Chapter 2

Isolation of Functional Mitochondria from Cultured Cells and Mouse Tissues

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Abstract

Mitochondria serve as the center stage for a number of cellular processes, including energy production, apoptosis, ion homeostasis, iron and copper processing, steroid metabolism, de novo pyrimidine, and heme biosynthesis. The study of mitochondrial function often requires the purification of intact and respiratory-competent organelles. Here, we provide detailed protocols to isolate functional mitochondria from various types of mammalian cells and mouse tissues, in both crude and pure forms. We introduce the use of nitrogen cavitation for the disruption of plasma membrane and the reproducible isolation of mitochondria-enriched fractions of high yield. Mitochondria that are isolated by these procedures are intact and coupled and can directly be used for several downstream analyses, such as measurements of oxygen consumption and calcium buffering capacity.

Key words Crude mitochondria, Pure mitochondria, Mitochondrial integrity, Organelle isolation, Nitrogen cavitation

1 Introduction

Mitochondria are double membrane organelles found in virtually all of eukaryotic cells. The organelle hosts several cellular processes, including but not limited to ATP synthesis. Over 200 metabolic reactions take place inside mitochondria, supporting numerous anabolic and catabolic pathways such as biosynthesis of iron-sulfur clusters and phospholipids and degradation of fatty acids and amino acids [1]. Besides, mitochondria are equipped with macromolecular machineries for the import of proteins and the exchange of inorganic ions and solutes. Given the central role of the organelle in cell biology, its dysfunction underlies a large set of common and rare human diseases [2].

Numerous mitochondrial processes, for example, the oxidative phosphorylation [3], the import of proteins [4], and the replication of mitochondrial DNA [5], have been elucidated at the subcellular level, using mitochondria-enriched fractions as starting...
material. This still remains the method of choice to investigate the effect of genetic, chemical, or pathological perturbations on mitochondrial-specific functions. Likewise, isolated mitochondria are instrumental to establish a direct and causal involvement of the organelle in a cellular phenotype. Finally, relatively pure preparations of mitochondria isolated from cells and tissues are often used for proteomics analyses that aim at studying composition, abundance, and modifications of the mitochondrial proteome under different conditions.

The most commonly used protocols for the isolation of mitochondria from cells and tissues include three main steps: (1) the selective disruption of plasma membrane to release intracellular organelles without compromising their integrity, (2) a differential centrifugation at low speed to remove cellular debris and large organelles such as nuclei, and (3) a high-speed centrifugation step of the supernatant to pellet lighter intracellular structures including mitochondria. However, available protocols may differ in the methods used to permeabilize the plasma membrane and in the speed of the differential centrifugation steps. While the latter should not compromise the quality and yield of the preparation, the first step is key to obtain intact mitochondria with high yields. On the one hand, an incomplete homogenization of the tissue may substantially decrease yields. On the other hand, the abrupt disruption of the cell membrane may compromise mitochondrial integrity and functions.

Mechanical homogenization procedures (e.g., mortar-pestle, bead beater, French press, cold shock) are often employed to generate cell and tissue lysates. The main drawback is that, by creating friction during sample tearing and ripping, these approaches generate extensive heat and shearing stress which can damage the organelles. Moreover, if performed manually by the user, tissue grinding can result in highly variable samples. The latter could be minimized by motorization of pestles.

In contrast, cell disruption by nitrogen cavitation, although less commonly used, has been shown to be an effective technique to extract intact and enzymatically active mitochondria from different sources [6–8]. This is achieved by a rapid decompression of the cell suspension placed in a cavitation chamber and subjected to high pressure in presence of nitrogen gas. Nitrogen, initially dissolved in the cell suspension under high pressure, comes out of solution when the pressure is released. As a result, bubbles are formed that will expand and rupture pressure-sensitive cellular structures such as plasma and nuclear membranes, while keeping smaller organelles intact. Afterwards, mitochondria are enriched by differential centrifugation. Cell lysis by nitrogen cavitation offers several advantages: (1) It is more reproducible than conventional homogenization methods because it is conducted under controlled and constant thermodynamic parameters (temperature and pressure).
It minimizes shearing stress, thus increasing the yield of functionally active mitochondria. (3) It enables the enrichment of mitochondria from small organs like the cortex, spinal cord, and other brain regions as it can be easily applied to very small volumes of cell suspension. Moreover, it can efficiently extract mitochondria from synaptic membranes which form during tissue grinding.

