96 μl of master mix into each tube (change tips with each tube) and place on ice.
- Vortex each tube, centrifuge briefly, and place each into the thermal cycler. Amplify using the following amplification program:

29 cycles:	1 min	94°C
	1 min	56°C
	3 min, add 1 sec/cycle	72°C
1 cycle:	10 min	72°C (final extension)
		4°C (hold).

- After completion, remove the tubes from the PCR machine, vortex, centrifuge, and place on ice.
- Run a 2- μl aliquot with 1 μl 5 \times loading buffer from each reaction on a 2% agarose gel.

The resulting smear migrates at \sim 500 bp. The efficiency of the amplification can be assessed by comparing the intensity of the resulting smear with concentration standards by gel electrophoresis or by standard spectrophotometry. The amplified product may be stored indefinitely at -20°C .

Label amplified products

15. Label PCR tubes according to labeling scheme corresponding to the software/hardware system used (i.e., ASI or Vysis). Remember to include a zero control for each color (master mix with no DNA sample).

Commercial probes for SKY and M-FISH are available from ASI and Vysis, however, some laboratories may prefer to produce their own probes to use with their system of choice. This labeling protocol can also be used to produce multi-color custom probe cocktails, whether they be whole-chromosome paints, or band-, locus-, or centromere-specific probes, the investigator simply substitutes the template DNA as required.

The critical difference between the two probe cocktails lies in the fact that SKY probes are both directly and indirectly labeled, thus requiring antibody detection, whereas the M-FISH probe cocktail is entirely directly labeled and does not require antibody detection.

Contact ASI for any changes in probe cocktail combinations as this will affect the reference library, which has been integrated into the SKY software. Furthermore, should the investigator choose to alter the probe combination, the investigator should make sure that the reference library is concomitantly altered to reflect the changes made in the probe. Contact ASI or refer to the user manual for instructions on how to change the combinatorial table (referred to as a .ctb file).

16. Thaw template DNA at 37°C, vortex, and centrifuge briefly. Dispense a 4- μ l aliquot of the appropriate DNA into each PCR tube, seal lid, and temporarily place at 4°C.
- 17a. *For SKY probes:* Label five 2-ml microcentrifuge tubes A through E and make the master mix for each fluorochrome according to Table 22.5.2 and in the following order: ddH₂O, 10 \times PCR buffer, 25 mM MgCl₂, 2 mM dNTP, and 100 μ M primer.

Table 22.5.3 lists the fluorochrome combinations for specific chromosomes.

- 17b. *For M-FISH probes:* Label five 2-ml microcentrifuge tubes A through E and make the master mix for each fluorochrome according to Table 22.5.4 and in the following order: ddH₂O, 10 \times PCR buffer, 25 mM MgCl₂, 2 mM dNTP, and 100 μ M primer.

Table 22.5.5 lists the fluorochrome combinations for specific chromosomes.

18. Dilute the Texas Red and spectrum-dUTPs 1:5 in sterile water immediately before use (1 mM final). Vortex the labeled fluor-dUTPs, centrifuge briefly, add each to the appropriate master mix, and place back on ice.

Table 22.5.2 SKY Master Mix

Reagent	Rhodamine (A)	Texas Red (B)	Biotin/Cy5 (C)	FITC (D)	Dig(Cy5.5) (E)
10 \times PCR buffer	120 μ l	110 μ l	130 μ l	140 μ l	120 μ l
25 mM MgCl ₂	96 μ l	88 μ l	104 μ l	112 μ l	96 μ l
2 mM dNTPs	60 μ l	55 μ l	65 μ l	70 μ l	60 μ l
Sterile ddH ₂ O	756 μ l	693 μ l	819 μ l	872 μ l	756 μ l
100 mM primer	58 μ l	44 μ l	52 μ l	56 μ l	58 μ l
Ampli Taq polymerase	12 μ l	11 μ l	13 μ l	14 μ l	12 μ l
x-dUPT	60 μ l	65 μ l (of 1:5 dilution)	65 μ l	80 μ l (of 1:5 dilution)	60 μ l
Total reactions	12	11	13	14	12

Table 22.5.3 Human SKY Labeling Scheme

Chromosome	Labeling scheme ^a
1	BCD
2	E
3	ACDE
4	CD
5	ABDE
6	BCDE
7	BC
8	D
9	ADE
10	CE
11	ACD
12	BE
13	AD
14	B
15	ABC
16	BD
17	C
18	ABD
19	AC
20	A
21	DE
22	ABCE
X	AE
Y	CDE

^aA, rhodamine; B, Texas Red; C, biotin/Cy5; D, FITC; E, digoxigenin/Cy5.5.

Table 22.5.4 M-FISH Master Mix

Reagent	Spectrum FarRed (A)	Spectrum Aqua (B)	Spectrum Green (C)	Spectrum Gold (D)	Spectrum Red (E)
10× PCR buffer	110 μl	100 μl	110 μl	110 μl	120 μl
25 mM MgCl ₂	88 μl	80 μl	88 μl	88 μl	96 μl
2 mM dNTPs	55 μl	50 μl	55 μl	55 μl	60 μl
Sterile ddH ₂ O	693 μl	630 μl	693 μl	693 μl	751 μl
100 μM primer	44 μl	40 μl	44 μl	44 μl	48 μl
Ampli Taq polymerase	11 μl	10 μl	11 μl	11 μl	12 μl
<i>x</i> -dUPT (1:5 dilution)	65 μl	60 μl	65 μl	65 μl	70 μl
Total reactions	11	10	11	11	12

Table 22.5.5 Human M-FISH Labeling Scheme^a

Chromosome	Labeling scheme ^b
1	D
2	E
3	B
4	CE
5	AD
6	C
7	A
8	AE
9	DE
10	ABD
11	BE
12	CD
13	BC
14	CDE
15	BDE
16	AC
17	ACE
18	ADE
19	ACD
20	ABE
21	BCD
22	BCE
X	AB
Y	BD

^aLike the SKY probes, contact Vysis for the latest probe combinations. Ensure that in-house probe matches combinatorial libraries established by the SpectraView software.

^bA, Spectrum Far Red; B, Spectrum Aqua; C, Spectrum Green; D, Spectrum Gold; E, Spectrum Red.

19. Mix the *AmpliTaq* enzyme carefully (tap with finger), centrifuge briefly, then add appropriate amounts to each master mix. Vortex the master mix tubes, centrifuge, and put back on ice.
20. Put labeled PCR tubes with aliquoted DNA in fluorochrome order (all As in one row, all Bs in the next, and so on).
21. Carefully open each tube in row A by handling only the outside of the tube. Vortex the master mix for spectrum-dUTPs, pipet 96 μ l into each tube (change tips with each tube) and put on ice. Repeat the same procedure for each remaining *x*-dUTP. Vortex each tube, centrifuge briefly, and put on ice again.

Table 22.5.6 Mouse SKY Labeling Scheme

Chromosome	Labeling scheme ^a
1	ACD
2	CE
3	AE
4	ABD
5	AC
6	BE
7	BD
8	AB
9	E
10	C
11	BC
12	CDE
13	D
14	ACE
15	AD
16	A
17	BCD
18	B
19	DE
X	ABC
Y	ADE

^aA, rhodamine; B, Texas Red; C, biotin/Cy5 antibodies (see recipe); D, FITC; E, digoxigenin/Cy5.5.

22. Start the thermal cycler, arrange the tubes in the PCR machine, close lid, and start the program using the following parameters:

29 cycles:	1 min	94°C
	1 min	56°C
	3 min, add 1 sec/cycle	72°C
1 cycle:	10 min	72°C (final extension)
	hold	4°C.

Analyze labeling results

23. After completion, remove the tubes from the thermal cycler, vortex, centrifuge, and place on ice.
24. Analyze a 2- μ l aliquot from each reaction on a 2% agarose gel.

