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Isolation of Mitochondria for Lipid Analysis

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Abstract

Diverse classes of lipids are found in cell membranes, the major ones being glycerolipids, sphingolipids, and sterols. In eukaryotic cells, each organelle has a specific lipid composition, which defines its identity and regulates its biogenesis and function. For example, glycerolipids are present in all membranes, whereas sphingolipids and sterols are mostly enriched in the plasma membrane. In addition to phosphoglycerolipids, plants also contain galactoglycerolipids, a family of glycerolipids present mainly in chloroplasts and playing an important role in photosynthesis. During phosphate starvation, galactoglycerolipids are also found in large amounts in other organelles, illustrating the dynamic nature of membrane lipid composition. Thus, it is important to determine the lipid composition of each organelle, as analyses performed on total cells do not represent the specific changes occurring at the organelle level. This task requires the optimization of standard protocols to isolate organelles with high yield and low contamination by other cellular fractions. In this chapter, we describe a protocol to isolate mitochondria from *Arabidopsis thaliana* cell cultures to perform lipidomic analysis.

Key words

Mitochondria isolation

Arabidopsis thaliana

Cell cultures

1. Introduction

The main lipids found in biological membranes of the eukaryotic cell are glycerolipids, sterols, and sphingolipids. Glycerolipids are found in all membranes whereas sterols and

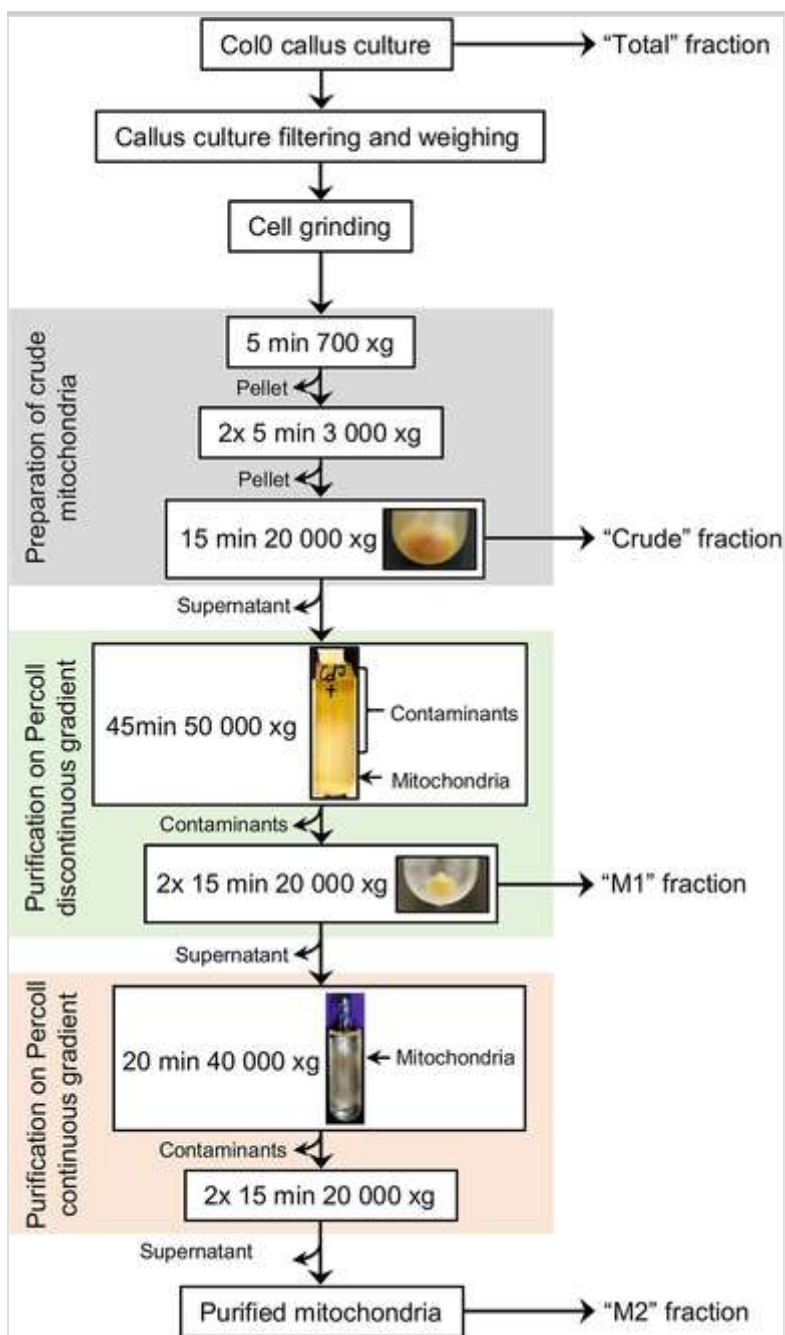
sphingolipids are mainly enriched in the plasma membrane [1, 2]. Glycerolipids are not evenly distributed among organelle membranes. Cardiolipin, a particular phospholipid with four acyl-chains, is present only in mitochondria where it plays an important role in the regulation of mitochondria morphology and respiration [3, 4]. In plant cells, galactoglycerolipids, a family of glycerolipids without a phosphate moiety in their polar head, are enriched in plastids where they play an important role in photosynthesis and membrane architecture [5, 6]. The membrane lipid composition can rapidly change in response to different stresses and stimuli. This is particularly true for plants that have to adapt to a variety of modifications of their environment, such as variations of light, temperature or nutrient availability [5, 7]. Modifications of lipid composition are often measured on total lipid extracts, which is a quick approach to study major changes but does not unravel the specific modifications occurring at the organelle level. Thus, it is important to be able to determine the lipid composition of each organelle. This requires protocols to isolate cellular organelles.