In this chapter, we provide in-depth protocols for isolating functional crude and pure mitochondria in high yield from cultured cells (e.g., HeLa, HEK293, mouse embryonic fibroblasts (MEF), human primary fibroblasts, and lung epithelial cells), as well as from soft (e.g., brain, spinal cord, liver, and kidney) and hard rodent tissues (skeletal muscle and heart) (Fig. 1). These protocols will allow the user to purify mitochondria with high yields that can be used for downstream functional assays such as respiration, swelling and calcium buffering, as well as for proteomics profiling studies. Finally, we also provide a protocol to measure mitochondrial Ca\(^{2+}\) uptake kinetics, as a fast and simple method to test integrity of isolated mitochondria.

## 2 Materials

### 2.1 Equipment

1. Cell disruption vessel 45 ml (Parr Instruments).
2. Commercial nitrogen cylinder.
3. Ultracentrifuge up to 45,000 \(\times g\) (Beckman Coulter).
4. Ultracentrifuge rotor for 3 ml tubes (Beckman Coulter, SW 55 Ti).
5. Refrigerated superspeed centrifuge (Sorvall, Evolution RC).
6. Centrifuge rotor, fixed angle (Sorvall, SS-34).
7. Centrifuge tubes 30 ml, glass.
8. Rubber adapter sleeves for ultracentrifuge tubes.
9. Dounce tissue grinder, all glass, 2 ml.
10. Mouse dissection and surgical tools.
11. Tissue grinder, PTFE pestle and glass tube, 45 ml.
13. Cell strainer for falcon tubes, pore size 70 \(\mu m\).
14. Stericup-GP sterile vacuum filtration system, pore size 0.22 \(\mu m\).
15. Glass rods.
16. 96-well fluorescence plate reader suitable for fast kinetic measurements with injection (BMG LABTECH, Clario star).
17. 96-well plate, black.
Fig. 1 Experimental approach to isolate crude and pure mitochondrial fractions. A crude mitochondrial fraction can be isolated by differential centrifugation and if required it can be loaded on a discontinuous Percoll density gradient to obtain pure mitochondria.
2.2 Reagents

1. 0.1 M ATP: dissolve 551.14 mg of adenosine 5’-triphosphate disodium salt hydrate (ATP) powder in 8 ml of distilled water. Adjust pH to 7.4 using KOH. Bring the solution to 10 ml with water, aliquot in 500 μl samples, and store at −20 °C.

2. 0.5 M EDTA-KOH: dissolve 18.61 g of ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate in 80 ml of distilled water, and adjust pH to 7.4 using KOH (see Note 1). Bring the solution to 100 ml with water and store at room temperature.

3. 0.5 M EGTA-KOH: dissolve 19.02 g of ethylene glycol-bis(2-aminoethylether) (EGTA) in 80 ml of distilled water and adjust pH to 7.4 using KOH (see Note 1). Bring the solution to 100 ml with water and store at room temperature.

4. 1 M HEPES-KOH: dissolve 119.15 g of HEPES in 400 ml of distilled water and adjust pH to 7.4 using KOH. Bring the solution to 500 ml with water and store at room temperature.

5. 1 M KCl: dissolve 37.28 g of KCl in 400 ml of distilled water. Bring the solution to 500 ml with water and store at room temperature.

6. 1 M Tris–HCl: dissolve 60.57 g of Tris in 400 ml of distilled water. Adjust the pH to 7.4 using HCl. Bring the solution to 500 ml with water and store at room temperature.

7. 1 M MgCl₂: dissolve 50.8 g of magnesium chloride hexahydrate (MgCl₂) in 200 ml of distilled water. Bring the solution to 250 ml with water and store at room temperature.

8. Isolation buffer (IB): 220 mM d-mannitol, 70 mM d-sucrose, 5 mM HEPES-KOH pH 7.4, 1 mM EGTA-KOH pH 7.4. Dissolve 40.08 g of d-mannitol and 23.96 g of d-sucrose in 900 ml of distilled water. Add 5 ml of 0.5 M HEPES-KOH and 2 ml of 0.5 M EGTA-KOH. Adjust with KOH pH to 7.4 at 4 °C. Bring the solution to 1 L with water. Sterilize the solution using sterile vacuum filter units with a pore size of 0.22 μm. Sterile IB solution can be stored at 4 °C for several months.

9. 5× isolation buffer (5× IB): 1.1 M D-mannitol, 350 mM d-sucrose, 25 mM HEPES-KOH pH 7.4, 5 mM EGTA-KOH pH 7.4. Dissolve 200.32 g of d-mannitol and 119.84 g of d-sucrose in 900 ml of distilled water. Add 25 ml of 1 M HEPES-KOH and 10 ml of 0.5 M EGTA-KOH. Adjust with KOH pH to 7.4 at 4 °C. Bring the solution to 1 L with water. Sterilize the solution using sterile vacuum filter units with a pore size of 0.22 μm. Sterile 5× IB solution can be stored at 4 °C for several months.