The resulting smear migrates at ~500 bp.

At this point, the custom probe cocktail is generated according to the labeling scheme. The chromosome paint for each chromosome will be added together for each of the 22 autosomes and 2 sex chromosomes according to Table 22.5.3 or 22.5.5, or according to Table 22.5.6 for mouse chromosome probes.

The total number of differently labeled DNAs for the ASI labeling scheme is 57, while the total number of labeled DNAs for the Vysis labeling scheme is 52.

Purify probe

25. To produce the SKY cocktail, add 3 μl of labeled, amplified product according to the labeling scheme into a 1.5-ml microcentrifuge tube. Add 20 μl of 1 mg/ml Cot-1 DNA and 1 μl of 10.0 mg/ml salmon sperm DNA.

This precipitation protocol outlines the amount of probe required for one slide to be hybridized. It is suggested that sufficient probe for five or ten assays should be precipitated together and stored as aliquots.

26. Add 1/10 vol of 3 M sodium acetate, 2.5 vol of 100% ethanol, mix well, and store overnight at -20°C or 1 hr at -80°C .
27. Centrifuge 20 min at $200\text{--}300 \times g$ (13,000 to 14,000 rpm), 4°C . Wash the pellet with cold 70% ethanol and repeat centrifugation. Dry the pellet either by air drying or vacuum.
28. Dissolve pellet in 15 to 20 μl hybridization buffer at room temperature.

If the probe does not readily dissolve back into solution, add more hybridization buffer.

29. Store the probe in aliquots sufficient for five to ten slide hybridizations at -20°C for long-term storage (>2 months), or at 4°C for short-term use (1 to 2 months).

IN SITU HYBRIDIZATION FOR SPECTRAL KARYOTYPING (SKY)

The following outlines the SKY protocol, which is simply FISH (UNIT 22.4) with different multi-color probes. The metaphase preparation is pre-treated with pepsin, formalin-fixed, and denatured. The prepared probe is denatured, allowed to pre-anneal and added to the denatured slide. After 48 hr, the slides are washed, detected with primary and secondary antibodies, counterstained, and visualized.

Materials

Metaphase slides (UNIT 22.2)

0.01 M HCl (see recipe), prewarmed to 37°C

10% (w/v) pepsin (see recipe)

1 \times PBS

1% formaldehyde/1 \times PBS/50 mM MgCl_2 (see recipe)

70% formamide/2 \times SSC (see recipe), prewarmed to 72°C

70%, 80%, and 100% ethanol

SKY kit (Applied Spectral Imaging) containing probe cocktail, block reagents, detection reagents, and counterstain *or* in-house custom-made probes in hybridization solution (see Basic Protocol 1)

Rubber cement

50% formamide/2 \times SSC, 45°C (3 Coplin jars)

1 \times SSC (see recipe), 45°C (3 Coplin jars)

0.1% Tween-20/4 \times SSC (see recipe), 45°C (7 Coplin jars)

Blocking solution (see recipe)

Avidin-Cy5 antibodies (see recipe for antibodies)

Mouse anti-digoxigenin (see recipe for antibodies)

Cy5.5 anti-mouse antibodies (see recipe for antibodies)

DAPI/antifade counterstain (see recipe)

Clear nail polish (do not use if planning to re-probe slide)

Phase-contrast microscope

37°C oven

Fume hood

22 \times 22-mm coverslips

BASIC PROTOCOL 2

Hybridization container
Fluorescent microscope and associated Applied Spectral Imaging Acquisition hardware and software (Applied Spectral Imaging)

Prepare slide

1. Using a phase-contrast microscope, determine the extent of cytoplasmic residue on the metaphase slide preparation (UNIT 22.2).
2. To 50 ml of prewarmed 0.01 M HCl, add 10 to 15 μ l of 10% pepsin. Allow the slide to incubate for 5 to 10 min at 37°C.
3. Wash the slide in 1 \times PBS for 5 min at room temperature. Then, wash in 1 \times PBS/50 mM MgCl₂ for 5 min at room temperature.
4. Incubate the slide in 1% formaldehyde/1 \times PBS/50 mM MgCl₂ for 10 min at room temperature in a well-ventilated area or fume hood.

Prepare the probe

- 5a. *For commercial probes:* From the commercially available SKY kit, pipet 10 μ l of probe cocktail (vial 1) for each slide to be hybridized. Heat denature the probe for 10 min at 72°C, then place 1 hr at 37°C.

This is sufficient probe to cover a 22 \times 22-mm area.

- 5b. *For in-house probes:* Heat denature prepared probe (labeled probe in hybridization buffer, see Basic Protocol 1) for 10 min at 72°C, then place 1 hr at 37°C.
6. Wash the slide in 1 \times PBS for 5 min at room temperature.
7. Pass slide through an ethanol dehydration series: 70%, 80%, and 100% ethanol for 5 min each. Allow slide to air dry after the final ethanol wash.
8. Denature the slide in 70% formamide/2 \times SSC for 1.5 to 2 min at 72°C.

The time required for denaturing will vary according to the age and quality of slide.

9. Promptly place the slide into 70% ethanol following denaturation and proceed through the dehydration series as in step 7.

Hybridize and wash slides

10. Add 10 μ l of the preannealed probe to the denatured slide. Apply a 22 \times 22-mm coverslip and seal with rubber cement.
11. Transfer the slides to a hybridization container that has been slightly dampened with a wet paper towel or gauze and place at 37°C.
12. After 48 hr, carefully peel rubber cement from slide and immerse in the first Coplin jar of 50% formamide/2 \times SSC at 45°C. Allow the coverslip to fall off and let stand for 5 min.
13. Remove slide and transfer to second Coplin jar of 50% formamide/2 \times SSC for 5 min. Repeat with third Coplin jar of 50% formamide/2 \times SSC.
14. Wash the slide three times in three separate Coplin jars of 1 \times SSC, 5 min each wash, at 45°C.
15. Briefly wash the slide in 0.1% Tween-20/4 \times SSC at 45°C.

Block and detect slides

- 16a. *For commercial probes:* Drain excess solution, but do not allow slide to dry. Add 80 μ l of blocking reagent (vial 2 from the SKY kit) to the slide. Apply coverslip and place back in hybridization container for 40 min at 37°C.

- 17a. Remove coverslip and add 80 μl of detection reagent (vial 3 from SKY kit) to the slide. Apply coverslip and place back in hybridization container for 40 min at 37°C.
- 16b. *For in-house probes:* Drain excess solution, but do not allow slide to dry. Add 80 μl of blocking solution, apply coverslip, and place back in hybridization container for 40 min at 37°C.
- 17b. Add 40 μl each of Avidin-Cy5 antibodies and mouse anti-digoxigenin antibodies. Apply coverslip and place back in hybridization container for 40 min at 37°C.
18. Remove coverslip and wash slide in three times in three separate Coplin jars of 0.1% Tween-20/4 \times SSC, 5 min each wash, at 45°C with gentle agitation.
- 19a. *For commercial probes:* Drain excess solution, but do not allow slide to dry. Add 80 μl detection reagent (vial 4 from the SKY kit) to the slides. Apply coverslip and place back in hybridization container for 40 min at 37°C.
- 19b. *For in-house probes:* Drain excess solution, but do not allow slide to dry. Add 80 μl of diluted Cy5.5 anti-mouse antibodies. Apply coverslip and place back in hybridization container for 40 min at 37°C.
20. Remove coverslip and wash slide three times in three separate Coplin jars of 0.1% Tween-20/4 \times SSC, 5 min each wash, at 45°C with gentle agitation.

Counterstain and visualize

21. Drain excess solution, but do not allow slide to dry. Add 40 μl of DAPI/antifade counterstain (vial 5 from SKY kit or from another source). Apply coverslip and seal with clear nail polish. However, do not seal with nail polish if there are plans to re-probe the slide (see Alternate Protocols 1, 2, and 3).