Mitochondria are organelles present in almost all eukaryotic cells and are involved in many cellular processes such as energy metabolism, synthesis of cofactors and amino acids or programmed cell death. Mitochondria are delineated by two membranes, the outer (OM) and the inner (IM) membranes. Phospholipids are the major constituents of mitochondrial membranes, and each membrane presents a specific lipid composition, with cardiolipin being enriched in the IM and phosphatidylinositol in the OM [3, 8]. As for the other cell compartments, mitochondrial lipid composition is tightly regulated and can be modified under specific conditions [9, 10]. A very prominent case for remodeling of mitochondrial lipid composition is induced by phosphate starvation in plants. In *Arabidopsis thaliana* cell cultures grown in the absence of phosphate, mitochondrial phospholipids are partially degraded to release phosphate. Galactoglycerolipids, barely detectable under normal growth conditions, become one of the most abundant mitochondrial lipid classes representing up to 18 mol% of total mitochondrial lipids [9, 11]. This example illustrates the necessity of analyzing the lipid composition of isolated mitochondria. Protocols for mitochondria purification are based on a succession of differential centrifugation steps followed by purification by gradient centrifugation. Several protocols to purify mitochondria starting from different *A. thaliana* plant materials (heterotrophic cell cultures [12, 13], seedlings [12], or leaves [14]) have been described in the literature. Two factors that have to be considered when choosing the starting plant material are (1) the yield of purified mitochondria and (2) the contamination of the mitochondrial fractions with other cell compartments. The yield of purification is often higher when using cell cultures because of the higher availability of the starting material. However, with the development of mass spectrometry to perform lipidomic analyses, the quantity of mitochondria required for a lipid analysis has dropped to amounts as low as around 200 µg of mitochondrial proteins. Cross-contaminations remain a concern, particularly in the case of lipid remodeling studies. It is now widely accepted that

mitochondria can establish membrane contact sites with many other organelles including plastids [15, 16]. As membranes establishing contact sites in vivo are tightly bound, they usually copurify together [17, 18]. This is the case for the endoplasmic reticulum fraction copurifying with mitochondria in yeast and mammals, called mitochondria-associated membranes (MAMs) [18, 19]. Thus, two types of contaminants can be present in mitochondrial fractions: (1) the “real” contaminants, corresponding to organelles or cell fractions that copurify with mitochondria because of their similar density, and (2) MAMs, which are associated to mitochondria in vivo and have a biological significance. The decrease of contaminants in *A. thaliana* mitochondria fractions can be achieved by using two successive purifications on Percoll gradients [9, 20]. In yeast and mammals, it has been shown that MAMs can be detached from mitochondria by lowering the pH of the isolation buffer from 7.4 to 6 [18, 19]. This protocol has never been tested in plants and the existence of MAMs remains unknown. Here, we describe a protocol to isolate mitochondria from *A. thaliana* callus cell cultures to perform lipidomic analysis (Fig. 1). The advantage of using callus cell cultures are (1) the low abundance of plastid membranes and proteins that are often a source of contamination when working with green tissue, (2) the purification of mitochondria with a high yield, (3) the possibility to easily control the composition of the cell culture media to study stress responses, and (4) the possibility to obtain cell cultures from mutants of interest.