10. Isolation buffer 1 muscle (IB₁_M): 100 mM KCl, 50 mM Tris–HCl pH 7.4, 2 mM EDTA-KOH pH 7.4. Mix 100 ml of 1 M KCl, 50 ml of 1 M Tris–HCl pH 7.4, and 4 ml of 0.5 M
EDTA-KOH with 800 ml of distilled water. Adjust with KOH pH to 7.4 at 4 °C and bring the solution to 1 L. Sterilize the solution using sterile vacuum filter units with a pore size of 0.22 μm. Sterile IB1M solution can be stored at 4 °C for several months.

11. 5× isolation buffer 1 muscle (5× IB1M): 500 mM KCl, 250 mM Tris–HCl pH 7.4, 10 mM EDTA-KOH pH 7.4. Mix 500 ml of 1 M KCl, 250 ml of 1 M Tris–HCl, and 20 ml of 0.5 M EDTA-KOH. Adjust with KOH pH to 7.4 at 4 °C and bring the solution to 1 L with distilled water. Sterilize the solution using sterile vacuum filter units with a pore size of 0.22 μm. Sterile 5× IB1M solution can be stored at 4 °C for several months.

12. 2× isolation buffer 2 muscle (2× IB2M): 200 mM KCl, 100 mM Tris–HCl pH 7.4, 4 mM EDTA-KOH pH 7.4, 10 mM MgCl2. Mix 50 ml of 1 M KCl, 25 ml of 1 M Tris–HCl, 2 ml of 1 M EDTA-KOH, and 2.5 ml 1 M MgCl2 with 150 ml of distilled water. Adjust with KOH pH to 7.4 at 4 °C and bring the solution to 250 ml. Sterilize the solution using sterile vacuum filter units with a pore size of 0.22 μm. Sterile IB2M solution can be stored at 4 °C for several months.

13. 0.5 M succinate: dissolve 2.95 g of succinic acid in 40 ml of distilled water. Adjust pH with KOH to 7.4 at RT and bring the solution to 50 ml with water. Aliquot the solution in 0.5 ml aliquots and store at −20 °C. Do not freeze and thaw.

14. 0.5 M malate: dissolve 3.35 g of L-(−)-malic acid in 40 ml of distilled water. Adjust pH with KOH to 7.4 at RT and bring the solution to 50 ml with water. Aliquot the solution in 0.5 ml aliquots and store at −20 °C. Do not freeze and thaw.

15. 0.5 M glutamate: dissolve 3.68 g of l-glutamic acid in 40 ml of distilled water. Adjust pH with KOH to 7.4 at RT and bring the solution to 50 ml with water. Aliquot the solution in 0.5 ml aliquots and store at −20 °C. Do not freeze and thaw.

16. 1 M KH2PO4: dissolve 6.80 g of KH2PO4 in 40 ml distilled water and adjust pH with KOH to 7.4 at RT and bring the solution to 50 ml with water. Sterilize the solution using filter with a pore size of 0.22 μm pore size. Sterile solution is stable for several months.

17. 2× respiration buffer (2× RB): 274 mM KCl, 20 mM HEPES-KOH pH 7.4, 5 mM MgCl2, 6 mM KH2PO4-KOH pH 7.4, 50 μM EDTA. Combine 68.5 ml of 1 M KCl, 5 ml of 1 M HEPES-KOH, 1.25 ml of 1 M MgCl2, 1.5 ml KH2PO4-KOH, and 25 μl of 0.5 M EDTA-KOH with 150 ml distilled water, and adjust pH to 7.4 at RT using KOH. Bring solution to 250 ml, and sterilize the solution using sterile vacuum filter units with a pore size of 22 μM. Sterile 2× RB solution can be stored at RT for several months.
18. Bicinchoninic acid assay (BCA) kit.
19. 100% Percoll solution (pH 8.5–9.5).
20. 420 μM Calcium Green-5 N Stock solution: Dissolve 0.5 mg Calcium Green-5 N powder with 1 ml distilled water. Aliquot solution to 15 μl aliquots and store at −20 °C protected from light. Do not freeze and thaw.
21. 1 mM Ru360 stock solution: dissolve 0.5 mg Ru360 with 909 μl distilled water to obtain 1 mM Ru360 stock solution. Aliquot solution to 15 μl aliquots and store at −20 °C.
22. 1× PBS (phosphate-buffered saline), commercially available.
23. Fatty acid-free bovine serum albumin (BSA).
24. EDTA-free protease inhibitor cocktail tablets for 50 ml medium.
25. Heparin.