Slides may be stored at -20°C and visualized for up to 6 months with sustained signal strength.

22. Acquire and analyze metaphase preparation using SKY labeling scheme with the hardware and software provided by Applied Spectral Imaging (<http://www.spectral-imaging.com/>).

See Figure 22.5.1 for a diagram of signal acquisition and Figure 22.5.2 for typical results.

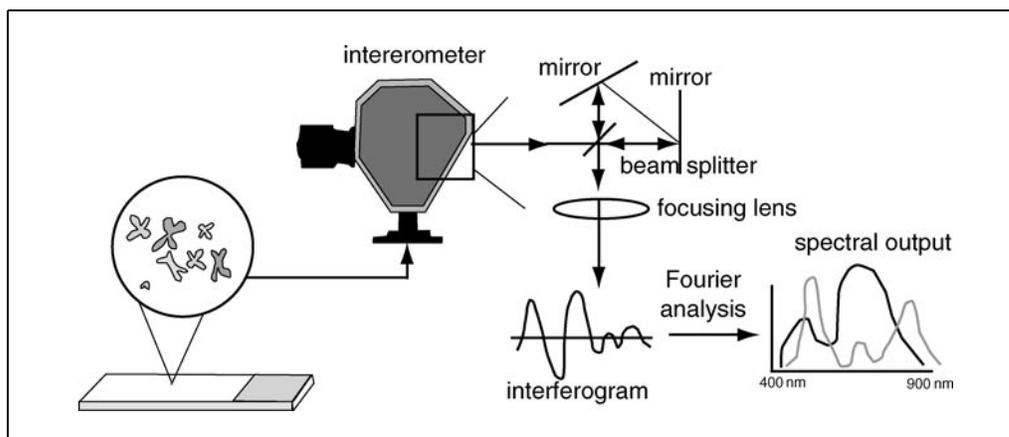


Figure 22.5.1 Schematic representation of spectral karyotyping image acquisition and image analysis. The metaphase, following hybridization with the SKYPaints is visualized by fluorescence microscopy. The light passes into the optical head containing the interferometer where 100 different images are taken. This information is Fourier transformed and processed using the SKYView software for analysis. (Adapted from the ASI manual.)

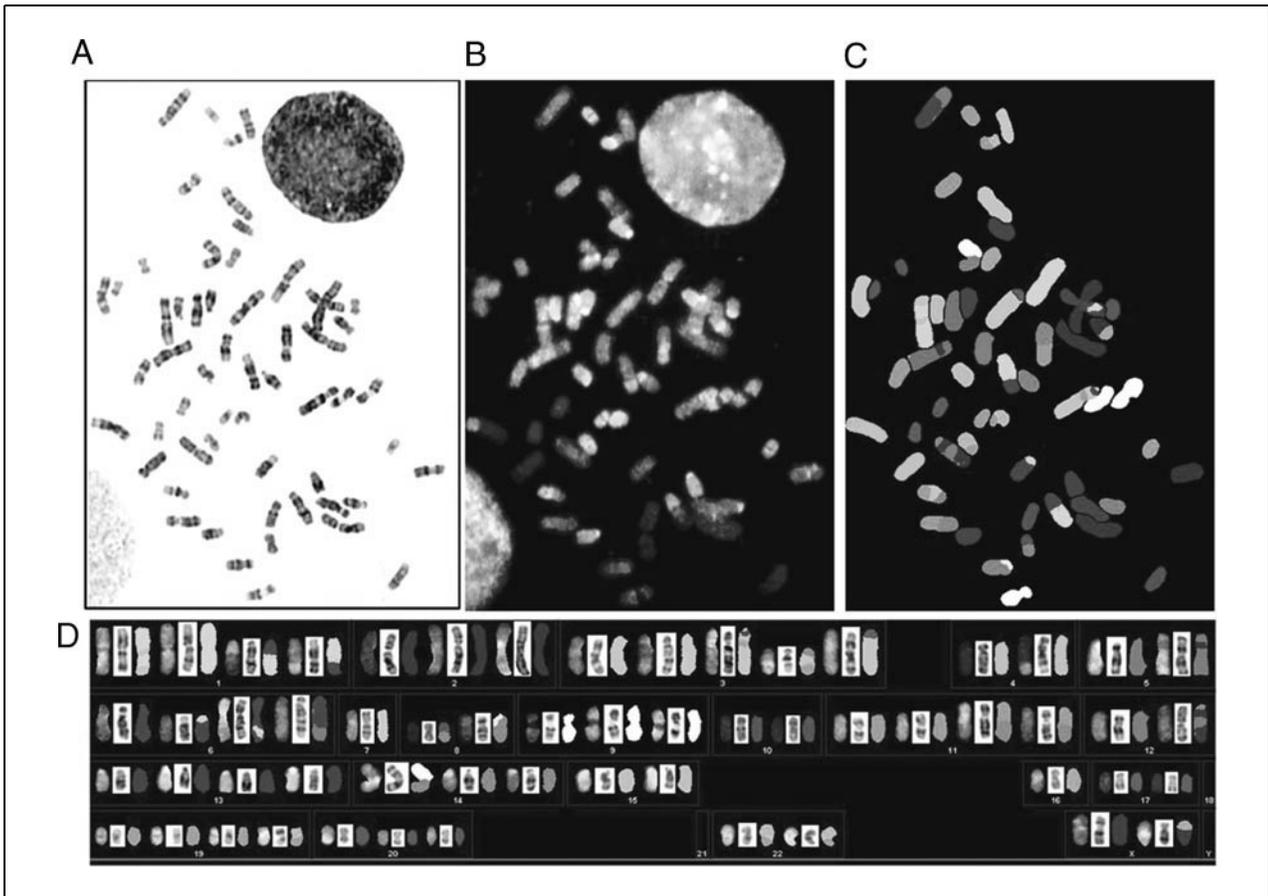


Figure 22.5.2 SKY analysis of a primary ovarian carcinoma. (A) Typical representations of SKY analysis. An inverted DAPI image reveals banding patterns similar to G-banding. (B) Red-Green-Blue (RGB) image of the hybridized spread as it would appear with the probe colors. (C) Classified or pseudo-colored image to help identify chromosomal aberrations not readily evident in the RGB image. Each chromosome is identified by a specific pseudo-color. A change in color along the length of a chromosome indicates a different chromosome. (D) SKY karyotype showing each representation of the chromosomes: RGB, inverted DAPI, and classified images. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to <http://www.currentprotocols.com/colorfigures>.*

**ALTERNATE
PROTOCOL 1**

IN SITU HYBRIDIZATION FOR M-FISH KARYOTYPING

The M-FISH protocol is very similar to the SKY protocol with one critical difference. Like SKY, the metaphase preparation is pretreated with pepsin, formalin-fixed, and denatured. The prepared probe is denatured, allowed to pre-anneal, and added to the denatured slide. After 48 hr, the slides are washed, counterstained, and ready for visualization. Because M-FISH probes are labeled using directly labeled dUTPs, there is no need for antibody detection.

Materials

- Chromosome spreads (UNIT 22.2)
- 70%, 80%, and 100% ethanol
- RNase A working solution (see recipe)
- 2× SSC (see recipe)
- 10% pepsin stock (see recipe)
- 0.01 M HCl (see recipe), 37°C
- 1× PBS (2 coplin jars)
- 1% formaldehyde/1× PBS/50 mM MgCl₂ (see recipe)
- 70% formamide/2× SSC (see recipe), 72°C
- Commercially available M-FISH probes (Vysis) *or* in-house custom probes

0.4× SSC/0.3% NP-40 (see recipe), 73°C
 2× SSC/0.1% NP-40 (see recipe)
 DAPI/antifade counterstain (DAPI III, Vysis or see recipe)
 50% formamide/2× SSC (see recipe), 45°C
 1× SSC (see recipe), 45°C
 0.1% Tween-20/4× SSC (see recipe), 45°C
 Clear nail polish (do not use if planning to re-probe slide)

Phase-contrast microscope
 Glass coverslips
 37°C dry oven or incubator
 Fume hood

Prepare slide

1. Prepare chromosome spreads as outlined in *UNIT 22.2*.
2. Under phase-contrast microscopy, determine the extent of cytoplasmic residue on the slide preparation.
3. Dehydrate the slide in a 70%, 80%, 100% ethanol series, 5 min each, and allow to air dry.
4. Apply 20 µl of the RNase A working solution to slide and coverslip. Incubate 30 min at 37°C.
5. Remove the coverslip and wash slide in 2× SSC for 5 min.