Fig. 1

Flow Chart of the mitochondria purification protocol. Photos were taken during the isolation procedure to illustrate crucial steps. The fractions collected for Western immunoblot analysis are indicated on the right



2. Materials

2.1. Cell Cultures and Media

1. MS medium: 4.41 g/L Murashige and Skoog (MS) salts and vitamins, 1.5% (w/v) sucrose, 1.2 mg/L 2,4-dichlorophenoxyacetic acid. The pH is adjusted to 5.7 with NaOH and the medium is autoclaved (*see Note 1*).
2. *Arabidopsis thaliana* Col0 plants are used.

2.2. Buffers and Equipment for Mitochondria Purification

1. Grinding buffer: 0.3 M mannitol, 15 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 8 with NaOH, 2 mM ethylene glycol-bis (2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) pH 8 with NaOH, 0.6% (w/v) polyvinylpyrrolidone K25 (PVP25). Just before use, 0.5% (w/v) bovine serum albumin (BSA), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM aminocaproic acid, and 1 mM benzamidine are added.
2. Washing buffer: 0.3 M mannitol, 10 mM MOPS-NaOH pH 7.4. Just before use, 1 mM PMSF, 5 mM aminocaproic acid, and 1 mM benzamidine are added.
3. CX2 buffer: 0.6 M mannitol, 20 mM MOPS–NaOH pH 7.2, 2 mM EGTA. Just before use, BSA is added to a final concentration of 0.2% (w/v) .
4. Percoll 40: 40% (v/v) Percoll (GE Healthcare Life Sciences), 1× of CX2 buffer.
5. Percoll 28: 28% (v/v) Percoll (GE Healthcare Life Sciences), 1× of CX2 buffer.
6. Percoll 23: 23% (v/v) Percoll (GE Healthcare Life Sciences), 1× of CX2 buffer.
7. Percoll 18: 18% (v/v) Percoll (GE Healthcare Life Sciences), 1× of CX2 buffer.
8. Mortar and pestle.
9. Sand.
10. 250 mL polycarbonate centrifuge bottles.
11. 35 mL polycarbonate centrifuge tubes.
12. Ultraclear 40 mL ultracentrifuge tubes.
13. 5 mL ultracentrifuge tubes.
14. Four and eight milliliter Potter-Elvehjem tissue grinder.
15. JA10 rotor (Beckman Coulter) in a Beckman Coulter Avanti J-E centrifuge (*see Note 2*).
16. JA25.50 rotor (Beckman Coulter) in a Beckman Coulter Avanti J-E centrifuge.
17. SW32-Ti swinging rotor (Beckman Coulter) in a Beckman Coulter Optima LE-80k ultracentrifuge or equivalent rotor with centrifuge.

18. HT-4 rotor (Sorvall) in a Sorvall Discovery M150 SE ultracentrifuge or equivalent centrifuge.

2.3. SDS-PAGE and Western Blot Analysis

1. Protein buffer: 8 M urea, 50 mM 2-amino-2-(hydroxymethyl) propane-1,3-diol (Tris) pH 6.8 with HCl, 1 mM EGTA. Add 10 mM DTT before use.
2. Loading buffer (4×): 0.24 M Tris pH 6.8, 40% (v/v) glycerol, 8% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) β-mercaptoethanol, 0.04% (w/v) bromophenol blue.
3. Precasted SDS-PAGE 4–12% gels with corresponding electrophoresis buffer are used according to the manufacturer's instructions.
4. Transfer buffer (10×): 0.25 M Tris and 1.92 M glycine, pH 8.3 with HCl. Store the 10× solution at 4 °C. Before use, dilute ten times the 10× solution in MilliQ water with 20% (v/v) ethanol to prepare the Transfer buffer.
5. Ponceau red: 0.2% (w/v) Ponceau red, 3% (v/v) trichloroacetic acid.
6. TBS (Tris buffered saline, 10×): 500 mM Tris–HCl pH 7.5 and 1.5 M NaCl. Dilute ten times the 10× solution in milliQ water to obtain the TBS buffer.
7. Bead-beater tissue homogenizer (*see Note 3*).
8. Tank for electrophoresis.
9. Blotting tank for protein transfer to membrane.
10. Nitrocellulose membrane (pore size 0.2 μm).
11. Antibodies: V-ATPase, Bip2 and Tic40 antibodies were purchased from Agrisera (www.agrisera.com). AtMic60 and PMD2 antibodies have been described [11].

