Isolation of Endoplasmic Reticulum, Mitochondria, and Mitochondria-Associated Membrane Fractions from Transfected Cells and from Human Cytomegalovirus-Infected Primary Fibroblasts

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ABSTRACT

Increasingly mechanistic virology studies require dependable and sensitive methods for isolating purified organelles containing functional cellular sub-domains. The mitochondrial network is, in part, closely apposed to the endoplasmic reticulum (ER). The mitochondria-associated membrane (MAM) fraction provides direct physical contact between the ER and mitochondria. Characterization of the dual localization and trafficking of human cytomegalovirus (HCMV) UL37 proteins required establishing protocols in which the ER and mitochondria could be reliably separated. Because of its documented role in lipid and ceramide transfer from the ER to mitochondria, a method to purify MAM from infected cells was also developed. Two robust procedures were developed to efficiently isolate mitochondria, ER, and MAM fractions while providing the substantial protein yields from HCMV-infected primary fibroblasts and from transfected HeLa cells. Moreover, this unit includes a protocol that allows visualization of the mitochondria network disruption that occurs in permissively infected cells by their optimal resolution in Percoll gradients. *Curr. Protoc. Cell Biol.* 37:3.27.1-3.27.23. © 2007 by John Wiley & Sons, Inc.

Keywords: subcellular fractionation • human fibroblasts • ER • mitochondria • MAM • HCMV • protein localization • sucrose gradient • Percoll gradient • differential centrifugation

INTRODUCTION

Fractionation, the mechanical separation and purification of subcellular compartments, is an invaluable tool for studying protein localization, trafficking, processing, and functions. Subcellular fractionation is also useful for the concentration of relatively low-abundance proteins, isolation of enzymatic complexes, or proteomic identification of organelle components. Increasingly mechanistic analyses of cellular biology and virology require dependable and sensitive methods for isolating purified organelles from which functional subcellular domains, such as translocation complexes, lipid rafts, and viral envelopment sites, can be studied. Moreover, functional contacts between distinct organelles have been identified and fractionated. For example, the contact sites between endoplasmic reticulum (ER) and mitochondria are being characterized as sites for exchange of calcium (Rizzuto et al., 1998) and lipids (Stone and Vance, 2000) between these organelles. In all, ~5% to 20% of the mitochondrial network surface within a cell is in close apposition to the

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ER (Rizzuto et al., 1998). The mitochondria-associated membrane (MAM) fraction, a subdomain of the ER, which consists of membrane tubules that provide direct physical contact between the ER and mitochondria, has been fractionated to high purity (Vance, 1990). Importantly, the purified MAM fraction is enriched in lipid synthetic enzymes, which produce phosphatidylserine and ceramide, and transport these products from the ER into mitochondria (Stone and Vance, 2000; Ardail et al., 2003; Bionda et al., 2004).

The authors' laboratory investigates human cytomegalovirus (HCMV) UL37 proteins, which dually target the ER and mitochondria of transfected and of HCMV-infected human foreskin fibroblasts (HFFs; Al-Barazi and Colberg-Poley, 1996; Goldmacher et al., 1999; Colberg-Poley et al., 2000; Mavinakere and Colberg-Poley, 2004a,b). The authors recently found that HCMV UL37 proteins traffic sequentially from the ER into the mitochondrial outer membrane (Mavinakere et al., 2006). Characterization of the subcellular localization and trafficking of HCMV UL37 proteins required establishing a protocol in which ER and mitochondria could be reliably separated from one another, despite their prevalent interconnection. Because of the documented role of MAM in lipid and ceramide transfer from the ER to mitochondria, the authors also sought to highly purify the MAM from transfected and from infected cells, adapting an established protocol from Vance (1990). The Vance protocol was originally used for the isolation of microsomes, mitochondria, and MAM from rat liver tissue (Vance, 1990). The following protocols were developed (see Basic Protocol 1) to maximally isolate mitochondria, ER, and MAM fractions while providing the best protein recoveries from HCMV-infected HFFs and from transfected, non-permissive HeLa cells. Basic Protocol 2 has proved especially valuable because it allows visualization of the mitochondria network disruption that occurs in permissively infected cells (McCormick et al., 2003) by their resolution in Percoll gradients.

BASIC PROTOCOL 1

DIFFERENTIAL SUCROSE GRADIENT ISOLATION OF ER AND MITOCHONDRIA

This protocol utilizes discontinuous sucrose gradients to band purified ER and mitochondrial organelles. Initially, cells are lysed mechanically with sonication and, then, a low-speed centrifugation (700 × *g*) is used to remove large cellular debris. Supernatant from this step is collected as a total lysed protein fraction. A subsequent 15,000 × *g* centrifugation crudely pellets mitochondria and separates it from ER and other organelles. The supernatant is loaded onto a three-layered sucrose gradient and purified ER is banded by centrifugation at 152,000 × *g*. Separately, the pellet is washed and loaded onto a two-layer sucrose gradient and purified mitochondria are banded using a 40,000 × *g* centrifugation. The high protein yields and considerable purity of banded organelles makes this fractionation of great utility for studies involving ER- or mitochondrial-resident proteins. The critical steps are shown in Figure 3.27.1.

Materials

Human foreskin fibroblasts (HFFs; Viromed SF cells)
HeLa cells (ATCC CCL-2)
HCMV (strain AD169 or desired strain) or DNA for transfection
2% and 10% (w/v) FBS
Lipofectamine 2000 (Invitrogen; *UNIT 20.6*)
Opti-MEM I (Invitrogen)
1.0, 1.3, 1.5, 1.7, and 2.0 M sucrose solutions (see recipes), sterile
1× PBS, pH 7.4 (*APPENDIX 2A*)
0.25% trypsin/EDTA
1× mannitol/Tris/EDTA (1× MTE) buffer (see recipe)
100 mM PMSF stock (see recipe)
Ultrapure water
70% ethanol

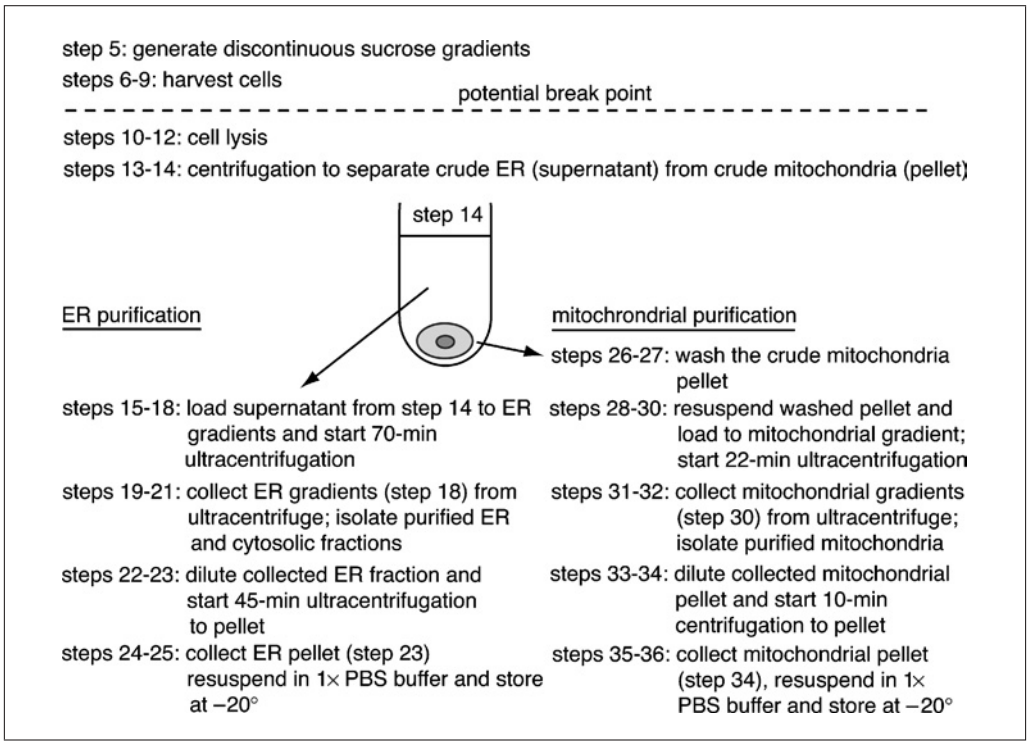

Figure 3.27.1 A flow chart for Basic Protocol 1 is shown. Basic Protocol 1, step 14, separates crude ER (supernatant) from crude mitochondria (pellet). Subsequent steps are grouped by the organelle which is to be purified for clarity and to provide a sense of continuity. To streamline the timing of the procedure and to reduce protein degradation, however, ER and mitochondrial purification steps should be carried out simultaneously.

175-cm^2 flasks (~0.8–2 × 10^7 cells/flask)
37°C incubator
Pre-sterilized (autoclaved) Beckman polyallomer centrifuge tubes: 14 × 89–mm (cat. no. 331372) or 11 × 60–mm (cat. no. 328874)
5-ml serological pipets
Aspirator
Sterile 15-ml conical tubes
Beckman GS-6R centrifuge with GH-3.8 swinging-bucket rotor
Analog sonicator with 1/8-in. microtip (Branson Ultrasonics model 250)
250-ml glass beakers
1.5-ml microcentrifuge tubes
14-ml polypropylene, round-bottom, snap-cap tubes (17 × 100–mm; Falcon cat. no. 2059)
Beckman J2-MI centrifuge with JA20.1 rotor
Beckman XL-90 ultracentrifuge with SW60 Ti and SW41 Ti rotors
1-ml syringes and 20-G needles
Parafilm
Beckman GS-15R centrifuge with F2402 rotor

Culture cells

1. For HCMV infection, seed two 175-cm^2 flasks of 60% to 70% density, actively growing primary HFFs. For transfection, seed two 175-cm^2 flasks of HeLa cells, 25% to 30% density and actively growing cells.