2.3 Working Solutions

Working solutions should be prepared fresh on the day of the experiment and should be kept on ice the whole time, unless indicated otherwise.

1. IB(++) buffer: add 500 mg of fatty acid-free BSA (0.5%) (see Note 2) and two tablets of protease inhibitor cocktail to 100 ml of IB. 100 ml of IB(++) are sufficient for the isolation of mitochondria from one mouse liver (∼2 g tissue).
2. IB1M(+) buffer: add 500 mg of fatty acid-free BSA to 100 ml of IB1M. 80 ml of IB1M(+) are sufficient for the isolation of mitochondria from ∼2 g of skeletal muscle tissue.
3. IB1M(++) buffer: add 500 mg of fatty acid-free BSA and two tablets of EDTA-free protease inhibitor cocktail to 100 ml of IB1M. 80 ml of IB1M(++) are sufficient for the isolation of mitochondria from ∼2 g of hind limb muscle tissue.
4. IB2M(++) buffer: mix 25 ml of 2× IB2M with 500 μl 100 mM ATP and 122.85 U of protease from Bacillus licheniformis and fill up to 50 ml with distilled water. If necessary, adjust the pH to 7.4 with KOH at 4 °C. 30 ml of IB2M(++) are sufficient for the isolation of mitochondria from ∼2 g of hind limb muscle tissue.
5. Heparin/PBS solution: Prepare 20 U heparin/ml PBS solution using heparin sodium salt from porcine intestinal mucosa (≥150 U/mg, dry basis). Depending on the perfusion system used, between 10 and 50 ml of heparin/PBS solution are required for the perfusion of a whole mouse.
6. 80% Percoll solution: mix 4 ml of 5× IB buffer with 16 ml of 100% Percoll solution.
7. 52% Percoll solution: mix 6.5 ml of 80% Percoll solution with 3.5 ml of 1× IB(++) buffer.
8. 26% Percoll solution: mix 3.25 ml of 80% Percoll solution with 6.75 ml of IB(++) buffer.

9. Respiration buffer (RB++): For 50 ml RB++, add 0.5 ml of each 0.5 M succinate, 0.5 M glutamate, and 0.5 M malate to 25 ml 2× RB. Add 11.9 μl of 420 μM Calcium Green-5 N and fill up to 50 ml with distilled water. Keep the solution at RT protected from light.

10. Ca²⁺ injection solution: add 8 μl of 0.5 M CaCl₂ stock solution to 10 ml of RB++. Keep the solution at RT and protected from light until use.

3 Methods

3.1 Isolation of Crude Mitochondria from Cultured Cells

The isolation of crude mitochondria from cells by nitrogen cavitation requires approximately 40 min. Working solutions as well as tissue grinder and cell disruption vessel should be kept ice-cold during the whole procedure.

1. Seed cells 2–3 days before the experiment. The number of cells that is required to isolate more than 1 mg of crude mitochondria may vary between cell types, and it usually reflects cell size and mitochondrial content. The typical yields for commonly used cell types are listed in Table 1 (see Note 3).

2. Remove the medium from the cells and wash once with 5–10 ml of room temperature PBS.

3. Remove PBS and detach the cells using a cell scraper. Collect the cells in a pre-weighted falcon tube. If needed, use few ml PBS to collect the remaining cells from the plate. Keep the sample on ice at all time.

4. Centrifuge cells at 600 × g for 5 min at 4 °C.

5. Discard the supernatant and weight out the cell pellet (see Note 4).

6. Resuspend the cells using 1 ml IB(++) per 10 mg of cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Culture surface (cm²)</th>
<th>Mitochondrial yield (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK-293</td>
<td>450</td>
<td>~1.5</td>
</tr>
<tr>
<td>HeLa</td>
<td>450</td>
<td>~2.25</td>
</tr>
<tr>
<td>MEF</td>
<td>450</td>
<td>~1.8</td>
</tr>
<tr>
<td>Human fibroblast</td>
<td>450</td>
<td>~0.4</td>
</tr>
</tbody>
</table>
7. Transfer the solution into a prechilled cell disruption vessel, add the magnetic stirrer bar into the vessel, and place it on a magnetic stirrer in a cold room. Close the vessel and attach the filling connection to a commercial nitrogen cylinder as in the instructions manual. To pressurize the vessel, open first the nitrogen cylinder to release nitrogen into the flexible nylon pressure hose. Then open carefully the valve on the head of the cylinder and adjust the pressure to 800 psi (see Note 5). Leave the sample in the vessel at 800 psi for 10 min.