This and the following incubations should use solutions in Coplin jars.

6. Add 10 to 15 µl of 10% pepsin to 50 ml prewarmed 0.01 M HCl and treat slide for 5 min. Then wash the slide in 1× PBS for 5 min.
7. Incubate the slide in 1% formaldehyde/1× PBS/50 mM MgCl₂ for 10 min at room temperature in a well-ventilated area or fume hood.
8. Wash the slide in 1× PBS for 5 min at room temperature.
9. Pass the slide through a 70%, 80%, and 100% ethanol series, 5 min each, and allow the slide to air dry.

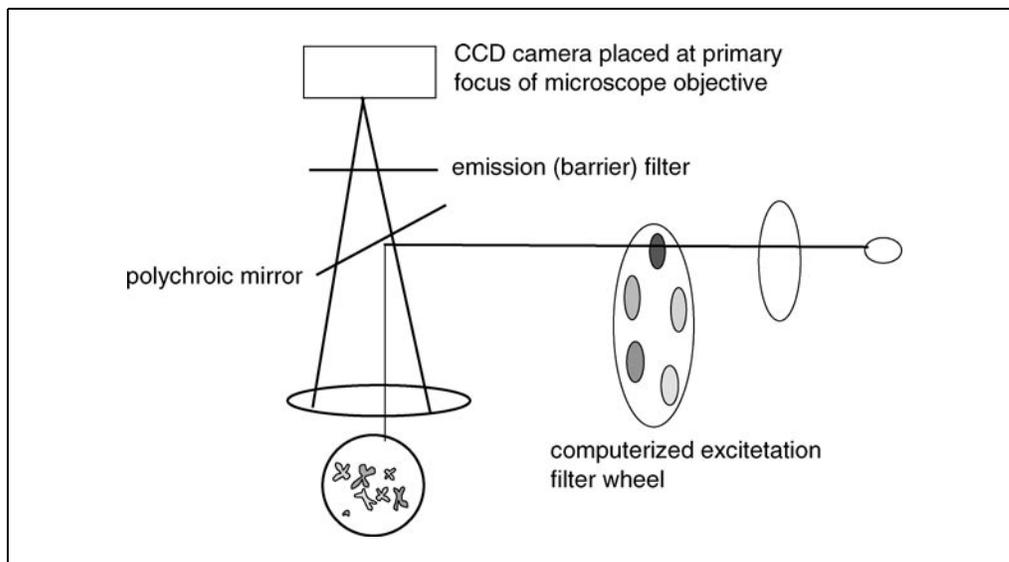


Figure 22.5.3 Schematic representation of M-FISH image acquisition and image analysis. M-FISH experiments involve the imaging of each dye or fluorochrome using a specific filter. The images are then stacked to produce the final merged image. (Adapted from the Vysis manual.)

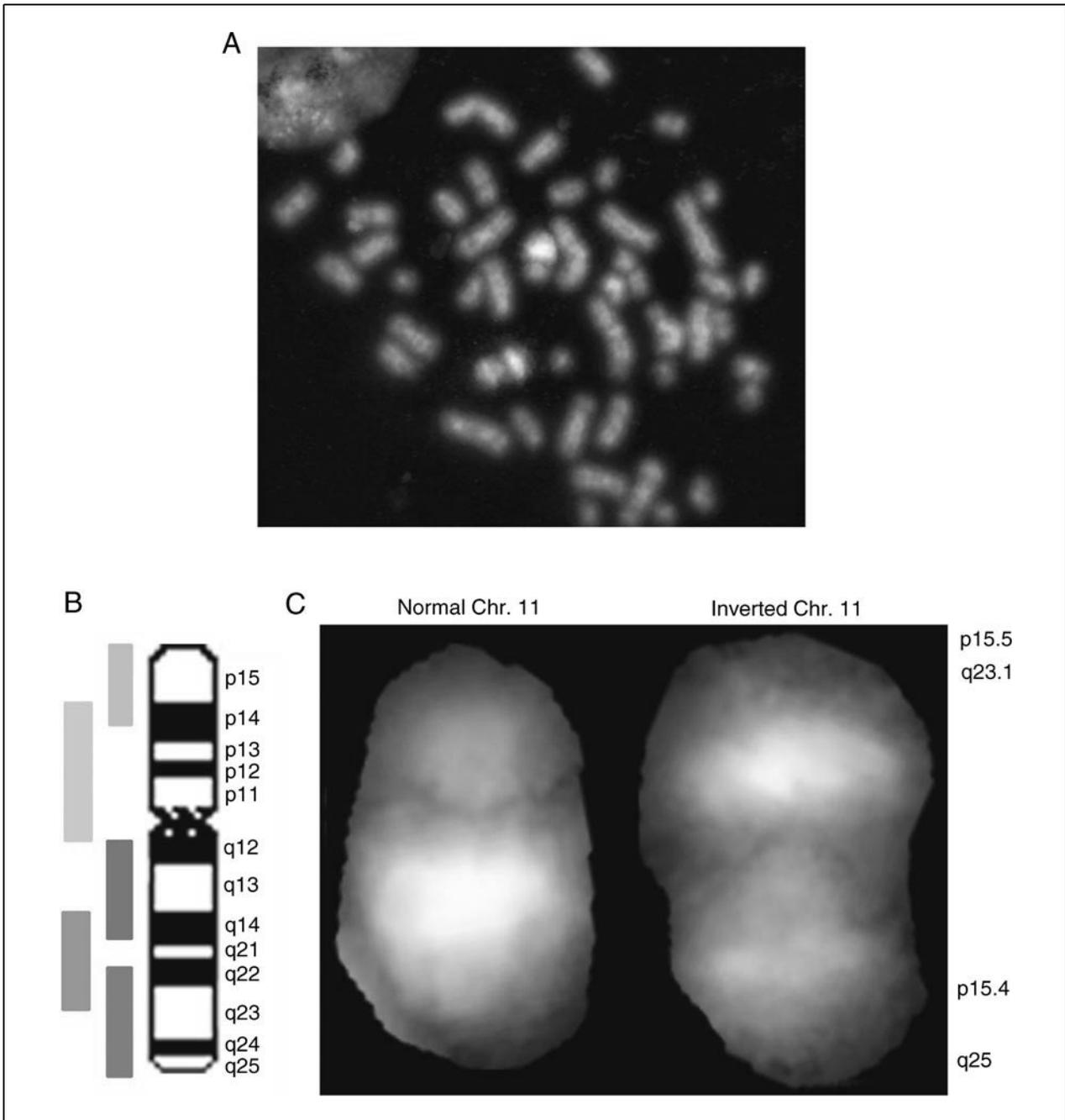


Figure 22.5.4 MBand using the 11Cytte probe and imaging system from Metasystems. **(A)** Metaphase spread from a patient possessing an inversion on chromosome 11, hybridized with the 11Cytte chromosome paint. Five different dyes are used to create the painting probe along the length of the chromosome in a specific order. **(B)** Ideogram of chromosome 11 showing the color order of the dyes along the length of the chromosome and the band regions they span. **(C)** The normal and inverted chromosome 11. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to <http://www.currentprotocols.com/colorfigures>.*

10. Denature the slide for 1.5 to 2 min in 70% formamide/2× SSC at 72°C.
11. Promptly place the slide into 70% ethanol following denaturation and proceed through the dehydration series as in step 9.
12. Remove the slide from the ethanol, add the recommended amount of denatured and pre-annealed commercial probe or 15 to 20 μl of in-house probe and hybridize for 48 hr at 37°C.