   For best results, use HFFs at or less than passage 18 as primary HFFs undergo senescence following 50 cell doublings or ~25 passages.

Transfer vital DNA to cells

To infect HFF cells with HCMV

2a. Twenty-four hr after seeding, infect permissive HFFs with HCMV (strain AD169 or desired strain), for 1 hr at 37°C in low-serum (2% FBS) medium, at a multiplicity of three plaque forming units (pfu) per cell (Mavinakere and Colberg-Poley, 2004a).

A cell density of ~80% the day of infection is best for maximal virus infectivity, as HCMV grows better in actively growing cells.

3a. After 1 hr, remove virus inocula by suction and overlay cells with complete medium (10% FBS). Proceed to step 4.

To transfect HeLa cells

2b. Transfect HeLa cells with Lipofectamine 2000 (*UNIT 20.6*) according to manufacturer's protocol. For each 175-cm^2 flask, use 20 μg DNA, 35 μl Lipofectamine 2000, and a total of 8.8 ml Opti-MEM I.

3b. Proceed to step 4.

A cell density of 80% to 90% is critical for maximal transfection efficiency using Lipofectamine 2000. If it is necessary to attain the correct cell density, incubate cultures for an extra day after seeding before transfecting.

Lipofectamine 2000 was found to give reliably higher transfection efficiencies in HeLa cells and HFFs than either Oligofectamine or Lipofectamine.

4. Incubate HCMV-infected cells according to the temporal class of examined viral protein. Allow transfected cells to incubate 24 hr at 37°C.

Depending on the abundance of the protein of interest or the kinetics of its movement through the cell, the incubation time may be adjusted to anywhere between 16 to 72 hr.

Prepare discontinuous sucrose gradients

5a. *For mitochondria gradients*. Dispense 1 ml of 1.7 M sucrose into a sterile 11 × 60–mm Beckman polyallomer ultracentrifuge tube. Mark the top of sucrose layer on the outside of the tube with an indelible felt-tip marker. Using a 5-ml serological pipet, carefully overlay with 1.6 ml of 1.0 M sucrose.

Hold the tube at a slight angle and slowly add top layer, to prevent sucrose layers from mixing.

5b. *For ER gradients*. Dispense 2 ml of 2.0 M sucrose to the bottom of a sterile 14 × 89–mm Beckman polyallomer ultracentrifuge tube. Using a 5-ml serological pipet, slowly layer 3 ml of 1.5 M sucrose onto the 2.0 M sucrose. Overlay with 3 ml of 1.3 M sucrose on top of the gradient.

The 14 × 89–mm polyallomer tubes used for ER gradients fit snugly into a polypropylene holder/dryer rack with 102 drying pins (made for 10- to 13-mm tubes).

Use the discontinuous sucrose gradients within 10 hr of preparation, for best results. The fine demarcation between sucrose layers may fade rapidly, but gradients remain stable at room temperature for several hours.

Harvest cells

6. Remove medium from cells by aspiration and wash the monolayers with 10 ml of 1× PBS, pH 7.4. Add 2 ml of 0.25% trypsin/EDTA and incubate 5 min at 37°C.

7. Inactivate the trypsin by adding 8 ml complete medium (10% FBS) to each flask and resuspend cells by vigorous pipetting. Transfer the resuspended cells from each flask into separate, sterile 15-ml conical tubes.

8. Pellet cells by centrifuging 5 min at 200 × *g* (1000 rpm in tabletop Beckman GS-6R centrifuge), 4°C. Aspirate the supernatant and resuspend the cell pellet in 10 ml of 1× PBS, pH 7.4.

9. Centrifuge cell suspension 5 min at 1400 × *g* (2500 rpm in tabletop Beckman GS-6R centrifuge), 4°C. Remove the supernatant by aspiration and store the cell pellet on ice (average pellet size is ~0.145 g).

   Cell pellets are stable on ice for up to 2 hr. Alternatively, the cell pellets can be frozen up to 1 month at –80°C at this stage and saved for later processing.

Lyse cells

10. Dispense 15 ml of 1× MTE and add fresh PMSF to a final concentration of 1 mM. Keep solution on ice. Add 2 ml of this solution to each 15-ml conical tube (step 9), and resuspend cell pellets.

11. Wash the sonicator microtip with ultrapure water and then with 70% ethanol. Blot dry with a Kimwipe before use. Set the sonicator for continuous pulse on a power setting of 3.5.

12. Place the 15-ml conical tube with resuspended cells into a 250-ml glass beaker partially filled with a slurry of ice and cold water. Submerge the cleaned, dry sonicator tip into the cell suspension to just above the bottom of the tube, avoiding touching the bottom. Sonicate the cell suspension on ice three times for 10 sec each, separated by 10-sec rest intervals. Keep the lysed cells on ice.

    Cell pellets from each flask should be kept separately up to this point to maximize the lysis efficiency. In step 14, however, combine the lysed cells from two 175-cm^2 flasks to maximize protein yields during fractionation.

    The 10-sec lysis condition had the best combination of high protein yields and fraction purity. If one wishes to sacrifice protein yield to obtain maximal purity of subcellular fractions, Basic Protocol 2 is recommended using homogenization lysis.

13. Centrifuge the lysed cells in 15-ml conical tubes 10 min at 1400 × *g* (2500 rpm in a tabletop Beckman GS-6R centrifuge), 4°C. Collect 100 µl of supernatant from each 15-ml conical tube, pool together duplicate samples into a single 1.5-ml microcentrifuge tube and label as "total protein." Store immediately at −20°C.

    Depending upon the cell lysis procedure, a pellet may not be seen at this step (corresponding to large cellular debris). A large pellet can be seen after gentle Dounce homogenization, while no pellet is usually visualized after long sonication times (see Table 3.27.1).

14. Decant remaining supernatant into a sterile, pre-chilled 14-ml polypropylene round-bottom snap-cap tube. Combine material from both 175-cm^2 flasks into a common chilled 14-ml tube. Centrifuge tubes without a snap-cap in the inner row of a JA20.1 rotor 10 min at 15,000 × *g*, 4°C.

    Use only polypropylene tubes for this procedure, as polystyrene tubes will crack and leak because of centrifugal stress.

    This centrifugation separates crude ER (supernatant) from crude mitochondria (pellet). Subsequent steps are grouped by the organelle which is to be purified for clarity and to provide a sense of continuity. To streamline the timing of the procedure and to reduce protein degradation, however, ER and mitochondrial purification steps should be carried out simultaneously.

Load ER gradient for ER purification

15. After centrifugation, transfer tubes to ice.

    There should be a large yellowish-brown pellet containing mitochondrial proteins.

Table 3.27.1 Cellular Markers for Verification and Assessment of ER, Mitochondria, and MAM Subcellular Fractions

Subcellular compartment	Marker	Antibody	Vendor	Reference
ER (microsomes)	DPM1	I-20	Santa Cruz Biotechnology cat. no. sc-15836	Maeda et al., 1998
	Calreticulin		Affinity Bioreagents cat. no. PA3-900	Johnson et al., 2001; Gelebart et al., 2005
Mitochondria	Grp75		Stressgen cat. no. SPS-825	Manning-Krieg et al., 1991
	COXII	K-20	Santa Cruz Biotechnology cat. no. sc-23984	Scheffler, 2001
MAM	mEFGP-huPPS-1	B-2 (anti-GFP)	Santa Cruz Biotechnology cat. no. sc-9996	
	FACL4		Abgent cat. no. AP2536b	Simmen et al., 2005

16. Using a micropipet, carefully withdraw 1.7 ml of the supernatant (containing crude ER) and layer it onto the top of the ER sucrose gradient, creating a new layer. Pipet slowly to keep from mixing the top layer of sucrose with the sample. Decant and discard any excess supernatant remaining in the 14-ml tubes containing the protein pellet, and then return the pellets to ice.
17. Transfer an additional 1.3 ml of ice-cold 1× MTE plus PMSF onto the top of the ER gradient.
18. Ultracentrifuge ER gradients 70 min at 152,000 × *g* (35,000 rpm in an SW41 rotor), 4°C. Set acceleration and deceleration profiles to 1 (transition speed of 170 rpm for 2 min).

    It is important to add the extra 1× MTE plus PMSF buffer on top of the samples to keep the polyallomer tubes from collapsing during ultracentrifugation.

Isolate ER fractions

19. Collect the ER gradient tubes from the ultracentrifuge. Withdraw the upper 1 ml of solution from the tube, using a micropipet, and transfer into a sterile 1.5-ml microcentrifuge tube. Label as "cytosol" and store immediately at −20°C.
20. Extract 0.4 to 0.6 ml volume of the large band at the interface of the 1.3 M sucrose gradient layer, using a 20-G needle and 1-ml syringe (Fig. 3.27.2).

    The samples can be stored for 1 to 2 months at –20°C prior to their use for western analysis.