8. Hold a 50 ml falcon tube at the lower valve of the vessel while depressurizing to collect the sample (see Note 6).

9. If the quality of mitochondria isolation is to be tested, save ~100 μl of sample for quality analysis (Fig. 2). This represents the whole cell (WC) fraction (see Note 7).

10. Centrifuge the whole cell sample at 600 × g for 10 min at 4 °C.

11. Carefully transfer the supernatant to a prechilled glass centrifuge tube without touching the pellet.

12. If the quality of mitochondria isolation is to be tested, save the pellet for quality analysis (Fig. 2). This represents the nuclei fraction (see Note 7).

13. Centrifuge the supernatant at 8000 × g for 10 min at 4 °C.

14. If the quality of mitochondria isolation is to be tested, save after centrifugation 500 μl for quality analysis. This represents the cytosolic (Cyto) fraction (see Note 7), while the pellet contains the mitochondrial fraction.

15. Discard the supernatant, and remove any loose or light-colored material surrounding the pellet, preferably by aspiration with a glass micropipette (see Note 8).
16. Carefully dislodge the pellet using a glass rod.

17. Resuspend the pellet in a small volume of IB(++) by slowly pipetting up and down first with a cut 200 μl pipette tip to minimize shearing forces and afterwards with an intact 200 μl pipette tip to obtain a homogeneous sample. The volume for resuspension depends on the pellet size and should be as small as possible (50–100 μl for mitochondria isolated from cells). Avoid the formation of bubbles during resuspension.

18. Quantify the concentration of crude mitochondria by BCA method (see Note 9).

### 3.2 Isolation of Crude Mitochondria from Soft Mouse Tissues

Timing for the isolation of crude mitochondria from soft mouse tissues depends on the number of processed tissues and animals. Approximately 1.5 h are necessary to isolate crude mitochondria from one mouse liver. The typical yields for several soft tissues are listed in Table 2 and refer to one mouse per tissue.

Working solutions as well as tissue grinder and cell disruption vessel should be kept ice-cold during the whole procedure.

1. Sacrifice the mouse by cervical dislocation and rapidly explant the organs of interest (see Note 10).

2. Weight out the organ and place it in a beaker on ice.

3. Add 1 ml IB(++)/0.1 g of tissue (see Note 11).

4. Mince the tissue in small pieces (2–4 mm) using scissors and wash several times with IB(++) to remove any contaminating blood (see Note 12).

5. Transfer the tissue pieces to a prechilled tissue grinder and homogenize with one stroke at 300 rpm (see Notes 13–15).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Weight (mg)</th>
<th>Mitochondrial yield (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>~1500</td>
<td>~15</td>
</tr>
<tr>
<td>Kidney</td>
<td>~350</td>
<td>~1.9</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>~60</td>
<td>~0.4</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>~75</td>
<td>~2.7</td>
</tr>
<tr>
<td>Cortex</td>
<td>~125</td>
<td>~4</td>
</tr>
<tr>
<td>Ventral midbrain</td>
<td>~40</td>
<td>~0.5</td>
</tr>
<tr>
<td>Striatum</td>
<td>~40</td>
<td>~0.5</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>~1800</td>
<td>~25</td>
</tr>
<tr>
<td>Heart</td>
<td>~160</td>
<td>~2</td>
</tr>
</tbody>
</table>
6. Transfer the homogenate into a prechilled cell disruption vessel, add the magnetic stirrer bar into the vessel, and place it on a magnetic stirrer in a cold room (see Notes 14 and 15). Close the vessel and attach the filling connection to a commercial nitrogen cylinder as in the instructions manual. To pressurize the vessel, open first the nitrogen cylinder to release nitrogen into the flexible nylon pressure hose. Then open carefully the valve on the head of the cylinder and adjust the pressure to 800 psi (see Note 5). Leave the sample in the vessel at 800 psi for 10 min.

7. Hold a 50 ml falcon tube at the lower valve of the vessel while depressurizing to collect the sample (see Note 6).

8. If the quality of mitochondria isolation is to be tested, save a 100 μl of sample for quality analysis. This represents the whole cell (WC) fraction (see Note 7).

9. Centrifuge at 600 × g for 10 min at 4 °C.

10. Transfer the supernatant to a prechilled glass centrifuge tube. Be careful to prevent contaminations from the cell pellet. If the quality of mitochondria isolation is to be tested, keep the pellet for quality analysis (see Note 7). This represents the nuclei fraction (Nu).

11. Centrifuge the supernatant at “centrifuge speed 1” as in Table 3 for 10 min at 4 °C (see Note 16).

12. If the quality of mitochondria isolation is to be tested, save after centrifugation 500 μl for quality analysis. This represents the cytosolic (Cyto) fraction (see Note 7), while the pellet contains the mitochondrial fraction.