Vysis rapid wash

- 13a. Carefully remove the coverslip from the slide and immerse in $0.4\times$ SSC/0.3% NP-40 for 2 min at 73°C .
- 14a. Wash the slide in $2\times$ SSC/0.1% NP-40 for 1 min at room temperature.
- 15a. Wash the slide in $1\times$ PBS for 2 min at room temperature.
- 16a. Air dry the slide and stain with DAPI/antifade counterstain. Store slides at -20°C .
Slides can be stored for several months at -20°C .
- 17a. Visualize slide using a fluorescent microscope and the associated hardware and software provided by Vysis.

Traditional wash protocol (for in-house probes)

- 13b. After 48 hr, carefully peel off rubber cement from slide and immerse in the first Coplin jar of 50% formamide/ $2\times$ SSC at 45°C . Allow the coverslip to fall off and let stand for 5 min. Remove slide and transfer to second Coplin jar for 5 min. Repeat with third solution.
- 14b. Wash the slide three times in three separate Coplin jars of $1\times$ SSC, 5 min each wash, at 45°C .
- 15b. Remove coverslip and wash slide in three washes of 0.1% Tween-20/ $4\times$ SSC, 5 min each wash, at 45°C with gentle agitation.
- 16b. Drain excess solution, but do not allow slide to dry and add $40\ \mu\text{l}$ of DAPI antifade counterstain. Apply coverslip and seal with clear nail polish. However, do not seal with nail polish if there are plans to re-probe the slide (see Alternate Protocols 2, and 3).

The slides are now ready for visualization. When not in use, store slides at -20°C .

- 17b. Analyze the slide using fluorescence microscopy with the appropriate filters and imaging system capable of integrating multiple fluorochromes.

See Figure 22.5.3 for a diagram of signal acquisition and Figure 22.5.4 for typical results.

PRETREATMENT OF PREVIOUSLY G-BANDED SLIDES FOR SKY OR M-FISH

This procedure can be used when a previously G-banded slide is needed for subsequent SKY or M-FISH analysis. The G-banded slide must be cleaned of residual oils using xylene. It is then destained using methanol. See Table 22.5.7 for guidelines for handling G-banded slides.

Materials

- G-banded metaphase slides (*UNIT 22.3*)
- Xylene
- 100% methanol
- 70%, 80%, and 100% ethanol
- $1\times$ PBS
- $1\times$ PBS/50 mM MgCl_2
- 1% formaldehyde/ $1\times$ PBS/50 mM MgCl_2 (see recipe)
- 70% formamide/ $2\times$ SSC (see recipe), 72°C
- Fume hood

ALTERNATE PROTOCOL 2

Table 22.5.7 General Guide for Processing Cytogenetic Preparations for G-Banding Analysis Followed by SKY or M-FISH

Banding steps	Slide 3 days to 1 month	Slide >1 month	In situ slide preparation 3 days to 1 month	In situ slide preparation >1 month
Trypsin (working solution)	10–20 sec with agitation	20–30 sec with agitation	40–60 sec with agitation	60–90 sec with agitation
Stain	50 sec	1+ min	50 sec	1+ min
Water	Brief rinse	Brief rinse	Brief rinse	Brief rinse
Water	Brief rinse	Brief rinse	Brief rinse	Brief rinse
Denaturing time	20–30 sec	45 sec–1 min	30–45 sec	1 min-?

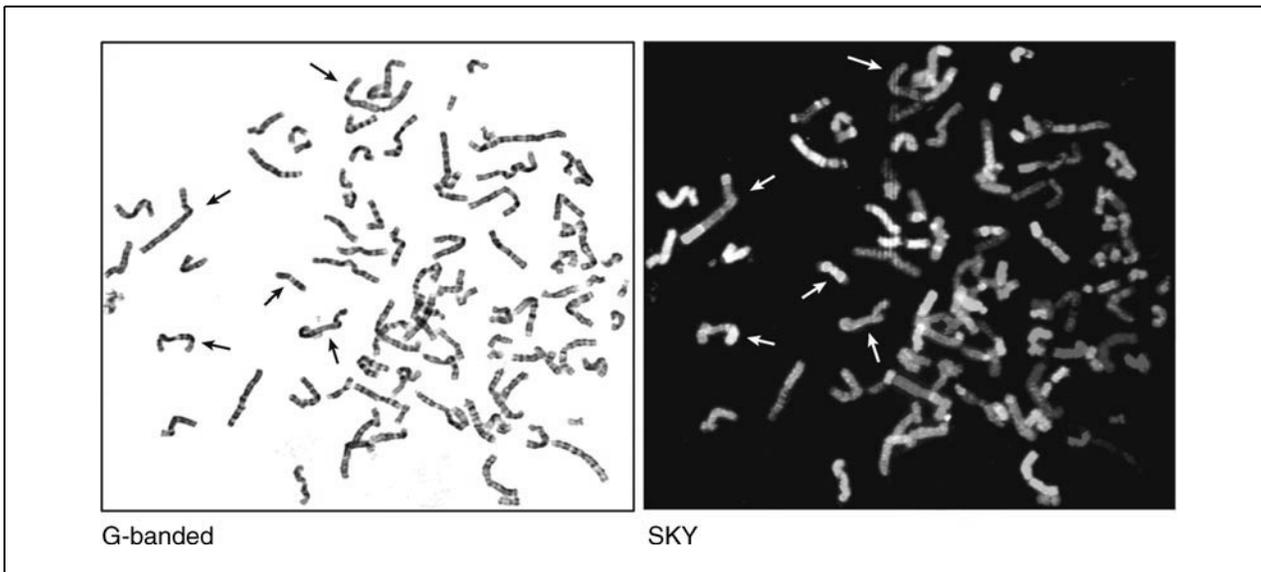


Figure 22.5.5 The results of sequential G-banding (A) and SKY (B) analysis of a rhabdomyosarcoma cell line. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to <http://www.currentprotocols.com/colorfigures>.*

Destain slide

1. For previously G-banded slide, wash in xylene for 5 min.
2. Destain with 100% methanol for 10 min.
3. Pass the slide through a 70%, 80%, and 100% ethanol series, 5 min each wash, and allow the slide to air dry after the final ethanol wash.
4. Rehydrate in 1× PBS for 5 min.

Denature slide

5. Incubate the slide in 1% formaldehyde/1× PBS/50 mM MgCl₂ for 10 min at room temperature in a well-ventilated area or fume hood.
6. Wash the slide in 1× PBS for 5 min at room temperature.
7. Pass the slide through a 70%, 80%, 100% ethanol dehydration series, 5 min each wash, and allow the slide to air dry after the final ethanol wash.

- Denature the slide in 70% formamide/2× SSC for 20 to 30 sec at 72°C.
- Promptly place the slide into 70% ethanol following denaturation and proceed through the dehydration series as in step 6.

Hybridize slide

- Proceed with probe denaturation and pre-annealing as outlined in the SKY (see Basic Protocol 2, step 6) and hybridization to slide (see Basic Protocol 2, step 11) or M-FISH protocols described above (see Alternate Protocol 1, step 12).
- Proceed with the respective protocol (SKY or M-FISH) following the appropriate post-hybridization detection and washing procedures (see Basic Protocol 2 or Alternate Protocol 1).

See Figure 22.5.5 for an example of SKY hybridization of previously G-banded chromosomes.