21. Remove the needle from the syringe, and transfer the extracted band to a sterile 11 × 60–mm Beckman polyallomer tube. Add an additional 3.6 to 3.8 ml of ice-cold 1× MTE plus PMSF buffer to dilute out the sucrose.

    It is important to remove the needle from the syringe before dispensing liquid into the new ultracentrifuge tube, to avoid shear forces that would damage the sample.

22. Cover the top of the tube with Parafilm and mix by inversion until the suspension looks homogeneous (until the highly viscous sucrose swirling within the tube is no longer distinguishable).
23. Ultracentrifuge 45 min at 126,000 × *g* (35,000 rpm in an SW60 Ti rotor), 4°C.

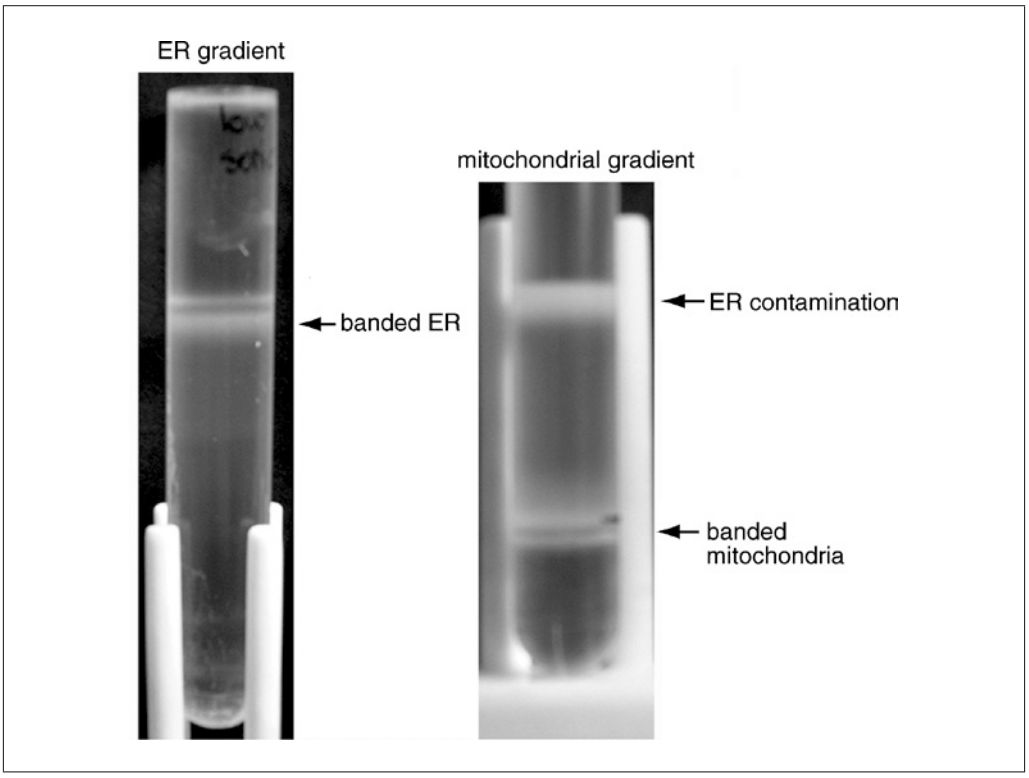

Figure 3.27.2 Representative pictures of visible bands seen upon ultracentrifugation as described in Basic Protocol 1. The ER and mitochondria bands are indicated on their respective gradients following ultracentrifugation.

24. Collect tube from ultracentrifuge. Decant and discard the supernatant.

    There will be a large, translucent pellet at the bottom of the tube.

25. Allow tubes to dry, inverted, for a few minutes. Resuspend the pellet in 100 µl of 1× PBS, pH 7.4, and label as "ER." Store immediately at −20°C.

    The samples can be stored for 1 to 2 months at −20°C prior to their use for western analysis.

Load mitochondria gradient for mitochondrial purification

26. For the mitochondrial pellet wash 1, return to the 14-ml tube on ice containing the protein pellets (step 16). Gently wash the inside sides of the tube with 0.5 ml of ice-cold 1× MTE plus PMSF, being careful not to disrupt the protein pellet. Wash the sides three to five times using the same aliquot of buffer. Decant and discard wash solution.

27. For mitochondrial pellet wash 2, use a fresh aliquot of 0.5 ml of ice-cold 1× MTE plus PMSF buffer to carefully wash the periphery of the protein pellet, which contains ER contaminant proteins. Wash three to five times using the same aliquot of buffer. Decant and discard the wash.

    The ER contaminant proteins make a large, loose ring around the more stable mitochondrial pellet. The mitochondrial pellet will appear more yellow in color, while the surrounding contamination appears whiter in color. A large amount of contaminant protein towards the bottom of the pellet will be seen.

28. Resuspend the washed mitochondrial pellet in 0.8 ml of ice-cold 1× MTE plus PMSF. Load slowly on top of the mitochondrial sucrose gradient so that it forms a new layer.

29. Top off gradient with an additional 0.8 ml of ice-cold 1× MTE plus PMSF buffer.

30. Ultracentrifuge the mitochondrial gradient 22 min at 40,000 × *g* (19,500 rpm in an SW60 Ti rotor), 4°C. Set acceleration and deceleration profiles to 1 (transition speed of 170 rpm for 2 min).

Isolate mitochondrial fractions

31. Collect mitochondrial gradients from the ultracentrifuge (step 30). Using a 1-ml syringe with a 20-G needle, extract a volume of 0.4 ml from the band at the interface of the 1.7 M and 1.0 M sucrose layers (Fig. 3.27.2).

    The mitochondrial band can vary in visibility and is often very thin and hard to see, which is why marking the location of the interface with an indelible felt tip marker before centrifugation is important.

32. Remove the needle and dispense the extracted volume into a sterile 1.5-ml microcentrifuge tube.

33. Add 1.1 ml of ice-cold 1× MTE plus PMSF, cap microcentrifuge tube, and mix well by inversion until suspension is homogeneous.

34. Centrifuge 10 min at 15,000 × *g*, 4°C (in tabletop Beckman GS-15R centrifuge).

35. Decant and discard supernatant (a small, stable yellowish-brown pellet should be visible).

36. Allow tube to dry, inverted, for a few minutes. Resuspend pellet in 30 µl of 1× PBS, pH 7.4, and label as "mitochondria." Store immediately at −20°C.

    The samples can be stored for 1 to 2 months at −20°C prior to their use for western analysis.

BASIC PROTOCOL 2

SEPARATION OF MITOCHONDRIA AND MITOCHONDRIA-ASSOCIATED MEMBRANE FRACTION

This procedure combines differential and Percoll gradient centrifugations. Its critical steps are underscored in Figure 3.27.3. During the first steps, the post-nuclear supernatant (PNS) is separated from nuclei and cellular debris by differential centrifugation at low *g* forces. The post-nuclear supernatant is then subjected to centrifugation at 10,300 × *g* during which the crude mitochondrial fraction is separated from the total microsomal fraction. The total microsomal fraction consists mainly of vesicles derived from rough and smooth ER and membranes from the Golgi apparatus and plasma membrane. The microsomal fraction is then recovered as a pellet after centrifugation of the total microsomal fraction at 100,000 × *g*. The post-microsomal (high-speed) supernatant is generally used as "cytosol" in downstream applications. The crude mitochondrial fraction is subjected to a density gradient fractionation through a self-generating Percoll gradient (Pertoft et al., 1978). Because both mitochondria and mitochondria-associated membrane (MAM) have similar densities, a second step of purification that includes a centrifugation at 6300× *g* follows their collection from the gradient. Finally, the supernatant containing MAM is subjected to centrifugation at 100,000 × *g* and the MAM is isolated as a pellet above the tight Percoll pellet.

NOTE: All solutions, glassware, centrifuge tubes, and equipment should be pre-cooled to 0°C to 4°C and kept on ice throughout the procedure.

Materials

Untransfected or transfected HeLa cells (3×10^7 cells, ten 100 × 20–mm tissue culture dishes, 100% confluent) or uninfected or HCMV-infected HFF cells (5×10^7 cells, four 850-cm^2 roller bottles, 90% confluent)

Phosphate-buffered saline (PBS; see recipe)