13. Discard the supernatant and carefully dislodge the pellet using a glass rod.

14. Resuspend the pellet with a pipette in the initial volume of IB(++) (see step 3). Avoid the formation of bubbles during resuspension.

---

Table 3

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Centrifugation speed 1</th>
<th>Centrifugation speed 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>10 min 8000 × g</td>
<td>2 min 2000 × g followed by 8 min 4000 × g</td>
</tr>
<tr>
<td>Liver</td>
<td>10 min 8000 × g</td>
<td>2 min 2000 × g followed by 8 min 4000 × g</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>10 min 12,000 × g</td>
<td>10 min 12,000 × g</td>
</tr>
<tr>
<td>Brain</td>
<td>10 min 12,000 × g</td>
<td>10 min 12,000 × g</td>
</tr>
</tbody>
</table>
15. Centrifuge the sample at “centrifugation speed 2” as in Table 3 for 10 min at 4 °C (see Note 16).

16. Discard the supernatant and resuspend the pellet with a small volume of IB(++) by slowly pipetting up and down first with a cut 200 μl pipette tip to minimize shearing forces and afterward with an intact 200 μl pipette tip to obtain a homogeneous sample. The volume for resuspension depends on the pellet size and should be as small as possible (e.g., 100–200 μl for the liver and ~50 μl for the brain tissues or spinal cord). Avoid the formation of bubbles during resuspension.

17. Quantify the concentration of the crude mitochondrial fraction by BCA method (see Note 9).

### 3.3 Isolation of Crude Mitochondria from Mouse Skeletal Muscle and Heart

Timing for the isolation of crude mitochondria from skeletal muscle or heart depends on the number of processed tissues and animals. Approximately 2 h are necessary to isolate crude mitochondria from one mouse heart. Working solutions as well as tissue grinder and cell disruption vessel should be kept ice-cold during the whole procedure. Skeletal muscle and heart are fibrous tissues, which cannot be easily homogenized without previous enzymatic digestion. To avoid that an uneven homogenization of the tissue clogs the cavitation vessel, we employ for mitochondrial isolation from muscle and heart tissue the following protocol:

1. Sacrifice the mouse by cervical dislocation and rapidly explant the organs of interest (see Note 10).

2. Weight out the organ and place it in a beaker on ice (see Note 11).

3. Mince the tissue in small pieces (2–4 mm) using scissors and wash it several times with IB1M(+) to remove any contaminating blood.

4. Decant any IB1M(+) and add 1 ml IB2M(++)/0.1 g of tissue.

5. Incubate for 3 min while stirring on ice.

6. Transfer the tissue pieces to a prechilled tissue grinder and homogenize with three strokes at 500 rpm. Use motorized system to ensure efficient homogenization and minimize variations between samples.

7. Transfer the homogenate into a falcon tube, and centrifuge at 600 × g for 10 min at 4 °C to remove plasma membrane and nuclei and not homogenized tissue.

8. Collect the supernatant which contains mitochondria, and filter it through a cell strainer of 70 μm pore size that is placed on a prechilled glass centrifuge tube.

9. Immediately centrifuge the sample at 10,400 × g for 10 min at 4 °C.
10. Discard the supernatant and dislodge the pellet using a glass rod. Resuspend the pellet with 300–500 μl volume of IB1M(++) by slowly pipetting up and down first with a cut 200 μl pipette tip to minimize shearing forces and afterward with an intact 200 μl pipette tip to obtain a homogeneous sample. Avoid the formation of bubbles during resuspension.

11. Dilute the sample with IB1M(++) at 1 ml/0.1 g of tissue.

12. Repeat steps 9–11 once to thoroughly wash out the remaining proteinases.

13. Centrifuge at 4000 × g for 10 min at 4 °C.

14. Discard the supernatant and dislodge the pellet using a glass rod. Resuspend the pellet with ~100 μl volume of IB1M(++) by slowly pipetting up and down first with a cut 200 μl pipette tip to minimize shearing forces and afterward with an intact 200 μl pipette tip to obtain a homogeneous sample. The volume for resuspension depends on the pellet size and should be as small as possible. Avoid the formation of bubbles during resuspension.

15. Quantify the concentration of crude mitochondria by BCA method (see Note 9).

3.4 Isolation of Pure Mitochondria by Discontinuous Percoll Gradient

Preparations of crude mitochondrial fractions usually contain other cellular structures as well. Lysosomes and endoplasmic reticulum are common contaminants which either co-sediment with mitochondria during differential centrifugation or are physically associated with them. To increase mitochondrial purity, the differential centrifugation step can be combined with a discontinuous Percoll density gradient. The advantage of a discontinuous gradient, over a continuous one, is that mitochondria concentrate as a sharp band at the interface between two Percoll steps, which facilitates their collection. Here, we provide a fast protocol which can be performed after obtaining a crude mitochondrial fraction. As an example, we apply this protocol to purify intact organelles from crude skeletal muscle mitochondria.