3. Methods

3.1. Preparation of Cell Cultures

1. 5 mL of callus of 7-day-old callus cultures (*see Note 4*) are used to inoculate 200 mL of media in a 1 L flask. For a typical experiment, five flasks are inoculated to obtain enough material for isolating one mitochondrial fraction.

2. Callus cells are grown for 4 days under continuous light (150 μ E) at 22 °C with agitation at 125 rpm.

3.2. Preparation of a Crude Mitochondria Fraction

1. To preserve mitochondria integrity and function, each step of purification has to be performed at 4 °C with all materials (mortar, tubes, rotors, ...) and buffers prechilled at 4 °C. Mitochondria have to be manipulated carefully. Cut-off pipette tips have to be used for resuspending organelle pellets (*see Note 5*).
2. Filter the cells of each flask through a circle of Whatman filter paper using a filtration unit. Cells are weighed and placed in a prechilled mortar (*see Note 6*). Typically, we obtain 20–25 g of callus from five flasks. A small aliquot of callus, corresponding to the total fraction, was collected at this step and stored at –80 °C for further analysis as crude fraction on SDS gels (*see Subheading 3.5*).
3. In a cold room, add grinding buffer (30 mL for 64 g of callus cells) and sand (15 mL, volume measured with a Falcon tube, for 64 g of callus) to the cells in the mortar. Grind the cells with a pestle until a homogenous suspension is obtained. In general, it takes 6–8 min to obtain a completely homogenized lysate.
4. Add grinding buffer (20 mL) to the lysate to facilitate the transfer into 250 mL centrifuge bottles for the JA10 rotor. Residual cells are washed out of the mortar with 30 mL aliquots of grinding buffer.
5. Centrifuge the cell lysates at $700 \times g$ for 5 min at 4 °C to pellet unbroken cells, heavy material and sand. Collect the supernatant with a 50 mL pipette and distribute it in 35 mL centrifuge tubes.
6. Centrifuge the supernatant at $3000 \times g$ for 5 min at 4 °C in 35 mL centrifuge tubes using a JA25.50 rotor. Transfer the supernatant to fresh 35 mL tubes and repeat this step once.
7. Transfer the supernatant to fresh 35 mL tubes and pellet the crude mitochondria by centrifugation at $20,000 \times g$ for 15 min at 4 °C in the JA25.50 rotor. Discard the supernatant.
8. Carefully resuspend the pellet in each 35 mL tube with 1 mL of washing buffer using a paint brush and transfer the crude mitochondria with a 1 mL pipette using cut pipette tips into an 8 mL Potter-Elvehjem tissue grinder.

9. Homogenize crude mitochondria by three gentle strokes. About 100 μL of this fraction, corresponding to the “crude” fraction, were collected at this step and stored at $-80\text{ }^{\circ}\text{C}$ for further analysis.
10. Adjust the crude mitochondria suspension volume to 4 mL with washing buffer.

3.3. First Purification of Mitochondria on a Discontinuous Percoll Gradient

1. Prepare two gradients for 20–25 g of callus cells.
2. Pour 5 mL of 40% Percoll at the bottom of a 40 mL ultracentrifuge tube. Then, gently add 20 mL of 23% Percoll and 10 mL of 18% Percoll (*see Note 7*). The three layers should be clearly visible.
3. Carefully add 2 mL of crude mitochondria suspension on the top of each gradient with a 1 mL pipette using cut pipette tips.
4. Centrifuge at $50,000 \times g$ for 45 min at $4\text{ }^{\circ}\text{C}$ in a SW32-Ti swing-out rotor, with acceleration “Max” and deceleration “Slow.”
5. After centrifugation, mitochondria are located at the 23–40% interface (Fig. 1).
6. Carefully aspirate and discard the upper part of the gradient.
7. Retrieve the mitochondria with a 1 mL pipette (around 4 mL) and place them in a 35 mL centrifuge tube (one tube per gradient). Fill the tube with washing buffer.
8. Centrifuge at $20,000 \times g$ for 15 min at $4\text{ }^{\circ}\text{C}$ in a JA25.50 rotor.
9. Carefully discard the supernatant (*see Note 8*) and pool the pellets in one 35 mL centrifuge tube. Fill the tubes with washing buffer and centrifuge again at $20,000 \times g$ for 15 min at $4\text{ }^{\circ}\text{C}$.
10. Discard the supernatant and carefully resuspend the pellet in 1 mL of washing buffer.
11. Transfer the mitochondria to a 4 mL Potter-Elvehjem tissue grinder and completely dissociate the mitochondria by three strokes. About 100 μL of this fraction, corresponding to the M1 fraction, were collected at this step and stored at $-80\text{ }^{\circ}\text{C}$ for further analysis. We usually obtain 1.3 mg of mitochondrial proteins in the M1 fraction from 20 to 25 g of callus.