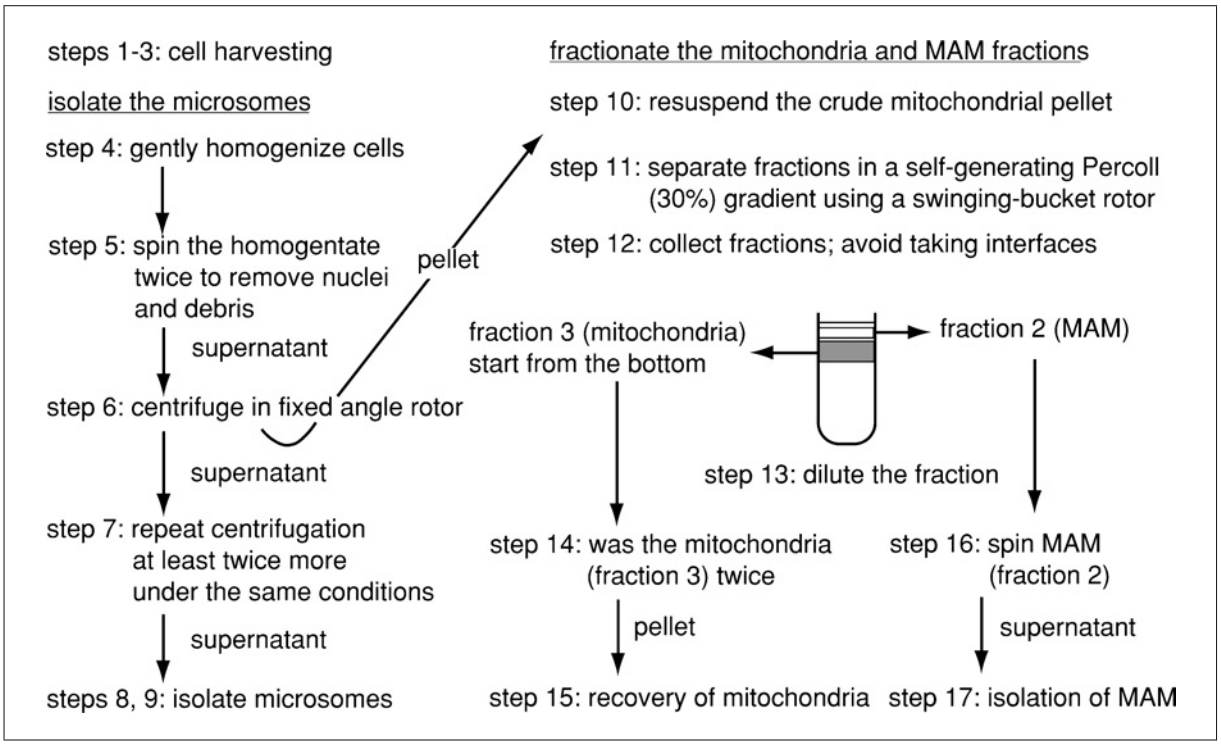

Figure 3.27.3 Schematic representation of Basic Protocol 2. Critical steps in the procedure, which improve purity of the microsomes, mitochondria, and MAM, are emphasized by bold font.

Sucrose homogenization medium (SHM; see recipe), ice cold
Protease inhibitors (optional but recommended, see recipe)
Mannitol buffer A (see recipe), ice cold
30% (v/v) Percoll suspension in mannitol buffer B (see recipe), ice cold
Mannitol buffer B (see recipe), ice cold

Beckman GS-15R tabletop centrifuge with swinging-bucket rotor (e.g., S4180)
14-ml polypropylene round-bottom centrifuge tubes (e.g., Falcon)
Potter-Elvehjem plastic-coated tissue grinder with ball-shaped Teflon pestle (e.g., Wheaton safe-grind type, cat. no. 358003)
Overhead stirrer for tissue grinder (Wheaton, cat no. 903475)
Phase-contrast microscope
1.5-ml microcentrifuge tubes
Beckman GS-15R centrifuge with fixed-angle rotor (e.g., F2402)
Centrifuge tubes, 1.5-ml microcentrifuge tubes
Beckman XL-90 ultracentrifuge with swinging-bucket rotor (e.g., SW 41)
Ultracentrifuge SW41 tubes (ultraclear tubes are highly recommended, e.g., 14 × 89–mm, cat. no. 344059)
1-ml syringes and 20-G needles

Harvest cells

1. Wash the cell monolayers with ~50 ml PBS at room temperature. Remove PBS by suction.
2. Harvest cells (90% to 100% confluent), using a sterile scraper, into 50 ml PBS and pellet cells by centrifuging 10 min at 1000 × *g*, room temperature, using a tabletop centrifuge and four 14-ml centrifuge tubes.
3. Decant the supernatant and resuspend cell pellets in 8 ml ice-cold sucrose homogenization medium containing freshly added protease inhibitors.

Isolate microsomes

4. Attach the pre-cooled pestle of a Potter-Elvehjem homogenizer to an overhead stirrer and gently homogenize the cells by ten up-and-down strokes at 500 rpm. Confirm that ≥90% cell breakage has occurred by examining the homogenate under a phase-contrast microscope.

   It is best to keep the cells on ice throughout the homogenization.

   To check the efficiency of the homogenization, pipet 2 to 3 μl of the homogenized suspension onto a glass slide, overlay with coverslip, and observe using a microscope. A shiny ring around the nuclei indicates that cells are still intact. If >90% of the nuclei do not have the shiny ring, proceed to the next step. Otherwise, repeat the homogenization at 1000 rpm.

5. Transfer homogenates to 1.5-ml microcentrifuge tubes. Pellet the nuclei, cell debris and any unbroken cells by low-speed centrifugation. Centrifuge the homogenates two times in a fixed-angle rotor (e.g., F2402) 5 min at 600 × *g*, 4°C.

   This step is more convenient using a 10-ml centrifuge tube. The centrifugation steps will then be under the same conditions using a different rotor (S4180) but the same centrifuge (Beckman GS-15R). However, it is still recommended that the homogenate should be transferred in 1.5-ml microcentrifuge tubes for the centrifugation that is described in step 6 because of the better separation of the pellet from the supernatant.

6. Transfer the supernatant into new 1.5-ml microcentrifuge tubes and centrifuge 10 min at 10,300 × *g*, 4°C, using the tabletop centrifuge (e.g., Beckman GS-15R) with a fixed-angle rotor (e.g., F2402) and retain the pellet for subsequent steps.

7. Centrifuge supernatant at least two additional times under the same conditions (10 min at 10,300 × *g*, 4°C) until a pellet is no longer visible. Save supernatant on ice for subsequent steps.

   These additional centrifugation steps under the same conditions are needed to ensure that the microsomal fraction will be efficiently separated from any residual mitochondrial membranes and MAM.

8. Pellet the microsomes by ultracentrifuging resultant supernatant 60 min at 100,000 × *g*, 4°C, using a swinging-bucket rotor (e.g., SW41).

9. Resuspend the pellet in 0.5 ml of sucrose homogenization medium.

Fractionate mitochondria and MAM fractions

10. Resuspend the pellet from step 6 in 0.3 ml of mannitol buffer A using two or three strokes in a Potter-Elvehjem homogenizer at 500 rpm, as above in step 4, and carefully layer on top of 10 ml of 30% Percoll suspension in a 14 × 89–mm SW41 ultraclear tube.

11. Centrifuge 65 min at 95,000 × *g*, 4°C, in an ultracentrifuge with a swinging-bucket rotor (e.g., a Beckman SW41).

    The generation of Percoll gradients is an automatic process occurring during centrifugation of the colloid. Therefore, the use of an acceleration program (e.g., 500 rpm for 3 min) in the beginning of the ultracentrifugation is recommended. Although Percoll gradients are considered to be very stable, a similar deceleration program would be the best choice, if such a program is available on the ultracentrifuge.

    A total of 1 to 5 mg of protein (~0.5 ml) on 10 ml of gradient material results in satisfactory separation of banded material without overloading the gradient.

12. Collect the banded organelles in 0.5- to 1-ml fractions (Fig. 3.27.4). To collect the fractions, attach a sterile metallic needle of appropriate diameter (e.g., 20-G) to a sterile 1-ml syringe and carefully puncture the wall of the tube just above fractions

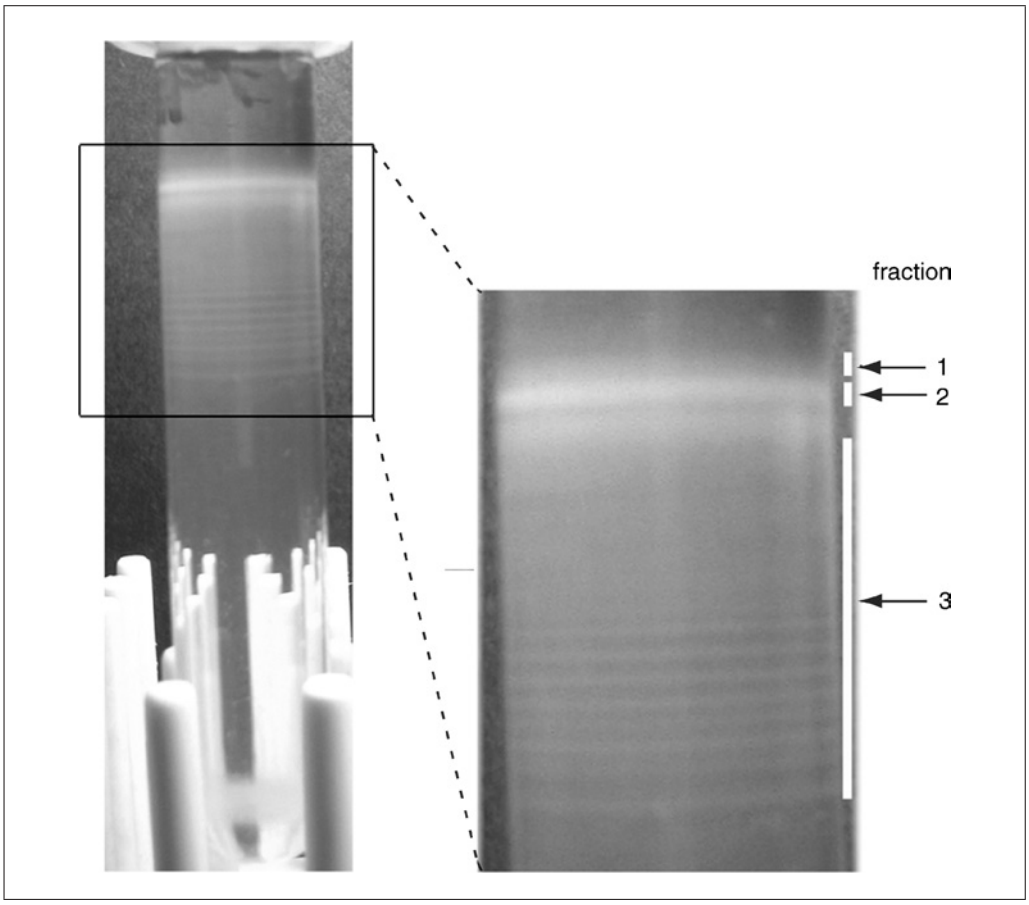

Figure 3.27.4 Separation of MAM and mitochondrial fractions using a self-generating Percoll (30%) gradient. Crude mitochondrial extract from HeLa cells was subjected to ultracentrifugation in a Percoll gradient (see Basic Protocol 2). Three fractions were isolated as indicated on the enlarged section of the gradient. Fraction 2 was identified as the MAM fraction, which is immediately above the top of the mitochondrial fraction (fraction 3). The heterogeneous mitochondrial fraction was conservatively taken.