FISH ANALYSIS USING LOCUS-SPECIFIC OR CHROMOSOME PAINTING PROBES FOLLOWING SKY/M-FISH HYBRIDIZATION

ALTERNATE PROTOCOL 3

This protocol can be used in conjunction with previously G-banded slides as well as SKY/M-FISH-hybridized slides. In some cases, investigators may wish to know the status of a specific DNA probe within the context of the SKY or M-FISH analysis that has been ascertained, thus it is particularly useful to be able to sequentially use locus-specific or chromosome paint probes on the same metaphase spread that was analyzed using SKY or M-FISH. The hybridized slides are washed free of mounting medium and dehydrated. The slides are redenatured and hybridized with the locus-specific or chromosome paint probe of interest. After hybridization, the slides are washed, the probe is detected with antibody (if needed), counterstained, and visualized.

Materials

Hybridized SKY or M-FISH slides (see Basic Protocol 2 or Alternate Protocol 1, respectively)

2× SSC (see recipe)

0.1% Tween-20/4× SSC (see recipe)

70%, 90%, and 100% ethanol

70% formamide/2× SSC (see recipe), 75°C

Labeled DNA probe (as prepared in *UNIT 22.4* or from a commercial supplier)

Rubber cement

Coverslips

Hybridization box

37°C dry oven or incubator

- For SKY or M-FISH hybridized slide, record the microscope coordinates of the metaphase spreads of interest.
- Carefully remove the coverslip and wash slide in 2× SSC for 5 to 10 min at room temperature. Gently agitate.
- Transfer slide to 0.1% Tween-20/4× SSC and wash for 5 min at room temperature with gentle agitation.
- Dehydrate the slide in a 70%, 90%, and 100% ethanol dehydration series, 5 min each wash. After the final ethanol (100%) wash, allow the slide to air dry.
- Denature the probe at 75°C and pre-anneal as needed or follow the manufacturer's instructions.

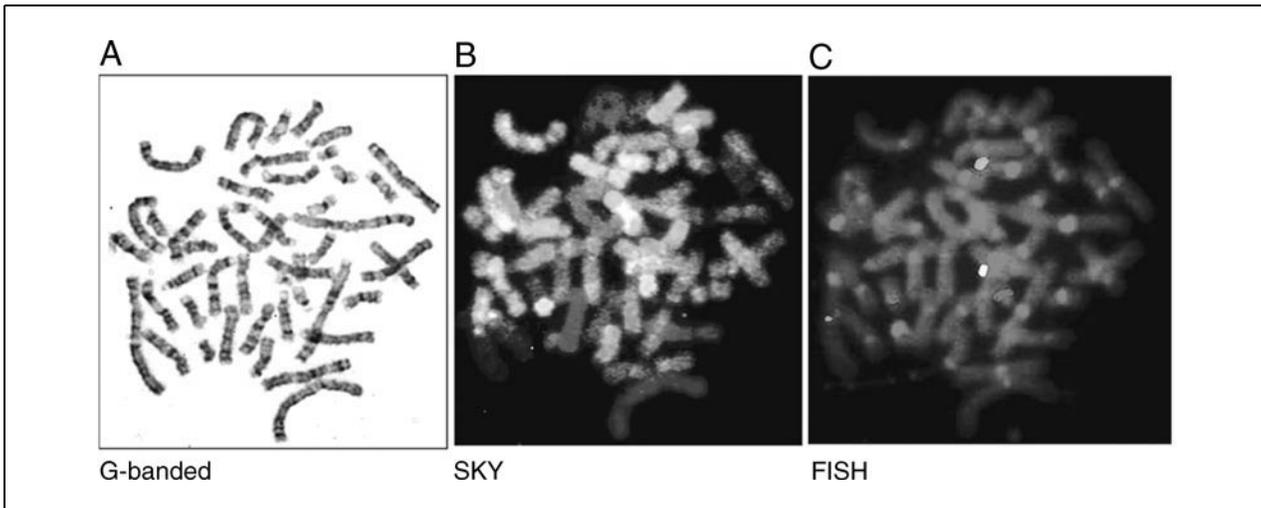


Figure 22.5.6 The results of sequential G-banding (**A**), SKY (**B**), and locus-specific FISH (**C**) for the BCR-ABL translocation in a CML sample. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to <http://www.currentprotocols.com/colorfigures>.*

6. Denature the slide in 70% formamide/2× SSC for 2 min at 75°C (for previously G-banded/SKY or M-FISH slides, denature for 1 min).
7. Immediately place the slide in 70% ethanol and continue with the dehydration series as in step 4.
8. Add the appropriate amount of the denatured probe to the denatured slide. Apply coverslip and ring with rubber cement. Hybridize at 37°C overnight.
9. Follow the necessary post-hybridization washes and detections as required (see *UNIT 22.4*)

See Figure 22.5.6 for typical results.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Agarose gel, 2% (w/v)

Dissolve 2 g of agarose into 100 ml of 1× TBE buffer (*APPENDIX 2A*) by warming the solution, e.g., in a microwave oven. After the agarose has been dissolved, add 5 ml of ethidium bromide. Pour into electrophoresis tray with combs and allow to solidify at room temperature.

Antibodies

Avidin-Cy5 antibodies (Amersham Bioscience): Reconstitute antibodies using water according to the manufacturer's instructions. Dilute 1:200 in blocking solution (see recipe).

Mouse anti-digoxigenin (Sigma): Reconstitute antibodies using water according to the manufacturer's instructions. Dilute 1:500 in blocking solution (see recipe).

Cy5.5 anti-mouse antibodies (Amersham Bioscience): Reconstitute antibodies using water according to the manufacturer's instructions. Dilute 1:200 in blocking solution (see recipe).

Store antibodies in 100- μ l aliquots up to 1 year at -20°C . Aliquots of working solutions may be stored for 2 to 3 months at 4°C protected from the light.

Blocking solution

1.0 g BSA (Sigma; final 1% w/v)
100.0 μ l Tween-20 (Sigma; final 0.1% v/v)
20.0 μ l 20 \times SSC (final 4 \times)
80.0 μ l H₂O
Store indefinitely at -20°C

DAPI/antifade medium

Dissolve the entire powder of DAPI (4',6-diamidino-2-phenylindole; Sigma) in water to a 100 $\mu\text{g/ml}$ stock solution. Store in 500- μ l aliquots up to 1 year at -20°C .

To combine DAPI in antifade medium, combine the following in order:

5.0 ml PBS
500.0 μ l 100 $\mu\text{g/ml}$ DAPI stock (final 1 $\mu\text{g/ml}$)
0.5 g *p*-phenylenediamine (Sigma; final 10 mg/ml)
Dissolve well then add
45.0 ml glycerol (final 90% v/v)

The resulting medium is very viscous. Transfer to a 50-ml Falcon tube and place on a rotator to ensure proper mixing (30 min). This product is light sensitive, so wrap the tube with foil. Store in 1-ml aliquots 1 year at -20°C . New medium must be made when an increasing amber tint appears.

This is also commercially available from Vectashield (Vector Laboratories).