3.4. Second Purification of Mitochondria on a Continuous Percoll Gradient

1. For further purification of mitochondria, carefully load 500 μL of M1 mitochondria on the top of a 3 mL continuous 28% Percoll gradient in a 5 mL ultracentrifuge tube.
2. Centrifuge the gradients 20 min at $40,000 \times g$ at 4°C in a HT-4 rotor with a slow break.
3. Mitochondria form a white band in the middle of the gradient (Fig. 1).
4. Discard the upper part of the gradient, collect the white band with a 1 mL pipette and transfer it to a 35 mL centrifuge tube and add washing buffer.
5. Centrifuge the mitochondria for 15 min at $20,000 \times g$ at 4°C in a JA25.50 rotor. Carefully remove the supernatant, resuspend the pellet in washing buffer and repeat the centrifugation once more.
6. Carefully resuspend mitochondria in 1 mL of washing buffer and transfer them to an Eppendorf tube.
7. Pellet the mitochondria by a centrifugation at $20,000 \times g$ for 8 min at 4°C in a microcentrifuge and discard the supernatant by pipetting.
8. Resuspend the mitochondria in 200 μL of washing buffer and store them at -80°C . This fraction corresponds to the M2 fraction. We usually obtain 0.8 mg of mitochondrial proteins in the M2 fraction from 20 to 25 g of callus.

3.5. Evaluation of Mitochondria Contamination by SDS-PAGE and Western Blot Analysis

Total proteins are extracted from callus samples, and together with protein aliquots taken during mitochondria isolation (“Crude,” “M1,” and “M2”), the samples are analyzed by SDS-PAGE and Western Blot using antibodies to proteins derived from different cell compartments to assess the purity and the degree of contamination derived from other cellular organelles.