1 and 2 and below fraction 3 (Fig. 3.27.4). Slowly and conservatively suction the fractions with a fan-like movement, avoiding other bands.

The MAM fraction (fraction 2) is the compact, white band, which is located immediately above the multi-band mitochondrial fraction (fraction 3), ~$^{1}/_{3}$ down the tube.

13. Transfer each sample to a 14 × 89–mm ultraclear SW41 tube. Dilute the fractions with ≥5 vol of ice-cold mannitol buffer B (~10 ml total) to dilute Percoll.

14. Wash the diluted mitochondrial band (fraction 3), at least two times, with 5 ml of ice-cold mannitol buffer B. Pellet mitochondria after each wash by centrifuging 10 min at 6300 × *g* (~6000 rpm using an SW41 rotor), 4°C.

    These washing steps are necessary to remove the Percoll from the fraction, which otherwise will interfere with subsequent analysis.

15. Resuspend the mitochondrial pellet after the last washing step in ice-cold mannitol buffer B at a concentration of 0.5 to 2 mg protein/ml (usually 0.05 to 0.2 ml).

16. Centrifuge the diluted MAM fraction (fraction 2) in SW41 ultraclear tubes 10 min at 6300 × *g* (~6000 rpm), 4°C.

    This step is needed to ensure that the mitochondria-associated membrane fraction will be detectably free from any residual mitochondria.

17. Ultracentrifuge the resulting supernatant 60 min at 100,000 × *g*, 4°C, to pellet the MAM.

    After the ultracentrifugation, the mitochondria-associated membranes form a loose pellet, which is located above the hard pellet of Percoll particles.

18. Resuspend the MAM in ice-cold homogenization buffer at a concentration of 0.5 to 2 mg protein/ml (usually 0.05 to 0.2 ml). Freeze the isolated fractions at −80°C or submit immediately to SDS-PAGE (*UNIT 6.1*) or proteomics analyses.

    The loose MAM pellet is carefully resuspended using a micropipette and transferred to a new 1.5-ml microcentrifuge tube. These fractions may be stored up to 6 months at –80°C.

REAGENTS AND SOLUTIONS

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

Mannitol buffer A

To 100 ml ultrapure H_2O, add:
9.13 g mannitol (0.25 M final)
38 mg EGTA (0.5 mM final)
10 ml 100 mM HEPES (5 mM final)
Adjust pH to 7.4 with 1 N NaOH
Add ultrapure H_2O to 200 ml
Sterilize by autoclaving for 20 min using a liquid cycle
Store up to 2 or 3 months at 4°C

Mannitol buffer B

To 100 ml ultrapure H_2O, add:
8.22 g mannitol (0.225 M final)
76 mg EGTA (1 mM final)
50 ml 100 mM HEPES (25 mM final)
Adjust pH to 7.4 with 1 N NaOH
Add ultrapure H_2O to 200 ml
Sterilize by autoclaving for 20 min using a liquid cycle
Store up to 2 or 3 months at 4°C

MTE solution, 1×

In 60 ml ultrapure H_2O, dissolve:
4.914 g D-mannitol (270 mM final)
121.1 mg Tris-base (10 mM final)
3.72 mg EDTA (0.1 mM final)
Adjust pH to 7.4 with 6 M HCl
Add ultrapure H_2O to 100 ml
Sterilize through a 0.22-μm vacuum filter
Store 3 to 6 months at room temperature

Percoll solution, 30% (v/v)

1 vol 90% (v/v) stock isotonic Percoll (see recipe)
2 vol mannitol buffer B (see recipe)
Sterilize by autoclaving for 20 min using a liquid cycle
Store 2 or 3 months at 4°C

Phosphate-buffered saline, pH 7.4

3 liters distilled water
32 g NaCl (140 mM final)
0.8 g KH_2PO_4 (1.5 mM final)
8.7 g $Na_2HPO_4 \cdot 7H_2O$ (8.1 mM final)
0.8 g KCl (2.7 mM final)
Adjust pH to 7.4 with 1 N NaOH
Add H_2O to 4 liters
Sterilize by autoclaving for 20 min using a liquid cycle
Store 2 or 3 months at 4°C

PMSF, 100 mM

In 500 μl absolute ethanol, dissolve:
8.71 mg PMSF
Store up to 1 year at −80°C

Protease inhibitors

PMSF (200 mM final):
0.348 g phenylmethylsulfonyl fluoride
10 ml 2-propanol or ethanol
Store up to 1 year at −20°C

Add to solutions as required so that the final concentrations are 1 mM.

Stock isotonic Percoll, 90% (v/v)

9 vol Percoll (Pharmacia cat. no. P1644)
1 vol 2.5 M sucrose (0.25 M final)
Sterilize by autoclaving for 20 min using a liquid cycle
Store 2 or 3 months at 4°C

Sucrose, 1.0 M

In 60 ml ultrapure H_2O, dissolve:
34.23 g sucrose
121.1 mg Tris-base (10 mM final)
3.72 mg EDTA (0.1 mM final)
Adjust pH to 7.6 with 6 M HCl
Add ultrapure H_2O to 100 ml
Sterilize by autoclaving 20 min using a liquid cycle
Store 3 to 6 months at room temperature
Open only in a laminar flow hood to avoid contamination

Sucrose, 1.3 M

In 60 ml ultrapure H_2O, dissolve:
44.50 g sucrose
121.1 mg Tris-base (10 mM final)
3.72 mg EDTA (0.1 mM final)
Adjust pH to 7.6 with 6 M HCl
Add ultrapure H_2O to 100 ml
Sterilize by autoclaving 20 min using a liquid cycle
Store 3 to 6 months at room temperature

Sucrose, 1.5 M

In 60 ml ultrapure H_2O, dissolve:
51.35 g sucrose
121.1 mg Tris-base (10 mM final)
3.72 mg EDTA (0.1 mM final)
Adjust pH to 7.6 with 6 M HCl
Add ultrapure H_2O to 100 ml
Sterilize by autoclaving 20 min using a liquid cycle
Store 3 to 6 months at room temperature

Sucrose, 1.7 M

In 60 ml ultrapure H_2O, dissolve:
58.19 g sucrose
121.1 mg Tris-base (10 mM final)
3.72 mg EDTA (0.1 mM final)
Adjust pH to 7.6 with HCl
Add ultrapure H_2O to 100 ml
Sterilize by autoclaving 20 min using a liquid cycle
Store 3 to 6 months at room temperature

Sucrose, 2.0 M

In 60 ml ultrapure H_2O, dissolve:
68.46 g sucrose
121.1 mg Tris-base (10 mM final)
3.72 mg EDTA (0.1 mM final)
Adjust pH to 7.6 with HCl
Add ultrapure H_2O to 100 ml
Sterilize by autoclaving 20 min using a liquid cycle
Store 3 to 6 months at room temperature

Sucrose homogenization medium (SHM)

To 100 ml ultrapure H_2O, add:
17.1 g sucrose (0.25 M final)
20 ml 100 mM HEPES (10 mM final)
Adjust pH to 7.4 with 1 N NaOH
Add ultrapure H_2O to 200 ml
Sterilize by autoclaving for 20 min using a liquid cycle
Store 2 or 3 months at 4°C

COMMENTARY

Background Information

Highly purified mitochondria are very difficult to obtain in appreciable amounts. The discontinuous sucrose gradient protocol (see Basic Protocol 1) is valuable for experiments that require higher yields of mitochondrial samples. It is often imperative to use the same pool of purified mitochondria to observe fraction purity, probe for the presence of mitochondrially localized viral proteins, and examine the post-translational modifications of those proteins. Obtaining enough purified mitochondria for all of these experiments by the Percoll protocol (see Basic Protocol 2) presented here would not be feasible. The discontinuous sucrose gradient protocol takes slightly less time, is more user-friendly, requires less starting material (number of cells), and produces higher yields of mitochondrial fractions. These mitochondrial fractions are well-purified and practical for almost any application. Also, the utility of starting with fewer cells is that one can compare more experimental conditions and controls within a single fractionation experiment. However, when one is willing to sacrifice protein yield for even higher purity mitochondrial fractions, or when one needs to isolate MAM fractions to compare to either

ER or mitochondria, then the Percoll protocol (see Basic Protocol 2) is the preferred method.