1. Transfer 1 ml of 80% Percoll solution into the ultracentrifuge tube. Mark the level of the solution (see Note 17).

2. Add 1.5 ml of 52% Percoll solution drop wise on top of the 80% Percoll solution. Hold the tube in a 45° angle to prevent mixing of the two solutions. Mark the level of the second solution.

3. Add 500 μl of 26% Percoll solution drop wise on top of the 52% Percoll solution. Hold the tube in a 45° angle to prevent mixing of solutions.

4. Dilute the freshly isolated, crude mitochondrial fraction with 500 μl of the corresponding IB solution supplemented with fatty acid-free BSA and protease inhibitors (IB(++) or IB1M(++) depending on the tissue of origin).
5. Place the diluted sample on top of the Percoll gradient. Balance the rotor carefully. In case of unequal sample number, use a balance tube (tube with the same weight as the sample tube).

6. Centrifuge at $44,000 \times g$ for 45 min at 4 °C.

7. Collect pure mitochondria at the interface between 26% and 52% Percoll gradient (Fig. 1) (see Note 18).

8. Dilute the sample at least tenfold with the corresponding IB solution (IB(++) or IB1M), and transfer it to a glass centrifuge tube.

9. Centrifuge at $8000 \times g$ for 10 min at 4 °C.

10. Decanted the supernatant. The pellet contains pure mitochondria.

11. Dislodge the pellet using a small glass rod. Resuspend the pellet with a small volume of IB solution (IB(++) or IB1M) by slowly pipetting up and down first with a cut 200 μl pipette tip to minimize shearing forces and afterward with a 200 μl pipette tip to obtain a homogeneous sample. Avoid formation of bubbles during resuspension.

12. Measure the concentration of the pure mitochondrial fraction by BCA method (see Note 9).

**3.5 Measuring Mitochondrial Calcium (Ca$^{2+}$) Buffering Capacity**

Measuring mitochondrial Ca$^{2+}$ buffering capacity is a fast and efficient method to assess the integrity of isolated mitochondria. The organelle takes up Ca$^{2+}$ via an inner membrane channel called the mitochondrial calcium uniporter (MCU) [9–11]. Ca$^{2+}$ uptake is driven by the mitochondrial membrane potential, which is generated by the oxidative phosphorylation system through pumping of protons across the inner mitochondrial membrane. Therefore, the integrity and coupling of isolated mitochondria will be reflected by their ability to buffer exogenously added Ca$^{2+}$ to the respiration medium.

First, mitochondria are diluted in a buffer containing a non-permeable Ca$^{2+}$ indicator such as Calcium Green-5 N (K$_d$ of 14 μM in the absence of magnesium). If the indicator binds Ca$^{2+}$, it exhibits an increase in fluorescence emission intensity with little shift in wavelength. By taking up Ca$^{2+}$, mitochondria compete with the dye, and as a result the intensity of the emitted light decreases.

1. Use a fluorescence microplate reader with the following settings: Ex/Em of 503/536 nm, kinetic reading mode and short time interval (e.g., reading of 24 wells with a 2 s interval).

2. Prime the injector with 400 μM Ca$^{2+}$ injection solution.

3. Dilute an appropriate amount of freshly isolated crude or pure mitochondria in RB++ to 1 mg/ml. This amount should be sufficient to test all of the conditions of interest in at least technical triplicates (see Note 19).

4. Pipette 90 μl of the mitochondria-containing solution per well into a black 96-well plate. As a positive control for mitochondrial Ca$^{2+}$ uptake, include 10 μM Ru360, a known MCU
Fig. 3 Ca\textsuperscript{2+} uptake kinetics in purified mitochondria from different mouse tissues. Intact and coupled mitochondria are isolated from mouse organs using nitrogen cavitation. MCU-dependent Ca\textsuperscript{2+} uptake kinetics are measured in 96-well plates with Calcium Green-5N following consecutive injections of 40 μM Ca\textsuperscript{2+} (red traces). Ca\textsuperscript{2+} uptake can be inhibited by the addition of 10 μM Ru360 (black traces).
inhibitor. If possible, use a multichannel pipette to minimize pipetting error.