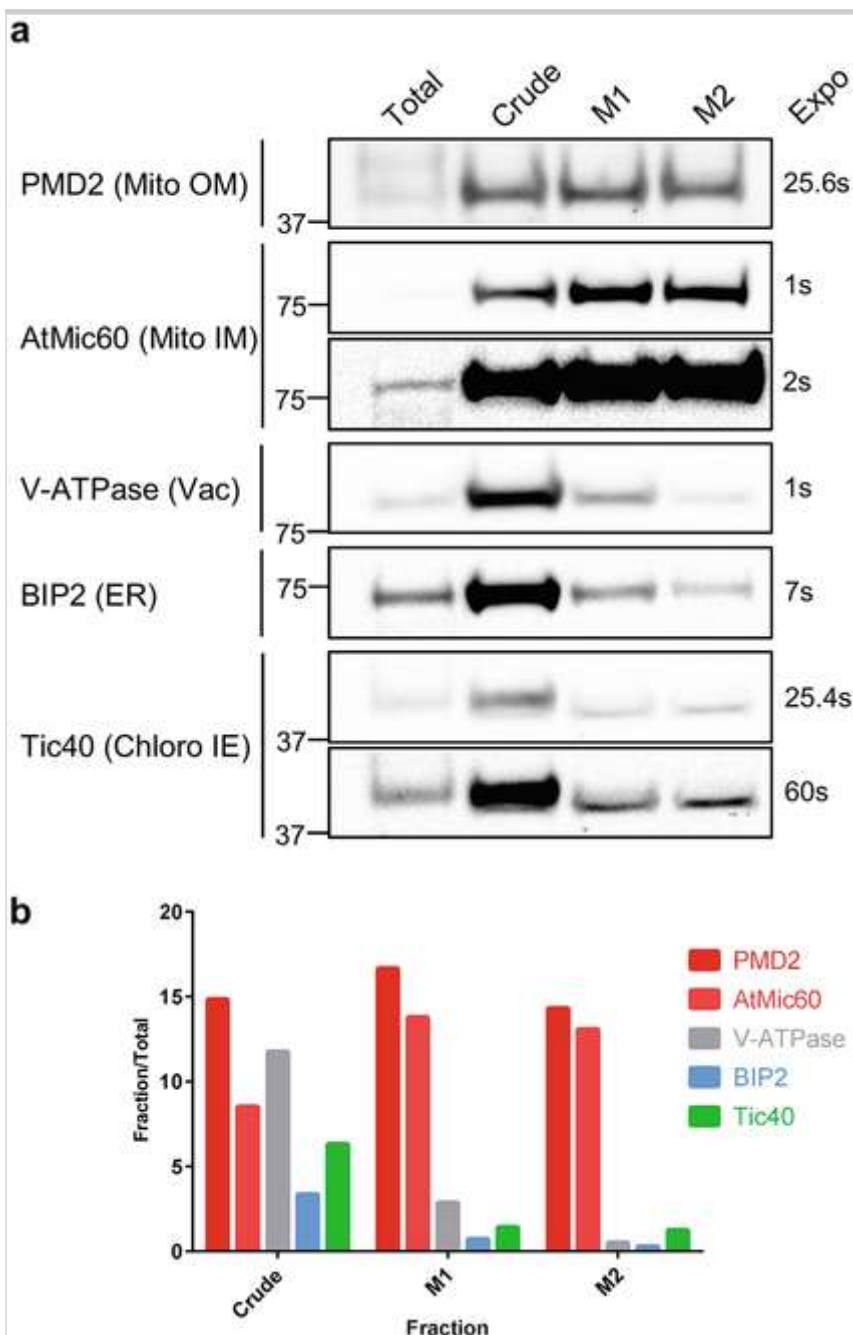
1. To extract total proteins from callus, place the callus in a resistant 2 mL bead-beater tissue homogenizer tube.
2. Add 200 μL of Protein buffer and around 200 μL of 0.5 mm acid-washed glass beads.
3. Heat the sample at 70°C for 5 min with 500 rpm agitation.

4. Grind the callus in a bead-beater tissue homogenizer at 4 °C: 3× 20 s at 10,000 rpm, pause 30 s.
AQ2
5. Repeat **steps 3** and **4**.
6. Centrifuge for 5 min at 20,000 × *g* at 4 °C in a microcentrifuge and transfer the supernatant to a fresh Eppendorf tube.
7. Thaw frozen samples (aliquots taken during mitochondria preparation) on ice for 15 min.
8. Determine the protein content of each sample (“Total,” “Crude,” “M1,” and “M2”) by Bradford protein assay [21].
9. Prepare samples for SDS-PAGE by mixing aliquots with 10 µg of proteins with loading buffer.
10. Heat protein samples 5 min at 95 °C.
11. A 4–12% gel is placed in an electrophoresis tank filled with electrophoresis buffer and is loaded with samples containing 10 µg of protein in each lane.
12. Electrophoresis is performed at 100 V until the bromophenol blue dye (migrating at the front) has reached the bottom of the gel (around 1 h 15 min).
13. After SDS-PAGE, the gel is removed from its cast and washed for 5 min in 25 mL of transfer buffer.
14. Prepare a sandwich, from the anode (pole +) to the cathode (pole –), composed of: a sponge, two Whatman filter papers, a nitrocellulose membrane (0.2 µm), the polyacrylamide gel, two Whatman filter papers, another sponge. Preincubate the sponges, the Whatman papers and the membrane in the transfer buffer before assembling the sandwich (*see Note 9*).
15. Place the sandwich in a tank with an ice block and fill with transfer buffer (*see Note 10*).
16. Perform the protein transfer at 90 V for 1 h.
17. Rinse the membrane two times in MilliQ water for 5 min.
18. Stain the membrane in 25 mL of Ponceau red for 5 min.

19. Wash several times with MilliQ water for a few seconds until the background of the membrane becomes clear.
20. Dry the membrane on a Whatman filter paper and take a photo of the protein profile. Ponceau red staining is used to assess equal loading of the proteins in each lane and the efficiency of the protein transfer to the membrane.
21. Destain the membrane by two washes of 10–15 min in 25 mL of TBS.
22. Proceed to classical immunostaining depending on the antibody used (Fig. 2).