Points of contact between the ER and mitochondria make up 5% to 20% of the total mitochondrial network (Rizzuto et al., 1998). These connections, as well as the ER and mitochondria organelles themselves, are quite variable and can undergo rapid changes in overall morphology, size, and composition (Bereiter-Hahn and Vöth, 1994; Collins et al., 2002). Factors such as calcium homeostasis, cell metabolism, and perceived stress can have dramatic impacts on both the form and function of these organelles. Thus, it is imperative to monitor fraction purity for each experiment by analyzing total, ER, mitochondria, and MAM pools by utilizing suitable organelle markers (Table 3.27.1). Not only will this ensure that the identity and purity of the fractions are sufficient for unequivocal conclusions, but appropriate controls can provide additional information about cell status at the time of fractionation.

Following lipofection, it is important to check transfected cells often. Lipofectamine 2000 was the least toxic, in the authors' hands, to both HeLa and HFF cells, but cell viability can still change significantly in response to overexpression of a transfected plasmid. When using control plasmids encoding innocuous or beneficial (anti-apoptotic) proteins, Lipofectamine 2000 can be left in the culture medium for up to 24 hr. However, for transfection of most plasmids, it is prudent to change Lipofectamine 2000 transfection medium after 4 hr, replacing it with complete cell medium so as to minimize the stress or apoptotic signals on the cells. Furthermore, overexpression of some plasmids may be more toxic to cells, in which case one must wash out the lipofection medium after 4 hr (as before) and, moreover, may have to decrease the total incubation time before cell harvesting. Prior to harvesting, monitor the cells for viability as cells that are heavily stressed or apoptotic are not reliable for fractionation experiments.

The need to obtain intact and highly purified subcellular fractions, including MAM, imposes the additional use of density gradient centrifugation to remove contamination by broken membranes or organelles of similar size as in Basic Protocol 2. Percoll is considered to be one of the best density gradient medium available for the separation of cells, organelles, viruses, and subcellular particles. Because of its low osmolarity (<25 mOs/kg water), Percoll forms a density gradient without generating an osmolarity gradient (Pertoft et al., 1978). Osmolarity of the gradient medium must be taken into consideration since cellular membrane-bound organelles such as mitochondria and MAM act as osmometers. A high external osmolarity will result in shrinkage of membrane-bound organelles while low osmolarities will result in swelling of organelles, hence altering their buoyant densities. Differences in the osmolarity of the gradient medium can explain differences in the size and apparent buoyant densities of subcellular compartments when different gradient media are used. Buoyant densities of organelles in sucrose rise as water is removed from their enclosed spaces. In contrast, organelles in Percoll gradients in physiological range (280 to 320 mOs/kg) have lower apparent buoyant densities than in sucrose (Pertoft et al., 1978).

Another unique feature of Percoll is that it can form self-generated gradients by centrifugation at moderate *g* forces. Fixed-angle rotors are most commonly used for Percoll gradients. The advantage of using fixed-angle or vertical rotors is that the path-length for formation of the gradient is shorter and the gradient forms more rapidly. Both Vance (1990) and Hovius et al. (1990) used a fixed-angle rotor to isolate purified mitochondria from a self-generated Percoll (30% v/v) gradient. Conversely, the authors used a swinging-bucket rotor for the formation of the Percoll gradient. There are two main reasons for this choice: first, because both fractions (mitochondria and MAM) have similar densities and they are banded within the density range of ~1.039 to 1.051 g/ml (Fig. 3.27.4), a better separation of this density range can be achieved by using a rotor with a longer path-length. Second, a good spatial separation between resolved mitochondria subpopulations, which differ slightly in density and size from one another, can be achieved (Collins et al., 2002). The latter feature is especially desirable in the case of HCMV-infected cells in which disruption of mitochondria networks (McCormick et al., 2003) can be visualized by density banding of mitochondrial species in these Percoll gradients.

Based upon the authors' experience, the transfection protocol does not affect the pertinent physical properties of the organelles and their constituents during fractionation. Thus, no difference should be expected between fractions that are isolated from either untransfected or transfected cells. However, HCMV infection markedly alters the fractionation protocol.

A change in the density of mitochondrial bands after HCMV infection has been observed. This density change in fraction 3 is detected as a significant increase of the mitochondrial markers in the pellet that is formed after the first centrifugation of the diluted MAM fraction at 6300 × *g* (see Basic Protocol 2, step 16). Usually, only background levels of the mitochondrial markers can be detected in this pellet. It appears that HCMV infection causes a shift of mitochondrial densities such that some mitochondria band in the density range of MAM. This is probably related to the documented disruption of mitochondria networks by HCMV. Therefore, it is highly recommended that the study of the MAM should always be based on the more rigorous fraction that is isolated after the final ultracentrifugation step at 100,000 × *g*, as it is described in Basic Protocol 2, step 17. This fraction is consistently devoid of mitochondrial contamination in both uninfected and HCMV-infected cells.

Critical Parameters and Troubleshooting

Sucrose gradient fractionation

Sometimes a mitochondrial band will not be seen after centrifugation, so make sure to mark the gradient interface between the 1.7 M and 1.6 M sucrose layers when preparing the gradients. If a band is not seen after centrifugation, insert the collecting needle into the side of the 11 × 60–mm Beckman tube at the marked gradient interface and carefully withdraw ~0.4 ml of liquid.

When collecting protein fractions with needles, the use of small-bore needles should be avoided as they increase the shear force on the extracted samples. Extract the protein band slowly into the syringe, then remove the needle before dispensing the protein fraction into a microcentrifuge tube for storage. This saves the sample from the shear force of flowing a second time through the needle.

Proteins can be degraded extremely rapidly. Once the cells have been lysed, work as quickly as possible. Also make sure to keep samples on ice at all times. Pre-cooling buffers to 4°C can also help. Furthermore, PMSF is a serine protease inhibitor. It does not inhibit other proteases, and does not even inhibit all serine proteases. If protein degradation appears to be a problem, it may be necessary to add a commercially available protease inhibitor cocktail to the 1× MTE buffer in addition to PMSF. Freeze fractionated samples immediately upon collection to avoid unwarranted degradation.

Differential pelleting by centrifugation

In the initial steps of Basic Protocol 2, we use differential centrifugation to separate the mitochondrial and MAM fractions from the microsomal fractions using a centrifugation at 10,300 × *g*. This centrifugation step proved to be critical. The method of Hovius et al. (1990) uses pelleting at a lower RCF (10,000 × *g*) to reduce the ER content in the resulting crude mitochondrial fraction. This lower centrifugal force will be insufficient to efficiently pellet the MAM with mitochondria and will decrease the subsequent yield of MAM on Percoll gradients. The proposed RCF (10,300 × *g*), however, greatly improves the yields of MAM in subsequent Percoll gradient.

Another critical step of the method involves the centrifugal separation of the mitochondrial-MAM fraction from the microsomal fraction. Care must be taken in this step to remove all traces of unpelleted mitochondrial-MAM. It is therefore recommended to use a fixed-angle rotor and at least two centrifugations under the same conditions (Fig. 3.27.3). In the authors' experience, if the above conditions are not followed, the supernatant microsomal fraction is still contaminated with detectable mitochondrial-MAM fraction.

Percoll density gradients

To generate Percoll gradients, stock isotonic Percoll (SIP) solution is prepared at the desired density in 0.25 M sucrose. To accurately measure buoyant density, samples are premixed with the gradient material. However, if samples are layered onto the top of the Percoll gradient, better resolution of subcellular particles from soluble proteins is attained. Soluble proteins tend to remain above the Percoll gradient and subcellular particles will sediment into the gradient, thus achieving better separation.

Percoll gradients continuously change during high-speed centrifugation. Therefore, depending on the centrifugation conditions (type of rotor, RCF, time of centrifugation), the density profile of the gradient and, consequently, the separation or the organelles will be altered. Thus, using density marker beads (Amersham Biosciences cat. no. 17–0459-01) to monitor densities in a duplicate gradient is recommended. Their use verifies the consistency of density gradient formation and greatly facilitates identification of the desired fractions.

In spite of their many advantages, Percoll particles are difficult to remove from purified fractions. A simple approach is to separate subcellular particles from Percoll-coated silica particles by high-speed centrifugation in a swinging-bucket rotor or fixed-angle rotor (Calaminus et al., 1979). The organelles can be separated from Percoll by centrifugation at 100,000 × *g* for 2 hr (in a swinging-bucket rotor) or 90 min (in a fixed-angle rotor). Percoll is in the tight pellet; whereas, the biological material remains loosely packed and can be gently resuspended.

Anticipated Results

Different lysis methods

The effects of various lysis conditions were evaluated using untransfected HeLa cells fractionated by discontinuous sucrose gradients (Table 3.27.2). Five conditions were surveyed: low sonication (three, 5-sec pulses), medium sonication (three, 10-sec pulses), high sonication (three, 15-sec pulses), homogenization (10 strokes with Dounce homogenizer), or freeze/thaw cycles (three cycles of 1-hr incubation at −80°C followed by a rapid thaw in a 37°C water bath).