CAUTION: DAPI is a potential carcinogen and should be handled with caution.

dNTP (dATP, dTTP, dGTP, dCTP) for secondary amplification, 2 mM

10.0 μ l 100 mM dATP (final 2 mM)
10.0 μ l 100 mM dCTP (final 2 mM)
10.0 μ l 100 mM dTTP (final 2 mM)
10.0 μ l 100 mM dGTP (final 2 mM)
460.0 μ l sterile H₂O (final 2 mM)
Store up to 1 year at -20°C .

dNTP (dATP, dGTP, dCTP, 1.5 mM dTTP) for labeling, 2 mM

10.0 μ l 100 mM dATP (final 2 mM)
10.0 μ l 100 mM dCTP (final 2 mM)
10.0 μ l 100 mM dGTP (final 2 mM)
7.5 μ l 100 mM dTTP (final 1.5 mM)
460.0 μ l sterile H₂O (final 2 mM)
Store up to 1 year at -20°C .

dNTP (dATP, dTTP, dGTP, dCTP), 10 mM

10.0 μ l 100 mM dATP (final 10 mM)
10.0 μ l 100 mM dCTP (final 10 mM)
10.0 μ l 100 mM dTTP (final 10 mM)
10.0 μ l 100 mM dGTP (final 10 mM)
60.0 μ l sterile H₂O (final 10 mM)
Store up to 1 year at -20°C .

x-dUTPs

Dilute 1 mM Texas Red 1:5 in sterile H₂O (13 ml Texas Red + 52 ml H₂O)
Dilute 1 mM spectrum red 1:5 in sterile H₂O (14 ml spectrum red + 56 ml H₂O)
Dilute 1 mM spectrum far red 1:5 in sterile H₂O (14 ml spectrum far red + 56 ml H₂O)
Dilute 1 mM spectrum aqua 1:5 in sterile H₂O (14 ml spectrum aqua + 56 ml H₂O)
Dilute 1 mM spectrum green 1:5 in sterile H₂O (14 ml spectrum green + 56 ml H₂O)
Dilute 1 mM spectrum gold 1:5 in sterile H₂O (14 ml spectrum green + 56 ml H₂O)
Store up to 1 year at –20°C

1% formalin/PBS/MgCl₂

2.7 ml 37% formaldehyde (Sigma; final 1% v/v)
100.0 ml 1× PBS/MgCl₂ (see recipe)
Make fresh

Discard in accordance with the regulations of the institution.

70% formamide/2× SSC

35.0 ml formamide (Invitrogen; final 70% v/v)
5.0 ml 20× SSC (final 2×)
10.0 ml H₂O
Make fresh

CAUTION: Formamide is a carcinogen and should be handled with caution. It should be discarded according to biohazard rules of the institution.

HCl, 0.01 M

0.5 ml 1 M HCl (final 0.01 M)
49.5 ml H₂O
Store at room temperature until ready for use

Hybridization buffer

500.0 μl high-grade formamide (final 50% v/v)
100.0 μl 20× SSC (final 2×)
100.0 μl dextran sulfate (final 10% v/v)
300.0 μl H₂O

Alternatively, this solution may be purchased from DAKO.

Loading dye, 5×

0.125 g bromophenol blue (final 0.25% w/v)
15.0 ml glycerol (final 30% v/v)
35.0 ml H₂O
Store for several months to 1 year at room temperature or 4°C

1× PBS/MgCl₂

950.0 ml 1× PBS (APPENDIX 2A)
50.0 ml 1M MgCl₂ (final 50 mM)
Store at room temperature until ready for use

Pepsin stock, 10%

100 mg pepsin powder (Sigma; final 10% w/v)
1.0 ml H₂O
Store in 20-μl aliquots up to 1 year at –20°C

RNase A stock solution

10.0 mg RNase A (DNase-free; final 10 mg/ml)

1.0 ml 2× SSC

Dispense into 10- μ l aliquots and store at -20°C

To make an RNase A working solution:

10.0 μ l 10 mg/ml RNase A stock solution

990.0 μ l 2× SSC

Vortex to mix. Do not freeze-thaw. Discard after use.

20× SSC

175.3 g NaCl

88.2 g sodium citrate

Adjust pH with 10 M NaOH to pH 7

Adjust volume to 1 liter with H₂O

Store for several months at room temperature

0.4× SSC/0.3% NP-40 solution

20.0 ml 20× SSC (final 0.4×)

950.0 ml H₂O

3.0 ml NP-40 (final 0.3% v/v)

Add up to 1 liter with H₂O

Store up to 6 months at room temperature

Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

2× SSC/0.1% NP-40 solution

100.0 ml 20× SSC (final 2×)

850.0 ml H₂O

1.0 ml NP-40 (final 0.1% v/v)

Add up to 1 liter with H₂O

Store up to 6 months at room temperature

Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

0.1% Tween-20/4× SSC

1.0 ml Tween-20 (Sigma; final 0.1% v/v)

200.0 ml 20× SSC (final 4×)

799.0 ml H₂O

Store at room temperature until ready for use

COMMENTARY

Background Information

Probe labeling

See Commentary on probe labeling in UNIT 22.4.

SKY

SKY is a combination of optical microscopy, high-resolution imaging, and the measurement of spectral emissions by Fourier spectroscopy (Malik et al., 1996; Fig. 22.5.1).

It is an interferometer-based method of image acquisition and analysis and has specific requirements for its correct operation. The ability for SKY to properly identify chromosomal segments relies on the proper calibration of the spectral reference library. Upon installation, the optical head is carefully aligned with the digital camera. Slides hybridized with each pure dye are then imaged to create the spectral reference library for that particular system

and microscope. Since SKY analysis is based on the recognition of distinct spectral signatures, a less than optimal signal intensity can still generate a coherent chromosome classification, provided that there are no shifts in the spectra. Furthermore, should shifts in the spectra occur, it is possible to create a new spectral library based on the spectral signatures of hybridized slides. The advantage of SKY acquisition is the use of only the SKYCube to detect all the dyes and a short exposure using the DAPI filter for inverted banding images, rather than the lengthier exposure of the slide to five different filters as with M-FISH. The SKY software provides the capability to manually identify the spectral properties of each pixel along the length of a chromosomal region and compare this to the reference library. Such analyses can be very helpful in determining the identity of chromosomes where identity is inconclusive.

The commercial SKY probes are derived from flow-sorted chromosomes that are amplified and labeled using DOP-PCR (Telenus et al., 1992). A 24-chromosome probe cocktail is generated by the combinations of five pure dyes—rhodamine, FITC, Cy5, Cy5.5, and Texas Red. This allows 2^{n-1} or 31 combinations. Thus, each chromosome has a unique spectral signature generated by the specific combination of the five pure dyes. The generation of a spectral image is achieved by acquiring ~100 frames of the same image that differ from each other only by their optical path differences. Once a spectral image is acquired, the SKYVIEW software compares the acquired spectral image against the combinatorial library containing the fluorochrome combinations for each chromosome to generate a classified image. The classified image pseudocolors the chromosomes to aid in the delineation of specific structural aberrations where the RGB (red-green-blue) display images (Figure 22.5.2), which displays the fluorescent colors of the chromosomes, may appear quite similar. For every chromosomal region, identity is determined by measuring the spectral emission at that point. Regions where sites for rearrangement or translocation between different chromosomes occur, are visualized by a change in the display color at the point of transition.

M-FISH

All other M-FISH methods rely on the use of fluorochrome-specific filters moved sequentially during the image capturing process (Speicher et al., 1996; Fig. 22.5.3). The identity

of each chromosome or chromosome region is based on which combination of the five fluorochromes hybridizes together in one location. As with SKY, the unique identification dye combinations are stored in a reference library within the software to which the test specimen is compared and aberrations are delineated in the same manner as described for SKY. Filter-based systems are more sensitive to the influences of high background and weak signal strength. Weak signal intensity or high background can misclassify a chromosome identity since the system may not consider the weak signal as true, thus choosing the next best chromosome filter pattern for identification. SKY, on the other hand, overcomes this obstacle since the fluorochrome spectral signature is the method of determining chromosome identity. Although a signal may be weak, its spectral output will remain the same. Nevertheless, both systems are widely used. Research laboratories are inclined to use SKY analysis, while clinical cytogenetic laboratories rely on M-FISH, since they already tend to have the filters and cytogenetic/FISH systems.