Fig. 2

Western blot analysis of different cell compartment markers. (a) To evaluate the enrichment of mitochondria and their contamination by other cell compartments at different purification steps, Western blot analysis was performed on “Total,” “Crude,” “M1,” and “M2” fractions sampled as described in the text and in Fig. 1. Aliquots containing 10 µg of proteins were loaded on each lane. PMD2 is a protein of the mitochondrial outer membrane (Mito OM). AtMic60 is a protein of the mitochondrial inner membrane (Mito IM). V-ATPase is a protein of the vacuole membrane (Vac). BIP2 is a protein of the endoplasmic reticulum (ER). Tic40 is a protein of the chloroplast inner envelope (Chloro IE). Observed molecular weights, in kDa, are indicated on the left and exposition times (Expo) on a chemiluminescence imager, in seconds (s), are indicated on the right. (b) Quantification of the Western blot analyses shown in (a). Band intensities were quantified using the Image J software and the values were plotted taking the “Total” fraction as a reference



3.6. Lipid Analysis by Thin-Layer Chromatography or HPLC-MS/MS

1. Lipids can be extracted from each fraction by the standard Folch [22] or Bligh and Dyer [23] method, described in Chapter 1 of this book.
2. Analyze the extracted lipids by thin layer chromatography (TLC) or High Pressure Liquid Chromatography coupled to mass spectrometry (HPLC-MS/MS) according to Chapters 4 or 9, respectively, of this book, or to published procedures [24].

4. Notes

1. To limit the contamination of plant cells by fungi and bacteria, media are prepared, poured into the culture flasks (200 mL per 1 L flask) and autoclaved. Plant cell cultures are manipulated in a laminar flow hood dedicated to plant cultures. The culture medium and the time of growth can be adapted to grow callus in the absence of phosphate or nitrate or in excess of a compound of choice.
2. We describe the work using rotors for Beckman Coulter and Sorvall centrifuges and ultracentrifuges. Rotors and centrifuges from other companies may be used as long as the rotation angles (fixed or swing-out), the centrifugation forces and the volumes of the tubes are comparable.
3. We used a PreCellys Evolution bead-beater tissue homogenizer (Montigny-le-Bretonneux, France).
4. *Arabidopsis thaliana* callus cell cultures are obtained from mesophyll tissue of 2-week-old *Arabidopsis* leaves, and grown on MS agar plates supplemented with 3% (w/v) sucrose, 1.2 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.8% (w/v) agar, pH 5.7 with NaOH. After 3–4 weeks of growth on solid media, callus cells are transferred from the plates into 1 L flask in 200 mL of MS medium under continuous light (150 μ E) at 22 °C and agitated with a rotary shaker at 125 rpm. Callus cells are sub-cultured every 7 days by letting the flask to sediment, through away the excess of media, and collect 5 mL of sedimented callus into a new flask with 200 mL of MS medium. Callus cultures are stable for at least 4 weeks in liquid culture.
5. Materials for mitochondria preparation (tubes, glassware, ...) have to be free of detergent. It is recommended to dedicate an extra set of material to mitochondria preparation and to wash the material with distilled water only.
6. After filtration, cells have to be maintained at 4 °C before grinding. Thus, a prechilled mortar is stored on ice close to the balance. Cells are immediately placed into the mortar after weighing.
7. We use a peristaltic pump to layer the 23% and 18% Percoll solutions over the 40% Percoll layer. The tubes containing the 5 mL of 40% Percoll are slightly inclined on ice and the exit tube of the pump is fixed at the top of the 32 mL tube. The entry tube of the pump is dived into a Falcon tube containing 20 mL of 23% Percoll and the pump is turned on. Usually, we use a flow rate of 1.5 mL/min to layer the 23% and 18% Percoll solutions. During the distribution with the pump, the solution has to slide onto the inner walls of the tube. A drop falling directly on the gradient could disturb it.
8. At this step, the mitochondria pellet is not stable. Discard the supernatant by carefully

pipetting the upper part of the tube. Leave around 5 mL in the tube to not lose mitochondria.

9. While preparing the sandwich for the Western blot, avoid trapping air bubbles between the different layers by applying a light pressure during each layer addition. A plastic pipette can be rolled on top of each Whatman layer to remove air bubbles. Each layer must be slightly larger than the gel size.
10. A wet transfer is preferred to a semidry transfer because we obtain better transfer efficiency for high-molecular-weight and highly hydrophobic proteins.

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