Homogenization, the gentlest lysis procedure, had the lowest ER and mitochondrial yields of the lysis protocols tested. The low-speed spin after lysis, to remove large cellular debris, produced large pellets of intact cells after homogenization, which decreased the material available for subsequent banding on ER and mitochondrial gradients.

To determine the purity of the fractionated mitochondria and ER from the various lysis procedures, an examination of the presence of ER (DPM1), MAM (FACL4), and mitochondrial (Grp75, COXII) markers in the banded ER and mitochondria fractions (Fig. 3.27.5) was done. Sonication resulted in good separation of ER and mitochondria although the anti-DPM1 antibody detected mitochondrial DPM (Gasnier et al., 1992) in this experiment. However, the other markers tested (FACL4, Grp75, and COXII) showed good separation of ER and mitochondria. Medium or high sonication disrupted the association between the MAM and mitochondrial compartments, as the MAM marker (FACL4) was detected in the ER fraction and was barely detected in the mitochondrial fraction. Conversely, the freeze/thaw lysis showed an equal distribution of MAM in both ER and mitochondrial fractions. Of the lysis procedures tested, homogenization was the most effective at preserving the association of the MAM with mitochondria. Nonetheless, the highest yields of ER and mitochondrial fractions were obtained from low and medium sonication procedures. Medium sonication was therefore chosen as the preferred lysis condition for discontinuous sucrose gradient fractionation (see Basic Protocol 1). This procedure produces superior yields of well-purified ER and mitochondrial fractions, with MAM membranes predominantly appearing in the ER fraction.

Purification of mitochondria and mitochondria-associated membranes

The ultracentrifugation of the crude mitochondrial extract in a self-generating Percoll (30%) gradient using a swinging-bucket rotor produces three distinct fractions (Fig. 3.27.4). Using density marker beads (Pharmacia), the authors found that MAM and mitochondria from cultured human cells span a range of densities between 1.039 and 1.051 g/ml (data not shown). The higher density beads ($\geq$1.069 g/ml) sediment near the bottom of the Percoll gradient. Because of this broad span of 1.039 to 1.051 g/ml densities, the self-generating 30% Percoll gradient is excellent for effective resolution of MAM from mitochondria.

Fraction 1 appears as a faint, diffuse layer just above fraction 2; whereas, fraction 2 is the more compact white in color band, less dense than mitochondria. Fraction 3 consists of multiple denser mitochondrial bands. Because the borders of the fractions usually are partially overlapping, care must be taken during the collection of the bands to minimize mixing of the samples. If a needle and syringe is used for this purpose, it is recommended that the collection should start from the top in the case of fraction 1 and from the bottom for fraction 3. In every case, the interface between the fractions should be carefully avoided.

The isolated fractions were verified by western blot analysis using known cellular protein markers for cytosol (Hsp70), microsomes (DPM1, calreticulin), MAM (mEGFP-huPSS-1), and mitochondria (COXII, Grp75) (Fig. 3.27.6 and Table 3.27.1). The MAM is physically associated and pelleted with mitochondria. Upon resolution in Percoll gradients, its position is consistent with previously reported relative densities of MAM (Vance, 1990). Most compellingly, the high relative abundance of mEGFP-huPSS-1 fusion protein in fraction 2 as well as the presence of the other ER markers (DPM1, calreticulin) indicate that it is an ER subcompartment, enriched for phosphatidylserine synthetic enzymes as

Table 3.27.2 Mitochondrial and ER Yields and Purity from Differentially Lysed HeLa Cells

		Average						
Lysis method	Yield samples	Initial cell pellet (g)	Total protein fraction (mg)	Mitochondria yield (μg)	ER yield (μg)	Yield	MAM detected in	Purity
Sonication								
Low (3 × 5 sec)	1, 2	0.153	9.87	415.86	842.6	High	ER + mitochondria	Good
Medium (3 × 10 sec)	1, 2	0.148	9.584	350.34	824.4	High	ER	Good
High (3 × 15 sec)	1, 2	0.146	8.778	163.14	820.5	Medium	ER	Good
Freeze/thaw	1, 2		7.634	ND[a]	195.2	Medium	ER + mitochondria	Good
Homogenization	1, 2	0.146	1.484	ND[a]	ND[a]	Low	MAM/ mitochondria	Excellent
	3, 4	0.147	1.392	ND[a]	ND[a]	Requires large scale-up	Mitochondria	

[a]ND, not determined as extract protein concentrations were below detection level of the BCA assay.

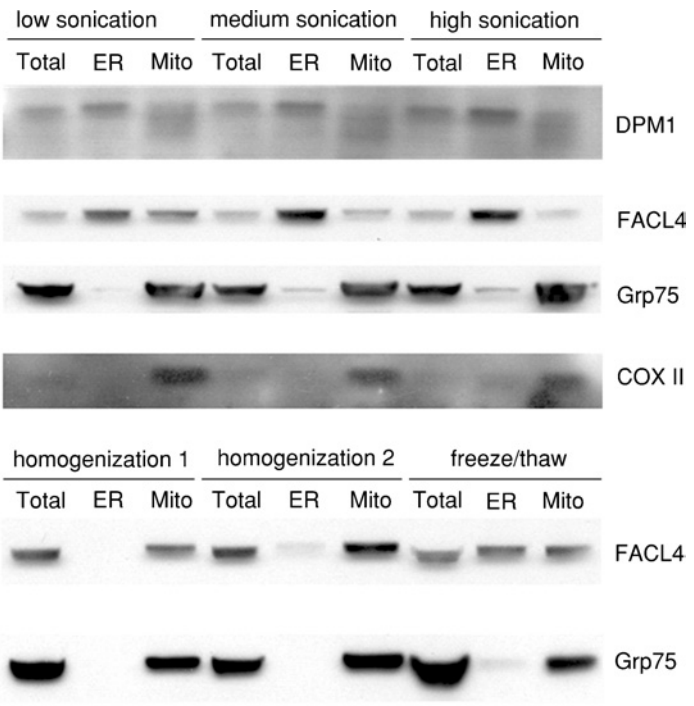

Figure 3.27.5 Western analyses of ER (DPM1), mitochondrial (Grp75, COXII), and MAM (FACL4) markers in HeLa cells lysed by different methods. HeLa cells were fractionated using discontinuous sucrose gradients (see Basic Protocol 1) as detailed in Table 3.27.2. Briefly, cells were lysed using sonication (three times, 5, 10, or 15 sec each), Dounce homogenization (ten strokes), or freeze/thaw cycles (three times 1 hr at –80°C, followed by rapid thawing). Twenty micrograms of total, ER, and mitochondrial protein fractions from each sonication condition were subjected to SDS-PAGE in 10% Bis-Tris NuPage gels (Invitrogen). For homogenization and freeze/thaw lysis conditions, 40 μl of each fraction were used (due to low protein concentrations). Proteins were then transferred to nitrocellulose membranes and probed for DPM1 (1:100 goat anti-DPM1, 1:2000 donkey anti-goat HRP). Membranes were stripped and reprobed for Grp75 (1:2000 mouse anti-Grp75, 1:2500 goat anti-mouse). Similarly, membranes were then stripped and reprobed sequentially for FACL4 (1:250 rabbit anti-FACL4, 1:3000 goat anti-rabbit HRP) then COX II (1:200 goat anti-COXII, 1:2500 donkey anti-goat HRP).

previously documented in rat liver tissue (Stone and Vance, 2000). Therefore, it has been concluded that fraction 2 contains the purified mitochondria-associated membranes from cultured human cells.

Western analyses identified fraction 3 as the purified mitochondria (Fig. 3.27.6). The parallel bands that can be observed within this fraction reflect mitochondria of slightly different sizes and densities. Mitochondria in the cell differ in size and morphology (Bereiter and Vöth, 1994; Collins et al., 2002). It should be noted that the position of the mitochondria in the gradient, exactly below fraction 2, is another indication that fraction 2 represents the mitochondria-associated membranes.

The identity of fraction 1 is less clear. Western analyses clearly indicate that it is ER-related as it shares markers (Fig. 3.27.6, calreticulin) with the ER and MAM. Nonetheless, fraction 1 notably differs in the presence of an alternative DPM species detected by the anti-DPM1 antibody. Moreover, fraction 1 has markedly less mEGFP-huPSS-1 than the MAM fraction. Because of its association with the MAM and mitochondria and the retention of some ER markers, fraction 1 could represent a transition between the rough ER and the MAM or from the ER to another secretory compartment.