M-FISH probes are generated in the same manner as the SKY probes, differing only by their dye combinations. Commercial M-FISH probes can be purchased from Vysis as SpectraVision probes. The fluorochromes used for this probe set includes Spectrum Far Red, Spectrum Red, Spectrum Gold, Spectrum Green, and Spectrum Aqua used in a combinatorial fashion. Metasystems also provides M-FISH probes in their 24Xcyte kit. Metasystems has generated band-specific probes (M BAND) along the length of a given chromosome. Since each chromosome has a specific five-color FISH assay, each chromosome analysis must be performed individually (Fig. 22.5.4). Therefore, this assay is particularly useful when a specific rearrangement has been identified by banding, SKY, or MFISH, and the investigator wishes to further examine the rearranged region in detail to delineate the breakpoint or determine whether a more complex inversion or deletion is also present.

Locus- or band-specific M-FISH

Clearly the use of M-FISH-based techniques has increased the amount of cytogenetic data that can be derived from a given specimen. The integration of locus-specific or band-specific FISH permits further analysis regarding specific breakpoint locations and the status of specific genes or chromosomal loci. The ability to sequentially carry out traditional

banding analysis, SKY, or M-FISH, and locus-specific FISH on the same metaphase spread eliminates the question of clonality by identifying aberrations with increasing specificity, thereby identifying unique aberrations of common aberrations.

Critical Parameters and Troubleshooting

The investigator is referred to *UNIT 22.4* for general troubleshooting issues associated with FISH experiments. However, the investigator should also consider the following factors.

The use of commercially available probes significantly reduces the preparatory work and problems associated with quality control. If the laboratory chooses to produce custom probes, careful planning and control experiments should be carried out to ensure that each chromosome probe has been efficiently labeled.

Both SKY and M-FISH involve the hybridization of whole chromosome paints, thus the risk of background is high. Slide age and pre-treatment are critical to minimizing background. Similarly, if antibodies are being used, they should be diluted to the proper concentration and maintained according to the manufacturer's instructions. The microscope and imaging software should be well maintained, and the user should be fully trained. Although SKY and M-FISH reveal the same information, the methods by which these data are generated are quite different.

SKY

Criteria for successful image acquisition and analysis should be provided by the companies supplying the microscope, imaging equipment, and analytical software. As mentioned previously, all parameters that influence basic FISH analysis will apply to multi-color FISH assays, regardless of the analysis format.

The overall signal intensity along the length of the chromosome should be high with minimal background. The acquisition times, which are usually automatically set by the software, should be relatively short, as longer exposure times will amplify signals arising from background and increase the contribution of electronic noise associated with all forms of digital imaging. Similarly, bright spots that arise as a result of insufficient antibody or probe washing can skew automatic exposure times. This results in a low exposure of the metaphase of interest and increases the risk of ambiguous or erroneous chromosomal assignments.

Interpretation of the analysis relies both on the familiarity of the investigator with the basic technicalities of FISH assays, chromosomal identification by banding methods, and an understanding of fluorescence microscopy. One of the greatest concerns in the analysis is distinguishing between true translocation events and some of the preparation artifacts that may be misinterpreted during M-FISH and SKY. Regions of repetitive sequences such as centromeric areas and telomeric chromosomal ends can often give false classifications due to the differential suppression of those regions either with unlabeled Cot-1 DNA or by the labeled probe. Through careful analysis of normal metaphase spreads and confirmation by locus-specific FISH, real translocations can be distinguished from artifacts. Another phenomenon that contributes to misclassification is the sandwich effect where the junction between a true chromosomal translocation produces an intermediate color. This intermediate color could be identified by the software as another chromosomal segment giving the false impression of a tiny insertion at the site of translocation. Since 24-color painting analysis relies on the combination of up to five different fluorochromes, it is implicit that the fluorescence of two different chromosomal junction regions may in some circumstances be misclassified as a third chromosome undergoing insertion and bearing the spectra or dye combinations of the two partner chromosomes that are actually undergoing a simple rearrangement. This phenomenon can also be seen in chromosomes that are overlapping. The region of overlap will produce a color pattern contributed by both chromosomes, and the software will identify the fluorescence at the region of overlap as that which most closely matches the reference library. In trying to determine whether a translocation is real or not, it is advisable to look for longer chromosomes for analysis in another metaphase. If the insertion is no longer present, then the misclassification is likely to be due to suboptimal hybridization and/or the sandwich effect. Obviously, it is necessary to confirm any uncertain rearrangement of interest using single whole chromosome paint or locus-specific FISH assays on another slide from the same preparation or by re-hybridizing the slide after elution of chromosomal paints.

M-FISH

As discussed for SKY, criteria for successful image acquisition and analysis should be provided by the companies supplying

the microscope, imaging equipment, and analytical software. However, some general points should be considered regardless of which method of image acquisition and analysis is used. The overall signal intensity along the length of the chromosome should be high with minimal background. This is more critical for filter-based systems because the analysis is based on the presence or absence of that fluorochrome. Any background or bleed-over into another filter can influence the outcome of the analysis. The acquisition times, which are usually automatically set by the software, should be relatively short, as longer exposure times will amplify signals arising from background and increase the contribution of electronic noise associated with all forms of digital imaging. Similarly, bright spots that arise as a result of insufficient antibody or probe washing can skew automatic exposure times. This results in a low exposure of the metaphase of interest and increases the risk of ambiguous or erroneous chromosomal assignments.

M-FISH is also subject to the sandwich affect. Caution should be taken when analyzing small insertions. Be sure that the sandwiched chromosome is not simply the mixture of the flanking chromosomal fluorescence. Analyzing longer chromosomes will help to alleviate this problem.

M-FISH imaging relies on the sequential acquisition of images through specific filters and detecting the combination and the presence/absence of the reference dyes hybridized to the metaphase spreads. This entails the acquisition of five different images that are then merged to form the final image. One of the most critical factors is the use of the proper filter sets designed specifically for the M-FISH probe cocktail being used. Careful consideration must be taken with respect to pixel shifts from one image to the next. Careful attention must also be paid to the order in which the images are taken because it is known that some fluorochromes/dyes emit a different wavelength upon degradation or exposure to UV. Such interaction between two fluorochromes could cause misclassification should, for example, a red fluorescing dye that degrades to a yellow emission after exposure to UV, be imaged before the yellow probe is exposed. Because the slides are exposed to different filters, there will be varying exposure times for each dye, and this may also differentially quench the signals from the other fluorochromes/dyes later in the exposure sequence. Investigators are urged to refer to the manufacturer's instructions for both

reagents and hardware/software for optimal results.

Like the SKY software, most M-FISH software packages allow the user to identify the fluorochromes that are present along the chromosome of interest and compare it to the combinatorial library. In the event of weak signals or small inconclusive regions of rearrangement, this function is very convenient.

Locus- or band-specific M-FISH

The use of SKY or M-FISH in combination with other cytogenetic or FISH methods provides more detailed information but also increases the damage to the DNA target. As with any FISH experiment, particularly with previously banded slides, the quality of the metaphase spreads, the age and pretreatment (for banding and SKY/M-FISH) influence the outcome for locus-specific FISH following these two assays. In many cases, the overall signal intensity of the locus-specific FISH probe will be weaker following banding and SKY/M-FISH. This is due to the fact that the target DNA has undergone a series of digestions and denaturations, thus compromising the quality of the DNA for hybridization. The overall background intensity will also increase since the slide was previously probed with a whole genome-painting probe. The denaturation of the slide prior to hybridization with the locus-specific probe should remove any bound probe and antibodies from the chromosomes, however, this is never complete. This is certainly the case if there is residual cytoplasm and a high density of cells on the slide. An indication that the DNA has been severely damaged is if it fails to take up the counterstain, such that the chromosomes appear a very faint blue and the centromeres are very bright.

Anticipated Results

In the best conditions, a given metaphase spread will classify perfectly.

If the DNA is not damaged, the resulting FISH should provide signals that can be clearly visualized by microscopy. These signals can be enhanced using imaging tools. For examples, see Figures 22.5.2, 22.5.4, 22.5.5, and 22.5.6.

Time Considerations

The time required to complete analysis depends on the complexity of the karyotype.

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