5. Mix well and incubate for 5 min at RT.

6. Place the plate inside the plate reader.

7. Start the assay and monitor basal fluorescence, followed by a repetitive injection of 10 μl from the 400 μM Ca\(^{2+}\) solution. Figure 3 shows typical Ca\(^{2+}\) uptake kinetics of crude mitochondria isolated from different mouse tissues.

### 4 Notes

1. EDTA and EGTA solutions are very acidic and dissolve poorly in water. Add KOH tablets till reaching a pH of ~7, and then continue adjusting the pH with a 5 M KOH solution.

2. BSA binds fatty acids. Those are released from the cells during the permeabilization process and can disrupt the integrity of mitochondrial membranes. Therefore, the addition of fatty acid-free BSA is critical for preserving mitochondrial integrity. It is recommended to wash out the BSA for proteomics.

3. The metabolic state of cells is influenced by the number of culturing passages and cell density. In order to minimize variation between samples, all cell lines to be processed should be cultured in parallel and have a similar passage number and a cell density corresponding to approximately 80% of the culture dish surface.

4. To weight out the cell pellet, balance the empty Falcon before use.

5. A pressure of 800 psi selectively permeabilizes the plasma membrane but not the mitochondrial membranes. Large sample volumes can absorb significant amounts of nitrogen leading to a drop of the pressure after several seconds. If necessary, readjust the pressure.

6. The permeabilization by nitrogen cavitation occurs at the decompression step. It is recommended to become completely familiar with the sample collection step before performing the experiment.

7. The enrichment of mitochondria from whole cells can be tested by Western blot analyses of whole cell (WC), nuclear (Nu), cytosolic (Cyto), and mitochondrial (Mito) fractions (Fig. 2). This step is particularly useful when testing an isolation protocol or the isolation from a specific tissue for the first time.

   Equal protein amounts of those fractions are decorated with antibodies that recognize bona fide cytosolic (e.g., actin), nuclear (e.g., lamine A/C), and mitochondrial proteins (e.g., OXPHOS subunits and porin). The integrity of the mitochondrial fraction can also be tested by protein immunoblot. To
this goal, loss of soluble matrix and intermembrane space proteins, such as cyclophilin D and cytochrome c, respectively, should be quantified.

8. Removing of loose or lighter-colored material surrounding the pellet can increase purity of the mitochondrial fraction but can also increase variation between samples. We recommend to skip this step if the sample is used in sensitive analysis such as mass spectrometry. In this case, the whole pellet should be carefully resuspended in the same volume of IB (without BSA and protease inhibitors). The sample should be centrifuged again at $8000 \times g$ for 10 min at 4 °C, and the resulting pellet should be resuspended in a small volume of IB (50–100 μl).

9. Other protein quantification methods can also be used (e.g., Bradford protein assay). It is recommended to test several dilution factors (25×, 50× and 100×) to obtain an accurate sample concentration. For reproducible results, ensure to use the same quantification method for all samples and the same standard for the calibration curve.

10. During the isolation procedure, blood cells are ruptured and release hemoglobin which sediments with the mitochondrial pellet and can compromise the functionality of mitochondria. When isolating mitochondria from organs that are well supplied by blood, such as the liver, it is recommended to euthanize the mice with CO$_2$ and to perform a whole body perfusion with heparin/PBS [12].

11. If necessary, organs can be kept for up to 2 h in ice-cold PBS before proceeding with the isolation protocol.

12. When isolating mitochondria from larger and compact tissue like the liver or kidney, a motorized homogenizer improves dislodging and minimizes variation between samples. When isolating mitochondria from small organs such as spinal cord or brain regions, it is not necessary to mince the tissue. Instead, use a small, prechilled glass tissue grinder with a loose pestle to dislodge the tissue and transfer the sample directly to the nitrogen cavitation vessel.

13. This step is performed with the aim of dislodging the tissue and not of permeabilizing the cells. The latter occurs during nitrogen cavitation.

14. Remove any large tissue pieces that remain after homogenization as they can clog the valve of the nitrogen cavitation vessel.

15. When working with small volumes of homogenate (less than 3 ml) place the sample in a test tube or a small beaker that can be placed inside the vessel cylinder. As a general rule, the test tube should be twice the size of the sample volume.

16. Sedimentation speed varies for mitochondria of different tissues.
17. During the centrifugation, mitochondria will sediment at the interface between 26 and 54% Percoll density. Demarking the interface will facilitate the identification of the right density band.

18. To collect mitochondria either aspirate with a long glass micro-pipette or remove layer by layer till reaching the mitochondria-containing band.

19. Mitochondria isolation buffer contains EDTA or EGTA which influence the calcium kinetics. Therefore, it is beneficial to work with highly concentrated mitochondria (25–50 mg/ml).

References


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