Cellular markers for verification of subcellular compartment identity and purity

To verify the purity of the isolated microsomal and mitochondria fractions, established organelle-specific markers that were previously used (Colberg-Poley et al., 2000; Mavinakere and Colberg-Poley, 2004a,b; Mavinakere et al., 2006), and others, were used for the same purpose. To unequivocally

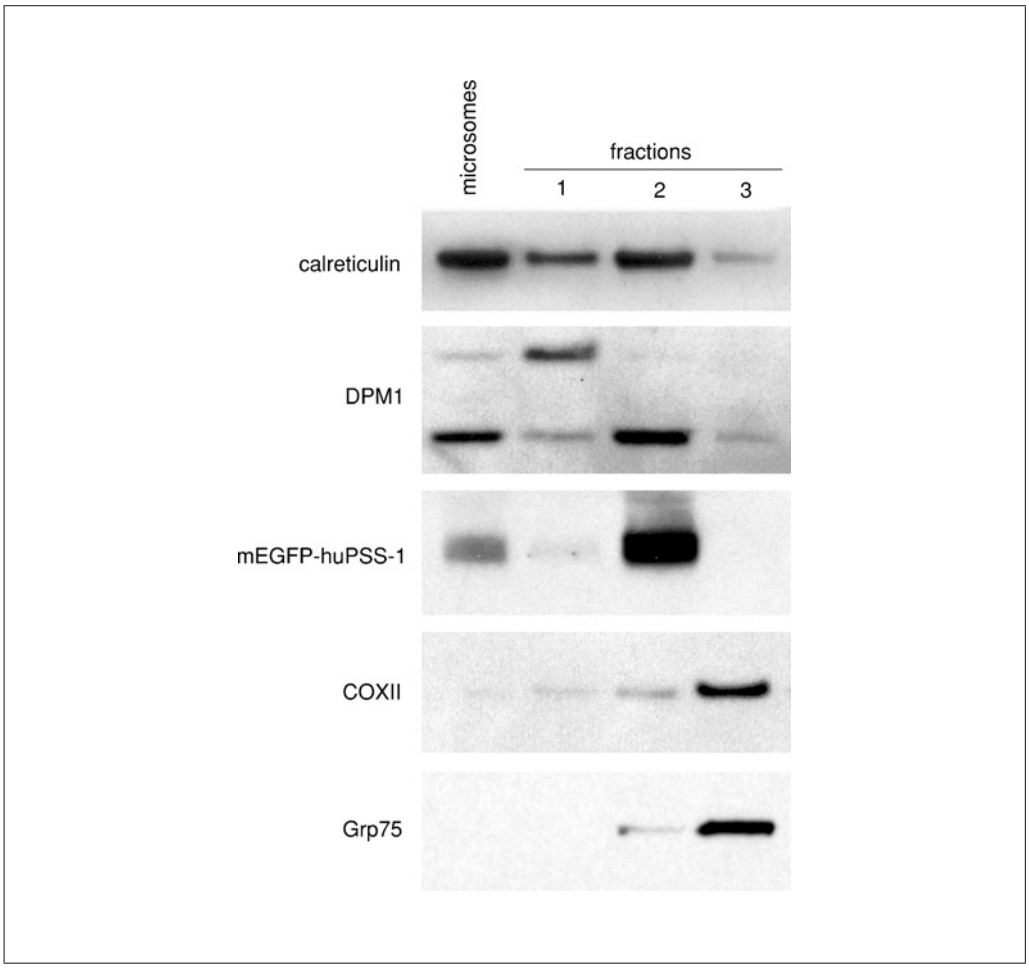

Figure 3.27.6 Western analyses of fractionated HeLa cells that stably express mEGFP-huPSS-1 fusion protein. Stably transfected cells were fractionated according to the procedure described in Basic Protocol 2 and subcellular fractions were isolated. Fractionated proteins (10 μg) were separated by 10% SDS PAGE and transferred onto nitrocellulose membranes using a semi-dry protein transfer apparatus (BioRad). Blotted proteins were probed against markers for microsomes (anti-DPM1, 1:100 or anti-calreticulin, 1:1000), MAM (mEGFP-huPSS-1 using anti-GFP, 1:100), and mitochondria (anti-COX, 1:100 or anti-Grp75, 1:2500) and with the corresponding horseradish peroxidase-conjugated secondary Ab (1:2000). Reactivity was detected using the chemiluminescent method (Amersham, GE Healthcare).

identify the MAM fraction (fraction 2) from Percoll gradients, phosphatidyl-serine synthase 1 (PSS-1), which has been shown to be enriched in the rat liver MAM (Stone and Vance, 2000), was chosen. However, neither commercial antibodies nor human PSS-1 cDNA clones were available. Therefore, the complete human PSS-1 cDNA was cloned from a HeLa cDNA library and its open reading frame was tagged with EGFP (data not shown). The cellular markers, antibodies, commercial sources and original references for ER, mitochondria and MAM are listed in Table 3.27.1. A HeLa cell transfectant stably expresses the fusion protein mEGFP-huPSS-1 fusion protein. The fractionated subcellular compartments from HeLa-PSS-1_{20} cells verified the identity of fraction 2 as the MAM by virtue of the selective presence of PSS-1 in fraction 2 but not in fraction 3 (Fig. 3.27.6).

Microsomes

Dolichol phosphate-mannose synthase 1 (DPM1). DPM synthase 1, a transmembrane protein, which catalyzes mannosyl transfer from GDP-mannose hydrophobic long-chain acceptor dolichol-phosphate, is present in the rough ER (Maeda et al., 1998). In human cells, DPM1, which is located at the cytosolic side of the ER, associates with DPM2 and DPM3 to form the complete multicomponent enzyme of human DPM synthase (Maeda et al., 2000). In addition, a mitochondrial DPM synthase is located on the cytosolic face of

the outer membrane of mitochondria (Gasnier et al., 1992). On occasion, the anti-DPM1 (I-20, Santa Cruz Biotechnology) antibody used detected these different DPM species in the ER or in mitochondrial fractions.

Calreticulin. Calreticulin is a Ca^{2+}-binding chaperone that contains an N-terminal amino acid signal sequence and a C-terminal KDEL ER retrieval sequence, which are responsible for its localization in the ER lumen (Gelebart et al., 2005). However, calreticulin has also been detected in cellular compartments, other than ER and including the cell surface (Johnson et al., 2001). Anti-calreticulin (Affinity Bioreagents) antibody detected calreticulin in the cytosol; whereas, other markers for the ER (anti-DPM1), MAM (anti-mEGFP-PSS-1), or mitochondria (anti-GRP75 and anti-COXII) did not.

Mitochondria

Grp75 (glucose regulated protein 75 kDa). Grp75 is a molecular chaperone that is localized in the mitochondrial matrix, where, in concert with Hsp60, it is thought to participate in the refolding of proteins translocated into this organelle (Manning-Krieg et al., 1991). Very recently, it was shown that Grp75 is also present in a macromolecular complex with VDAC and IP_3Rs at the ER-mitochondria interface (Szabadkai et al., 2006).

COXII (cytochrome c oxidase subunit II). COXII is one of three mitochondrial DNA–encoded subunits (MTCO1–3) of respiratory complex IV. Complex IV localizes to the mitochondrial inner membrane and is the terminal enzyme of the electron transport chain (Scheffler, 2001). Although COXII is mainly localized in the mitochondria, recently, it was shown that the protein is present at extra-mitochondrial sites (Sadacharan et al., 2005).

Mitochondria-associated membranes

mEGFP-huPSS-1 (monomeric-enhanced green fluorescent protein- human phosphatidylserine synthase). Both the PSS-1 and PSS-2 enzymes are involved in phosphatidylserine biosynthesis in mammalian cells. It has been shown that PSS-1 is highly enriched in MAM of rat liver and is largely excluded from the bulk of the ER (Stone and Vance, 2000). The fusion protein mEGFP-huPSS-1 has the same localization like the wild-type protein in human cells.

FACL4 (fatty acid CoA ligase, long chain 4). FACL4 converts long-chain fatty acids into fatty acyl-CoA esters for use in synthesizing complex lipids (Piccini et al., 1998). Similar to PSS-1, it is enriched in MAM compartments and is a marker for MAM fractions in western blot analyses (Simmen et al., 2005).

Time Considerations

Basic Protocol 1 can easily be accomplished within 1 full working day. The time required heavily depends on the number of transfection or infection conditions being tested. It is best to limit the scale of the experiment to ultracentrifuge availability (i.e., each ultracentrifuge rotor can only hold six tubes). For a point of reference, twelve 175-cm^2 flasks (six duplicate sets of experimental conditions) can be processed at once using a single rotor for each ultracentrifugation set, taking a total of ~6 to 7 hr. Preparation and sterilization of buffers is done prior to the day of fractionation. PMSF is added fresh to 1× MTE buffer just before use. Creation of sucrose gradients and harvesting of cell pellets are done first. Cell pellets are stable on ice for up to 2 hr, providing a convenient resting point. Alternatively, cell pellets can be frozen, providing a stopping point in the protocol. After sonication of cells, there are no more stopping points in the protocol, and one should work as quickly as possible. The ultracentrifugation for the ER fraction is relatively long (70 min), and this should be started first. After beginning this ultracentrifugation, go back and perform the pellet washes and load the gradients for the mitochondrial fractions. Both centrifugations will probably finish around the same time. Again, work with the ER fractions first, as they require an additional 45 min ultracentrifugation. Then, go back and collect banded mitochondria from the other tubes.

There are no points at which Basic Protocol 2 can be stopped. All of the fractions that are isolated through this procedure must be stored at −80°C upon recovery. All the solutions can be made ahead of time and stored for 1 to 2 days at 4°C or for longer period (e.g., 2 to 3 months) frozen at −20°C. Note that PMSF or other protease inhibitors should be added immediately prior to use.

Cell preparation and homogenization can be accomplished in 30 min. The separation of crude mitochondrial fraction and total microsomal fraction should take an additional ~30 min. The estimated time of both the isolation of the fractions from the Percoll gradient and the recovery of the pure microsomal pellet is ~2 hr. Another 90 min are needed for the isolation of pure mitochondria and MAM fractions. The total time of this protocol is

estimated to be ~4 hr. Functional assays should be carried out as soon as possible after the preparation, but for simple marker enzyme assay material can be stored up to 16 hr at 0° to 4°C without much loss of activity.

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